THERMODYNAMICS OF OXIDATION-REDUCTION REACTIONS IN MAMMALIAN NITRIC OXIDE SYNTHASE ISOFORMS

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1Abbreviations: NO, nitric oxide; eNOS, endothelial nitric oxide synthase (NOSIII); iNOS, inducible nitric oxide synthase (NOSII); nNOS, neuronal nitric oxide synthase (NOSI); NHA, N-hydroxy-L-arginine; BH4, tetrahydrobiopterin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.
Abstract

The three mammalian nitric oxide synthases produce NO from arginine in a reaction requiring 3 electrons per NO, which are supplied to the catalytic center from NADPH through reductase domains incorporating FAD and FMN cofactors. The isoforms share a common reaction mechanism and requirements for reducing equivalents but differ in regulation; the endothelial and neuronal isoforms are controlled by calcium/calmodulin modulation of the electron transfer system, while the inducible isoform binds calmodulin at all physiological Ca\(^{2+}\) concentrations and is always on. The thermodynamics of electron transfer through the flavin domains in all three isoforms are basically similar. The major flavin states are FMN, FMNH\(_1\), FMNH\(_2\), FAD, FADH\(_1\), and FADH\(_2\). The FMN/FMNH\(_1\) couple is high potential (~-100 mV) in all three isoforms, and is unlikely to be catalytically competent; the other three flavin couples form a nearly isopotential group clustered around -250 mV. Reduction of the flavins by the pyridine nucleotide couple at ~-325 mV is thus moderately thermodynamically favorable. The ferri/ferroheme couple in all three isoforms is ~-270 mV in the presence of saturating arginine. Ca\(^{2+}\)/calmodulin has no effect on the potentials of any of the couples in eNOS or nNOS. The pH dependence of the flavin couples suggests the presence of ionizable groups coupled to the flavin redox/protonation states.
Introduction

Nitric oxide (NO) is an important molecular messenger in a variety of signal transduction pathways (1-4). Nitric oxide synthases (NOS) comprise a family of complex, modular enzymes, including three isoforms expressed in mammals. The endothelial and neuronal isoforms (eNOS and nNOS) are signal generators under the control of calcium/calmodulin (5); a third isoform, iNOS, is induced during immune response (6-9) and produces much higher levels of NO even at basal levels of calcium.

The generation of NO from arginine and oxygen by nitric oxide synthases requires the delivery of three pyridine nucleotide derived electrons to the catalytic site per mol of NO formed. The electron transfer system in endothelial nitric oxide synthase (eNOS, NOSIII) utilizes one flavin mononucleotide (FMN) and one flavin adenine dinucleotide (FAD) cofactor (10-15) to deliver electrons derived from reduced nicotinamide dinucleotide phosphate (NADPH) to the site of oxygen chemistry, a protoporphyrin IX derived heme with cysteinyl thiolate axial ligation (11,12,16,17). The electron transfer pathway is closely related to the NADPH P450 reductase/P450 system, and significant sequence homology exists between the flavoprotein binding domains in the two systems (10).

The details of electron transfer differ significantly in FAD/FMN flavoprotein reductases related by homology and function. The two flavin cofactors have the capacity to accept four electrons. In mammalian P450 reductase, extensive studies have revealed that the system operates between the one and three electron reduced states (18,19). The FMN/FMNH\(^+\) couple has a relatively high potential (−50 to -100 mV), and is not a kinetically competent reductant in the catalytic cycle. The FMNH\(^-\)/FMNH\(_2\) and FAD/FADH\(^+\) couples are intermediate in potential between the pyridine nucleotide pool and the physiological acceptor, and are the primary functional one electron couples in the catalytic cycle. The FADH\(^-\)/FADH\(_2\) couple is too negative to be readily reduced by NADPH. Recently, the homologous reductase domains of cytochrome
P450 BM3, which contains both flavin and P450 heme domains in a single polypeptide, have been shown to cycle primarily between the one and two electron reduced states; in this system, the order of the FMN potentials is reversed, and the FMN semiquinone stability constant is slightly less than unity. Both FMN one electron couples appear to be good enough reductants to participate in the catalytic cycle (20).

In enzymes of the P450 superfamily, it is well known that binding of 'type I' substrates shifts the spin state equilibrium of ferriheme towards the high spin form, while simultaneously raising the midpoint potential (21-23). This is an important step in the catalytic cycle, since it makes the heme a competent acceptor of pyridine nucleotide derived electrons. The phenomenon has been especially well studied in P450cam, and has been discussed in terms of 'spin state control' of redox equilibria (23). An increase in the reduction of NOS ferriheme by NADPH has been reported after arginine addition (24), suggesting that a similar coupling of substrate binding and heme midpoint potential plays a role in that system as well.

