INVESTIGATING THE STABILITY OF NITRIC OXIDE SYNTHASE QUATERNARY STRUCTURE TO DENATURANT AND TEMPERATURE

by

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Abstract

A limitation to investigations of homodimeric protein dissociation is that the signals produced from methods such as fluorescence and circular dichroism represent both dissociation and protein unfolding that may be occurring simultaneously within a sample. Although size exclusion chromatography examines the state of a protein’s quaternary structure, complicated overlapping peaks representative of oligomer and monomer can result. To address these limitations the mixed dimer system has been adopted to investigate the dissociation of a homodimeric protein. A mixed dimer is a species in which each subunit of a homodimeric protein is associated with a different affinity tag. The two tags used are the His$_6$-tag and the Glu$_7$-tag. Such a mixed dimer will bind to a metal chelating column such as Ni-NTA so long as the dimer is intact. Denaturant- or temperature induced dimer dissociation can be detected by the amount of Glu$_7$-tagged subunit present in the unbound fraction after the protein is passed over an Ni-NTA resin. SDS PAGE and densitometry assess the amount of Glu$_7$-tagged subunit present in those unbound fractions. The experimental conditions necessary to implement this method were developed, and then applied to mammalian inducible nitric oxide synthase (iNOS) and Staphylococcus aureus NOS (SaNOS). With respect to both urea and temperature, the stability of SaNOS is higher than that of iNOS in spite of the bacterial enzyme having a much smaller dimer contact surface. We have also used the mixed dimer method to estimate an equilibrium dissociation constant ($K_D$) for iNOS dissociation of no greater than 2.3μM. This value is compared to the results obtained for iNOS by analytical ultracentrifugation, which can characterize protein complexes and their stoichiometry.
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# Table of Contents

Abstract .................................................................................................................. i
Acknowledgements ............................................................................................... ii
Table of Contents ................................................................................................... iii
List of Figures ......................................................................................................... vii
List of Tables ......................................................................................................... x
List of Abbreviations ............................................................................................. xi

Chapter 1 Introduction ............................................................................................ 1
  1.1 Oligomeric Protein Structure ......................................................................... 1
  1.2 Protein Structure ............................................................................................ 1
    1.2.1 Primary Structure ................................................................................... 2
    1.2.2 Secondary Structure .............................................................................. 4
    1.2.3 Tertiary Structure ................................................................................. 4
    1.2.4 Quaternary Structure ............................................................................ 6
  1.3 Protein Folding ............................................................................................... 7
  1.4 Forces Driving Protein Folding ...................................................................... 9
  1.5 Protein Unfolding .......................................................................................... 12
  1.6 Investigations of Protein Unfolding ............................................................... 15
  1.7 Dimer Dissociation Studies using Mixed Dimers ......................................... 24
  1.8 Applications of the Mixed Dimer System ..................................................... 27
  1.9 Nitric Oxide Synthases .................................................................................. 28
    1.9.1 Biological Importance of NO ................................................................. 28
1.9.2 NOS Structure ................................................................. 32
1.9.3 Dimerization ................................................................. 39
1.9.4 The NOS Reaction ......................................................... 46
1.9.5 UV-Visible Spectroscopic Properties of NOS ......................... 46

Chapter 2 Objectives .................................................................. 52

Chapter 3 Materials and Methods ................................................ 53

3.1 Homodimeric Protein Expression and Purification ...................... 53

3.1.1 Bacterial Growth Conditions ............................................ 53

3.1.2 Wild-Type COD\text{INOS-His}_{6} homodimer Purification .......... 54

3.1.3 Wild-type COD\text{SaNOS-His}_{6} Homodimer Purification ........ 55

3.1.4 Wild-Type COD\text{INOS-Glu}_{7} Homodimer Purification ......... 55

3.1.5 Wild-type COD\text{SaNOS-Glu}_{7} Homodimer Purification .......... 56

3.2 Mixed Dimer Protein Expression and Purification ....................... 57

3.2.1 Bacterial Growth Conditions and Cell Lysis ......................... 57

3.2.2 Wild-Type COD\text{INOS-His}_{6}/COD\text{INOS-Glu}_{7} Mixed Dimer Purification ....................................................... 57

3.2.3 Wild-Type COD\text{SaNOS-His}_{6}/COD\text{SaNOS-Glu}_{7} Mixed Dimer Purification ....................................................... 58

3.3 Determination of Protein Purity and Concentration .................... 59

3.4 Investigation of Dimer Dissociation ......................................... 59

3.4.1 Initial Ni-NTA column .................................................... 59

3.4.2 Dimer Dissociation ....................................................... 60

3.4.3 SDS PAGE and Densitometry ......................................... 61

3.4.4 Calculation of $C_m$ and $T_m$ values .................................. 61
3.5 Determination of “on” or “off” Column Dissociation using Alkaline Phosphatase
3.6 Investigation of His$_6$-tag stability at Elevated Temperatures
3.7 Investigation of His$_6$-tag Binding Upon Protein Aggregation
3.8 Investigation of SaNOS Thermal Denaturation by UV-Visible Spectroscopy
3.9 Investigation of Possible Rapid Exchange of Subunits
3.10 Investigation of the Effect of Dilution on Dissociation and Estimation of $K_D$ in the absence of Denaturant
3.11 Investigation of $K_D$ by analytical ultracentrifugation

Chapter 4 Results and Discussion

4.1.1 Protein Purification
4.1.2 Determination of Protein Purity
4.2 The Mixed Dimer System – Control Investigations
4.2.1 Determination of “on” or “off” Column Dissociation Using Alkaline Phosphatase
4.2.2 Dissociation of the COD$_{iNOS}$-His$_6$ homodimer
4.2.3 Dissociation of the COD$_{iNOS}$-Glu$_7$ Homodimer
4.2.4 Choice of Stain for SDS PAGE
4.3 Denaturant-Induced Dissociation of COD$_{iNOS}$-His$_6$/COD$_{iNOS}$-Glu$_7$ and COD$_{SaNOS}$-His$_6$/COD$_{SaNOS}$-Glu$_7$ mixed dimers
4.4 Temperature-Induced Dissociation of COD$_{iNOS}$-His$_6$/COD$_{iNOS}$-Glu$_7$ and COD$_{SaNOS}$-His$_6$/COD$_{SaNOS}$-Glu$_7$ mixed dimers
4.5 Rapid Exchange of Subunits
4.6 Effect of Dilution on Dissociation and Estimation of $K_D$
4.7 Investigation of $K_D$ by analytical ultracentrifugation
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.8 Limitations to the Mixed Dimer Method</td>
<td>115</td>
</tr>
<tr>
<td>Chapter 5 Concluding Summary</td>
<td>117</td>
</tr>
<tr>
<td>References</td>
<td>118</td>
</tr>
<tr>
<td>Appendix 1</td>
<td>121</td>
</tr>
<tr>
<td>Appendix 2</td>
<td>124</td>
</tr>
<tr>
<td>Appendix 3</td>
<td>125</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1: The peptide bond .................................................................3
Figure 2: Common elements of secondary structure............................5
Figure 3: Models of protein folding pathways ..................................8
Figure 4: The structure of urea ............................................................14
Figure 5: Fluorescence properties of amino acids .............................17
Figure 6: Circular dichroism properties of the peptide bond and aromatic amino acids .................................................................18
Figure 7: A typical protein unfolding curve in the presence of denaturant......20
Figure 8: Size exclusion chromatography profile the iNOS oxygenase domain after incubation in denaturant ..................................................23
Figure 9: Creation and purification of mixed dimers ............................26
Figure 10: Dissociation of a mixed dimer ............................................29
Figure 11: Schematic of the proposed model for NO-induced increase in IRE-binding activity in the IRF .................................................................31
Figure 12: The domain structure of NOS .............................................33
Figure 13: Overall fold of the NOS oxygenase domain .........................34
Figure 14: The heme binding pocket of the NOS oxygenase domain .........36
Figure 15: Overall fold of the NOS reductase domain ..........................37
Figure 16: Dimerization in NOS .........................................................40
Figure 17: The binding pockets for H\textsubscript{4}B and arginine in NOS ............42
Figure 18: The N-terminal hook regions of NOS ..................................44
Figure 19: Overlapped structures of the SaNOS and eNOS dimers ............45
Figure 20: The NOS catalyzed reaction ..............................................47
Figure 21: Electron configuration of NOS heme iron ............................50
Figure 22: UV-Visible spectra of NOS ................................................................. 51

Figure 23: COD_{iNOS}-Glu_{7} homodimer separation by anion exchange chromatography ................................................................. 70

Figure 24: COD_{iNOS}-His_{6}/COD_{iNOS}-Glu_{7} separation by anion exchange chromatography ................................................................. 72

Figure 25: COD_{SaNOS}-His_{6}/COD_{SaNOS}-Glu_{7} separation by anion exchange chromatography on low pressure LC ......................................... 73

Figure 26: COD_{SaNOS}-His_{6}/COD_{SaNOS}-Glu_{7} separation by Q column anion exchange chromatography on an HPLC ..................................... 75

Figure 27: UV visible spectra of COD_{iNOS}-His_{6}/COD_{iNOS}-Glu_{7} and COD_{SaNOS}-His_{6}/COD_{SaNOS}-Glu_{7} .................................................. 76

Figure 28: SDS PAGE of COD_{iNOS}-His_{6} urea-induced dissociation .......................................................... 80

Figure 29: SDS PAGE of COD_{iNOS}-His_{6} urea-induced dissociation when purified with captopril ............................................................... 83

Figure 30: SDS PAGE of COD_{iNOS}-Glu_{7} urea-induced dissociation .......................................................... 85

Figure 31: SDS PAGE of COD_{iNOS}-His_{6} urea-induced dissociation with 20 mM imidazole ............................................................... 88

Figure 32: Standard curve created by staining the SDS PAGE gel with Coomassie Blue stain ............................................................... 90

Figure 33: Standard curve created by staining the SDS PAGE gel with SYPRO Ruby stain ............................................................... 92

Figure 34: Urea-induced dissociation of COD_{iNOS}-His_{6}/COD_{iNOS}-Glu_{7} ........................................... 93

Figure 35: Urea-induced dissociation of COD_{iNOS}-His_{6}/COD_{iNOS}-Glu_{7} –H_{4}B – Arg ............................................................... 97

Figure 36: SaNOS dimer interface .......................................................... 99

Figure 37: Temperature-induced dissociation of COD_{iNOS}-His_{6}/COD_{iNOS}-Glu_{7} ........................................... 101

Figure 38: SDS PAGE of temperature-induced dissociation of COD_{iNOS}-His_{6} ........................................... 102

Figure 39: SDS PAGE of temperature-induced dissociation of COD_{iNOS}-His_{6} ........................................... 103
Figure 40: UV visible spectrum of the COD$_{SaNOS}$-His$_6$/COD$_{SaNOS}$-Glu$_7$
incubated at increasing temperatures ........................................... 107

Figure 41: Temperature-induced dissociation of COD$_{SaNOS}$-His$_6$/COD$_{SaNOS}$-Glu$_7$
–Arg +H$_4$B ................................................................. 108

Figure 42: Determination of Rapid Mixing of Subunits ........................................... 111

Figure 43: Dissociation of the COD$_{iNOS}$-His$_6$/COD$_{iNOS}$-Glu$_7$ at different
concentrations of total protein .......................................................... 112
List of Tables

Table 1: Susceptibility of NOS oxygenase dimers to urea-induced dissociation......94

Table 2: Susceptibility of NOS oxygenase dimers to thermally-induced dissociation.............................................105

Table 3: % Composition of COD$_{\text{NOS-His}_6}$/COD$_{\text{NOS-Glu}_7}$ mixed dimer according to analytical ultracentrifugation.........................................................114
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>Arg</td>
<td>L-arginine</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>Cap</td>
<td>Captopril</td>
</tr>
<tr>
<td>COD&lt;sub&gt;iNOS&lt;/sub&gt;</td>
<td>Core oxygenase domain, inducible nitric oxide synthase</td>
</tr>
<tr>
<td>COD&lt;sub&gt;SanOS&lt;/sub&gt;</td>
<td>Core oxygenase domain, <em>Staphylococcus aureus</em> nitric oxide synthase</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
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<tr>
<td>Glu&lt;sub&gt;7&lt;/sub&gt;</td>
<td>Polyglutamate tag</td>
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<tr>
<td>H&lt;sub&gt;4&lt;/sub&gt;B</td>
<td>(6R)5,6,7,8-tetrahydrobiopterin</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Hexahistidine tag</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilized metal ion affinity chromatography</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>LPLC</td>
<td>Low pressure liquid chromatography</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel-nitrilotriacetic acid</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOHA</td>
<td>N-hydroxy-L-arginine</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PNPP</td>
<td>Para-nitrophenyl phosphate</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>TCEP</td>
<td>Tris(2-carboxyethyl)phosphine hydrochloride</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific broth</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris-(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</tbody>
</table>
Chapter 1: Introduction

1.1 Oligomeric Protein Structure

Protein complex formation is a common mechanism responsible for the function and regulation of many physiological processes. Some of the advantages conferred by oligomerization include; i) enhanced functioning through the creation of scaffolds, ii) improved regulation through allosteric regulation, iii) regulatory flexibility, and iv) increased resistance to degradation or denaturation (Mayssam and Imperiali 2005). The tendency of a protein to oligomerize can be strong or weak, or it can vary based on stimulus or environmental conditions (Mayssam and Imperiali 2005). Disruption of the stable complex by exogenous forces causes dissociation and results in loss of function or regulation. Dissociation can therefore serve as a point of protein regulation, making it critical to understand the conditions under which proteins will dissociate and subsequently become inactive (Siddhanta, Presta et al. 1998). It is important to consider the overall protein structure when considering dissociation of an oligomeric protein.

1.2 Protein Structure

Proteins are complex organic macromolecules possessing various hierarchical levels of structure. The overall three-dimensional fold of a protein is a result of various forces acting together to produce the native protein structure. Many proteins can function only once properly folded. The most basic level of structure is the primary structure of a protein.
1.2.1 Primary Structure

The primary structure of a protein encodes all the information necessary for the upper levels of protein folding. Since the function of a protein relies on its ability to fold into a stable structure, the primary structure also plays a vital role in protein function. The primary structure is comprised of a linear, unbranched polymer chain consisting of repeating amino acid units held together by a peptide bond, as seen in figure 1 (Fersht 1999). The basic repeating unit of the peptide backbone, termed the peptide group, is identical, as all 20 amino acids contribute the same central $\alpha$-carbon linked to a hydrogen, an amino group, and a carboxylic acid group (Creighton 1984). The peptide bond linking amino acids is formed through a condensation reaction between a carboxylic acid group and an amino group of neighbouring amino acids. Also attached to the $\alpha$-carbon of the amino acids are varying side chains. The side chains of the respective amino acids vary in size, shape, charge, hydrogen bonding ability, and hydrophobic character. The side chains can also possess different functional groups including alcohols, thiols, thioethers, carboxylic acids, amides, and basic groups. These functional groups possess different properties which they impart on the polypeptide chain as a whole (Creighton 1984). The functional groups all contribute to determination of the folded structure and the overall properties of a protein. The length of a polypeptide chain and the sequence of amino acids are specific to each protein. Variations in the length and sequence of the polypeptide chain as well as the individual abundance of each amino acid helps determine upper levels of folding structure.
Figure 1: The peptide bond connects each amino acid residue, the functional units of the peptide backbone. The central α-carbon is labelled and is linked to a hydrogen, an amino group, a carboxylic acid group, and a variable R group side chain. The peptide bond (shown in pink) occurs between the amino group of one amino acid and the carboxylic acid group of the neighbouring amino acid. (Berg, Tymoczko et al. 2007)
1.2.2 Secondary Structure

Linked amino acids provide the basic elements for the formation of secondary structure formation. Secondary structure refers to the general three-dimensional fold of local segments of the peptide backbone (Creighton 1984). These structures result in the formation of regular patterns within the peptide backbone (Fersht 1999). Secondary structures form when the carbonyl oxygen and amide protons within the peptide group, near to each other in the polypeptide chain, form hydrogen bonds resulting in the formation of localized structures including α-helices, β-sheets and β-turns, as seen in figure 2 (Fersht 1999). Although it is the atoms involved in the peptide bond that are responsible for the stabilization of a secondary structure, the side chains of the amino acids within a polypeptide segment influence the propensity of that segment to fold into a particular secondary structure. Size, charge, shape, length, identity, and localization of the amino acids within the polypeptide chain all affect the ability of a peptide segment to fold into a local secondary structure (Creighton 1984). Once folded, the segments of secondary structure can associate with one another to form higher structural elements.

1.2.3 Tertiary Structure

Tertiary structure represents the overall topology of a single folded polypeptide chain and results from the close packing of secondary structure segments into common patterns (Fersht 1999). Common groupings of secondary structure can occur amongst proteins. These are termed motifs or supersecondary structure examples of which include a β-barrel, β-sandwich, Jellyroll, Greek key, and α-turn-α structure (Fersht 1999). Short polypeptide segments function to link together sections of secondary structure.
Figure 2: Common elements of secondary structure including the α-helix (a) β-sheet (b) and β-turn (c). The peptide backbone is shown in red, the O of the CO group involved in the peptide bond is seen in blue, and the H of the NH group involved in the peptide bond is seen in green. Hydrogen bonds (shown as dashed lines) between NH and CO groups involved in the peptide bond stabilize the structures.
Fragments of the polypeptide not involved in secondary structures can be non-repetitive well-defined structures, termed regions of random coil, or flexible disordered regions (Creighton 1984). In association, all of these regions contribute to the tertiary structure of a folded polypeptide. Individual polypeptide chains can also associate together to form a complex.

1.2.4 Quaternary Structure

Quaternary structure refers to the grouping together of two or more polypeptide chains into a complex. Each peptide chain is referred to as a subunit or monomer within the multi-subunit or oligomeric complex. Complexes can be composed of varying numbers of monomeric units. Those comprised of different monomers are termed heteromeric whereas those comprised of repeating units of the same monomer are termed homomeric. Complex formation can also involve the recruitment of other elements that are not amino acids but are necessary for protein activation or regulation.

