Thiols and Neuronal Nitric Oxide Synthase: Complex Formation, Competitive Inhibition, and Enzyme Stabilization†

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ABSTRACT: To elucidate how thiols affect neuronal nitric oxide synthase (nNOS) we studied the binding of thiols to tetrahydrobiopterin (BH_4)-free nNOS. Dithiothreitol (DTT), 2-mercaptoethanol, and L- and D-cysteine all bound to the heme with K_d values varying from 0.16 mM for DTT to 41 mM for L-cysteine. DTT, 2-mercaptoethanol, and L-cysteine yielded absorbance spectra with maxima at about 378 and 456 nm, indicative of bisthiolate complexes; the maximum at 426 nm with D-cysteine suggests binding of the neutral thiol. From the results with 2-mercaptoethanol we deduced that in 2-mercaptoethanol-free, BH_4-free nNOS the sixth heme ligand is not a thiolate. DTT binding to nNOS containing one BH_4 per dimer was biphasic. Apparently, the BH_4-free subunit bound DTT with the same affinity as the BH_4-free enzyme, whereas the BH_4-containing subunit exhibited a >100-fold lower affinity, indicative of competition between DTT and BH_4 binding. Binding of DTT to the BH_4-containing subunit was suppressed by L-arginine, whereas high-affinity binding was not affected, suggesting that L-arginine binds only to the BH_4-containing subunit. DTT competitively inhibited L-citrulline production by nNOS containing one BH_4 per dimer ($K_i \approx 11$ mM). Comparison of DTT binding and inhibition suggests that the heme of the BH_4-free subunit is not involved in catalysis. Thermostability of nNOS was studied by preincubating the enzyme at various temperatures prior to activity determination. At nanomolar concentrations, nNOS was stable at 20 °C but rapidly deactivated at higher temperatures ($t_{1/2} \approx 6$ min at 37 °C). At micromolar concentrations, inactivation was 10 times slower. Absorbance and fluorescence measurements demonstrate that inactivation was not accompanied by major structural changes. The stabilization of nNOS by thiols was illustrated by the fact that omission of 2-mercaptoethanol during preincubation for 10 min at 30 °C led to an activity decrease of up to 90%.

The important signaling and cytotoxic molecule NO is produced from the amino acid L-arginine by several isoforms of nitric oxide synthase [EC 1.14.13.39; NOS;[1] for recent reviews see Masters (1994), Griffith and Stuehr (1995), and Mayer (1995)]. L-Arginine oxidation occurs at a heme moiety located in the N-terminal oxygenase domain of the enzyme. The electrons required for reductive activation of molecular oxygen are shuttled in a calmodulin-dependent fashion from the donor NADPH through a FAD- and FMN-containing reductase domain to the heme. Electron transfer through the reductase domain requires the binding of Ca^{2+}/calmodulin; in addition, L-arginine oxidation requires the binding of (6*R*)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin, BH_4) (Mayer & Werner, 1995), probably at a site in the vicinity of the heme. Purified neuronal nitric oxide synthase (nNOS) always contains heme and BH_4 in a ratio of 2:1, with each dimer containing two hemes and one tightly bound BH_4 at most (List et al., 1996). Notwithstanding, dimers apparently half-saturated with BH_4 were stimulated 2-fold in the presence of the exogenously added cofactor, suggesting that the enzyme is able to bind two BH_4 molecules per dimer. We recently found that the BH_4 stoichiometry is due to strong anticooperativity between the binding of two BH_4 molecules (Gorren et al., 1996), whereas binding of BH_4 and L-arginine is cooperative.