Previous studies of independently expressed reductase and oxygenase domains using potentiometric and stoichiometric titration have produced important information about the midpoint potentials of the heme and flavin components (25-27). These results provide indications of redox equilibria in intact enzymes, and indicate that at least in separate domains heme and flavin potentials are calmodulin independent. We have reported preliminary results from NOS holoenzymes (e.g., (28,29)); here we describe important aspects of the redox behavior and coupling to protonation of three NOS mammalian isoforms.
Methods

Materials. All chemicals used for purification were obtained from Sigma Chemical Co. NNA and BH$_4$ were from Research Biochemicals International (Natick, ME).

Expression and purification of iNOS. The human iNOS $\Delta 70$ enzyme fused to a histidine tag at the amino end was expressed in E. coli and purified by a combination of metal chelating and affinity chromatography as previously described (30).

Expression and purification of bovine eNOS. Bovine eNOS was expressed in E. coli and purified as previously described (31) except that we use ER2556 cells and break them using a French Press. Protein samples were frozen at -80 C at 30 $\mu$M. Enzyme sample concentrations were determined on the basis of heme concentration.

Expression and purification of rat nNOS. Rat nNOS was expressed in E. coli and purified as previously described (32), with the minor modifications noted for bovine eNOS.

Potentiometric titrations. Titrations were carried out under nitrogen essentially as described by Dutton (33) using a titration vessel with a platinum/calomel combination electrode calibrated by titrating redox mediators of known potential. Titrations were monitored optically with an Aminco DW2000 scanning dual wavelength spectrophotometer. Mediators used in the default system were: methyl viologen, 5uM; benzyl viologen, 5 uM; safranine o, 2uM; AQ 2 sulfonate, 10 uM; AQ 2,6 disulfonate, 10 uM; resorufin, 2 uM; pyocyanine, 2 uM; phenazine, 5 uM; and 1,4 hydroxynaphthoquinone, 5 uM.

Titrations of optical bands were simulated using summed contributions from two heme redox states and six flavin redox states. At any wavelength these contributions are of the form:

$$OD_{heme\_ox} = c_{heme} \cdot \varepsilon_{heme\_ox} \cdot 10^{(Eh-Emheme)/RT} / (1 + 10^{(Eh-Emheme)/RT})$$

$$OD_{heme\_red} = c_{heme} \cdot \varepsilon_{heme\_red} / (1 + 10^{(Eh-Emheme)/RT})$$
In these expressions $R$ is the gas constant, $F$ is the Faraday constant, and $T$ is the Kelvin temperature. The value of $RT/F$ at 25°C is approximately 59.6 mV. The variables of the form $\varepsilon_{\text{FMN}}$ represent extinction coefficients of the redox states of the hemes and flavins at wavelengths chosen for analysis; OD values are the contributions of individual components to the optical density. Variables of the form $c_{\text{heme}}$ represent the total concentration of all heme (or, equivalently, flavin) states, including redox, spin, and conformational states, in rapid equilibrium on the time scale of redox equilibration in the experiment. $E_h$ is the standard potential relative to the hydrogen electrode as measured potentiometrically. The midpoint potentials of the one electron couples are specified by the parameters $E_{\text{heme}}$, $E_{1\text{FMN}}$, and $E_{2\text{FMN}}$. $E_{1\text{FMN}}$, $E_{2\text{FMN}}$, $E_{1\text{FAD}}$, and $E_{2\text{FAD}}$ are the midpoint potentials for the first and second one electron reductions of the FMN and FAD. In some cases multiple heme components which differ in concentration, extinction coefficient, and midpoint potential have been introduced for simulation purposes.

Analogous expressions are included for the three FAD reduction states. Both the apparent midpoint potentials (e.g., $E_{\text{heme}}$, $E_{1\text{FMN}}$) and the extinction coefficients at wavelength $\lambda$ are dependent on experimental conditions such as ligand concentration. Each redox state is composed of multiple ligation, conformational, and protonation states which behave as a single state in the potentiometric titration as long as they are in rapid equilibrium on the time scale of the experiment (minutes). Simulation of a titration at any wavelength is accomplished by stepping the $E_h$ and calculating the total and component O.D.s at each potential. Simulations
can be simplified by choosing wavelengths at which the extinction coefficients of several states are negligible, or by choosing isosbestic points so that the absorbance from one or more chromophores is independent of potential.