Cofactors or prosthetic groups are various chemical components that are integral to the structure and activation of many proteins (Creighton 1984). Common cofactors include, heme, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), or nicotinamide adenine dinucleotide phosphate (NADP). Proteins can also possess metals as prosthetic groups. These metals commonly serve enzymatic roles but can also serve structural roles in maintaining protein structure (Creighton 1984). Some more commonly incorporated metals are zinc, iron, copper, and magnesium. The overall result of protein folding is the formation of a stable protein structure or complex.
1.3 Protein folding

All the information necessary to determine the proper native fold of a protein is contained within the primary sequence, but the process of folding a polypeptide chain into the native protein conformation has many possible pathways. Ultimately, a two-state folding pathway where initially the unfolded polypeptide chain exists in solution, followed by complete conversion to the native folded state is impossible (Dagget and Fersht 2003). Various mechanisms have been postulated to explain the process of protein folding as seen in figure 3. The nucleation-growth mechanism suggests that initially local secondary structures form, providing a nucleus from which tertiary and quaternary structures can form (Anfinsen 1973; Fersht 1999; Dagget and Fersht 2003). However, the nucleation-growth mechanism does not account for the formation of folding intermediates within the process. The framework model proposes that grouping pre-formed secondary structures together simply provides the native protein fold, as seen in figure 8. Conversely, the hydrophobic collapse model proposes that condensation of the hydrophobic regions of a polypeptide chain provides a confined region within which the native state can form (Dagget and Fersht 2003). In effect, proteins may employ a combination of these methods to achieve a native folded conformation. Termed the nucleation-condensation mechanism, long-range hydrophobic interactions act to stabilize otherwise weak secondary structure formations. These stabilized secondary structures within the hydrophobic core are then a framework for the remainder of the native state to form around (Dagget and Fersht 2003). Although the nucleation-condensation model originates from studies on smaller proteins, the principles can also be applied to larger
Figure 3: Schematic view of protein folding showing the framework and the hydrophobic collapse models. The nucleation-condensation model is a combination of the two models. (Dagget and Fersht 2003)
multi-domain proteins and the formation of quaternary structure by considering the domains individually (Fersht 1999; Dagget and Fersht 2003). The underlying forces driving protein folding are ultimately governed by differences in free energy (Chothia and Finkelstein 1990).

1.4 Forces Driving Protein Folding

For proteins with known structures, the process of protein folding occurs to form the most stable structure possible. The main forces driving protein folding include hydrophobic and electrostatic interactions, and the formation of hydrogen bonds (Fersht 1999). Underlying all these forces are the changes in free energy associated with protein folding, where the native folded state possesses a minimum of free energy (Anfinsen 1973; Lattman and Rose 1993).

The formation of hydrophobic interactions is driven by the need to sequester hydrophobic regions from an aqueous environment while hydrophilic regions remain exposed. Exposure of hydrophobic regions to an aqueous environment requires water molecules to arrange around the region reducing the entropy of the system (Schmid 1989). Burying of hydrophobic regions away from the aqueous environment into the interior of a protein upon folding is driven by the regaining of entropy of the water (Fersht 1999). Consequently, proteins existing within an aqueous environment will adopt a hydrophobic core with hydrophilic regions exposed to the environment. However, proteins that exist in a hydrophobic environment, for example proteins that span a biological membrane, fold to adopt the opposite orientation, where hydrophobic regions are exposed and hydrophilic regions are buried within the interior.
Electrostatic interactions occur between charged molecules. Ion-dipole, or dipole-dipole interactions are electrostatic interactions that occur between an ion and a neutral molecule that has a dipole, or between two molecules with dipoles. Interactions such as these are possible within a protein due to the charges created on amino acids with ionisable side chains at different pH’s (Berg, Tymoczko et al. 2007). The energy involved in electrostatic interactions decreases with increasing distance between charges. The process of protein folding reduces the distance between molecules involved in electrostatic interactions and therefore reduces the energy involved in those interactions (Fersht 1999).

Hydrogen bonds are formed when two electronegative atoms, one of which is usually oxygen, share the same proton. Hydrogen bonds are of sufficient energy that they can play integral roles in stabilizing a protein’s native fold and consequently, play a vital role in holding upper levels of folding structures together, specifically secondary structures (Fersht 1999).

In addition to the interactions that stabilize the secondary and tertiary interactions within an individual polypeptide chain, additional factors drive the formation of a quaternary structure and complex formation. Steric constraints require that, for polypeptide chains to associate together, the surfaces that are to interact must be complementary to each other (Archakov, Govorun et al. 2003). To aid in producing complementary surfaces, complex formation can be accompanied by structural changes to enable favourable interactions to occur. H-bonds that occur at the subunit interface contribute to the specificity and complementarity of subunits for their partner (Archakov, Govorun et al. 2003). The contact surface areas for the association of subunits into a
complex can be as compact as the interior of a monomeric protein, and more than 65% of a protein’s stability can be provided by its quaternary interactions (Neet and Timm 1994; Fersht 1999). Overall, differences in the free energy of the monomeric and oligomeric states drive oligomer formation.

The formation of a protein dimer can be represented as:

\[ A + B \rightleftharpoons AB \]  

Equation 1

In the case of a homodimeric protein where two identical subunits associate to form a dimer, Equation 1 simplifies to:

\[ A + A \rightleftharpoons A_2 \]  

Equation 2

Where the equilibrium constant for the dissociation \((K_D)\) and association \((K_A)\) of the homodimer are ratios of the concentration of each species given at equilibrium, represented in:

\[ K_D = \frac{[A]_2}{[A]^2} = \frac{1}{K_A} \]  

Equation 3

The relationship between the equilibrium dissociation constant \((K_D)\) and the free energy of dissociation \((\Delta G_D)\) is:

\[ \Delta G_D = -RT \ln K_D \]  

Equation 4

where \(R\) is the gas constant and \(T\) is the temperature (in K). Typical free energies of folding for globular proteins range between -5 to -15 kcal/mol representing the stability of the folded state (Lattman and Rose 1993). Overall, there is a reduction in free energy
associated with the formation of a complex. The noncovalent interactions that are formed along with the reduction in free energy associated with hydrophobic free energy favour complex formation (Chothia and Finkelstein 1990; Neet and Timm 1994). However, compared to the free energy associated with the individual interactions that form during protein folding, the reduction in free energy is minimal, representing the inherent instability of the folded state (Berg, Tymoczko et al. 2007). This stable structure can be disrupted by elevated temperature or denaturant leading to complex dissociation and protein unfolding (Lattman and Rose 1993).

1.5 Protein Unfolding

Protein denaturation is the process by which the native folded protein structure unfolds. The noncovalent interactions that hold the protein together are altered, or lost, and therefore the native fold is disrupted. Common methods to induce denaturation of proteins include thermal denaturation or the addition of a chemical denaturant. Thermal denaturation involves increasing the temperature of a protein solution, which weakens the noncovalent interactions that hold the native fold together. Long-range interactions are those first affected by increases in temperature. This results in a more flexible structure where regions can be exposed to the solvent. As H-bonds begin to break, secondary structures are destabilized and hydrophobic regions become exposed to the solvent. The hydrophobic regions aggregate to minimize exposed surface area. Denaturing agents such as urea or guanidinium hydrochloride disrupt the noncovalent interactions that hold the folded protein together, thereby causing its denaturation (Pace, Shirley et al. 1989; Berg, Tymoczko et al. 2007). One example of a chemical denaturant is urea whose
structure can be seen in figure 4. The structure of urea enables it to hydrogen bond with the protein serving its function as a denaturant that disrupts the noncovalent interactions within proteins (Berg, Tymoczko et al. 2007). Overall increasing the temperature of a protein solution or the addition of denaturant causes the native fold to be destabilized causing unfolding of the protein.

The process of protein denaturation can be approximated as a two-state process, where only the native folded protein and unfolded polypeptide chain exist in solution (Neet and Timm 1994). However, on the pathway from native folded to unfolded state there are transition states through which the protein passes (Li and Dagget 1994). The transition states are high energy forms of the native stable structure where the forces stabilizing the native state have been disrupted (Dagget and Fersht 2003). Studies on small single domain proteins have shown that upon denaturation the inner hydrophobic core is the first element to be disrupted followed by breaking of hydrogen bonds that hold the secondary structural elements together (Li and Dagget 1994). Upon disruption of the hydrophobic core the increased volume is not compensated for, and consequently leads to the overall loss of tertiary resulting in a completely unfolded state (Li and Dagget 1994). In order for denaturation of multi-subunit complexes to occur fully, dissociation of the complex must also occur. The process by which a natively folded complex dissociates and becomes denatured is characteristic of the individual protein.

Investigations of dissociation and unfolding of oligomeric proteins must consider not only stabilizing interactions within the monomer but also those that occur between monomers to stabilize the complex (Neet and Timm 1994). Because a large proportion of a protein’s stability can be provided by its quaternary interactions, oligomeric proteins
Figure 4: Structure of urea which allows it to serve its function as a denaturant that disrupts the noncovalent interactions within proteins.
can display a 2-state unfolding process where only folded oligomers and unfolded monomers exist, or a 3-state unfolding process where the native oligomer, native monomers, and unfolded monomers exist (Neet and Timm 1994). Which process predominates is determined by the stability of the monomeric intermediate species that can be formed. If the monomeric intermediate formed can be sufficiently stabilized by the intra-monomeric interactions, then a 3-state model may occur (Neet and Timm 1994). For example, the $\Delta G$ of dissociation of the human pituitary growth hormone is 4.7 kcal/mol, whereas the $\Delta G$ of unfolding is 27.8 kcal/mol suggesting that the isolated monomer can be stabilized prior to unfolding allowing a 3-state unfolding process to occur (Neet and Timm 1994). If the interactions are not sufficient to stabilize the monomer, then the unfolding process will approach a 2-state transition (Neet and Timm 1994). For example, the $\Delta G$ of dissociation of the bacteriophage p22 arc repressor is 9.7 kcal/mol, equivalent to the $\Delta G$ of unfolding of 9.5 kcal/mol indicating that the isolated monomer is not stabilized to any extent and a 2-state unfolding process predominates (Neet and Timm 1994). Overall, the transition between a oligomeric and monomeric state is an important transition in terms of protein regulation.

1.6 Investigations of Protein Unfolding

Methods that use changes in protein fluorescence and circular dichroism exploit the ability of proteins to absorb UV light to investigate protein structure. The ability of a protein to absorb light can be attributed largely to the aromatic amino acid residues and the $\pi - \pi^*$ transitions of the delocalized electrons that can occur. When the electrons within the aromatic residues are excited and then revert from the first excited state back
to the ground state, fluorescence emission is observed. Energy is lost as excited electrons transition between the excited state and the ground state. Consequently the energy of the emitted fluorescence is always less then the absorbed energy causing the emission spectra to be shifted to longer wavelengths, as seen in figure 5 (Schmid 1989). The emission properties of each aromatic amino acid are different, with phenylalanine fluorescing maximally at 282nm, tyrosine at 303nm, and tryptophan at 348nm as seen in figure 5. The fluorescence emissions for each of the amino acids are sensitive to changes in the environment, making fluorescence a good method for probing conformational changes. Changes in the emission wavelength of fluorescence upon folding are very large and are manifested as increases or decreases in intensity or shifting in wavelength (Schmid 1989).

In circular dichroism (CD) spectroscopy, the presence of secondary structure, higher levels of folding, protein structure can be investigated due to the asymmetry of the environment surrounding the peptide bond. CD in the far-UV region (170 nm-250 nm) probes the peptide bond directly and is used to estimate the secondary structure content and monitor changes in secondary structure (Schmid 1989). For example, the presence of an α-helix produces a strong band in the CD spectrum as seen in figure 6. CD in the near-UV region (250nm-300nm) probes the aromatic side chains. In a sample containing denatured, unfolded protein, the near-UV CD spectrum is usually not dominated by any characteristic peaks. However, upon protein folding, the aromatic residues within the protein are exposed to an asymmetric environment causing changes to be observed in the near-UV CD spectrum as seen in figure 6 (Schmid 1989). Consequently, differences between folded and unfolded
Figure 5: The energy of the emitted fluorescence is always less than the absorbed energy causing the emission spectra to be shifted to longer wavelengths. (a) The absorption (solid line) and emission spectra (dashed line) of tryptophan showing a shift of the emitted wavelength to 348nm. The emission properties of each aromatic amino occur at different wavelengths (b) phenylalanine at 282nm, tyrosine at 303nm and tryptophan at 348nm. (a) (Boyer 2000) (b) (Schmid 1989)
Figure 6: (a) Circular dichroism spectra in the far UV region of a poly-lysine peptide fragment in α-helical (α), β-sheet (β) and random coil (r) conformations. The presence of an α-helix or β-sheet produce strong bands in the CD spectrum compared to that of the random coil. (b) CD in the near UV aromatic region of RNase from red deer in the native (-) and unfolded (---) conformations. Upon protein folding the aromatic residues are exposed to an asymmetric environment causing changes to be observed in the spectrum. (a) (Creighton 1984) and (b) (Schmid 1989).
structures are generally pronounced and can be used to observe the level of protein unfolding within a sample.

Analysis of the transition between folded and unfolded state can produce unfolding curves as seen in figure 7. Typical curves possess three regions; (i) the pre-transition region (ii) the transition region and (iii) the post-transition region (Schmid 1989). The pre-transition region illustrates the state of the folded protein prior to disruption, and the effect of denaturing conditions on the folded state of the protein. The transition region illustrates the transition between the folded and unfolded state. The post-transition region illustrates the state of the unfolded protein and the effect of further perturbation on the unfolded protein (Schmid 1989). Although figure 7 is a curve representative of protein unfolding, a method investigating solely protein oligomer dissociation may produce a similar curve, possibly with multiple transition regions depending on the number of monomers involved in the oligomer. Initially, prior to complex disruption the pre-transition region illustrates the protein complex in its native state and under early dissociating conditions. The transition region illustrates the dissociation of the complex, and the post-transition region illustrates the individual monomers. The sharp transition region that exists in both curves illustrates that complex dissociation and protein unfolding are cooperative processes, where conditions that initiate the dissociation and unfolding processes will cause complete dissociation or unfolding respectively (Berg, Tymoczko et al. 2007). Under the influence of denaturant $C_m$ refers to the denaturant concentration where half of the protein present has dissociated into monomer. Under the influence of temperature $T_m$ is where
Figure 7: The unfolding curve of RNase T1 in the presence of denaturant. Three regions are evident (i) the pretransition region (ii) the transition region (iii) the post transition region (Pace, Shirley et al. 1989; Barry and Matthews 1999)
half of the protein present has dissociated into monomers. This point can be calculated by extrapolation of the pre- and post-transition curves (Pace 1986; Chen and Matthews 1994). The midpoint of the curve will be specific and characteristic to each protein, or condition under which it is investigated. $C_m$ or $T_m$ values can be determined and compared under varying conditions to observe the effect of different conditions, such as the presence of substrates or cofactors, or mutations on dimer dissociation and the behaviour of a protein. However, to investigate complex dissociation as a process separate from protein denaturation it is necessary to develop a method that probes dissociation alone and not in association with protein unfolding.

Fluorescence and CD provide information about the overall state of the proteins in a sample and do not probe the state of the individual protein complexes within that sample (Kalnine and Schachman 2002). Specifically, the process of complex dissociation into monomers and the process of unfolding may occur simultaneously within a protein sample, causing folded oligomers and unfolded monomers to coexist within a sample (Kalnine and Schachman 2002). Alternatively, the oligomer may dissociate to give folded monomers. Consequently, both 2- and 3-state dissociation and unfolding processes may be occurring to different extents within a protein sample. The resulting profiles are therefore representative of the average contributions of each species (Kalnine and Schachman 2002). The folded monomers within the sample will have similar CD and fluorescence spectra to the complex because the aromatic residues and elements of secondary structure are often within the same environment within the folded protein, even after dissociation of the complex. It is also possible that complexes that have begun to unfold but have not yet dissociated are present in solution. In this case the
CD and fluorescence spectra are altered because of the partially unfolded environment. However, dissociation has not occurred and the complex persists in solution. To investigate complex dissociation alone there must be a way to discriminate between the dissociated monomers and their complex counterpart.

In the case of a heteromeric complex, each monomer is different and therefore maybe distinguishable from each other on the basis of size or other properties. In a homomeric complex the monomers are identical and cannot be distinguished from each other. One method of discriminating between the monomers and their homomeric complex uses high-resolution size exclusion chromatography (Panda, Rosenfeld et al. 2002). Proteins are incubated under various conditions such as different concentrations of denaturant, causing dissociation of the complex into monomers. Following this, the relative monomer:oligomer ratio is determined from the chromatogram. An example of such a size exclusion chromatogram is seen in figure 8. Peaks on the resulting profiles are cut out separately and weighed on a microbalance to determine the monomer:oligomer ratio. Problems with this method include i) use of high concentrations of denaturants such as urea may be damaging to the high-resolution gel chromatography apparatus ii) the temperature of dissociation can not be easily manipulated iii) resulting overlapping monomer and oligomer peaks may be difficult to resolve and iv) the method is not easily modified to detailed studies of the dissociation process, only a limited number of conditions can be investigated at once. An alternative method is to generate a heteromeric oligomer from a homomeric one, and then discriminate between either monomer.
Figure 8: Size exclusion chromatography profile of iNOS oxygenase domain after incubation with 3M urea. Peaks representing the dimer and monomer are indicated. (Panda, Rosenfeld et al. 2002)
1.7 Dimer Dissociation Studies using Mixed Dimers

The mixed dimer system provides a means of discriminating between each subunit of a homodimeric protein. The mixed dimer system involves the creation of heterodimers out of homodimers by attributing a different affinity tag to each monomer (McDonald, Taylor et al. 2003). The two tags used are the polyhistidine tag (His$_6$) and the polyglutamate tag (Glu$_7$) (McDonald, Taylor et al. 2003). The His$_6$-tag is a small hydrophilic tag that has a high affinity for a nickel-chelate resin in an interaction that is insensitive to denaturants such as urea. It can be placed at either the carboxy or amino terminus of a protein. Due to the nature of the tag, the resulting protein usually retains the physical properties and structure of the original protein (McDonald, Taylor et al. 2003). The His$_6$-tag allows for isolation of a protein through used of immobilized metal affinity chromatography (IMAC). In IMAC, an immobilized chelator forms coordinate bonds to a metal ion. More commonly used metal ions include Ni$^{2+}$, Cu$^{2+}$ or Zn$^{2+}$. The metal ion, once bound to the chelator, also has free sites available to coordinate to the His$_6$-tag of the target protein. Once the His$_6$-tag is coordinated to the metal, the target protein is isolated from others within the sample. The target protein can then be eluted by competition with imidazole, whose structure resembles the histidine ring.