The amino acid sequence of the reductase domain places it in the family of P450 reductases (Bredt et al., 1991). The oxygenase domain, although not genetically related to cytochrome P450, spectroscopically (McMillan et al., 1992; Stuehr & Ikeda-Saito, 1992; White & Marletta, 1992) and functionally (Klatt et al., 1993; Marletta, 1994; Ghosh et al., 1995) resembles cytochrome P450. Most of the spectroscopic similarities between the NOS and cytochrome P450 hemes derive from the identity of the axial ligand, which is a cysteinyl sulfur in both cases (Chen et al., 1994; Richards & Marletta, 1994; Sari et al., 1996). Thus, reduced and oxidized NOS, as well as its complexes with CO, NO, and CN^- exhibit spectroscopic properties very similar to those of cytochrome P450 (McMillan et al., 1992; Stuehr & Ikeda-Saito, 1992; White & Marletta, 1992; Wang et al., 1993; Matsuoka et al., 1994; Wang et al., 1994; Sono et al., 1995). As with cytochrome P450, addition of the substrate L-arginine causes a blue-shift of the Soret maximum (type I difference spectrum), indicative of a low-to-high spin transition (McMillan & Masters, 1993; Matsuoka et al., 1994; Rodríguez-Crespo et al., 1996), but unlike many cytochrome P450s (Dawson & Sono, 1987) NOS is already to a large extent in the high-spin state in the absence of substrate. The latter property appears to be largely due to the presence of BH_4, as BH_4-free nNOS contains a far greater fraction of low-

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[1] Abbreviations: NOS, nitric oxide synthase; nNOS and iNOS, neuronal and inducible isoforms of NOS, respectively; BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin); DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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spin heme (Gorren et al., 1996). Typical heme ligands such as imidazole (McMillan & Masters, 1993), elicit an absorbance red-shift (type II difference spectrum), diagnostic of a high-to-low spin transition. Binding of thiols to sulfur-ligated hemoproteins such as cytochrome P450 or chloroperoxidase gives rise to an unusual spectrum with a double Soret band with maxima at 370−390 and 450−470 nm (Sono et al., 1982, 1984). Studies of such complexes as well as of model compounds (Ruf & Wende, 1977) revealed that these so called hyperporphyrin spectra are common to bisthiolate−heme complexes. With native NOS such complexes were thus far not observed, but hyperporphyrin spectra were observed for urea-denatured iNOS in the presence of dithiothreitol (DTT) (Abu-Soud et al., 1995).

Because of their stabilizing effects thiols are usually added to NOS purification and storage buffers, and, in most cases, to the enzymatic assay as well [see Hofmann and Schmidt (1995) and Komori et al. (1995) and references therein)]. Contradictory results on the effects of thiols on NOS activity have been reported [see Hofmann and Schmidt (1995) and Komori et al. (1995) and references therein)]. In view of their wide use in NOS assays and the demonstrated ability to bind to the heme of NOS under some conditions, it seemed appropriate to investigate the interactions of thiols with NOS more closely. In the present study we report on the spectral properties of complexes of nNOS with several thiols, we reinvestigate the effects of thiols on enzyme activity, and we describe the thermal stability of nNOS and how it is affected by thiols and several other compounds.

MATERIALS AND METHODS

Materials. L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) was purchased from MedPro (Amersham, Vienna, Austria). Dithiothreitol, 2-mercaptoethanol, and D- and L-cysteine were obtained from Sigma (Vienna, Austria).

Enzyme Purification and Activity Assay. Recombinant rat nNOS was purified from baculovirus-infected insect cells as described previously (Harteneck et al., 1994; Mayer et al., 1996). BH_4-free nNOS was obtained from Sf9 cells treated with 2,4-diamino-6-hydroxypyrimidine according to a published procedure (List et al., 1996). Enzyme concentrations are expressed as the concentration of the monomer, assuming a molecular mass of 160 kDa. Throughout this paper the terms "BH_4-free nNOS" and "BH_4-containing nNOS" are used to refer to the enzyme with 0 and 1 equiv of BH_4 per NOS dimer, respectively. NOS activity was determined as the formation of L-[2,3,4,5-^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine (Mayer et al., 1994b).

Optical Absorbance and Fluorescence Spectroscopy. Absorbance spectra were measured with a Hewlett-Packard 8452A diode array spectrophotometer. Emission fluorescence spectra between 300 and 400 nm were measured with a Shimadzu RF-5000 spectrofluorophotometer at an excitation wavelength of 290 nm (excitation slit, 5 nm; emission slit, 5 nm).