The redox titration of the flavin cofactors at a single pH thus can be described in terms of six flavin redox states, neglecting protonation, if the flavins are well described as independent electron carriers. Inclusion of cooperativity between the flavins requires nine states, but the current titrations could be well described without cooperative effects. The pH dependence of the system was modeled by inclusion of two protonation states for each flavin redox state; one state was insufficient to simulate the results. Simulation of the dependence of midpoint potential on pH was accomplished by incrementing the pH and calculating the apparent midpoint potential at each pH as previously described (34). The pH dependence of midpoint potentials is derived from ionizable groups with different pK values in the oxidized and reduced states. For a one electron couple with a single associated ionizable group, the pH dependence is of the form:

$$Em = E_0 + \frac{RT}{F} \log_{10} \left( \frac{1 + 10^{(pH-pKR)}}{1 + 10^{(pH-pKO)}} \right).$$

For a one electron couple with two such groups, in the absence of cooperativity the expression is of the form:

$$Em = E_0 + \frac{RT}{F} \log_{10} \left( \frac{1 + 10^{(pH-pKR_1)} + 10^{(pH-pKR_2)} + 10^{(pH-pKR_3)} + 10^{(pH-pKR_4)}10^{(pH-pKR_3)}}{1 + 10^{(pH-pKO_1)} + 10^{(pH-pKO_2)} + 10^{(pH-pKO_3)}10^{(pH-pKO_2)}} \right).$$

The factor RT/F introduces characteristic pH dependences which are multiples of 59.6 mV except in the vicinity of a pK. Interactions between the ionizable groups designated by the subscripts 1 and 2 can be introduced by multiplying the cross terms by factors $\delta_{ox}$ and $\delta_{r}$ which modify the equilibrium constant for the protonation of one group when the other is protonated.
Results

Potentiometric titration of nNOS holoenzyme Changes in the redox state of cofactors are evaluated using their spectral features in the UV-visible region. The optical spectrum of the enzyme is dominated by the Soret band of the ferriheme at 400 nm. The Soret band is sensitive to redox and spin state, and in nNOS as isolated includes contributions from high and low spin heme. Oxidized flavins absorb at 380, 470, and 520 nm; the 380 nm band is obscured by the heme Soret band, and the 520 nm band is visible as a prominent shoulder on the 470 nm band. The disappearance of these bands is a measure of the reduction of FMN and FAD. Both flavin semiquinones contribute bands at 600-650 and 520 nm. The band at 650 nm is overlapped by a charge transfer band from high spin ferriheme. We routinely take spectra at a few points during an initial reductive titration of the sample, followed by a more detailed oxidative titration.

Spectra taken at the same potential during the oxidative and reductive phases are nearly identical except for a small dilution correction. NNOS and eNOS have each been titrated more than ten times with reproducible results.

Figure 1 shows spectra obtained during a titration at pH 7. The reduction of the heme causes the Soret maximum to shift with a midpoint potential of slightly above -300 mV. As the potential is lowered the oxidized flavin bands in the 450-500 nm region are bleached, and a reduced heme alpha band complex becomes prominent near 560 nm. Spectra shown have had mediator contributions, obtained from independent titrations, subtracted. The maximum corrections for mediators in the spectral and potential regions covered are about 0.25 O.D. units in the Soret due to the sharp viologen bands at 400 nm, and 0.1 O.D. units elsewhere. The spectra of fully oxidized and fully reduced enzyme so corrected are very similar to the spectra of oxidized and reduced enzymes without mediators, and titrations were conducted with concentrations of individual mediators 10-fold lower without significantly altering the spectra produced. The cost
of lowering the mediator concentration is visibly slower equilibration and greater uncertainty in potential measurement.

The region with the highest information content includes the overlapped bands at 600-650 nm. Because these bands are due to flavin semiquinones and high spin ferriheme, they contain information about all five one-electron Em values. Equivalent information can be extracted by simulations of other wavelengths, but the blue neutral semiquinone absorbance is followed most easily in the 600-650 nm region. The semiquinones also absorb near 520 nm.