Like the His$_6$-tag, the Glu$_7$-tag is a small hydrophilic tag. Addition of glutamic acid residues ($pK_a$ 4.25) effectively alters the pI of the protein while leaving the functional and structural properties of the target protein unaltered. This confers a more negative charge on the protein compared to its charge in the absence of the tag. Consequently, a protein possessing a Glu$_7$-tag adheres more strongly to an anion exchange resin than a less charged species, resulting in isolation of the target protein.
Being composed of acidic residues, the Glu$_7$-tag is also likely to be on the surface of the overall folded protein structure, placing it in a location amenable to serving as an affinity tag. This tag has been placed successfully at the carboxy-terminus of a protein of interest (McDonald, Taylor et al. 2003). A mixed dimer results from a dimeric complex in which one subunit possesses a His$_6$-tag and the other possesses a Glu$_7$-tag.

In the mixed dimer strategy two plasmids, each possessing a separate selectable marker, are used to co-express the two versions of the subunit of a protein homodimer, each possessing a separate affinity tag. Growth under double antibiotic selection results in three possible species; a His$_6$-tagged homodimer in which both subunits possess a His$_6$-tag, a Glu$_7$-tagged homodimer, and the mixed dimer species in which one subunit possess a His$_6$-tag and the other possesses a Glu$_7$-tag. Sequential IMAC and anion exchange chromatography isolates the mixed dimer (McDonald, Taylor et al. 2003). Initially IMAC isolates only those species possessing a His$_6$-tag. Subsequently, anion exchange chromatography separates those species possessing a Glu$_7$-tag from those that do not. Accordingly, a dimer containing two separately tagged subunits is isolated. This permits discrimination of subunits within the dimer and can be applied to mutagenic and dissociation studies. A scheme of mixed dimer purification is seen in figure 9.
Figure 9: Illustration of the creation and isolation of a mixed dimer. The first step is co-transformation of bacterial cells with two vectors encoding the protein of interest bearing a His$_6$- or a Glu$_7$-tag. The second step is the growth of the bacteria under dual antibiotic resistance, and expression of the protein. Three dimer species are possible. Mixed dimer species possessing a His$_6$-tag and a Glu$_7$-tag are isolated using Immobilized metal affinity chromatography (IMAC) using the Ni-NTA resin, and anion exchange chromatography (McDonald, Taylor et al. 2003).
1.8 Applications of the Mixed Dimer System

Within any protein or protein complex, there are critical elements for its stabilization, regulation and function. These critical elements may include the active site of the enzyme, sites for cofactors, prosthetic groups, or metal binding, or dimerization domains among others. Certain amino acid residues play vital roles in achieving these processes. Amino acid residues thought to be involved in these roles are typically those targeted for mutagenic studies. Mutation of these residues to others that have different properties often illustrates the specificity and necessity of the vital amino acid residues. In typical mutagenesis studies on homodimeric proteins, mutation of a residue in one subunit of a homodimeric complex results in the identical mutation within the other subunit, maintaining the homodimeric nature of the protein. Although informative, these dual mutations do not illustrate how mutation of a single subunit will affect the properties of the complex as a whole. Utilizing the mixed dimer system allows a mutation to be made within a single subunit of a homodimeric complex. This creates a heterodimer where the effect of the single mutation on the complex as a whole can be investigated. The mixed dimer system can also be adapted to assess the stability of protein homodimers.

Dissociation of a mixed dimer produces one free subunit that is His$_6$-tagged and one that is Glu$_7$-tagged. The different affinity properties of each tag allow the two subunits to be discriminated. After incubation of the protein under denaturing conditions, passing the dissociation solution over a Ni-NTA column will allow only the His$_6$-tag to bind to the column. If the denaturant concentration is such that the dimer has not dissociated, association of the His$_6$-tagged subunit with the resin will also hold the Glu$_7$-
tagged subunit in place, and all protein will remain on the column, as seen in figure 10. However, if the denaturant concentration is sufficient to cause dimer dissociation, the Gluγ-tagged subunit will not bind and it will be detected in the unbound fraction as seen in figure 10. The amount of protein in this unbound fraction, which can be determined by SDS-PAGE and densitometry measurements, will indicate the extent of dimer dissociation, and will allow the $C_m$ or $T_m$ values to be determined. Advantages of this method over previous ones used to investigate the stability of homodimeric proteins include i) it uses only Ni-NTA chelate resin and urea, making it inexpensive and readily available ii) thermal dissociation can also be investigated iii) unlike in size exclusion chromatography, overlapping monomer and dimer fractions that require interpretation are not produced iv) only a small amount of protein is necessary v) the method examines dimer dissociation alone because the only way protein will be observed in the unbound fractions is upon dissociation of the dimer. It is possible to apply this method for investigating dimer dissociation to homodimeric proteins such as nitric oxide synthase.

1.9 Nitric Oxide Synthases

1.9.1 Biological Importance of NO

Nitric oxide (NO) is a small free radical produced within various tissues and has many diverse functions. Well known is the role of NO in muscle relaxation and vasodilation. Stimuli that function to dilate blood vessels, such as acetylcholine and ATP, act on receptors on endothelial cells to stimulate the production of NO (Snyder 1992). This causes NO to be released and diffuse to neighbouring smooth muscle cells. NO binds to the heme iron in guanylyl cyclase with a very high affinity (Snyder 1992).
Figure 10: On the left, the scheme displaying incubation of a mixed dimer in conditions insufficient to dissociate the dimer. Association of the His$_6$-tagged subunit with the Ni-NTA resin holds the Glu$_7$-tagged subunit in place. On the right, the scheme displaying incubation of a mixed dimer in conditions sufficient to dissociate the dimer. Association of the His$_6$-tagged subunit with the Ni-NTA resin does not hold the Glu$_7$-tagged subunit in place, and it is detected in the unbound fraction.
Binding of NO causes conformational changes in guanylyl cyclase enhancing its catalytic activity and thereby influencing the formation of cyclic guanosine 3’5’-monophosphate (cGMP) (Snyder 1992). cGMP acts on cGMP-dependent protein kinases stimulating their activity, thereby causing muscle relaxation (Snyder 1992). In the brain NO also acts through cGMP to participate in neurotransmission (Snyder 1992).

NO has also been implicated in aspects of mRNA translation (Weiss, Goossen et al. 1993). NO reacts with the Fe$_4$S$_4$ cluster of iron regulatory proteins, which control translation of mRNAs involved in iron metabolism, forming iron-nitrosyl complexes (Weiss, Goossen et al. 1993). Complex formation triggers conformational changes in the iron regulatory factor that facilitate the binding of iron-responsive elements located on the 5’ or 3’ untranslated region of the mRNA as seen in figure 11. The effect of this coordination is translation repression of mRNAs containing the iron-responsive element and the biosynthesis of cellular iron storage proteins (Weiss, Goossen et al. 1993).

NO also plays a role in the destruction of parasites and tumour cells within the body (Nathan and Hibbs 1991). Mitochondrial inhibition and damage are two of the principle means by which NO exerts its cytotoxic effects (Brown 1999). During an immune response, NO is present at higher (μM) sustained levels (Brown 1999). At these elevated concentrations NO can inhibit mitochondrial respiratory chain complexes such as aconitase through association and alteration of the Fe$_4$S$_4$ clusters (Drapier and Hibbs 1986; Brown 1999). NO can also react with superoxide (O$_2^−$) to produce peroxynitrite (ONOO$^−$) which can react with mitochondrial proteins and DNA causing protein modification and cross-linking, and damage to DNA (Brown 1999). Because of the diverse actions of NO, its production must be highly regulated. The enzymes
Figure 11: The IRF is the circular object which can exist in 2 different conformations, one with low IRE-binding activity (dark grey) and one with NO bound and a higher IRE-binding activity. The binding of NO exerts a direct effect on the Fe₄-S₄ cluster inducing a conformational change in the IRF. (Weiss, Goossen et al. 1993)
responsible for production of NO are termed nitric oxide synthases (NOS).

NOS proteins exist in at least three mammalian isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Alderton, Cooper et al. 2001) and bacterial isoforms such as those found in Bacillus subtilis, (BaNOS) (Kunst, Ogasawara et al. 1997) Staphylococcus aureus, (SaNOS) (Kuroda, Ohta et al. 2001) and Deinococcus radiodurans (White, Eisen et al. 1999). The mammalian isoforms possess at least 51-57% sequence identity along with a similar genomic structure (Alderton, Cooper et al. 2001).

1.9.2 NOS Structure

Mammalian NOS isoforms possess a bidomain structure where each monomer is composed of an N-terminal oxygenase domain and a C-terminal reductase domain linked through a calmodulin binding site, as seen in figure 12 (Crane, Arvai et al. 1997), whereas the bacterial isoforms lack the reductase domain and calmodulin binding site (Bird, Ren et al. 2002). Oxygenase and reductase domains can be expressed as separate recombinant proteins that fold and function individually (Siddhanta, Presta et al. 1998).

The NOS oxygenase domain possess an elongated structure with a novel α-β fold that resembles a left-handed baseball catchers mitt as seen in figure 13 (Crane, Arvai et al. 1997). Overall the structure possesses 10 α-helices and 9 antiparallel β-sheets (Crane, Arvai et al. 1997). The antiparallel β-sheets predominate within the oxygenase domain, with projecting β-hairpins, and bordering α-helices (Crane, Arvai et al. 1997). This unique structure is specific to NOS proteins and is notably different from
Figure 12: Scheme of the domain structure of NOS. Subunit 1 (grey) and 2 (white) are indicated and binding sites for NADPH, FMN, and FAD in the reductase domain, the linking region of calmodulin binding, and binding sites for H₄B Fe and Arg in the oxygenase domain are shown. (Andrew and Mayer 1999)
Figure 13: a) The overall fold of the NOS oxygenase domain monomer. The structure resembles a baseball catcher's mitt with heme binding pocket (blue) seen in the palm of the catcher's mitt. b) The 10 α-helical (blue) and 9 antiparallel β-sheet (orange) structures that comprise the oxygenase domain are indicated. A heme moiety, located in the heme binding pocket is represented by white bonds where oxygen is coloured red and nitrogen is coloured blue. The heme binding pocket dominated by β-sheets and not α-helices as is more common in other heme based oxygenases and catalases (Crane, Arvai et al. 1997)
other heme-based oxygenases, such as cytochrome P450 which tend to be mainly \( \alpha \)-helical in content (Alderton, Cooper et al. 2001). The oxygenase domain is the location of the active site of the protein, and therefore contains a binding site for heme. Upon dimerization binding sites for the cofactor 6R-tetrahydrobiopterin (H\( _4 \)B), and the substrate L-arginine are also created within the oxygenase domains.

The location of heme binding is within the distal pocket of the oxygenase domain as seen in figure 13 (Crane, Arvai et al. 1997). The hydrophobic binding pocket is comprised mainly of \( \beta \)-sheets 8 and 9 which differs from other heme-based oxygenases, catalases, and peroxidases whose heme binding pockets possess primarily \( \alpha \)-helical regions (Crane, Arvai et al. 1997). The hydrophobic residues in this region are highly conserved. Direct interactions with the heme occur mainly through \( \alpha \)-helix 3 and \( \beta \)-loop 2 where Cys\( _{194} \) axially coordinates to the heme iron as seen in figure 14 (residues numbered according to the iNOS sequence). In addition, Trp\( _{188} \) and Phe\( _{363} \) (numbering corresponding to iNOS) sandwich the porphyrin ring of heme to enhance stabilization of the bound cofactor (Crane, Arvai et al. 1997). A sixth coordinate bond to the heme can be provided by another ligand such as CO, O\( _2 \), water or imidazole (Rousseau, Li et al. 2005).

The reductase domain is responsible for the transfer of electrons from NADPH to the oxygenase domain (Garcin, Burns et al. 2004). This is accomplished through sequential electron transfer from NADPH, to FAD, FMN and finally to the heme (Marletta 1993). The reductase domain within NOS can be further divided into three different subdomains: the N-terminal FMN binding portion and the C-terminal FAD- and NADPH binding portions as seen in figure 15 (Zhang, Martasek et al. 2001). The FMN
Figure 14: Direct interactions between the heme moiety and near by residues stabilize heme in its location. Cys$^{194}$ axially coordinates to the heme iron, and Trp$^{188}$ and Phe$^{363}$ participate in ring stacking to sandwich the porphyrin ring and enhance stabilization. The sixth coordinate bond to the heme iron is provided by imidazole (IM1). (Crane, Arvai et al. 1997)
Figure 15: The overall fold of the NOS reductase domain. The different domains are indicated including the N-terminal FMN binding region (orange) the FAD binding region (purple) and the C-terminal NADPH binding region (red). Also shown are the hinge (pink) and connecting domain (blue) linking regions. (Garcin, Burns et al. 2004)
binding domain is composed of a five-stranded parallel β sheet flanked by five α-helices (Zhang, Martasek et al. 2001; Garcin, Burns et al. 2004). The FAD binding portion is a β-barrel motif composed of six β-strands with a single α-helix at the N-terminus. The NADPH binding portion has the αβα structural motif, with a five-stranded β-sheet sandwiched between two α-helices (Garcin, Burns et al. 2004). NADPH is held in place through hydrogen bonding, hydrophobic stacking, and ionic interactions with surrounding residues (Zhang, Martasek et al. 2001). Overall the reductase domain possesses substantial sequence identity to cytochrome P450 reductase (Zhang, Martasek et al. 2001; Knudsen, Nishida et al. 2003). The reductase domain is also the location of many of the regulatory control elements of NOS including an autoinhibitory lock in the FMN-binding domain, and a C-terminal extension in the FAD-binding domain (Craig, Chapman et al. 2002). The oxygenase and reductase domains are linked through a calmodulin-binding helix.

Calmodulin is a small protein that senses intracellular Ca$^{2+}$ levels and binds to Ca$^{2+}$, resulting in structural changes that lead to calmodulin binding to a target protein (Li and Poulos 2005). The calmodulin-binding region within NOS is a 20-25 residue α-helical segment (D503 to M533 in iNOS and R492 to G511 in eNOS) that links the oxygenase and reductase domains (Lee, Beckingham et al. 2000; Craig, Chapman et al. 2002; Li and Poulos 2005). The peptide segment participates in extensive hydrophobic contacts with calmodulin in the classical “1-5-8-14” motif where the bulky hydrophobic residues that mediate calmodulin binding are located at positions 1, 5, 8, and 14 (Aoyagi, Avari et al. 2003; Li and Poulos 2005). All three mammalian NOS isoforms bind calmodulin, however iNOS binds Ca$^{2+}$/calmodulin or calmodulin in a irreversible Ca$^{2+}$-
independent manner, whereas eNOS and nNOS reversibly bind Ca\(^{2+}\)/calmodulin in a Ca\(^{2+}\)-dependent manner (Knudsen, Nishida et al. 2003). Calmodulin binding causes structural rearrangements of the oxygenase and reductase domains, although it mainly influences the reductase domain, that aid electron transport from the reductase to the oxygenase domains (Groves and Wang 2000; Craig, Chapman et al. 2002).

1.9.3 Dimerization

All NOS isoforms are homodimeric and the formation of this stable dimer is essential for activity in all isoforms (Chen, Panda et al. 2002; Li and Poulos 2005). Dimerization occurs via a large interface of the oxygenase domains involving more than 85 residues per monomer, and buries 15% of the total surface area of each monomer as seen in figure 16 (Crane, Arvai et al. 1998; Li and Poulos 2005). This interface is largely hydrophobic in character, and over 90% of it borders the concave face of the monomer which includes the heme binding site (Crane, Arvai et al. 1997). Consequently the solvent exposure of the heme distal pocket is reduced upon dimerization (Crane, Arvai et al. 1997). The only mandatory element for dimerization to occur is the presence of heme within the oxygenase domain. Upon dimerization the majority of the monomer structure is maintained with the exception of the regions that are buried upon dimerization (Crane, Arvai et al. 1998). The buried regions refold to create binding pockets for the pterin cofactor and to form the substrate binding channel (Crane, Arvai et al. 1998).
Figure 16: Dimerization of NOS monomers involves a large interface of the oxygenase domains. a) upon dimerization the heme residues within the cupped palm of the catchers mitt fold are orientated inwards towards the dimerization surface. b) The solvent exposed surface area of the NOS dimer. Each monomer is colour coded monomer A in red and monomer B in blue. Dimerization buries 15% of the total surface area per monomer. (Crane, Arvai et al. 1998)
The dimerization interface also contains binding sites for the pterin cofactor 6R-tetrahydrobiopterin (H₄B), and the substrate L-Arginine. Upon formation of a symmetrical dimer two H₄B molecules bind at the heart of the dimer interface (Crane, Arvai et al. 1998). Two helical lariats as seen in figure 17 within the NOS structure self-associate around the pterin. These helical lariats participate in hydrogen bonding and ring stacking with the pterin to stabilize its conformation (Crane, Arvai et al. 1998; Li and Poulos 2005). Trp⁴⁵⁷ from one subunit and Phe⁴⁷⁰ from the other subunit are involved in ring stacking with H₄B (Li and Poulos 2005). The pterin also interacts with one of the two propionate groups of the heme through hydrogen bonding (Rousseau, Li et al. 2005). This hydrogen bond is essential as it stabilizes the pterin cofactor close to the heme, which facilitates electron transfer from H₄B to the heme during the catalytic cycle (Li and Poulos 2005). Buried within the hydrophobic interface, H₄B also acts as a factor that stabilizes the dimer, and acts to form the binding site for the substrate (Crane, Arvai et al. 1998; Li and Poulos 2005).