Thiol Binding Studies. For absorbance measurements, nNOS samples were diluted to a final concentration of approximately 5 μM. On account of the 2-mercaptoethanol in the enzyme stock solutions, these samples always contained 3−6 mM 2-mercaptoethanol. For optical titrations, spectra were measured 30 s after addition of the ligand or, in case of slow equilibration (*vide infra*), after 15 min. DTT binding rates were determined in separate experiments by continuously monitoring the increase of the peak (468 nm) minus the trough (420 nm) absorbance difference induced by addition of DTT.

Optical Studies of Thermal Inactivation. Immediately prior to the absorbance and fluorescence thermal inactivation studies, the enzyme was dialyzed for 3 h (Pierce Microdialyzer System 100) against 100 mM KP_i, pH 7.4, to remove 2-mercaptoethanol. Subsequently the enzyme was incubated at the indicated temperatures and spectra were measured at 2−5 min intervals. The intensity of the fluorescence of nNOS at zero incubation time decreased at higher temperatures (unpublished observations), probably because of the increased mobility of the tryptophans. Therefore, as a control, the samples obtained after 30 min incubation were cooled down to 20 °C and compared with samples that had not been heated.

RESULTS AND DISCUSSION

Binding of Thiols to BH_4-Free nNOS

BH_4-free nNOS readily bound DTT, giving rise to an absorbance spectrum with Soret maxima at 378 and 456 nm (Figure 1A). We already suspected that the shoulders at 370−380 and 456 nm in the absorbance spectrum of BH_4-free nNOS are due to the formation of a bisthiolate complex with the 2-mercaptoethanol derived from the nNOS stock solutions. It therefore came as no surprise that BH_4-free nNOS also bound 2-mercaptoethanol (maxima at 376 and 456 nm, Figure 1B), though with much lower affinity than DTT. The absorbance spectrum of BH_4-free nNOS in the complete absence of thiols could not be measured directly, because the enzyme is not stable under those conditions, but we could reconstruct the spectrum from the samples containing 6 mM and 0.17 M 2-mercaptoethanol and the K_d value of 15 mM determined below (Figure 1B). Since the reconstructed spectrum exhibited a strong absorbance at 418 nm and a weak broad shoulder at about 390 nm but was devoid of bisthiolate contributions at 380 and 456 nm, we can definitively rule out that in BH_4-free nNOS a cysteine thiolate serves as the sixth heme ligand. The bisthiolate complex observed in electron paramagnetic resonance spectra of BH_4-free nNOS (McMillan et al., 1996) is most likely caused by complex formation with the 2-mercaptoethanol from the enzyme stock solutions.

D-Cysteine also bound to nNOS, but instead of the hyperporphyrin spectrum typical of bisthiolate ligation, one band with a maximum at 426 nm appeared (Figure 1C). Similar spectra have been found with a number of thiols in the case of cytochrome P450 and were ascribed to the binding of the neutral thiol rather than the thiolate (Sono et al., 1982). Remarkably, formation of the low-spin compound was only observed with the D-form, whereas with L-cysteine the resulting spectrum (maxima at 370 and 454 nm) closely resembled that obtained with DTT and 2-mercaptoethanol (not shown).

Titrations of the optical changes revealed large differences in affinity between the thiols (Figure 2). Whereas for DTT a dissociation constant of 0.16 ± 0.01 mM could be estimated, titration of the 2-mercaptoethanol-induced absorbance changes yielded a K_d of 15 ± 1 mM. Glutathione did not bind to the enzyme up to a concentration of 20 mM,