A plot of the absorbance at 650 nm during a titration is shown in Figure 2 together with a simulation, which includes the individual contributions of heme, FAD and FMN. The simulation makes use of the known properties of the heme and flavin chromophores. The oxidized and reduced flavins do not contribute in this region, and the heme contribution is solely from the charge transfer band of the high spin ferric state; this allows us to set the extinction coefficients of the five non-contributing states to zero. The high potential absorbance is thus primarily due to oxidized heme, establishing the extinction coefficient for simulation purposes. The flavin semiquinone extinction coefficients, together with the potentials E1 and E2 for each flavin, determine the magnitude and potential dependence of the flavin radical contribution. In a typical titration 15-20 data points contribute to the determination of the five unknown parameters, including the heme midpoint potential. Initial simulations assume equivalent contributions from FMN and FAD semiquinones in that the concentrations and extinction coefficients are set equal. Fine adjustments to simulations sometimes produce the best fit with one flavin in slight excess (10% or less) over the other.

At low potentials the bands in this region disappear, but as the potential is raised heme oxidation and FAD radical formation contribute to the absorbance. At slightly higher potential FMN semiquinone formation becomes significant; since the FMN radical is more stable than the FAD radical the maximum intensity of the band is reached above the Em values of the
FMN/FMNH\textsuperscript{\textdagger} and FADH/FADH\textsubscript{2} couples, which are similar. Since the FMN radical is quite stable the maximum intensity plateaus before falling to the intensity of the heme charge transfer band as FMN is formed.

The potentials of the five one-electron couples for nNOS are given in Table I. The flavin couples are -250 mV for the FAD/FAD\textsuperscript{\textdagger} couple (E\textsubscript{1FAD}), -260 mV for the FADH/FADH\textsubscript{2} couple (E\textsubscript{2FAD}), -220 mV for the FMNH/FMNH\textsubscript{2} couple (E\textsubscript{2FMN}), and -120 mV for the FMN/FMNH\textsuperscript{\textdagger} couple (E\textsubscript{1FMN}). The midpoint potential (Em\textsubscript{7,0}) of the heme is -290 mV when fitted as a single component, but can also be well described by two components as described later.

Similar results are obtained from the titration of the other bands. The titration of the flavin absorbance around 480 nm is shown in Figure 3. The data are well described by the heme and flavin potentials given above, but the contribution of the radicals is not significant enough to determine the E\textsubscript{2} potentials of FMN and FAD from this spectral region.

A simulation of nNOS titration data at 560 nm is shown in figure 4. The 560 nm band can be simulated in much the same way as the 650 nm band, but the contributions from the overlap of the 520 nm semiquinone band are weak, so the flavin potentials are less reliable. As indicated by the simulation, the titration is consistent with the Table I values for the flavin potentials. The data are described better by including two heme components, one of which is significantly lower in potential than the best single heme component fits obtained at other wavelengths.

Figure 5a and 5b show plots of the same titration monitored in the Soret region. The heme Soret band titration has a midpoint consistently 15-20 mV above the value obtained from the 650 nm band when fit as a single component as in Figure 5a. While this difference is at the edge of resolution primarily because of the range of acceptable simulation fits to the data, the consistently lower potential obtained in comparison to other bands in the same titration makes us confident in the discrepancy. As shown in figure 5b, a two (heme) component fit significantly
improves the simulation. The majority component in the simulation shown has a midpoint of -250 mV, while the minority component, accounting for about 20% of the heme contribution, has a potential of -350 mV; reasonable fits can be obtained with potentials down to -400 mV for the low potential component because of endpoint uncertainty for the minor component.

The heme component of the 650 nm band is the result of high spin ferriheme only, while the formation of the 560 nm band can be the sum of the reduction of ferric high spin and low spin components. A reasonable hypothesis explains the titration in terms of a majority, primarily high spin component titrating at -250 to -270 mV with the 650 nm band, and a minor primarily low spin component titrating at ~-350 mV, which contributes at 650 nm only to the extent that it equilibrates rapidly with a high spin form.

In the absence of arginine the titration of (primarily low spin) nNOS heme is heterogeneous, and the midpoint potential is around -350 mV. Titrations of nNOS preparations without arginine or tetrahydrobiopterin are very heterogeneous. This can be observed spectrally in the Soret and elsewhere and in the titration of the heme as multiple components (data not shown). The heme is primarily low potential and low spin, and heterogeneity reflects the existence of a variety of states not in rapid equilibrium.

In general agreement with previous results obtained with reductase domains (25) and with our results from eNOS holoenzyme, no effect on either heme or flavin potentials was observed from the addition of Ca\(^{2+}\)/calmodulin to the titrations. This is significant since NO synthesis is controlled by regulation of electron transfer from NADPH to heme.