Dimerization creates a deep funnel shape that facilitates diffusion of the substrate L-Arginine to the active site near the hydrophobic dimer interface (Crane, Arvai et al. 1998). Throughout this funnel L-Arg participates in hydrogen bonding to secure its location as seen in figure 17 (Crane, Arvai et al. 1998). Specifically, the hydrogen bond that occurs with the carboxylate oxygen of Glu³⁷¹ is of critical importance in substrate binding (Crane, Arvai et al. 1998). L-Arg also interacts extensively with hydrophilic side chains that are directly implicated in dimer formation, thereby participating in dimer stability (Crane, Arvai et al. 1998). L-Arg is linked to H₄B through extensive hydrogen bonds mediated by the propionate group of the heme.
Figure 17: Dimerization creates binding pockets for H$_4$B within the NOS oxygenase domain.  a) H$_4$B (white bonds) interacts with the N-terminal hook region (red), the N-terminal pterin binding segment (yellow), and the helical lariat that self associates around the pterin (blue). b) Aromatic residues contributed by the helical lariat, and neighbouring subunit participate in hydrogen bonding to stabilize H$_4$B (H4B, centre, edge on grey bonds) in its conformation. Side chains of one subunit are indicated in yellow and those contributed by the other subunit are indicated in green. Trp$^{457}$ from one subunit (yellow) and Phe$^{470}$ from the other subunit (green) participate in ring stacking to stabilize the conformation of H$_4$B. The hydrogen bonding that occurs to stabilize arginine (top green ARG) in its location is also indicated. Hydrogen bonding involving the side chains of a single subunit (yellow) stabilize arginine. (Crane, Arvai et al. 1998)
In addition, in the mammalian isoforms, but not those of bacteria, the oxygenase domain possesses a 49-residue N-terminal hook region composed of antiparallel β sheets as seen in figure 18 (Crane, Arvai et al. 1998). This region can adopt either a swapped structure, where the hooks interact with the partner subunit, or an unswapped structure, where the hooks interact mainly with their own subunit (Crane, Rosenfeld et al. 1999). In the unswapped structure a zinc atom is tetrahedrally coordinated to two cysteines residues, Cys$^{104}$ and Cys$^{109}$, from each subunit for stability (Crane, Rosenfeld et al. 1999). The stability of the unswapped dimer is a result of not only coordination with the zinc atom itself, but also burying of surface area. In the swapped structure, zinc is absent and Cys$^{109}$ residues from each subunit form an intersubunit disulfide bond that links the two subunits, while Cys$^{104}$ becomes exposed to the solvent (Crane, Rosenfeld et al. 1999). Stability that is lost in the absence of zinc, is compensated for in the domain swapping and the burying of three times more surface area per monomer (Crane, Rosenfeld et al. 1999). Furthermore, mutations that alter the swapped conformation drastically (D92A or N83A in iNOS) have more effect on reducing dimer stability than those mutations that alter the unswapped structure (mutation of Trp$^{84}$ in iNOS) (Crane, Rosenfeld et al. 1999). Deletion of the N-terminal region altogether leads to monomeric and inactive species (Li and Poulos 2005). This N-terminal hook region is absent in the bacterial isoforms as seen in figure 19, making the sources of their dimer stability of specific interest (Bird, Ren et al. 2002). Formation of a stable dimer may be an important regulatory mechanism in NOS making a means of investigating iNOS dimer dissociation a vital component in understanding its function (Siddhanta, Presta et al. 1998; Li and Poulos 2005).
Figure 18: The mammalian isoforms possess an N-terminal hook region composed of antiparallel β-sheets. Each subunit is coloured differently, one in green and one in yellow. In the swapped conformation Cys$^{109}$ from each subunit participate in a disulfide bond at the dimer interface to stabilize the dimer. In the unswapped conformation Cys$^{104}$ and Cys$^{109}$ are tetrahedrally coordinated to a zinc atom (orange) at the dimer interface. (Li, Hayden et al. 2006)
Figure 19: Overlapped SaNOS and bovine eNOS dimers. The subunits are colored blue and green for SaNOS, and red and orange for bovine eNOS. The N-terminal hook region belonging to eNOS in red and orange is absent in SaNOS. (Bird, Ren et al. 2002)
1.9.4 The NOS Reaction

Overall, the NOS reaction is a two-step process where the first step involves hydroxylation of one of the terminal guanidino nitrogens on the side chain of arginine to produce the intermediate \(\text{N-hydroxy-L-arginine (NHA).}\) This is followed by further oxidation of that same nitrogen to produce citrulline and NO (Marletta 1993; Hurshman and Marletta 2002). The overall reaction utilizes 1.5 moles of NADPH, and 2 moles of oxygen per mole of product formed (Andrew and Mayer 1999; Alderton, Cooper et al. 2001).

In the first step of the reaction, upon arginine binding, NADPH is oxidized by hydride loss, and the electrons are transferred singly by the flavin cofactors to the heme in the oxygenase domain (Groves and Wang 2000). One mole of \(\text{O}_2\) per mole of arginine is consumed as arginine is hydroxylated into NHA (Alderton, Cooper et al. 2001). The involvement of FAD and FMN in the transfer of electrons is essential as they allow the electrons derived from NADPH to be transferred one at a time, by the formation of stable intermediates, to the heme (Alderton, Cooper et al. 2001).

In the second step, a single electron derived from NADPH is consumed along with a second mole of \(\text{O}_2\) in the further oxidation of the intermediate NHA to citrulline and nitric oxide (NO) (Andrew and Mayer 1999). A scheme of the NOS catalyzed reaction is seen in figure 20.

1.9.5 UV-Visible Spectroscopic Properties of NOS

NOS, being a heme-containing protein, absorbs light in the UV-visible region. The heme group is composed of an iron atom surrounded by a porphyrin, which possess
Figure 20: A scheme of the NOS catalyzed reaction. In the first step one mole of oxygen and 1 mole of NADPH per mole of substrate are consumed to form the intermediate $N^\alpha$-hydroxy-L-arginine. In the second step a second mole of oxygen along with a single electron derived from NADPH are used in the oxidation of the intermediate to citrulline and nitric oxide (NO).
extensively delocalized π electrons. Light possessing sufficient energy can promote these electrons from a ground electronic state (\(\pi\)) to a higher energy electronic state (\(\pi^*\)) (Boyer 2000). Transitions that occur in the near UV region between 390nm and 450nm, termed the Soret transition, represent the \(\pi\) to \(\pi^*\) transitions within the porphyrin ring. Those transitions occurring in the visible region around 650nm represent the ligand to metal charge transfer band between the \(\pi\) orbital of the cysteine ligand and the \(d\)-orbital of the heme iron in the iron III state (Harris and Bertolucci 1978). Although the transitions between 390nm and 450nm are due to the porphyrin ring and not the metal, this transition is sensitive to the oxidation state, coordination, and spin state of the iron (Rousseau, Li et al. 2005).

The iron within the heme moiety has two common oxidation states; the 3+ oxidation state where iron possesses 5 \(d\) electrons, and the 2+ oxidation state where iron possesses 6 \(d\) electrons. In each of these oxidation states it is possible to have two different spin states, which refers to the distribution of electrons within the \(d\)-orbitals (Winter 1994). The \(d\)-orbitals are not equivalent in energy but are split into two levels. The extent of the splitting is dependent on the ligand environment surrounding the metal. Strong field ligands cause a large splitting of the \(d\)-orbitals and therefore a large energy difference between the higher and lower levels (Winter 1994). The association of a strong field ligand with a metal will most likely result in pairing of the electrons in the lower set of orbitals as the energy required to promote electrons to higher energy levels is too great (Winter 1994). This electron configuration is referred to as the low spin state. The association of a weak field ligand with a metal will most likely result in unpaired electrons, where some have been promoted to the higher energy levels (Winter 1994).
This is because the energy required to promote the electrons to the higher energy level is not as great as with a strong field ligand. This electron configuration is referred to as the high spin state. The coordination of the metal refers to the type and number of these interactions to the metal (Winter 1994).

The resting state of the heme iron in NOS is its ferric (3+) state, high spin, referring to the five valence electrons in the 3 \( d \)-orbitals being maximally unpaired as seen in figure 21 (Rousseau, Li et al. 2005). The high spin state occurs when the heme iron is 5-coordinate or has a weak field ligand at the sixth position (Rousseau et al, 2005). This occurs in the presence of L-Arg and H\(_4\)B (Rousseau, Li et al. 2005). When the heme iron is in the high spin state the Soret band absorbs maximally at 396nm as seen in figure 22 (Rousseau, Li et al. 2005). The low spin state of the heme iron occurs when the 5 valence electrons in the 3 \( d \)-orbital are maximally paired (Rousseau, Li et al. 2005). This occurs when a strong field sixth ligand, such as NO, is bound causing a large \( d \)-orbital splitting (Rousseau et al, 2005). When the heme iron is bound to NO and is in the low spin state the Soret band is present at 439nm (Rousseau, Li et al. 2005). Consequently, the absorption spectra of NOS can be used to characterize the environment of the heme and the active site ligands.
Figure 21: The electron configuration of the 3 $d$-orbitals of the heme iron in NOS. On the left, the low spin state of the heme iron, as is seen when a strong field sixth ligand such as NO is bound. On the right, the high spin resting state of the heme iron as is seen when the iron is 5-coordinate, or has a weak field ligand at the sixth position.
Figure 22: The absorption spectra of NOS can be used to characterize the environment of the heme and the active site ligands.  a) Optical spectra of high spin state iNOS heme in the presence of L-arginine and H₂B. The Soret band absorbs maximally at \(~396\text{nm}\)  

b) Optical spectra of low spin state iNOS heme when bound to NO. The Soret band absorbs maximally at \(~439\text{nm}\). (Diagram modified from Rousseau et al, 2005).
Chapter 2: Objectives

The objectives of this research were two-fold. The primary aim of this research was to develop the mixed dimer system for investigating dimer stability with respect to denaturant- and temperature-induced dissociation using the core oxygenase domain of inducible nitric oxide synthase (residues 75-500) (COD\textsubscript{iNOS}) and then to apply this method to other proteins such as the core oxygenase domain of \textit{Staphylococcus aureus} nitric oxide synthase (COD\textsubscript{SaNOS}). We show that the mixed dimer method for investigating dimer dissociation can successfully be applied to these proteins. Furthermore, we compare the stability of the COD\textsubscript{iNOS} and COD\textsubscript{SaNOS} mixed dimers with respect to denaturant- and temperature-induced dissociation. In addition, this research has used the mixed dimer system to investigate the effects of dilution on the dissociation of COD\textsubscript{iNOS} with the overall goal of estimating an equilibrium dissociation constant ($K_D$) for dimer dissociation under non-denaturing conditions.
Chapter 3: Materials and Methods

3.1 Homodimeric Protein Expression and Purification

3.1.1 Bacterial Growth Conditions

pET-based vectors encoding the His\textsubscript{6}-tagged version of the NOS core oxygenase domain (COD\textsubscript{iNOS-His\textsubscript{6}}) and those expressing the Glu\textsubscript{7}-tagged version of the NOS core oxygenase domain (COD\textsubscript{iNOS-Glu\textsubscript{7}}) had previously been constructed by J. McDonald (McDonald, Taylor et al. 2003). Vectors encoding the His\textsubscript{6}-tagged version of the COD of \textit{Staphylococcus aureus} NOS (COD\textsubscript{SaNOS-His\textsubscript{6}}) and those expressing the Glu\textsubscript{7}-tagged version of the SaNOS COD (COD\textsubscript{SaNOS-Glu\textsubscript{7}}) had previously been constructed in our lab. Electrocompetent \textit{Escherichia coli} strain BL21(DE3) cells were transformed with 2 ng of plasmid vector using a BTX electroporation system Electro Cell Manipulator 600. The cells were allowed to recover at 37°C for 1 hour with aeration. A 100 μL aliquot of this transformation was plated onto LB agar supplemented with 50 μg/mL ampicillin (COD\textsubscript{iNOS-His\textsubscript{6}} and COD\textsubscript{SaNOS-His\textsubscript{6}}) or 30μg/mL kanamycin (COD\textsubscript{iNOS-Glu\textsubscript{7}} and COD\textsubscript{SaNOS-Glu\textsubscript{7}}). Incubation at 37°C overnight resulted in extensive colony growth on the plate. A single colony was picked and used to inoculate a 2 mL starter LB culture supplemented with the appropriate antibiotic. The starter culture was grown at 37°C until it was visibly turbid. Once turbidity in the 2 mL starter culture was evident, indicating bacterial growth, 500 μL was used to inoculate each of 4 large-scale 500 mL cultures containing Terrific Broth (TB) supplemented with the appropriate antibiotic for a total of 2 L of growth medium. Large-scale cultures were allowed to grow at 30°C for 48 h. Harvesting of the cell cultures was achieved by centrifugation at 10 000 g for 15 minutes.
3.1.2 Wild-Type COD_{INOS}-His\textsubscript{6} homodimer Purification

After harvesting the cells by centrifugation the cells were resuspended in 2 volume equivalents of Buffer A (50 mM Tris-HCl, 0.5 M NaCl, 5 mM L-Arginine, 100\mu M H\textsubscript{4}B 0.1 mM captopril, pH 7.5). If protein lacking L-Arg or H\textsubscript{4}B was desired, these components were excluded from Buffer A and all subsequent steps. Cells were lysed using a Biospec Bead-beater, 0.1 mm glass beads, and 4 x 30 second pulses with 60 seconds of incubation on ice in between pulses. Centrifugation at 10 000 g for 10 minutes removed most of the cells debris and glass beads, and subsequent centrifugation at 17 000 g for 30 minutes resulted in a clarified solution that could be applied to further purification procedures.

The COD\textsubscript{INOS}-His\textsubscript{6} homodimer was purified on an Ni-NTA column. Ni-NTA resin was poured into a 20 mL plastic column (BioRad) using a resin volume proportional to the anticipated amount of protein in solution (usually 2 mL of Ni-NTA for COD\textsubscript{INOS}-His\textsubscript{6} homodimer purification from an initial culture volume of 2 L). The column was then washed with at least 2 column equivalents of Buffer A. The protein solution was applied to the Ni-NTA column. Application, washing, and elution of the protein from the Ni-NTA column was performed using a peristaltic pump with a 1 mL/min flow rate. After application to the Ni-NTA resin the column was washed sequentially with 10 mL of Buffer A and 5 mL of Buffer A containing 20 mM imidazole. Bound protein was eluted with Buffer A containing 150 mM imidazole. The collected protein was concentrated by ultrafiltration using Amicon Ultra Centrifugal Filtration Devices (Millipore) to a total volume of 500 \mu L. Imidazole was removed from the protein using a 10 mL column pre-
packed with 10 mL of Bio-Gel P6DG Desalting resin (BioRad) that had been equilibrated with Buffer A.

3.1.3 *Wild-type COD$_{SaNOS}$-His$_6$ Homodimer Purification*

Purification of the COD$_{SaNOS}$-His$_6$ homodimer was conducted in the same manner as for the COD$_{iNOS}$-His$_6$ homodimer with the following exceptions; Buffer G (50 mM HEPES, 0.15 M NaCl, 5 mM L-Arginine, 0.1mM captopril pH 7.5) was used instead of Buffer A in all cases. Following cell lysis and prior to application to the Ni-NTA column, 35% and 50% ammonium sulphate precipitation was performed. Approximately 10 mL of Ni-NTA resin was used in purification of the COD$_{SaNOS}$-His$_6$ homodimer from an initial culture volume of 2 L.

3.1.4 *Wild-Type COD$_{iNOS}$-Glu$_7$ Homodimer Purification*

Cell lysis and centrifugation conditions were identical to those previously described for the COD$_{iNOS}$-His$_6$ homodimer with the exception that Buffer B (50 mM Tris-HCl, 10% glycerol, 0.5 mM DTT, 100 μM H$_4$B, 5 mM L-Arg pH 7.5) was used instead of Buffer A. The COD$_{iNOS}$-Glu$_7$ homodimer was purified as previously described using ammonium sulphate precipitation, anion exchange chromatography, and hydrophobic interaction chromatography (McDonald, Taylor et al. 2003). Following centrifugation ammonium sulphate was added to 30% saturation to the supernatant. The solution was stirred and incubated on ice for 1 hr. Centrifugation at 12 000 g and 4°C for 15 minutes removed the precipitated undesired proteins from solution. The resulting supernatant was brought to 60% saturation with (NH$_4$)$_2$SO$_4$ and incubated with stirring on
ice for 1 hr. The COD$_{\text{INOS}}$-Glu$_7$ homodimer precipitated in the 60% fraction and consequently was present in the pellet following subsequent centrifugation. The pellet was dissolved in a minimal volume of Buffer C (same components as Buffer B but at pH 6.8) and dialyzed over a 24-hour period against Buffer C. The resulting protein solution was loaded directly onto an anion exchange Q column. For low-pressure liquid chromatography a 5 mL Econo-Pac High Q column (BioRad) that had been pre-equilibrated with Buffer C was used. The COD$_{\text{INOS}}$-Glu$_7$ homodimer was eluted over a linear salt gradient of 0-0.5 M NaCl over 60 mL, regulated by a BioRad Econo-Gradient system. The COD$_{\text{INOS}}$-Glu$_7$ homodimer eluted at approximately 0.26 M NaCl. Fractions that contained protein and appeared red-brown due to the heme present within the NOS protein were collected and pooled. Solid (NH$_4$)$_2$SO$_4$ was added to a concentration of 1 M. The protein was applied to a Phenyl Sepharose column that had been pre-equilibrated with Buffer C supplemented with 1 M (NH$_4$)$_2$SO$_4$. The column was washed with 3 column equivalents of Buffer C supplemented with 1M (NH$_4$)$_2$SO$_4$. The column was then washed with three column equivalents of decreasing concentrations of (NH$_4$)$_2$SO$_4$ in Buffer C (0.75 M, 0.5 M, 0.25 M, 0 M). The COD$_{\text{INOS}}$-Glu$_7$ homodimer eluted in the 0 M fraction.

3.1.5 Wild-type COD$_{\text{SaNOS}}$-Glu$_7$ Homodimer Purification

Purification of the COD$_{\text{SaNOS}}$-Glu$_7$ homodimer was conducted in the same manner as for the COD$_{\text{INOS}}$-Glu$_7$ homodimer with the following exceptions; Buffer H (50 mM HEPES, 10% glycerol, 0.5 mM DTT, 5 mM L-Arginine, 0.15 M NaCl, 0.1 mM captopril, pH 7.5) was used instead of Buffer B and Buffer I (same components as Buffer H but
without NaCl and at pH 6.5) instead of Buffer C. For ammonium sulphate precipitation 35% and 50% fractionation was employed.

3.2 Mixed Dimer Protein Expression and Purification

3.2.1 Bacterial Growth Conditions and Cell Lysis

Bacterial inoculation, growth, and initial culture harvesting conditions for the COD$_{\text{iNOS}}$-His$_6$/ COD$_{\text{iNOS}}$-Glu$_7$ mixed dimer and the COD$_{\text{SaNOS}}$-His$_6$/ COD$_{\text{SaNOS}}$-Glu$_7$ mixed dimer, where one subunit possesses a His$_6$-tag and one subunit possesses a Glu$_7$-tag, were identical to those previously described for homodimeric protein expression except that both ampicillin (50 μg/mL) and kanamycin (30 μg/mL) were used for antibiotic resistance.