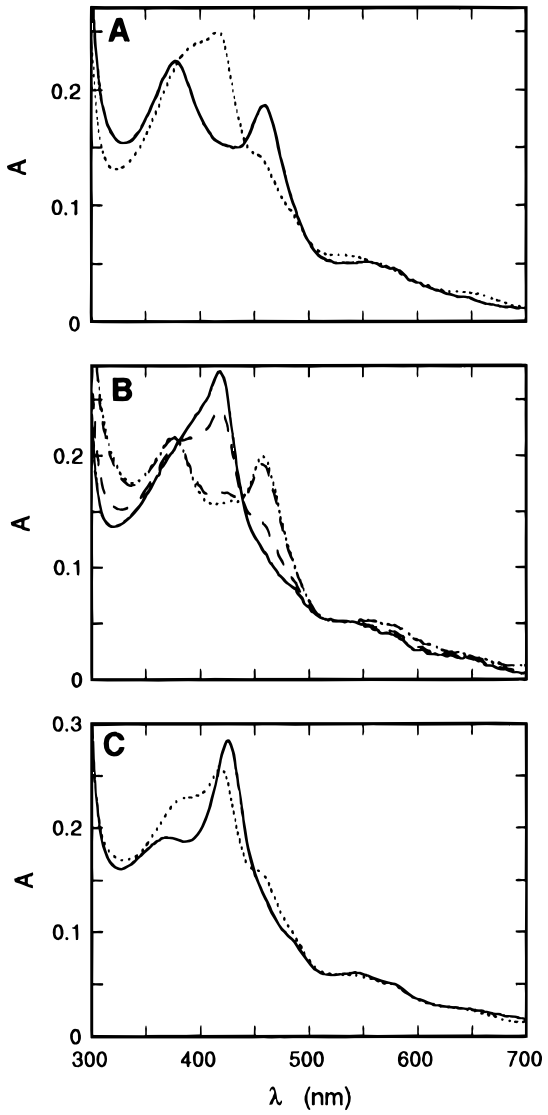


FIGURE 1: Effect of thiols on the absorbance spectrum of BH_4-free nNOS. (A) Effect of DTT. Absorbance spectra were measured before (dashed line) and after (solid line) the addition of 10 mM DTT. (B) Effect of 2-mercaptoethanol and the calculated spectrum of thiol-free BH_4-free nNOS. From the spectrum of BH_4-free nNOS without additions (containing 6 mM 2-mercaptoethanol) and the spectrum after addition of 0.17 M 2-mercaptoethanol, spectra of 2-mercaptoethanol-free and 2-mercaptoethanol-saturated nNOS were constructed, applying at all wavelengths the equation $A = [BH_4\text{-free nNOS}]\{\epsilon_{+2ME}[2ME]/(K_d + [2ME]) + \epsilon_{-2ME}K_d/(K_d + [2ME])\}$, in which A is the absorbance, [BH_4-free nNOS] and [2ME] are the total enzyme and 2-mercaptoethanol concentrations, ϵ_{-2ME} and ϵ_{+2ME} are the absorbance coefficients of BH_4-free nNOS and its complex with 2-mercaptoethanol, and K_d is the equilibrium dissociation constant for 2-mercaptoethanol (15 mM, Figure 2). The solid line is the calculated spectrum of BH_4-free nNOS, and the dotted line is the spectrum of the 2-mercaptoethanol-saturated enzyme. The dashed lines are the observed spectra with and without 0.17 M 2-mercaptoethanol. (C) Effect of D-cysteine. Absorbance spectra were measured before (dashed line) and after (solid line) the addition of 20 mM D-cysteine. Experimental conditions for all spectra: 4.7 μM BH_4-free nNOS; 50 mM triethanolamine/HCl (pH 7.0); 25 °C.

probably because of steric constraints (not shown). Surprisingly, L-cysteine had a 10-fold lower affinity than D-cysteine (K_d values of 3.6 ± 0.5 and 41 ± 4 mM for D- and L-cysteine, respectively; Figure 2). The origin of the striking difference between the two stereoisomers is unclear but may involve a site on NOS that binds L-amino acids (Sennequier & Stuehr,