**Potentiometric titration of iNOS holoenzyme** The spectra of iNOS during potentiometric titration are similar to the nNOS spectra shown in Figure 1 and have been omitted to save space. As in nNOS, oxidized flavin bands are clearly visible around 500 nm in oxidized samples. As the potential is lowered, oxidized flavin bands decrease in intensity, to be replaced by longer wavelength species characteristic of blue neutral flavosemiquinones at 600-650 nm. This species
decreases as the potential is lowered further. A ferriheme charge transfer band contributes to the absorbance in the 600-650 nm region; it is visible without the flavin contribution in very oxidized samples, and decreases as the heme is reduced. In the Soret region the initial change during reduction is due to the loss of the bands of the oxidized flavins, followed by a red shift in the heme Soret maximum concurrent with the latter stages of flavin reduction.

In Figure 6 the absorbance at 650 nm is plotted as a function of Eh. The results are similar to those obtained with nNOS. The simulation of the data again includes contributions of heme, FAD and FMN. The maximum intensity at intermediate potentials is due to FMN semiquinone formation; since as in nNOS the FMN radical is more stable than the FAD radical, producing maximum semiquinone concentration above the Em values of the FMN/FMNH and FADH/FADH₂ couples. The maximum intensity of the 650 nm band again has a broad peak as the potential is raised before falling to the intensity of the heme charge transfer band as oxidized FMN is formed.

The potentials of the five one electron couples for iNOS are given in Table I. The flavin couples are -240 mV for the FAD/FADH couple (E_{1FAD}), -270 mV for the FADH/FADH₂ couple (E_{2FAD}), -245 mV for the FMNH/FMNH₂ couple (E_{2FMN}), and -105 mV for the FMN/FMNH couple (E_{1FMN}). The midpoint potential (Em₇,0) of the heme is -270 mV. These results are consistent with measurements at other wavelengths (plots not shown). Two titrations of iNOS gave consistent results, but because of limitations on the amount of available material the data are of lower quality than the nNOS and eNOS results.

Potentiometric titration of eNOS: pH dependence of holoenzyme midpoint potentials

Potentiometric titration of eNOS produced absorbance spectra very similar to the spectra of nNOS shown in Figure 1, and have been previously presented by us in preliminary form (28). Figure 7 shows plots of the titration of the 600 and 650 nm bands of eNOS holoenzyme at three
pH values. The data can be readily simulated using the potentials shown in Table I, which are generally similar to the nNOS and iNOS results.

The most notable feature of these plots is the extension of the potential range at which radicals can be observed on the low potential side of the titration as the pH is raised from 7 to 9. High quality simulations, generally consistent with these values but suggesting the presence of a minor low potential heme component, can be readily produced at other wavelengths; e.g., for 407 nm, 480 nm, and 560 nm. At pH 9 a slight decrease in the intensity of the heme Soret band occurs during the time course of a titration (about four hours); the loss in intensity cannot be accounted for by dilution.

Figure 8a shows the pH dependence of the midpoint potentials of the eNOS FAD couples in the range from pH 7 to pH 8. Both one electron couples are pH dependent; since the dominant form of the radical in this region is the blue neutral semiquinone, the primary species observed are FAD, FADH\textsubscript{1}, and FADH\textsubscript{2}. The pH dependence is slightly less than RT/F, expected for a ratio of 1 proton per electron (~45 mV/pH unit rather than 59.6 mV/pH unit); the theoretical lines were obtained by including pK values of 6.7 and 7.4 associated with the oxidized flavin, 7.4 and 9.2 associated with the semiquinone, and 9 and 10 associated with the reduced flavin.

The semiquinone pK of 9.2 may reflect the transition from the neutral to the anionic form; we can detect no spectral evidence of red semiquinone, and the maximum concentration of the blue species rises as the pH is raised, but it is difficult to rule out small contributions from the red species arising from pK values above the pH at which we were able to work. The 7.4 value cannot reflect deprotonation of the isoalloxazine ring, since this would result in observation of the red species as a majority at pH 8 and pH 9. Neither of the pK values associated with the oxidized flavin are likely to be associated with the isoalloxazine ring system, but may instead reflect protonation of nearby groups linked to flavin redox/protonation state. A reduced flavin pK value of 9 is necessary to account for the pH dependence; this may reflect the transition
between FADH$_2$ and FADH-. The second reduced pK was included for simulation purposes so that the number of sites is the same in all redox states; any value above 10 is consistent with the data.