3.2.2 Wild-Type COD$_{\text{iNOS}}$-His$_6$/ COD$_{\text{iNOS}}$-Glu$_7$ Mixed Dimer Purification

Cell lysis and centrifugation conditions were identical to those previously described for the COD$_{\text{iNOS}}$-His$_6$ homodimer. The COD$_{\text{iNOS}}$-His$_6$/ COD$_{\text{iNOS}}$-Glu$_7$ mixed dimer, where each subunit possess a different affinity tag, was purified as previously described with the following exceptions; (McDonald, Taylor et al. 2003). Following centrifugation, the supernatant was directly bound and eluted from an Ni-NTA column as previously described for the COD$_{\text{iNOS}}$-His$_6$ homodimer. The eluate was collected, pooled, and desalted as in previous steps. To equilibrate the protein into an appropriate buffer for anion exchange chromatography, following desalting into Buffer A, as in COD$_{\text{iNOS}}$-His$_6$ homodimer purification, the protein was applied to a second desalting column equilibrated with Buffer D (same as Buffer A but without NaCl and at pH 6.8).
For low-pressure liquid chromatography a 5mL Econo-Pac High Q column (BioRad) was used. After loading, the protein was washed with 5 mL of Buffer D at a rate of 1 mL/min, and then eluted over a linear NaCl gradient from 0 M to 0.5 M NaCl in Buffer D over a volume of 50 mL at a rate of 1mL/min. The COD\textsubscript{iNOS-His\textsubscript{6}} homodimer eluted at 0.2 M NaCl and the COD\textsubscript{iNOS-His\textsubscript{6}}/COD\textsubscript{iNOS-Glu\textsubscript{7}} mixed dimer eluted at 0.3 M NaCl.

### 3.2.3 Wild-Type COD\textsubscript{SaNOS-His\textsubscript{6}}/COD\textsubscript{SaNOS-Glu\textsubscript{7}} Mixed Dimer Purification

Cell lysis and centrifugation conditions were identical to those previously described for the COD\textsubscript{iNOS-His\textsubscript{6}} homodimer with the exception that Buffer E (50 mM HEPES, 0.15 M NaCl and 0.1 mM captopril pH 7.5) was used instead of Buffer A. Purification of the wild-type COD\textsubscript{SaNOS-His\textsubscript{6}}/COD\textsubscript{SaNOS-Glu\textsubscript{7}} mixed dimer was conducted in the same manner as the COD\textsubscript{iNOS-His\textsubscript{6}}/COD\textsubscript{iNOS-Glu\textsubscript{7}} mixed dimer with the following exceptions; in all cases where Buffer A was used to purify the COD\textsubscript{iNOS-His\textsubscript{6}}/COD\textsubscript{iNOS-Glu\textsubscript{7}} mixed dimer, Buffer E was used instead. To elute the protein from the Ni-NTA resin, Buffer E containing 0.5 M imidazole was used. For anion exchange Buffer F (25 mM Bis-Tris, 0.1 M NaCl pH 6.5) was used and the protein was eluted over a linear NaCl gradient from 0.1 M to 1 M NaCl in Buffer F over a volume of 50 mL at a rate of 1mL/min. Alternatively, anion exchange of the COD\textsubscript{SaNOS-His\textsubscript{6}}/COD\textsubscript{SaNOS-Glu\textsubscript{7}} mixed dimer was conducted by HPLC using a MonoQ HR 10/10 column. Baseline resolution between peaks was not possible and therefore the mixed dimer was isolated by pooling only the desired fractions and passing them through the column several times. The COD\textsubscript{SaNOS-His\textsubscript{6}} homodimer eluted at 0.3 M NaCl and the COD\textsubscript{SaNOS-His\textsubscript{6}}/COD\textsubscript{SaNOS-Glu\textsubscript{7}} mixed dimer eluted at 0.44 M NaCl.
3.3 Determination of Protein Purity and Concentration

The UV visible spectrum of the protein was observed using a NanoDrop ND 1000 spectrophotometer. The absorbance at 396 nm ($A_{396}$) (400 nm in the absence of either H$_4$B or Arg and 420 nm in the absence of both H$_4$B and Arg) was noted and a concentration was calculated using a value of 74 000 M$^{-1}$ cm$^{-1}$ as the extinction coefficient. The $A_{280}$ was also noted. The ratio of $A_{396}/A_{280}$ was used as an indication of the purity of the sample. A ratio greater than 0.6 was accepted as a pure protein sample.

3.4 Investigation of Dimer Dissociation

3.4.1 Initial Ni-NTA column

Immediately prior to exposing the mixed dimer to dissociation conditions it was applied to an initial 1mL Ni-NTA column equilibrated with Dissociation Buffer (50 mM Tris-HCl, 0.5 M NaCl, 100 μM H$_4$B, 0.5 mM L-Arginine, 0.1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) pH 8). When mixed dimer dissociation was investigated in the absence of arginine or H$_4$B these were excluded from the dissociation buffer and all subsequent steps. The Ni-NTA- column bearing the bound mixed dimer was washed with Dissociation Buffer containing 20 mM imidazole, and then eluted in Dissociation Buffer containing 150 mM imidazole for the COD$_{iNOS}$ homodimer and mixed dimer, and 0.5 M imidazole for the COD$_{SaNOS}$ mixed dimer. Imidazole was removed from the mixed dimer using a prepacked 10 mL of Bio-Gel P6DG Desalting resin (BioRad) that had been equilibrated with Dissociation Buffer. The resulting mixed dimer was concentrated to a minimum volume (~100 μL). This initial Ni-NTA column was done to ensure that only mixed dimer that still possessed a His$_6$-tag
was used to investigate dimer dissociation. This step took no more than 1 hr to complete and protein treated in this way was immediately used in dimer dissociation experiments. The UV Visible spectrum of the protein was observed and the concentration calculated.

3.4.2 Dimer Dissociation

Each dissociation solution was prepared in a volume of 50 μL and contained 2μg/μL of mixed dimer in Dissociation Buffer and 20 mM imidazole. For urea-induced dissociation each solution had a different urea concentration (0 M, 2 M, 2.67 M, 3.3 M, 4M, 5.3 M). For thermally-induced dissociation all solutions were 0 M urea. Wash solutions were also made containing the same components as the mixed dimer solutions but lacking the protein. Mixed dimer dissociation solutions were incubated for 3 hrs. For urea induced dissociations, all solutions were incubated at 4°C, for temperature induced dissociations solutions were incubated at varying temperatures (4°C, 20°C, 25°C, 30°C, 37°C, 45°C). All wash solutions were incubated at 4°C. Dissociations under each condition were performed in duplicate.

A separate empty spin column (Sigma) was used for each urea concentration or temperature point being investigated. 250 μL of Ni-NTA resin was added to each of the required number of spin columns. The resin was equilibrated with the appropriate wash buffer and then incubated for the remainder of the 3 hrs at 4°C.

After the incubation period each mixed dimer solution was applied to the corresponding Ni-NTA spin column. The columns were microfuged at 8000 rpm and 4°C for 15 seconds. The columns were washed twice by applying 100 μL of the appropriate wash buffer to the resin and microfuging as before. The pooled eluate at this
point was termed the unbound fraction. When fractions bound to the Ni-NTA column were investigated the resin was resuspended in 500 μL Dissociation Buffer containing 150 mM imidazole (0.5M imidazole for SaNOS). Microfuging performed as described above pelleted the resin. The resin was washed a second time with 500 μL of Dissocation Buffer containing a high concentration of imidazole and pelleted. The pooled eluate at this point was termed the high imidazole eluted fraction.

3.4.3 SDS PAGE and Densitometry

The extent of dimer dissociation was investigated using SDS PAGE to analyze each of the unbound fractions. Gels were stained using either Coomassie Blue Simply Safe Stain (Invitrogen) or the fluorescent stain SYPRO Ruby Protein Gel Stain (Invitrogen). When SYPRO Ruby stain was used the gel was imaged using a UV transilluminator at a 250-350 nm excitation wavelength range. The resulting bands were quantified using densitometry on a Chemigenius 2 gel imaging system (Stratagene) by comparison to protein standards (COD$_{iNOS}$) loaded in adjacent lanes.

3.4.4 Calculation of $C_m$ and $T_m$ values

The quantity of protein in each fraction was converted to the fraction of total protein present in each dissociation reaction by dividing the quantity of protein present within the unbound fraction by the total mass of protein originally present in the sample. These values were plotted on the dependent y-axis and urea or temperature was plotted on the independent x-axis. This resulted in sigmoidal-shaped curves with a pre-dissociation minimum and a transition region. Lines corresponding to the initial and final
plateau were drawn. The post-dissociation maximum plateau line was drawn corresponding to a fraction of 0.5. This the maximum fraction that can be observed in the unbound fraction as only half of the total number of subunits present are Glu7-tagged. \( C_m \) and \( T_m \) values were estimated from the midpoint between the pre- and post-dissociation plateau lines (Pace 1986; Chen and Matthews 1994).

3.5 Determination of “on” or “off” Column Dissociation using Alkaline Phosphatase

In principle, dimer dissociation experiments could also be performed on the column. To determine if the stability of proteins may be altered upon coordination of the His6-tag to the Ni-NTA resin, His6–tagged Alkaline Phosphatase homodimer was used. Alkaline Phosphatase (AP) is a homodimeric protein responsible for hydrolysing phosphate groups from many types of molecules (Birtikati, Besson et al. 1999). Its activity can be conveniently observed when a phosphate from p-nitrophenyl phosphate (PNPP) is hydrolysed to produce p-nitrophenyl (PNP) which possesses a characteristic yellow colour. AP is only active in dimer form, but it is also thermostable which allows it to maintain activity at elevated temperatures. AP-His6 homodimer had previously been expressed and characterized in our lab.

The AP-His6 homodimer was first bound to an Ni-NTA column. Following this, sufficient washes of the column with Dissociation Buffer were conducted to ensure that any loosely-bound or unbound protein was removed before incubation began. Dissociation Buffer (50 \( \mu \)L) was added to each column and the columns were incubated at varying temperatures (4°C, 37°C and 80°C) for 3 hrs. Following incubation, the resin was pelleted by microfuging at 8000 rpm and 4°C for 15 seconds. The columns were then
washed 2 times by applying 100 μL of the Dissociation Buffer to the resin and microfuging as before. The eluate at the point was termed the unbound fraction. In addition, protein bound to the column was eluted by treatment with 150 mM imidazole. This fraction was termed the high imidazole eluted fraction. The high imidazole eluted fraction was of specific interest as it denoted the stability of the dimer while coordinated to the Ni-NTA resin. Activity assays were conducted on both unbound and eluted fractions by combining a small amount of substrate (PNPP) with 20 μL of each fraction. Production of a yellow colour indicated AP activity. In addition a Detergent Compatible (DC) assay was conducted to assay for total protein concentration. The DC assay is similar to the Lowry assay which is based on the reaction of the nitrogen atoms within the peptide bond with alkaline copper sulphate, and reduction of tryptophan and tyrosine residues by a folin reagent, both reactions producing a characteristic blue/purple colour and allowing protein concentration to be determined (Peterson 1979; Boyer 2000).

3.6 Investigation of His₆-tag stability at Elevated Temperatures

To determine the stability of the His₆-tag at elevated temperatures the COD₁NOS-His₆ homodimer, in which both subunits possess a His₆-tag and therefore have the ability to bind to the Ni-NTA resin regardless of the extent of dimer dissociation, was used. The initial Ni-NTA column, dimer dissociation, and SDS PAGE and densitometry procedures described above were conducted using the COD₁NOS-His₆ homodimer. Initially the stability of the His₆-tag of the COD₁NOS-His₆ homodimer was investigated at 37°C.
3.7 Investigation of the effect of Protein Aggregation on His<sub>6</sub>-tag/Ni-NTA Interactions

In a subsequent trial, the ability of the His<sub>6</sub>-tag in protein aggregates, which may result from elevated temperatures, to bind to the Ni-NTA resin was investigated. If the His<sub>6</sub>-tag was unable to bind the Ni-NTA resin due to burying of the tag upon protein aggregation, denaturation of aggregates using urea should restore their ability to bind the column. A sample of COD<sub>NOS</sub>-His<sub>6</sub> homodimer was prepared in a total of 500 μL containing 2 μg/μL of protein, and was incubated at 45°C. Following incubation, 50 μL of this solution was passed over an Ni-NTA spin column as in the dimer dissociation protocol previously described. As turbidity in the remaining 450 μL was observed, the sample was microfuged to pellet the aggregates. The resulting aggregated pellet was dissolved in Dissociation Buffer containing 8 M urea to unfold any of these aggregates. The dissolved pellet corresponding to completely unfolded aggregates was subsequently applied to an Ni-NTA column and the unbound fraction collected. All unbound fractions were assessed for protein content using SDS PAGE, densitometry and the fraction of total protein present was determined.

3.8 Investigation of SaNOS Thermal Denaturation by UV-Visible Spectroscopy

A 3 mL sample containing COD<sub>SaNOS</sub> protein in Dissociation buffer was made to the same concentration as those used in the dissociation experiments described above (2 μg/μL). The sample was placed in a quartz cuvette and the UV visible spectrum between 700 nm and 280 nm was recorded using the Cary 400 Bio UV visible spectrophotometer (Varian). The temperature of the sample was raised within the spectrophotometer, allowed to incubate at the appropriate temperature for 5 minutes, and another spectrum
was recorded. The UV visible spectrum was observed between 45°C and 50°C and at 55°C. When precipitate was observed in the sample, the 3mL sample was microfuged at room temperature for 3 minutes before the spectrum was recorded and then the temperature treatment was continued.

3.9 Investigation of Possible Rapid Exchange of Subunits

The rapid exchange of subunits within a sample of mixed dimer could possibly regenerate homodimeric species, in which the Glu7-tagged homodimer would be present in the unbound fraction. To determine if protein present in the unbound fraction was due to rapid reassortment of subunits to recreate homodimers, equal amounts of each homodimer in Dissociation Buffer were mixed, and the sample was immediately passed over an anion exchange column. If rapid reassortment had occurred then some mixed dimer would have been generated and could be detected in anion exchange chromatography. The remainder of the sample was incubated at room temperature for 1 hr and then a sample was passed over an anion exchange column. Peak locations were compared for evidence of mixed dimer formation from individual homodimers.

3.10 Investigation of the Effect of Dilution on Dissociation, and Estimation of \( K_D \) in the absence of Denaturant

The initial Ni-NTA experiments were conducted in the same manner as previously stated for the COD\textsubscript{INOS-}His\textsubscript{6}/ COD\textsubscript{INOS-}Glu\textsubscript{7} mixed dimer. Each dissociation solution was 50 \( \mu \)L in volume but the total protein concentration was varied. The concentrations investigated were 2 \( \mu \)g/\( \mu \)L, 1 \( \mu \)g/\( \mu \)L, 0.5 \( \mu \)g/\( \mu \)L, and 0.25 \( \mu \)g/\( \mu \)L. The dimer dissociation, SDS PAGE, and densitometry procedures were conducted in the same
manner as previously stated. The fraction of total protein present was also determined. This was termed the fraction of protein in monomer. The fraction of protein present in dimer was calculated according to the equation:

\[ f_m + f_D = 1 \]  

Equation 5

Fraction of dimer present was plotted on the y-axis and total protein concentration plotted on the x-axis. An estimate of \( K_D \) was determined from this plot by determining the point where the fraction of dimer present was 0.5.

3.11 Investigation of \( K_D \) by Analytical Ultracentrifugation

Analytical centrifugation (AUC) is a method for characterizing interacting proteins in dilute solutions (Balbo and Schuck). In AUC the sample is subjected to centrifugal force and is continuously optically monitored throughout centrifugation using UV Visible spectrophotometry for macromolecular redistribution. This allows the macromolecular redistribution of the sample to be monitored as the applied centrifugal field is altered. By monitoring the sample at a wavelength of 280nm, the protein concentration can be assessed as a function of centrifugal force. The rate of sedimentation by centrifugation of any molecule is proportional to its molecular weight where faster sedimenting complexes migrate through the solution of slower sedimenting ones (Balbo and Schuck; Creighton 1984). Therefore, AUC can characterize protein complexes with respect to their stoichiometry and differentiate between multiple coexisting complexes of different stoichiometries (Balbo and Schuck). When a combination of monomer and dimer are present in solution, the respective content of each can be assessed as a function of centrifugal force by AUC.
COD_{iNOS-His6/COD_{iNOS-Glu7} mixed dimer was analyzed by AUC under three different conditions; in the presence of both substrate and cofactor (+Arg +H_4B), in the presence of only the cofactor (-Arg +H_4B), and in the absence of both the substrate and the cofactor (-Arg –H_4B). For each of these conditions three different protein concentrations were prepared base on the absorbance at 280nm, A_{280}=0.8, A_{280}=0.6, and A_{280}=0.4. Ultracentrifugation was conducted by Kim Munro at Queen’s University, Kingston, Ontario, on the Beckman Optima XL-I ultracentrifuge.
Chapter 4: Results and Discussion

4.1.1 Protein Purification

After growth, cell culture pellets for all proteins ranged between 3 -5 g per litre initial culture volume. The red-brown colour of the pellets indicated the presence of heme and consequently NOS. The persistence of the red-brown colour throughout the purification scheme allowed the location of NOS to be monitored. Accordingly, after cell lysis, all protein solutions appeared red-brown although COD$_{SaNOS}$ cultures appeared a much darker red than the COD$_{iNOS}$ cultures indicating higher levels of expression.

When homodimeric His$_6$-tagged proteins (COD$_{iNOS}$-His$_6$ homodimer and COD$_{SaNOS}$-His$_6$ homodimer) were purified using the Ni-NTA column, all NOS protein bound to the resin as was evident in the dark red-brown bands that appeared on the resin, and no His$_6$-tagged protein was observed in the flow through from the column as was evident by the absence of colour. This was expected for His$_6$-tagged proteins. The flow through from the column appeared light yellow representing other bacterial proteins which do not bind to the resin. Addition of 150 mM (for COD$_{iNOS}$ or 0.5 M for COD$_{SaNOS}$) imidazole containing buffer to the column resulted in the dark red-brown band moving through the resin and eluting off the column.