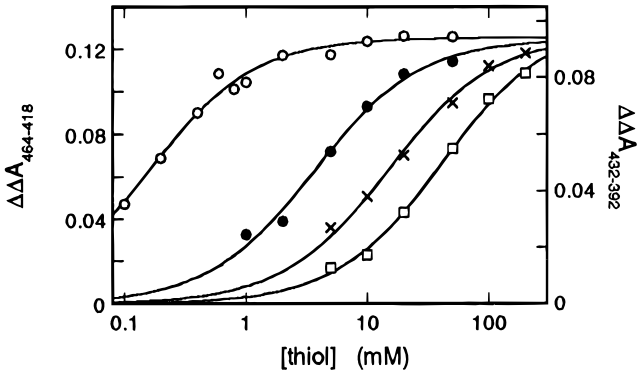


FIGURE 2: Titration of BH_4-free nNOS with thiols. Absorbance spectra of BH_4-free nNOS were measured at various thiol concentrations. Plotted are the changes in the peak-to-trough absorbance difference of the Soret band as a function of the thiol concentration for DTT (open circles), 2-mercaptoethanol (crosses), L-cysteine (open squares), and D-cysteine (closed circles). For DTT, L-cysteine, and 2-mercaptoethanol the peak and trough were at 464 and 418 nm, for D-cysteine at 432 and 392 nm. The lines drawn through the data points were fitted to the function $\Delta\Delta A = \Delta\Delta A_{max}[thiol]/(K_d + [thiol])$. The fitting parameters are listed here for DTT ($K_d = 0.16 \pm 0.01$ mM, $\Delta\Delta A = 0.126 \pm 0.002$), for D-cysteine ($K_d = 3.6 \pm 0.5$ mM, $\Delta\Delta A = 0.094 \pm 0.003$), for 2-mercaptoethanol ($K_d = 15 \pm 1$ mM, $\Delta\Delta A = 0.126 \pm 0.003$), and for L-cysteine ($K_d = 41 \pm 4$ mM, $\Delta\Delta A = 0.133 \pm 0.004$). Experimental conditions: 4.7 μM BH_4-free nNOS; 50 mM triethanolamine/HCl (pH 7.0); 25 °C.

1996). Binding of L-cysteine to this site might not cause large absorbance changes but would be expected to sterically hinder the binding of a second L-cysteine to the heme. Since D-cysteine would bind to the heme only, this may explain the great difference in affinity for the heme between the stereoisomers. The presence of a site in the heme pocket that specifically binds L-amino acids agrees with previous reports that the enzyme does not bind D-arginine (McMillan & Masters, 1993; Marletta, 1994). It can, however, not be excluded that the difference in affinity is peculiar to the geometry of the heme pocket.

Competitive Inhibition of nNOS by DTT

The observation of thiol binding to nNOS suggests that, like other heme ligands such as imidazole (Mayer et al., 1994a), thiols can act as L-arginine-competitive enzyme inhibitors. In most previous reports, however, no clear inhibitory effect of thiols was found (Hofmann & Schmidt, 1995). Since we suspected that this apparent inability of thiols to inhibit NOS activity is due to the high affinity of NOS for BH_4 in the presence of L-arginine (Gorren et al., 1996), we decided to reinvestigate the effect of thiols on the activity of nNOS in the absence of exogenous BH_4, which would lower the residual BH_4 concentration to approximately 3 nM, instead of the 10 μM present under standard assay conditions. Under these conditions we indeed observed concentration dependent inhibition by DTT (Figure 3A). For L-arginine concentrations between 4 and 50 μM, we observed an increase in IC_{50} from 18 ± 5 mM to 39 ± 1 mM indicating that DTT inhibition is competitive. In the case of purely competitive inhibition IC_{50} will equal $(1 + [S]/K_m)K_i$. A plot of IC_{50} vs [Arg] for six different L-arginine concentrations between 4 and 50 μM could be fitted to a straight line (not shown). From this plot and Figure 3A one can estimate, in the absence of exogenous BH_4, an apparent K_i for DTT of 11 ± 2 mM, a V_{max} of 470 ± 50 nmol of