Figure 8b shows the pH dependence of the midpoint potentials of the eNOS FMN couples. The pH dependence of the semiquinone/reduced FMN couple is simulated with a single pK of 8 for the semiquinone and 9.5 for the reduced form. As in the case of FAD, the protonation associated with the semiquinone is not reflected in a red/blue transition in this pH range. Unexpectedly, the oxidized FMN/semiquinone couple appears to be essentially pH independent. This implies that at all pH values in this range no protonations accompany reduction to the semiquinone; hence, a group associated with the oxidized flavin must have a pK~8, similar to that of the semiquinone. A caveat is that this couple appears to equilibrate more slowly than the other flavin couples, perhaps reflecting a mismatch with the potentials of the FMN couples and a mechanism related lack of accessibility to mediators. We point out that the reductase domains of NOS, because of their complex control mechanism, can sometimes take hours or days to transfer electrons, and that the FMN/FMNH$_2$ couple is not part of the catalytic cycle.

The midpoint potential of the heme is pH independent in this range even in arginine replete preparations. This implies that any pH dependence of arginine binding is independent of the redox state of the heme. In the absence of arginine the heme titration is heterogeneous; the overall half reduction potential is typically about -320 mV at pH 7, but there is a small high potential component even in preparations to which no arginine has been added.

Similar experiments with nNOS holoenzyme yielded nearly identical results. As with eNOS, the heme and the FMN oxidized/semiquinone couples are pH independent, and the flavin semiquinone is predominantly in the blue neutral state in the pH range from 7 to 9. As in eNOS, the other three flavin couples are pH dependent, but in nNOS the pH dependence of these couples is ~55 mV/ pH unit, close to RT/F. No specific pK values were required to fit the data.
The predominant states are FAD, FADH\(^+\), FADH\(_2\), FMN, FMNH\(^+\), and FMNH\(_2\). The data for iNOS are not as good, but it is clear that at high pH three of the flavin couples are shifted to lower potential, indicating that the majority protonation states are the same.

The lack of pH dependence for the FMN oxidized/semiquinone couple suggests the presence of a group coupled primarily to the protonation state, rather than the oxidation state, of the isoalloxazine ring. Such a group would be protonated in the oxidized state but deprotonated in the reduced and neutral semiquinone states; this suggests in turn that its protonation is stabilized by H bonding to a ring nitrogen.
Discussion

Potentiometric titration of NOS isoforms provides information about the thermodynamics of electron transfer and associated protonation/deprotonation events, and in addition about the coupling of redox processes to ligand binding. Since control of NO synthesis is exerted through electron transfer from NADPH to heme through the flavin cofactors, understanding the thermodynamics of this process is important in a full description of regulation.

The electrochemistry of the three mammalian NOS isoforms appears to be quite similar. A modest shift in the potential of the heme on arginine binding makes the final step from FMNH$_2$ to heme nearly isopotential, and indeed the FeIII/FeII heme couple and three of the four flavin couples form a nearly isopotential group slightly higher in potential than the pyridine nucleotides which reduce them. The exception is the high potential FMN/FMNH$^-$ couple, which is about 120 mV too positive to be an effective electron donor to heme.

The NAD$^+/$/NADPH couple, at about -325 mV, is well positioned to reduce the three flavin couples in the isopotential group, and the high potential heme component is no more than thirty mV lower in potential than the FMN couple which reduces it. This slightly uphill reaction can be readily pulled by oxygen or pushed by excess NADPH. The potential shift on substrate binding is relatively small compared to the effects seen in P450 systems (21-23). The potential shift observed in eNOS varied between 40 and 60 mV with saturating arginine, while nNOS preparations exhibited shifts as large as 80mV. The simplest interpretation implies that the Kd for arginine in reduced eNOS is 5-10 times smaller than in the oxidized enzyme; while a similar estimation suggests that the arginine Kd is ~20 times smaller in reduced nNOS than in oxidized nNOS. This almost certainly reflects isoform differences in the linkages between ligand binding, conformation, and dimerization rather than simple affinities of the reduced state. Interactions
between ligands and their effects on redox equilibria in NOS is a complex subject which will be treated elsewhere.

The heme potentials measured in NOS holoenzymes are slightly lower than those observed in independently expressed oxygenase domains. This is apparently an effect of interactions between the oxygenase and the reductase domains, corresponding to a slight stabilization of the oxidized state. In nNOS, the potential of the arginine replete heme (-220 mV; ref. 27) is ~ 30 mV higher than the value obtained for the majority holoenzyme component. The arginine induced shift is much smaller in the isolated domains, suggesting that the removal of restrictions imposed by the reductase domains stabilizes the reduced, arginine free state relative to the oxidized state.