Purification of the COD$_{iNOS}$-Glu$_7$-tagged and COD$_{SaNOS}$-Glu$_7$-tagged homodimers was accomplished with ammonium sulphate precipitation, anion exchange chromatography, and hydrophobic interaction chromatography. As in purification of the His$_6$-tagged homodimers, after cell lysis the protein solution was red-brown in colour. The supernatant remained red-brown and a faint yellow pellet was observed after subjecting the protein solution to initial ammonium sulphate precipitation conditions
(30% for COD\textsubscript{iNOS}-Glu\textsubscript{7} and 35% for COD\textsubscript{SaNOS}-Glu\textsubscript{7}). This indicated that the protein remained in solution whereas unwanted proteins were precipitated. After higher ammonium sulphate precipitation (60% for COD\textsubscript{iNOS}-Glu\textsubscript{7} and 50% for COD\textsubscript{SaNOS}-Glu\textsubscript{7}) the supernatant was no longer coloured, and the pellet was a dark red-brown indicating precipitation of the target protein. Resuspension of the pellet in Buffer C (or buffer I for COD\textsubscript{SaNOS}-Glu\textsubscript{7} homodimer) resolubilized the Glu\textsubscript{7}-tagged species homodimer for dialysis.

A typical chromatograph that resulted from anion exchange chromatography of COD\textsubscript{iNOS}-Glu\textsubscript{7} homodimer contained many peaks as is seen in figure 23. However, only one peak had red-brown colour associated with it. Fractions corresponding to this peak were pooled as containing COD\textsubscript{iNOS}-Glu\textsubscript{7}-tagged or COD\textsubscript{SaNOS}-Glu\textsubscript{7}-tagged homodimer respectively. For further purification of Glu\textsubscript{7}-tagged species the homodimers were applied to a phenyl sepharose hydrophobic interaction chromatography. Upon application to the column a red-brown band resulted at the top of the column. Upon washings with decreasing ammonium sulphate concentrations the band remained tightly bound at the top of the column, until its elution in 0M (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}.

Purification of the COD\textsubscript{iNOS}-His\textsubscript{6}/COD\textsubscript{iNOS}-Glu\textsubscript{7} and COD\textsubscript{SaNOS}-His\textsubscript{6}/ COD\textsubscript{SaNOS}-Glu\textsubscript{7} mixed dimers was accomplished using both Ni-NTA resin chromatography and anion exchange chromatography. A red-brown band appeared on the Ni-NTA resin upon application of mixed dimer cultures. In addition, the flow through from the column appeared red-brown under conditions where the column was not saturated. The band on the column represented those species containing a His\textsubscript{6}-tag (His\textsubscript{6}-tagged homodimers and mixed dimers) and the coloured flow through represented those species not possessing
Figure 23: COD$_{iNOS}$-Glu$_7$ homodimer separation by anion exchange chromatography on LPLC 5mL Q column. Samples were injected onto the column in Buffer C and subsequently eluted over a linear NaCl gradient of 0-0.5M. The peak corresponding to red-brown colour is indicated with a red bracket and corresponds to fractions 25mL to 43mL.
any His$_6$-tag (Glu$_7$-tagged homodimers). After application and sequential washing steps with low concentrations of imidazole, the flow-through from the Ni-NTA column became clear, indicating that all Glu$_7$-tagged species had been eliminated leaving only species containing a His$_6$-tag. These species were eluted using higher imidazole concentrations and elution was evident in the advance and elution of the red-brown band through the column.

Anion exchange of the COD$_{iNOS}$-His$_6$/COD$_{iNOS}$-Glu$_7$ mixed dimer was conducted on an LPLC system. This produced a chromatograph with 2 peaks, separated by near-baseline resolution. A sample chromatograph of COD$_{iNOS}$-His$_6$/COD$_{iNOS}$-Glu$_7$ separation is seen in figure 24. The first peak corresponds to the COD$_{iNOS}$-His$_6$ homodimer and the second peak corresponds to the COD$_{iNOS}$-His$_6$/COD$_{iNOS}$-Glu$_7$ mixed dimer as the presence of the Glu$_7$-tag confers a greater affinity for the anion exchange column (McDonald, Taylor et al. 2003). Fractions corresponding to each peak were pooled separately with the transition region between the two peaks being excluded.

Anion exchange of the COD$_{SaNOS}$-His$_6$/COD$_{SaNOS}$-Glu$_7$ mixed dimer was initially conducted on an LPLC. However, it was noted that near-baseline resolution between the two peaks could not be achieved as is seen in figure 25. Consequently, the two species could not be separated on an LPLC. To improve the selectivity in peak separation, anion exchange was attempted on an HPLC. The column type and gradient conditions were altered in attempts to improve the peak resolution and the best results were obtained using a higher resolution MonoQ HR 10/10 column and successive separation steps.

In successive anion exchange columns, only fractions corresponding to the desired peak are pooled, and then re-applied to the column. In this way, the sample
Figure 24: COD\textsubscript{iNOS}-His\textsubscript{6}/COD\textsubscript{iNOS}-Glu\textsubscript{7} mixed dimer separation by anion exchange chromatography on LPLC 5mL Q column. Samples were injected onto the column in Buffer D and subsequently eluted over a linear NaCl gradient of 0-0.5M. Fractions corresponding to the first peak, 18mL-25mL, contained a red-brown colour and were pooled as containing the COD\textsubscript{iNOS}-His\textsubscript{6} homodimer. Fractions corresponding to the second peak, 30mL-38mL, also contained a red-brown colour and were pooled as containing the COD\textsubscript{iNOS}-His\textsubscript{6}/COD\textsubscript{iNOS}-Glu\textsubscript{7} mixed dimer.
Figure 25: COD$_{5aNOS}$-His$_6$/COD$_{5aNOS}$-Glu$_7$ mixed dimer separation by anion exchange chromatography on LPLC. Samples were injected onto the column in Buffer F and subsequently eluted over a linear NaCl gradient of 0-1M. Peaks corresponding to the COD$_{5aNOS}$-His$_6$ homodimer (a) and the COD$_{5aNOS}$-His$_6$/COD$_{5aNOS}$-Glu$_7$ mixed dimer (b) are indicated. Baseline resolution is not achieved between the two peaks, and resulting fractions could not be separated and pooled.
becomes enriched in the desired protein while the other is depleted. Initial application of a sample containing His6-tagged homodimer and mixed dimer to a Mono Q column resulted in peaks that were of equal intensity. Pooling only those fractions corresponding to the mixed dimer (the second peak) and application to a second Mono Q column resulted in enrichment of mixed dimer within the sample. Repeated pooling of only the mixed dimer fractions and application to a third and final Mono Q column further enriched the sample to a maximum composition of 80% mixed dimer and 20% His6-tagged homodimer. Peaks representative of each step in the successive anion exchange separation is seen in figure 26. Successive anion exchange columns using the Mono Q HR 10/10 column were deemed the most successful means of COD_{SaNOS}-His6/COD_{SaNOS}-Glu7 mixed dimer separation.

4.1.2 Determination of Protein Purity

The purity of all protein samples was determined using UV-visible spectroscopy. The Soret peak in the UV visible spectrum is characteristic of the heme present within NOS and can therefore be used to determine the state of the heme within the protein and assess the purity of the sample. Sample spectra characteristic of COD_{iNOS} and COD_{SaNOS} are seen in figure 27. The A_{396}/A_{280} of COD_{iNOS} from homodimer and mixed dimer purifications were typically 0.6 indicating a successful purification of the protein of interest. The purity ratio of COD_{SaNOS} from homodimer and mixed dimer purifications were typically >0.7. SDS PAGE also confirmed purity of the samples. Once the concentration and purity of the NOS protein was determined, dissociation of the species could be investigated.
Figure 26: COD$_{\text{SaNOS}}$-His$_6$/COD$_{\text{SaNOS}}$-Glu$_7$ mixed dimer separation by Mono Q column anion exchange chromatography on an HPLC. Samples were injected onto the column in Buffer F and subsequently eluted over a linear NaCl gradient of 0-1M. Peaks corresponding to COD$_{\text{SaNOS}}$-His$_6$ homodimer (a) and COD$_{\text{SaNOS}}$-His$_6$/COD$_{\text{SaNOS}}$-Glu$_7$ mixed dimer (b) are indicated. The red trace corresponds to the initial separation where peaks are of approximately equal intensity. The pink trace corresponds to the second sequential separation where the peaks are approximately 30:70 mixed dimer, and the blue trace corresponds to the third and final separation where the peaks are maximally separated corresponding to 20:80 mixed dimer.
Figure 27: UV visible spectra of (i) COD\textsubscript{iNOS-\textit{His6}/COD\textsubscript{iNOS-Glu7}} mixed dimer and (ii) COD\textsubscript{SaNOS-\textit{His6}/COD\textsubscript{SaNOS-Glu7}} mixed dimer showing the characteristic soret peak at 396nm when the dimer contains arginine and H\textsubscript{4}B. The peak ratio for iNOS is 0.6 and the peak ratio for SaNOS is 0.8 indicating successful purifications.
4.2 The Mixed Dimer System – Control Investigations

Before implementing the mixed dimer method for investigating dimer stability to COD\textsubscript{INOS}-His\textsubscript{6}/COD\textsubscript{INOS}-Glu\textsubscript{7} or COD\textsubscript{SANOS}-His\textsubscript{6}/COD\textsubscript{SANOS}-Glu\textsubscript{7} mixed dimers, several control investigations were required to ensure that the extent of dimer dissociation was accurately quantified.

4.2.1 Determination of “on” or “off” Column Dissociation Using Alkaline Phosphatase

One matter that was addressed was whether dimer dissociation should be conducted “on” or “off” of the column. On-column refers to a procedure where the mixed dimer is initially bound to the Ni-NTA resin which is then exposed to dissociating conditions. In off-column the mixed dimer is first incubated in dissociating conditions followed by passage over the Ni-NTA resin. The advantage of “on” column dissociation is that it can be used with a gradient of denaturant elution. The aspect of concern is whether initial coordination with the Ni-NTA resin would have a destabilizing affect on the protein. To address this question, temperature-induced dissociation of a thermostable His\textsubscript{6}-tagged homodimeric protein was conducted “on” column to determine if association with the Ni-NTA resin reduced the homodimer’s thermostability.

The homodimeric enzyme alkaline phosphatase (AP) is responsible for hydrolysing phosphate groups from many types of molecules (Birtikati, Besson et al. 1999). AP is only active in dimer form, and it is also thermostable which allows it to maintain activity at elevated temperatures. To determine if the stability of a protein may be altered upon coordination of the His\textsubscript{6}-tag to an Ni-NTA resin, AP-His\textsubscript{6} homodimer was first coordinated to the Ni-NTA resin, and then incubated at 4°C, 37°C and 80°C.
Activity of the high imidazole eluted fractions was of interest as it indicated whether or not the protein remained dimeric and active while bound to the Ni-NTA resin. As it is thermostable, AP should remain dimeric and all activity should be detected in the high imidazole eluted fractions. When assayed for activity, high imidazole eluted fractions corresponding to 4°C and 37°C turned yellow indicating hydrolysis of PNPP to PNP and consequently intact AP homodimer was still present and active. However, when the high imidazole eluted fraction corresponding to 80°C was assayed for activity no yellow colour was evident. In addition, determination of the total protein present by DC assay indicated that all high imidazole eluted fractions from 4°C, 37°C and 80°C possessed comparable amounts of protein. As AP is active only as a homodimer, inactive samples suggested that dissociation of the AP-His₆ homodimer may have occurred. In solution the AP dimer remains active up to a temperature of 90°C. Inactivity of the 80°C samples suggested that coordination of the His₆-tag with the Ni-NTA resin may have altered the stability of the protein resulting in dimer dissociation or inactivating conformational changes at temperatures lower than expected. Consequently, it was concluded that all future dissociation incubations should be conducted “off” column, followed by passage over the Ni-NTA column to allow coordination of the His₆-tagged subunits. In this way, the possibility that coordination to the Ni-NTA column alters dimer stability would be avoided.

4.2.2 Dissociation of the COD₂NOS-His₆ homodimer

Another matter that was addressed was the preservation of the His₆-tag throughout the purification scheme, and the ability of the tag to bind to the Ni-NTA resin
under dissociating conditions. A critical element for success of the mixed dimer method of investigating dimer stability is the presence and stability of a His$_6$-tag and its coordination to the Ni-NTA resin. Degradation of the tag or loss of binding ability would result in more protein than expected in the unbound fraction. To address the maintenance of the His$_6$-tag following purification and the ability of the tag to coordinate to the Ni-NTA resin under dissociation conditions, the dissociation of the His$_6$-tagged homodimer was investigated. In using the His$_6$-tagged homodimer every subunit had the ability to bind the Ni-NTA resin. Even upon dimer dissociation all protein should coordinate to the Ni-NTA resin and none should be present in the unbound fractions. Any protein present in the unbound fraction indicated tag loss or inability to bind the resin under dissociation conditions.

To confirm that the COD$_{iNOS}$-His$_6$ homodimer was intact and able to bind the Ni-NTA under the intended dissociation conditions it was first subjected to dissociation using increasing concentrations of urea as a denaturant. As both subunits have the ability to bind the Ni-NTA resin, no protein should be observed in the unbound fraction. As seen in figure 28, even in the absence of urea, a significant amount of protein was observed in the unbound fraction of 0 M and 3 M urea. Subsequent applications of the COD$_{iNOS}$-His$_6$ homodimer to larger volumes of Ni-NTA resin at longer incubation periods did not restore binding ability, which indicated that resin saturation could not be the reason for this observation. This inability to bind the Ni-NTA resin was most likely due to loss of the C-terminal His$_6$-tag from a portion of the protein. Cleavage of the protein chain at the C-terminus, possibly by an *E.coli* dipeptidyl carboxypeptidase seemed likely (Deutch and Soffer 1987).
Figure 28: SDS PAGE of COD$_{iNOS}$-His$_6$ tagged homodimer urea-induced dissociation. Lanes corresponding to both 0M and 3M urea possess bands indicating that protein did not bind the column, consistent with tag loss.
Since this is the location of the His$_6$-tag, cleavage of this site would result in reduction or elimination of the His$_6$-tag, preventing the protein from binding to the Ni-NTA resin.

When C-terminal cleavage has occurred to varying extents within a sample, three possible species may be present; i) a dimer in which both subunits retain the His$_6$-tag ii) a dimer where both subunits have lost the His$_6$-tag iii) a dimer where only 1 subunit has lost its His$_6$-tag. If neither subunit has lost the His$_6$-tag, both have the ability to bind the Ni-NTA resin, and neither will be observed in the flow through. If both subunits have lost the His$_6$-tag, neither will be able to bind the Ni-NTA resin, and the dimer will be present in the flow through. If only one subunit has lost its His$_6$-tag, dissociation of the dimer would be required for protein to be observed in the flow through. Consequently, this protein would be present in the flow through corresponding to 3 M urea but not in the 0 M urea.

The original methodology employed involved incubation of the dimer in varying concentrations of urea for 3 hours at room temperature (approximately 25°C). However, the dimer has been shown to be destabilized by incubation at temperatures greater than 4°C (Mitchell, Erwin et al. 2005). Specifically, when the protein is incubated at 37°C and the passed through a size exclusion column, only 60% of the protein is present in dimer form (Mitchell, Erwin et al. 2005). In our experiments, destabilization, and subsequent dissociation of the dimer may have resulted from dissociation at room temperature. This, in combination with loss of the His$_6$-tag may account for the protein observed on the SDS-PAGE gel.

In figure 28 the bands present on the SDS-PAGE gel corresponding to the 0 M urea concentration represent that protein which has; a) lost the His$_6$-tag from both
subunits causing inability of the protein to bind the resin or b) has lost a His$_6$-tag on one subunit and dissociated due to temperature. In figure 28 the band present on the SDS-PAGE gel corresponding to the 3 M urea concentration represents that protein which has; a) lost the His$_6$-tag from both subunits causing inability of the protein to bind the resin or b) has lost a His$_6$-tag on one subunit and dissociated due to temperature or denaturant.

To minimize the extent of C-terminal cleavage, the experimental conditions were altered to include a dipeptidyl carboxypeptidase inhibitor, captopril, to limit C-terminal cleavage during protein purification. Dissociations where the effect of denaturant was being investigated were also conducted at 4°C to minimize dissociation due to temperature. Captopril is a potent inhibitor of the dipeptidyl carboxypeptidase present in *E. coli*, as a concentration of only 0.04μM is required to inhibit 50% of its activity (Deutch and Soffer 1987). As seen in figure 29 these modifications greatly decreased the amount of protein present in the unbound fraction. To further reduce the amount of protein in the unbound fraction in the absence of urea, the protein was first bound to and eluted from an initial Ni-NTA column immediately before incubation in denaturant. This step ensured that only protein which still had the ability to bind to the Ni-NTA resin was utilized in the dissociation experiment, and that which was unable to bind the Ni-NTA resin, due to a possible small extent of tag loss, was eliminated. Use of the initial Ni-NTA column resulted in less than 10% of the total protein being present in the unbound fraction in absence of urea.
Figure 29: SDS PAGE of COD\textsubscript{INOS}-His\textsubscript{6} tagged homodimer urea-induced dissociation when the COD\textsubscript{INOS}-His\textsubscript{6} tagged homodimer was purified in the presence of captopril and the dissociation was conducted at 4°C. Significantly, less protein is present in the unbound fractions suggesting the His\textsubscript{6}-tag has remained intact. Molecular size standards are seen on the left side.
4.2.3 Dissociation of the COD\textsubscript{INOS-}Glu\textsubscript{7} Homodimer

In addition to successful coordination of the His\textsubscript{6}-tag to the Ni-NTA resin, another critical element for success of the mixed dimer method of investigating dimer stability is the successful dissociation of the Glu\textsubscript{7}-tagged subunit. Although the Glu\textsubscript{7}-tag has no affinity for an Ni-NTA resin, and passage of the intact COD\textsubscript{INOS-}Glu\textsubscript{7} homodimer over an Ni-NTA resin results in all the protein remaining unbound to the resin and appearing in the unbound fraction, the same may not be true for the monomeric subunits. To accurately quantify the fraction of dissociated protein, the Glu\textsubscript{7}-tagged monomer must not have an affinity for the Ni-NTA resin, and the dissociated Glu\textsubscript{7}-tagged subunit must not bind to the Ni-NTA resin, so that it will all appear in the unbound fraction. To ensure that the Glu\textsubscript{7}-tag was unable to bind the Ni-NTA resin, and address and the ability of the Glu\textsubscript{7}-tagged subunit to remain unbound once the dimer had dissociated, the dissociation of the Glu\textsubscript{7}-tagged homodimer was investigated. Upon dissociation any reduction in the amount of protein present in the unbound fraction indicated adhesion of the dissociated subunit to the Ni-NTA resin.