There are no detectable effects of arginine binding on the flavin midpoint potentials. As indicated by titrations of NOS reductase domains, the thermodynamics of the system are closer to P450 reductase than the BM3 reductase domains. The NOS isoforms differ from P450 reductase in that the FADH/FADH₂ couple is nearly isopotential with the FAD/FADH and FMNH/FMNH₂ couples as indicated in Table I.

As in titrations of reductase domain constructs (25), no significant effects of calcium/calmodulin on any of the midpoint potentials were observed. This is consistent with the concept of control through domain alignment, since the electron transfer tunneling rate must be regulated by distance rather than thermodynamics (35,36). The nature of the intervening protein is not a significant factor in the modulation of electron transfer (37). Consequently, the major determinants of the electron transfer rate are the thermodynamic parameters, shown here to be invariant, and the distance between redox centers. Since the heme and FMN moieties of NOS cannot be docked to within 25Å using the solved structures of reductase domains, significant domain realignment must occur to enable catalytic rates of electron transfer.

As we previously proposed (35), the FMN binding domain probably plays a central role in these realignments. Two domain enzymes homologous to the FAD and NADPH binding
domains of NOS are reduced by ferredoxins or flavodoxins; the latter are homologous to the NOS FMN binding domains, and serve as an electron shuttle between FAD and redox components in other systems (e.g., Photosystem I). In our proposal the FMN binding domain of NOS acted as a ‘tethered shuttle’. After releasing from its site on the FAD/NADPH domain duplex, to which it remains covalently attached by a polypeptide linker, the FMN bearing edge of the domain can approach the heme site on the oxygenase domain, to which it is also connected through the calmodulin binding site. Calmodulin binding enables the shuttle; this probably involves facilitation of the release of the FMN binding domain from the FAD/NADPH domain duplex. An obvious potential trigger for the redox modulation of interdomain interactions is the redox linked protonation of groups in domain interfaces.

The pH dependence of nNOS and eNOS establishes that the primary forms of the three redox states of both flavins are the neutral species between pH 7 and 9; the extra proton present when FMN is oxidized is probably not directly associated with the flavin. The effects of ionizable groups in the polypeptide which are coupled to flavin redox/protonation state are detectable, especially through the pH independent midpoint potential of the FMN oxidized/semiquinone couple. The pK values used to produce the simulations in figure 8 represent the simplest fit to the data, but pH dependences which differ slightly from multiples of RT/F can be produced by several ionizable groups weakly coupled to the redox and/or protonation states of the flavins.

Given the other constraints on the system, notably the persistence of the blue neutral semiquinone up to pH 9, the pH dependence data imply the existence of a group which is protonated in the FMN oxidized state and deprotonated in the presence of the blue neutral FMN semiquinone. This can only be due to coupling of the ionizable group to FMN protonation, as might arise from H bonding between an isoalloxazine ring position and the group in at least one redox/protonation state. Given the fact that oxidized FMN is not expected to participate in catalysis, this is of limited importance.
In conclusion, the behavior of the NOS isoforms during potentiometric titrations indicates a basic similarity between the NOS reductase domains and P450 reductase. The overall similarity between the isoforms is so great that differences in activity and control cannot be assigned to thermodynamic parameters, but probably instead reflect isoform differences in either domain alignments or in the rate of transition between states with different alignments.
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<td>-232 mV</td>
<td>-280 mV</td>
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Table I: Midpoint potentials at pH 7 for arginine and biopterin replete mammalian NOS holoenzymes and related systems. Heme potentials in the single column reflect the best single component fits to the data. Heme low values are the highest consistent with titration data in the Soret and 560 nm region. NNOS reductase domain data is from ref. 25; numbers in parenthesis are in the presence of CaM unless otherwise indicated. NNOS oxygenase domain (heme) data is from ref. 26. P450 reductase data is from ref. 18, and BM3 data is from ref. 20.
Figures

Figure 1 Spectra of arginine and tetrahydrobiopterin replete nNOS recorded on an Aminco DW2000 spectrophotometer during a potentiometric titration at pH 7.0, 23C; potentials are shown above in mV. Spectra of mediator dyes have been subtracted electronically. Enzyme concentration was 1.6 uM.