To ensure that the intact COD\textsubscript{INOS-}Glu\textsubscript{7} homodimer did not have affinity for the Ni-NTA resin, and to investigate whether the dissociated COD\textsubscript{INOS-}Glu\textsubscript{7} monomer had an affinity for the Ni-NTA resin the COD\textsubscript{INOS-}Glu\textsubscript{7}-tagged homodimer was incubated under dissociating conditions and then passed over the Ni-NTA resin. As seen in figure 30 when the homodimer remains intact the protein does not bind to the Ni-NTA column and is detected in the unbound fraction. However, upon dissociation with 5 M urea, the intensity of the unbound fraction band is decreased. This indicates that the dissociated COD\textsubscript{INOS-}Glu\textsubscript{7} monomer has some affinity for the Ni-NTA resin.
Figure 30: SDS PAGE of COD$_{\text{NOS}}$-Glu$_7$ tagged homodimer urea-induced dissociation. On the left, unbound and high imidazole eluted fractions corresponding to incubation in 0M and 5M urea but in the absence of imidazole or IAC. The 5M unbound fraction band intensity is much less compared to the 0M. In the middle, unbound and high imidazole eluted fractions corresponding to incubation in 0M and 5M urea and in the presence of 10mM imidazole in the dissociation solution. The 5M unbound band intensity is comparable to the 0M unbound band intensity. On the right, unbound and high imidazole eluted fractions corresponding to incubation in 0M and 5M urea in the presence of a 2-fold excess of IAC. The 5M unbound band intensity is reduced compared to the 0M band intensity. Molecular weight standards are seen on the left side.
Dissociation of a homodimer into monomers exposes regions that were previously buried in the dimer interface. These freshly exposed regions may possess amino acid residues capable of adhering to the Ni-NTA resin and thereby reducing the amount of protein in the unbound fraction. Li et al. (2006) reported that upon dissociation of the iNOS oxygenase domain dimer, two cysteine residues, Cys$^{104}$ and Cys$^{109}$, and previously buried residues between positions 82-97 and 393-404 were exposed to the solvent. These Cys residues may be in the correct position to coordinate to the Ni-NTA resin and thus prevent the Glu$_7$-tagged subunit from being present in the unbound fraction (Ross and Burrows 1998). In addition, regions 82-97 and 393-404 contain 3 and 1 Histidine residues respectively, making coordination of these exposed regions to the Ni-NTA resin a possibility (Li, Hayden et al. 2006). To eliminate these interactions, iodoacetic acid may be employed to alkylate the cysteine residues thereby blocking their ability to coordinate to the Ni-NTA resin, while a low concentration of imidazole can be included in the dissociation solution to reduce weak binding of the histidines upon dissociation of the dimer.

Iodoacetic acid (IAC) functions by irreversibly alkylating exposed thiols, thereby blocking them and preventing further complex formation with the Ni-NTA resin (Hollecker 1989). An excess of IAC will result in complete alkylation of exposed cysteine residues (Hollecker 1989). Histidine residues use their imidazole side chains to adhere to the Ni-NTA resin, consequently, free imidazole in solution competes for binding on the Ni-NTA resin. Low concentrations of imidazole serve to inhibit non-specific and weak binding to the Ni-NTA resin where as high concentrations of imidazole elute target proteins. Figure 30 illustrates COD$_{iNOS}$-Glu$_7$-tagged homodimer dissociation
in the absence of either IAC treatment of the protein or imidazole, in the presence of 10 mM imidazole, and in the presence of a 2-fold excess of IAC. In the presence of IAC, the 5 M urea band intensity is still reduced in comparison to that of 0 M urea indicating that inclusion of IAC in the dissociation solution does not reduce interaction of the dissociated COD$_{\text{INOS}}$-Glu$_7$ tagged monomer with the Ni-NTA resin. In contrast, the presence of imidazole is successful at reducing interaction of the dissociated COD$_{\text{INOS}}$-Glu$_7$-tagged monomer to the Ni-NTA resin as is evident in the increased band intensity of the 5 M urea band comparable to that of 0 M urea. This suggests that coordination of exposed histidine residues was responsible for adhesion of the COD$_{\text{INOS}}$-Glu$_7$ tagged monomer to the Ni-NTA resin. However, 10 mM imidazole is not wholly successful in eliminating all adhesion of the dissociated COD$_{\text{INOS}}$-Glu$_7$ tagged monomer. When the concentration of imidazole included in the dissociation solution is increased to 20 mM, both unbound and high imidazole eluted band intensities are of comparable intensity for 0 M and 5 M urea indicating that 20 mM urea is successful at eliminating coordination of the dissociated COD$_{\text{INOS}}$-Glu$_7$ tagged monomer to the Ni-NTA resin. Furthermore, inclusion of 20 mM imidazole does not impede the ability of the His$_6$-tag protein to bind the Ni-NTA resin as is seen in figure 31. Therefore, these conditions were optimal for investigating the dissociation of the mixed dimer.
Figure 31: SDS PAGE of COD\textsubscript{INOS}-His\textsubscript{6} tagged homodimer urea-induced dissociation in the presence of 20 mM imidazole. No protein is observed in the 0M unbound fraction and only a small amount is observed in the 5M unbound fraction. Molecular weight standards are seen on the left side.
4.2.4 Choice of Stain for SDS PAGE

Another critical element for success of the mixed dimer method of investigating dimer stability is the accurate determination of the amount of protein in the unbound fraction. This concern was addressed by investigating the linearity and accuracy of protein standards treated with different stains for SDS PAGE gels. The stain that produced the most linear standard curve was deemed a more accurate stain for determining the mass of protein in the unbound fractions.

Coomassie Blue is the most common method of protein staining in SDS PAGE (Williams 2001). Coomassie Blue SimplyBlue Safe stain is a water soluble version of Coomassie Blue and does not require methanol or acetic acid fixing or destaining. Coomassie Blue binds to basic and aromatic amino acid residues including arginine, lysine, histidine, tyrosine, tryptophan, and phenylalanine (Williams 2001). Interaction of the dye with the protein is dependent on Van der Waals forces and hydrophobic interactions (Williams 2001). However, as seen in figure 32, there is variability associated with the points when a standard curve is created using Coomassie Blue. Other more sensitive fluorescent stains produce a standard curve with less variability.

The Coomassie Blue stain which was previously used to stain the SDS PAGE gels was replaced with SYPRO Ruby protein gel stain. SYPRO Ruby stain is a fluorescent stain that contains ruthenium as part of an organic complex (Berggren, Chernokalskaya et al. 2000). The stain interacts noncovalently with the SDS coating around the proteins within the gel (Williams 2001). SYPRO Ruby is excited in the mid UV range between 250-350 nm and emits maximally at 610nm (Berggren, Chernokalskaya et al. 2000). It is a very sensitive protein stain, which allows smaller amounts of protein to be detected.
Figure 32: Standard curve created by staining the SDS PAGE gel with Coomassie Blue stain and performing densitometry on respective bands. The $R^2$ value indicates variability within the standard curve. Insert: SDS PAGE gel of COD$_{iNOS}$-His$_6$ tagged homodimer standard masses of protein.
compared to Coomassie Blue. SYPRO Ruby stain also has a linear dynamic range that spans three orders of magnitude (Berggren, Chernokalskaya et al. 2000). As seen in figure 33 the standard curve that results from staining with SYPRO Ruby possesses lower variability than that stained with Coomassie blue. The large linear range and reduced variability implicates SYPRO Ruby as a more accurate method for quantitative measurements of protein content.

4.3 Denaturant-Induced Dissociation of the COD\textsubscript{INOS}-His\textsubscript{6}/COD\textsubscript{INOS}-Glu\textsubscript{7} and COD\textsubscript{SaNOS}-His\textsubscript{6}/COD\textsubscript{SaNOS}-Glu\textsubscript{7} mixed dimers

With the possible complicating factors such as tag loss and non-specific adhesion of the Glu\textsubscript{7}-tagged subunit accounted for, the mixed dimer method can be employed as a means of investigating dimer stability of COD\textsubscript{INOS}-His\textsubscript{6}/COD\textsubscript{INOS}-Glu\textsubscript{7} or COD\textsubscript{SaNOS}-His\textsubscript{6}/COD\textsubscript{SaNOS}-Glu\textsubscript{7} mixed dimers.

The denaturant-induced dissociation of the COD\textsubscript{INOS}-His\textsubscript{6}/COD\textsubscript{INOS}-Glu\textsubscript{7} mixed dimer in the absence of substrate (Arg) and presence of the cofactor (tetrahydrobiopterin, H\textsubscript{4}B) is illustrated in figure 34. This is a typical dimer dissociation curve (additional dissociation curves, not presented in the results and discussion, for the data in table 1 are present in appendix 1). The shape of the plot is sigmoidal, where initially at low denaturant concentrations, the fraction of protein present in the unbound fraction is minimal, indicating that very little dimer dissociation has occurred. As denaturant concentrations increase, an increase in the fraction of protein present in the unbound fraction is observed, indicating dissociation of the dimer. The fraction of protein present
Figure 33: Standard curve created by staining the SDS PAGE gel with SYPRO Ruby stain and performing densitometry on respective bands. The $R^2$ value indicates more agreement with a linear standard curve. Insert: SDS PAGE gel of COD$_{\text{INOS}}$-His$_6$ tagged homodimer standard masses of protein.
Figure 34: Urea-induced dissociation of COD\textsubscript{NOS-His\textsubscript{6}}/COD\textsubscript{NOS-Glu\textsubscript{7}} mixed dimer in the absence of arginine and with H\textsubscript{4}B present. Fraction of total protein present at each urea concentration is represented. To determine $C_m$ values pre- and post-dissociation lines corresponding to the minimum and maximum plateaus were drawn and $C_m$ values were derived from the midpoint of the curve. Insert: SDS PAGE of the COD\textsubscript{NOS-His\textsubscript{6}}/COD\textsubscript{NOS-Glu\textsubscript{7}} mixed dimer urea-induced dissociation in the absence of arginine and with H\textsubscript{4}B present.
Table 1: Susceptibility of NOS oxygenase dimers to urea-induced dissociation. Abbreviations: Arg = L-arginine, 0.5 mM; H₄B = tetrahydrobiopterin, 0.1 mM.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Conditions</th>
<th>Cₘ, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>(+Arg +H₄B)</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>(-Arg +H₄B)</td>
<td>4.4</td>
</tr>
<tr>
<td>SaNOS</td>
<td>(+Arg +H₄B)</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>(-Arg +H₄B)</td>
<td>5.5</td>
</tr>
</tbody>
</table>
in the unbound fraction eventually approaches a plateau indicating maximum dimer
dissociation has occurred.

To determine $C_m$ values for dissociation curves the data was treated by linear
extrapolation of the pre- and post-dissociation curves (Pace 1986). The midpoint of the
curve was determined from these lines. Table 1 presents $C_m$ values obtained for iNOS
and SaNOS under the conditions stated. The $C_m$ values obtained for
COD$_{\text{iNOS}}$-His$_6$/COD$_{\text{iNOS}}$-Glu$_7$ mixed dimer are higher than previous estimates in which
only three denaturant concentrations (0, 3 and 5 M urea) were used in the estimation of
dimer dissociation conditions by size exclusion chromatography (Panda, Rosenfeld et al.
2002). This increased stability may be accounted for by the presence of imidazole, which
is essential to restrict adhesion of the dissociated COD$_{\text{iNOS}}$-Glu$_7$-tagged monomer.

Small molecules such as imidazole can promote dimerization. The structural
constraints of these molecules are minimal and thus they do not greatly disrupt the overall
protein structure (Sennequier, Wolan et al. 1999). Small molecules can affect
dimerization in at least two ways. In the first, the small molecules may have a high
affinity for the dimeric structure but a lower affinity for the monomer. This suggests that
the binding sites for the molecule are incompletely formed in the monomer. In this
scenario, initially two monomers associate to form a loose dimer. This loose dimer then
binds to the small molecule, which stabilizes the dimer. In NOS most small molecules
that stabilize the dimer bind near the distal heme pocket although they do not ligate
directly to the heme (Sennequier, Wolan et al. 1999). Molecules such as these include
H$_4$B and arginine.
However, imidazole can act as a ligand to metals and it binds directly to the NOS monomer heme iron where it remains bound to the heme after the dimer has formed (Sennequier, Wolan et al. 1999). Two molecules of imidazole can bind within the distal heme pocket, as a ligand to the heme iron and the other binding to the carboxylate of a glutamic acid residue (Glu$^{371}$) which, under native conditions, binds to arginine (Sennequier, Wolan et al. 1999). The ability of imidazole to bind to the monomer heme maybe critical to its functioning as a promoter of dimerization because imidazole derivatives that cannot bind heme do not promote dimerization (Sennequier, Wolan et al. 1999). However, those imidazoles that can interact with the NOS dimer stabilize it (Panda, Rosenfeld et al. 2002). Therefore, $C_m$ values appear elevated in comparison to previous estimates because of the stabilizing effects of imidazole.

Prior research has also indicated that the presence of arginine has a stabilizing effect on the dimeric structure (Crane, Arvai et al. 1998). However, our $C_m$ values indicate that the stability of the iNOS dimer is not significantly altered in the absence of arginine. When arginine is absent, the residues that typically participate in binding arginine to hold it in place, most importantly Glu$^{371}$, are free. Consequently, Glu$^{371}$ is free to bind imidazole. This may further stabilize the dimer, resulting in an apparent stability over the NOS dimer in the presence of arginine.

The stability of the COD$_{iNOS}$-His$_6$/COD$_{iNOS}$-Glu$_7$ mixed dimer in the absence of both arginine and H$_4$B was also investigated. The lack of H$_4$B has a significant effect as seen in figure 35. No clear trend is observed in the dissociation of the COD$_{iNOS}$-His$_6$/COD$_{iNOS}$-Glu$_7$ mixed dimer in the absence of both arginine and H$_4$B. The presence
Figure 35: Urea-induced dissociation of COD$_{iNOS}$-His$_6$/COD$_{iNOS}$-Glu$_7$ mixed dimer in the absence of both arginine and H$_4$B. Fraction of total protein present at each urea concentration is represented. No clear trend is observed within the data, hence no $C_m$ value can be determined.
of arginine and H₄B are required to form a stable dimer in iNOS and in their absence, iNOS is present as a mixture of monomer and loosely formed dimer (Alderton, Cooper et al. 2001). Since a stable dimer is not formed, $C_m$ values for the dissociation of the dimer cannot be calculated or compared to that of iNOS under other conditions.

In comparison, although the stability of SaNOS to denaturant-induced dissociation has not been reported, it appears to be slightly more resistant to denaturant-induced dissociation compared to iNOS. This increased stability occurs even in the absence of the N-terminal hook region present in the mammalian forms of NOS. In the absence of the N-terminal hook there is a reduction in dimer interface and buried surface area of the SaNOS dimer (Bird, Ren et al. 2002). However, variations in the SaNOS sequence allow for stronger and additional interactions at the dimer interface (Bird, Ren et al. 2002). One example of this is the presence of a proline at position 323 where mammalian NOS isoforms possess a glycine. This Pro$^{323}$ effectively reduces the conformational flexibility and increases hydrophobic packing. In addition the interaction between Phe$^{262}$ from one subunit with Tyr$^{273}$ and Tyr$^{276}$ of the other subunit is stronger in SaNOS than in mammalian NOS isoforms. These residues at the dimer interface of the SaNOS dimer are seen in figure 36. Finally, there are various additional hydrogen bonds that are present in SaNOS that help to stabilize the dimer (Bird, Ren et al. 2002).
Figure 36: The dimer interface of SaNOS. The main chains are shown as ribbons with the A chain coloured green and the B chain coloured blue. The side chains of key residues involved in the interface interactions are shown as balls and sticks, those belonging to the A chain are coloured orange and those belonging to the B chain are coloured cyan. The yellow dashed lines represent hydrogen bonding between the two chains. (Bird, Ren et al. 2002)
4.4 Temperature-Induced Dissociation of the COD\textsubscript{iNOS}-His\textsubscript{6}/COD\textsubscript{iNOS}-Glu\textsubscript{7} and COD\textsubscript{SaNOS}-His\textsubscript{6}/COD\textsubscript{SaNOS}-Glu\textsubscript{7} mixed dimers

The temperature-induced dissociation curve of the COD\textsubscript{iNOS}-His\textsubscript{6}/COD\textsubscript{iNOS}-Glu\textsubscript{7} mixed dimer in the presence of Arg and H\textsubscript{4}B is shown in figure 37. A value greater than 0.5 for the fraction of total protein present is observed at 45°C. The maximum fraction of total protein that can be present in an unbound fraction is 0.5, as this represents the fraction of Glu\textsubscript{7}-tagged subunit of the mixed dimer. A fraction greater than this indicates that some His\textsubscript{6}-tagged species is present in the unbound fraction. It was also possible that elevated temperatures resulted in degradation or loss of the His\textsubscript{6}-tag.

Incubation of the COD\textsubscript{iNOS}-His\textsubscript{6} homodimer at 37°C demonstrated the stability of the His\textsubscript{6}-tag at elevated temperatures. As shown in figure 38, no band is present in the unbound fraction indicating that all the protein present was able to bind to the Ni-NTA resin. Furthermore, there is a very intense band corresponding to the fraction of protein that bound the column and then was eluted with high concentrations of imidazole. This indicated that at 37°C temperature the His\textsubscript{6}-tag remained intact and maintained its ability to bind to the Ni-NTA column. However, when the COD\textsubscript{iNOS}-His\textsubscript{6} homodimer is incubated at 45°C the ability of the His\textsubscript{6}-tag to bind the column is diminished.

When the COD\textsubscript{iNOS}-His\textsubscript{6} homodimer was incubated at 45°C the sample became visibly turbid. After passage over a Ni-NTA column, the resulting unbound fraction contained protein as is indicated in figure 39. This indicated that at 45°C some fraction of the protein was unable to bind to the Ni-NTA resin despite both subunits possessing a His\textsubscript{6}-tag. At elevated temperatures as proteins begin to unfold, there is an attempt to bury
Figure 37: Temperature-induced dissociation of COD$_{\text{INOS}}$-His$_6$/COD$_{\text{INOS}}$-Glu$_7$ mixed dimer in the presence of both arginine and H$_4$B. Fraction of total protein present at each temperature is represented. To determine $T_m$ values pre- and post-dissociation lines corresponding to the minimum and maximum plateaus were drawn and $T_m$ values were derived from the midpoint of the curve. Insert: SDS PAGE of the COD$_{\text{INOS}}$-His$_6$/COD$_{\text{INOS}}$-Glu$_7$ mixed dimer temperature-induced dissociation in the presence of both arginine and H$_4$B.
Figure 38: SDS PAGE of temperature-induced dissociation of COD$_{\text{INOS}}$-His$_{6}$ homodimer. The absence of protein in the unbound lane and a very intense band in the high imidazole eluted lane indicates all protein bound to the column and was consequently, eluted. Molecular weight standards are seen on the left side.
Figure 39: SDS PAGE of the temperature-induced dissociation of COD$_{iNOS}$-His$_6$ homodimer. A band is present in the lane corresponding to the unbound fraction when the sample was incubated at 45°C. Upon dispersion of the aggregated pellet and reapplication to an Ni-NTA resin, no protein appears to be in the unbound fraction. Molecular weight standards are seen on the left side.
exposed hydrophobic groups while exposing polar groups to the solvent (Creighton 1984). This can result in the formation of aggregates. As aggregates form, burying of the His\textsubscript{6}-tag below the surface may limit its accessibility to the Ni-NTA resin, causing the species to be observed in the unbound fraction. Dispersion of the aggregates by complete protein unfolding would consequently re-expose the His\textsubscript{6}-tag and restore binding ability.

As seen in figure 39 when the aggregated protein pellet was completely unfolded before passage over a Ni-NTA column, no band is present in the lane corresponding to the unbound fraction. Consequently, aggregation of the COD\textsubscript{iNOS}-His\textsubscript{6} homodimer by incubation at 45°C masked the His\textsubscript{6}-tag. The His\textsubscript{6}-tag was unmasked by complete unfolding of the protein and binding was restored. Therefore, a fraction of 0.5 was still used as the maximum fraction of protein in the unbound fraction and any value above this point represented aggregated protein possessing masked His\textsubscript{6}-tags. Similar to the denaturant-induced dissociation curves, values of $T_m$, the temperature where half the dimers have been dissociated, were obtained by linear extrapolation of the pre- and post-dissociation curves (Chen and Matthews 1994). The midpoint of the curve was determined from these lines. Table 2 presents $T_m$ values for iNOS and SaNOS under the conditions stated (additional dissociation curves, not presented in the results and discussion, for data in table 2 are present in appendix 2). The $T_m$ value for COD\textsubscript{iNOS} is in agreement with Mitchell and Irwin et al (2005) who reported, using gel filtration chromatography, that at 37°C only 60% of the protein present was in dimer form. Initially, the SaNOS dimer appears to be much more stable than the iNOS dimer towards thermally-induced dissociation.
Table 2: Susceptibility of NOS oxygenase dimers to thermally-induced dissociation. For SaNOS with both Arg and H_{4}B present there was no evidence of significant dimer dissociation up to 45 °C, at which point the protein began to precipitate.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Conditions</th>
<th>$T_m$ (in °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>(+Arg +H_{4}B)</td>
<td>28-29</td>
</tr>
<tr>
<td>SaNOS</td>
<td>(+Arg +H_{4}B)</td>
<td>&gt;45</td>
</tr>
<tr>
<td></td>
<td>(-Arg +H_{4}B)</td>
<td>~44</td>
</tr>
</tbody>
</table>
The absence of significant SaNOS dissociation in the presence of arginine and H$_4$B prior to the protein denaturation which begins at 45 °C suggested the formation of a very stable dimer whose dissociation may not be distinct from denaturation. Investigation of SaNOS denaturation was conducted by monitoring the UV visible spectrum between 700 nm and 280 nm. The Soret peak at 396 nm (in the presence of Arg and H$_4$B) is associated with the π-π* transitions of the heme, and is sensitive to the surrounding protein environment making it a good method of monitoring denaturation of the protein. Protein denaturation disrupts the heme-binding pocket and it may result in its release from the protein. After incubation of COD$_{\text{SaNOS}}$-His$_6$/COD$_{\text{SaNOS}}$-Glu$_7$ at 45°C the sample did not appear turbid, and as seen figure 40 the Soret peak remains intact at 45°C, indicating that the protein has not yet begun to denature. Further increases in temperature caused turbidity of the sample and reduced the intensity of the Soret peak. At 55°C the sample did not appear red-brown, and the Soret peak was completely diminished. This indicated that below 45°C the protein remained intact, but temperatures greater than 45°C initiate protein unfolding and aggregation. This in association with the absence of a band representing dimer dissociation prior to 45°C suggested the formation of a dimer whose dissociation may not be distinct from denaturation.

In contrast to denaturant-induced dissociation, when arginine is removed from the incubation during temperature-induced dissociation of COD$_{\text{SaNOS}}$, visible dimer dissociation is evident as the band intensity corresponding to 37°C and 45°C temperature points is increased as seen in figure 41. This indicates that with respect to temperature-induced dissociation, imidazole has not stabilized the dimer. The inability of imidazole to stabilize the dimer to temperature-induced dissociation is a result of how temperature
Figure 40: UV visible spectrum of the COD$_{\text{SanOS-His}6}$/COD$_{\text{SanOS-Glu7}}$ incubated at increasing temperatures. The soret peak at 396nm is intact at 45°C representing intact protein. Increasing temperatures cause the intensity of the soret peak to become reduced. Incubation at 55°C results in total elimination of the Soret peak.
Figure 41: Temperature-induced dissociation of COD\textsubscript{SaNOS-His\textsubscript{6}}/COD\textsubscript{SaNOS-Glu\textsubscript{7}} mixed dimer in the absence of arginine and the presence of H\textsubscript{4}B. Fraction of total protein present at each temperature is represented. To determine $T_m$ values pre- and post-dissociation lines corresponding to the minimum and maximum plateaus were drawn and $T_m$ values were derived from the midpoint of the curve. Insert SDS PAGE of the COD\textsubscript{SaNOS-His\textsubscript{6}}/COD\textsubscript{SaNOS-Glu\textsubscript{7}} mixed dimer temperature-induced dissociation in the absence of arginine and the presence of H\textsubscript{4}B.
acts to destabilize the dimer in comparison to the action of denaturing agents. Denaturants act to destabilize the dimer by preferentially interacting with the surface of a protein. Individual monomers have more exposed surface area than a dimer. Therefore, an additive that interacts more favourably with protein surfaces, especially hydrophobic surfaces that are buried upon dimerization, than the solvent will be a denaturant (Creighton 1984). Interaction of the denaturant with the surface area of a dimer at a concentration sufficient to cause dissociation may not affect other interactions. The disruption of the dimer by urea therefore does not affect binding of imidazole to the heme or glutamic acid residue and consequently the stabilizing effects of imidazole are observed. Conversely, increasing the temperature of a protein sample weakens all the interactions within the protein possibly including that of the imidazole to the heme and Glu$^{371}$. Therefore, the stabilizing effects of imidazole may not be observed. Thus, with respect to temperature-induced dissociation, it may be possible to observe the stabilizing effects of arginine.

### 4.5 Rapid Exchange of Subunits

Incubation of two homodimeric species together in solution may result in rapid exchange of subunits to create mixed dimers, while rapid exchange between subunits of a mixed dimer could result in generation of some homodimeric species. If this had occurred during investigations of dimer dissociation, passage of the sample over an Ni-NTA resin would result in His$_6$-tagged homodimers remaining bound to the column and Glu$_7$-tagged homodimers from passing through the column. In such a case the fraction of protein present in the unbound fraction would not be representative of the
thermodynamics of dimer dissociation but the kinetics of subunit exchange. To ensure that the protein bands observed in the unbound fractions were due to dimer dissociation and not rapid exchange of subunits to recreate homodimers, equal amounts of CODSaNOS-His$_6$ homodimer and CODSaNOS-Glu$_7$ homodimer were incubated at room temperature together and separated by anion exchange chromatography. If rapid exchange of subunits was occurring, peaks corresponding to the homodimeric species would decrease in intensity over time and a third peak representing the mixed dimer species would appear. However if no rapid exchange was occurring, the chromatograph would appear unchanged over time. As can be seen in figure 42, the homodimers each possess characteristic peaks, which are both present upon initial mixing of the samples. Furthermore, incubation of the samples for 1 hour at room temperature does not result in reduction of the homodimer peaks, or the appearance of a third peak representing creation of a mixed dimer. Consequently, no rapid mixing is observed and bands observed on SDS PAGE gels following dissociation experiments are representative of dimer dissociation.

4.6 Effect of Dilution on Dissociation and Estimation of $K_D$

The $K_D$ for the dimer-monomer equilibrium can be calculated from a plot of the fraction of dimer versus the total protein concentration (Graziano, McGrath et al. 2006). A plot such as this is shown in figure 43. The shape of the plot is hyperbolic starting from a minimum at ~0.7 corresponding to a total protein concentration of 0.25 μg/μL and quickly reaching a plateau at a maximum of ~0.95 at a total protein concentration of 2μg/μL. $K_D$ is estimated from the half-maximal point of this line as 2.3 μM.
Figure 42: MonoQ column anion exchange chromatography on an HPLC of COD$_{SaNOS}$-His$_6$ homodimer and COD$_{SaNOS}$-Glu$_7$ homodimers. COD$_{SaNOS}$-His$_6$ homodimer and COD$_{SaNOS}$-Glu$_7$ were mixed in equal amounts, injected onto the column in Buffer F initially following mixing and after 1 hour incubation at room temperature, and subsequently eluted over a NaCl gradient of 0-1M. The red trace represents t=0 when the sample was passed down a Q column immediately after mixing, and the pink trace represents t=1 hour, after the same sample was incubated at room temperature for 1 hour and then passed down the Q column. Both traces contain the same peaks, and no third peak representative of the mixed dimer formation has appeared.
Figure 43: Dissociation of the COD$_{\text{NOS}}$-His$_6$/COD$_{\text{NOS}}$-Glu$_7$ mixed dimer at different concentrations of total protein. Fraction of dimer is plotted as a function of total protein concentration. $K_D$ was estimated as the total protein concentration where half the dimers present have dissociated into monomers ($f_D=0.5$)
4.7 Investigation of $K_D$ by Analytical Ultracentrifugation

The results from analytical ultracentrifugation indicated that the oligomeric behaviour of the CODiNOS-His$_6$/CODiNOS-Glu$_7$ mixed dimer was not a simple monomer-dimer relationship. Strong evidence suggested the presence of monomer, dimer, trimer, and tetramer species. The percent contribution of each species under the conditions investigated is presented in table 3. In the presence of both arginine and H$_4$B when the dimer is most stable, there is still a small fraction of monomer, trimer, and tetramer formation. However, as the dimer is destabilized by the sequential removal of arginine and H$_4$B the contribution of dimer is decreased and the contribution of monomer, trimer, and tetramer are increased. This supports the stabilizing effect of arginine and the possibility that imidazole may be providing increased stability in the dissociation experiments. However, due to the complicated monomer, dimer, trimer, tetramer relationship a simple $K_D$ for the dissociation of dimer into monomer cannot be established.

Although the results from AUC suggest the presence of trimeric and tetrameric species, the functioning unit of NOS is its dimeric form. The trimeric and tetrameric forms may represent partially unfolded species that have aggregated together. Due to the nature of the interactions that hold these aggregates together they may to disperse at low concentrations of urea. Consequently, they are not implicated in the aforementioned dissociation experiments.
Table 3: %Contribution of monomer, dimer, trimer, and tetramer within COD\textsubscript{INOS-His\textsubscript{6}}/COD\textsubscript{INOS-Glu\textsubscript{7}} mixed dimer AUC samples under the conditions indicated

<table>
<thead>
<tr>
<th></th>
<th>-Arg –H\textsubscript{4}B</th>
<th>-Arg + H\textsubscript{4}B</th>
<th>+Arg +H\textsubscript{4}B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>17 %</td>
<td>19 %</td>
<td>7 %</td>
</tr>
<tr>
<td>Dimer</td>
<td>45 %</td>
<td>43 %</td>
<td>75 %</td>
</tr>
<tr>
<td>Trimer</td>
<td>24 %</td>
<td>17 %</td>
<td>9 %</td>
</tr>
<tr>
<td>Tetramer</td>
<td>10 %</td>
<td>12 %</td>
<td>4 %</td>
</tr>
</tbody>
</table>
4.8 Limitations to the Mixed Dimer Method

Although the mixed dimer method of investigating dimer dissociation is a direct and accurate method it has some limitations. With respect to investigations of denaturant-induced dissociation, $C_m$ values derived are estimates of the midpoint of dissociation in the presence of 20 mM imidazole. As is illustrated with NOS, imidazole can stabilize a dimer, and therefore increase $C_m$ values above what would be observed in the absence of imidazole. Consequently, only apparent $C_m$ values are obtained. However, if the protein of interest does not interact with imidazole, then $C_m$ values obtained represent the true denaturant concentration required to produce 50% dimer dissociation.

In addition, for the mixed dimer method to be used as an effective means of monitoring denaturant-induced dissociation of a homodimeric protein, the protein must first assemble to a stable dimer. As illustrated with iNOS in the absence of arginine or H$_4$B, if a stable dimer is not formed no clear trend in the dissociation is observed and $C_m$ values cannot be calculated.

With respect to temperature-induced dissociation, the His$_6$-tag must remain exposed to coordinate to the Ni-NTA resin. Aggregation of the protein that causes masking of the His$_6$-tag eliminates the ability of the tag to coordinate to the resin. Therefore, temperature-induced dissociation can only be investigated where protein aggregation does not occur. This places a maximum limit on the temperatures that can be investigated which will depend on the protein being studied.

Conversely, when the effect of dilution on dissociation is investigated with the aim of determining a $K_D$, there is a lower detection limit. Different methods of staining
SDS PAGE gels have different minimum detection limits (Williams 2001). This minimum detection limit places a restriction on the total protein concentrations that can be assayed. Consequently, only those total protein concentrations that, when equilibrium is reached between dimer and monomer, produce monomer concentrations within the detectable range can be investigated.

Although limitations to the mixed dimer method for investigation dissociation of homodimeric proteins exists, valuable information regarding the conditions that cause dimer dissociation is obtained making it a valuable and important technique.
Chapter 5: Concluding Summary

Within the limits described above the mixed dimer system is accurate method of examining denaturant- and temperature-induced dissociation of a homodimeric protein. The method examines dimer dissociation alone and is not complicated by signals due to protein unfolding. The method is also rapid and inexpensive as it only involves incubation in dissociating conditions for a short time followed by passage over Ni-NTA resin and SDS PAGE densitometry. The mixed dimer method was applied iNOS and SaNOS to assess dimer stability, and to iNOS to estimate value for $K_D$. The SaNOS dimer was found to be more stable to denaturant- and temperature-induced dissociation. The dissociation constant estimated for the dissociation of the iNOS dimer was 2.3 μM. Using analytical ultracentrifugation it was not possible to obtain a $K_D$ for comparison as a more complicated oligomeric system exists between the species within a sample.
References


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"Crystal Structure of SaNOS, a Bacterial Nitric Oxide Synthase Oxygenase Protein from Staphylococcus aureus." **Structure** 10: 1667-1696.


Protein Expression and Purification 27(1): 115-127.


Appendix 1: Dissociation curve used to calculate $C_m$ values in table 1.

Figure 44: Urea-induced dissociation of COD$_{	ext{NOS}}$-His$_6$/COD$_{	ext{NOS}}$-Glu$_7$ mixed dimer in the presence of both arginine and H$_4$B. Fraction of total protein present at each urea concentration is represented. To determine $C_m$ values pre- and post-dissociation lines corresponding to the minimum and maximum plateaus were drawn and $C_m$ values were derived from the midpoint of the curve.
Figure 45: Urea-induced dissociation of COD$_{SaNOS}$-His$_6$/COD$_{SaNOS}$-Glu$_7$ mixed dimer in the presence of both arginine and H$_4$B. Fraction of total protein present at each urea concentration is represented. To determine $C_m$ values pre- and post-dissociation lines corresponding to the minimum and maximum plateaus were drawn and $C_m$ values were derived from the midpoint of the curve.
Figure 46: Urea-induced dissociation of COD$_{SaNOS}$-His$_6$/COD$_{SaNOS}$-Glu$_7$ mixed dimer in the absence of arginine and the presence of H$_4$B. Fraction of total protein present at each urea concentration is represented. To determine $C_m$ values pre- and post-dissociation lines corresponding to the minimum and maximum plateaus were drawn and $C_m$ values were derived from the midpoint of the curve.
Appendix 2: Dissociation curve used to calculate $T_m$ values in table 2.

Figure 47: Temperature-induced dissociation of COD$_{SaNOS}$-His$_6$/COD$_{SaNOS}$-Glu$_7$ mixed dimer in the presence of both arginine and H$_4$B. Fraction of total protein present at each temperature is represented. To determine $T_m$ values pre- and post-dissociation lines corresponding to the minimum and maximum plateaus were drawn and $T_m$ values were derived from the midpoint of the curve. Insert SDS Page of the COD$_{SaNOS}$-His$_6$/COD$_{SaNOS}$-Glu$_7$ mixed dimer temperature-induced dissociation in the presence of both arginine and H$_4$B.
Appendix 3: Summary of buffer recipes

**Buffer A** 50 mM Tris-HCl, 0.5 M NaCl, 5 mM L-arginine, 100 μM H₄B 0.1 mM captopril, pH 7.5

**Buffer B** 50 mM Tris-HCl, 10% glycerol, 0.5 mM DTT, 100 μM H₄B, 5 mM L-arginine pH 7.5

**Buffer C** 50 mM Tris-HCl, 10% glycerol, 0.5 mM DTT, 100 μM H₄B, 5 mM L-arginine pH 6.8

**Buffer D** 50 mM Tris-HCl, 5 mM L-arginine, 100 μM H₄B 0.1 mM captopril, pH 6.8

**Buffer E** 50 mM HEPES, 0.15 M NaCl and 0.1 mM captopril pH 7.5

**Buffer F** 25 mM Bis-Tris, 0.1 M NaCl pH 6.5

**Buffer G** 50 mM HEPES, 0.15 M NaCl, 5 mM L-arginine, 0.1 mM captopril pH 7.5

**Buffer H** 50 mM HEPES, 10% glycerol, 0.5 mM DTT, 5 mM L-arginine, 0.15 M NaCl, 0.1 mM captopril, pH 7.5

**Buffer I** 50 mM HEPES, 10% glycerol, 0.5 mM DTT, 5 mM L-arginine, 0.1 mM cap, pH 6.5

**Dissociation Buffer** 50 mM Tris-HCl, 0.5 M NaCl, 100 μM H₄B, 0.5 mM L-arginine, 0.1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) pH 8