Figure 2 Plot of the intensity of the 650 nm band of nNOS (OD650-OD695) simulated with contributions from the high spin ferriheme charge transfer band and the blue neutral semiquinones of FMN and FAD. The baseline has been corrected by subtraction of the most reduced spectrum. Fitting parameters are: FAD, E1 = -250, E2 = -260, ε = .02; FMN, E1 = -120, E2 = -220, ε = .02; heme, Em = -290, ε = .013. Maximum FAD semiquinone corresponds to 38%; maximum FMN semiquinone corresponds to 77%.

Figure 3 Plot of the intensity of the 480 nm band of nNOS simulated with contributions from heme, the oxidized flavins, and the blue neutral semiquinones of FMN and FAD. Potentials are those given in Table I. Fitting parameters are: FAD, E1 = -250, E2 = -260, ε = .025; FMN, E1 = -120, E2 = -220, ε = .028; heme, Em = -290, ε = .043.

Figure 4 Plot of the intensity of the 560 nm band of nNOS simulated with contributions from reduced heme and the blue neutral semiquinones of FMN and FAD. Potentials are those given in Table I. Fitting parameters are: FAD, E1 = -250, E2 = -260, ε = .009; FMN, E1 = -120, E2 = -220, ε = .009, heme; Em = -320, ε = -.067.
Figure 5 Plot of the intensity of the Soret band of nNOS simulated with contributions from heme, FMN and FAD. a) Single component fit with heme potential of -270 mV. Fitting parameters are: FAD, $E_1 = -250$, $E_2 = -260$, $c_\varepsilon = .015$; FMN, $E_1 = -120$, $E_2 = -220$, $c_\varepsilon = .015$; heme, $E_m = -270$, $c_\varepsilon = -.113$.

b) Two component fit with potentials of -250 and -350 mV; the low potential component accounts for 20% of the observed change in O.D. due to heme. Fitting parameters are: FAD, $E_1 = -250$, $E_2 = -260$, $c_\varepsilon = .015$; FMN, $E_1 = -120$, $E_2 = -220$, $c_\varepsilon = .015$; heme, high potential $E_m = -250$, $c_\varepsilon = -.091$; low potential, $E_m = -350$, $c_\varepsilon = -.022$.

Figure 6 Plot of the intensity of the 650 nm band of iNOS (OD650-OD695) simulated with contributions from the high spin ferriheme charge transfer band and the blue neutral semiquinones of FMN and FAD. Potentials are those given in Table I. Contributions from FAD and FMN are $c_\varepsilon = .0018$, and for heme $c_\varepsilon = .0011$. Enzyme concentration was 0.2 uM. Maximum semiquinone concentration for FMN is 88%, and for FAD 47% at pH 7.

Figure 7 Plots of the 650 nm band of eNOS holoenzyme vs. potential during potentiometric titrations at pH 7, 8, and 9. Simulations include contributions from the high spin ferriheme charge transfer band and the blue neutral semiquinones of FMN and FAD. Potentials used in the simulation are plotted against pH in figure 8. Absorbance unit scales for pH 7 are 0 to .06 (enzyme concentration 1.6 uM) and 0 to .12 for pH 9 (enzyme concentration 3.2 uM). Maximum FMN semiquinone concentration ranged from 87% at pH 7 to 92% at pH 9. The maximum FAD concentration is 47% at pH 7 and 40% at pH 9.

Figure 8 (left) pH dependence of the midpoint potentials of the FAD couples in eNOS holoenzyme. The data sets and accompanying simulations marked as E1 and E2 represent the pH dependences of
the two one electron midpoint potentials of FAD for the first and second reductive steps. Simulations include pK values given in the text: 6.7 and 7.4 (oxidized flavin), 7.4 and 9.2 (semiquinone), and 9 and 10 (reduced flavin). For completeness the 2 electron midpoint, which is the average of the two one electron potentials, is also shown as E12.

(right) pH dependence of the midpoint potentials of the FMN couples in eNOS holoenzyme. The data sets and accompanying simulations marked as E1 and E2 represent the pH dependences of the two one electron midpoint potentials of FMN for the first and second reductive steps. Simulations include a single pK of 8 (oxidized and semiquinone) and 9.5 (reduced FMN); see text for discussion. For completeness the 2 electron midpoint, which is the average of the two one electron potentials, is also shown.

References

nNOS ph7

- Fe III heme
- FAD radical
- FMN radical
- simulation 650
- 650 nm

OD

Eh

figure 2
figure 3
figure 6
figure 7b
figure 7c
figure 8
Thermodynamics of oxidation-reduction reactions in mammalian nitric oxide synthase isoforms


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