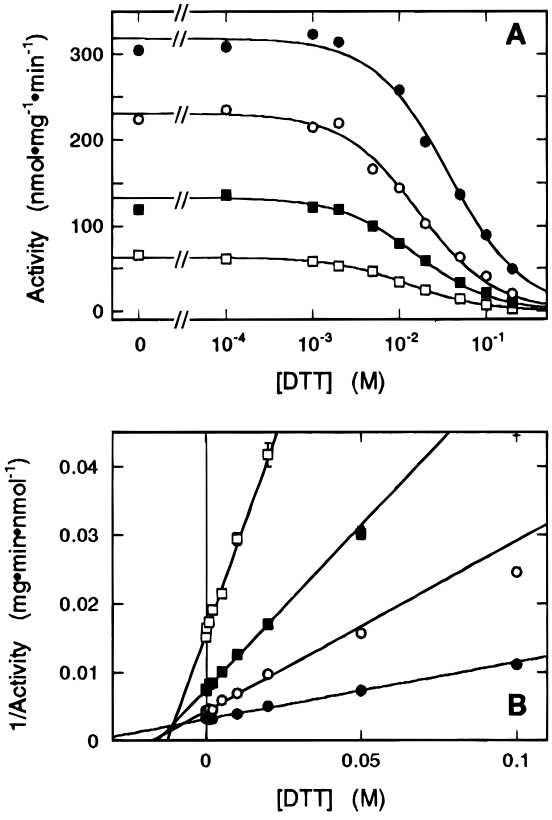


FIGURE 3: Inhibition of nNOS by DTT in the absence of exogenous BH_4. (A) Concentration response curves of the inhibition by DTT of BH_4-containing nNOS (approximately 1 equiv of BH_4 per dimer) in the presence of 4 (open squares), 10 (closed squares), 20 (open circles), or 50 (closed circles) μM L-arginine. The lines drawn through the data points were fitted to the function Activity = $\text{Activity}_{max}/(IC_{50} + [DTT])$, in which Activity_{max} is the activity in the absence of DTT and IC_{50} is the DTT concentration exerting half-maximal inhibition. (B) Dixon plot of the same data. The lines are best fits to the weighted data. To allow visual inspection of the points of intersection the seven outward most data points ([DTT] = 0.2 M and/or 1/activity > 0.45 mg min nmol^{-1}) are not in the figure; they were, however, included for the fit. The plot yields a K_i of 11 ± 4 mM. Experimental conditions: 6 nM nNOS, 4, 10, 20, or 50 μM [^{3}H]arginine (~50 000 cpm), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 0.5 mM $CaCl_2$, 10 μg/mL of calmodulin, 0.2 mM CHAPS, 2.4 mM 2-mercaptoethanol, 0.125 mM EDTA, and 50 mM triethanolamine/HCl (pH 7.0) were incubated at 37 °C for 10 min, followed by separation and detection of [^{3}H]citrulline.

L-citrulline mg^{-1} min^{-1}, and a K_m of 21 ± 5 μM. Comparable values for V_{max} and K_m were obtained in direct determinations of the L-arginine concentration dependence of the activity of nNOS in the absence of exogenous BH_4 (not shown). A K_i^{app} for DTT of 11 mM was also obtained from a Dixon plot (Figure 3B). This value should not be regarded as the K_d of DTT, since it is still strongly affected by the residual BH_4. The true affinity of NOS for DTT, as determined from the titration of the spectral changes caused by DTT is 2 orders of magnitude greater. From these data one can estimate the BH_4 dissociation constant of the high-affinity pteridine site of nNOS with the equation $K_i^{app}(DTT) = K_d(DTT)\{1 + [BH_4]/K_d(BH_4)\}$. Substituting $K_i^{app}(DTT) = 11$ mM, $K_d(DTT) = 0.16$ mM, and $[BH_4] \cong 3$ nM, one arrives at a value in the order of 10^{-11} M for binding of the first BH_4 molecule to pteridine-free nNOS. This extremely low dissociation constant might explain why one BH_4 molecule remains tightly bound to dimeric NOS during enzyme purification and is not readily replaced by pterin-based NOS inhibitors (Klatt et al., 1994; Werner et al., 1996).

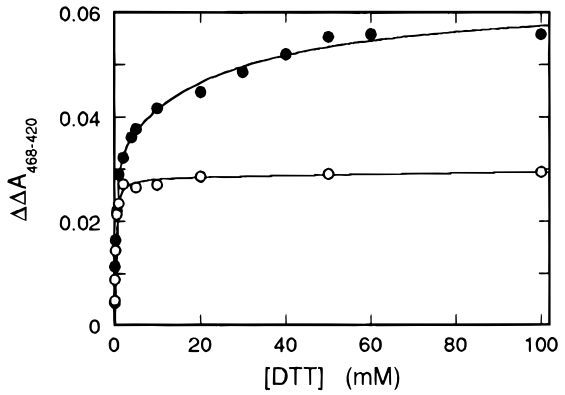


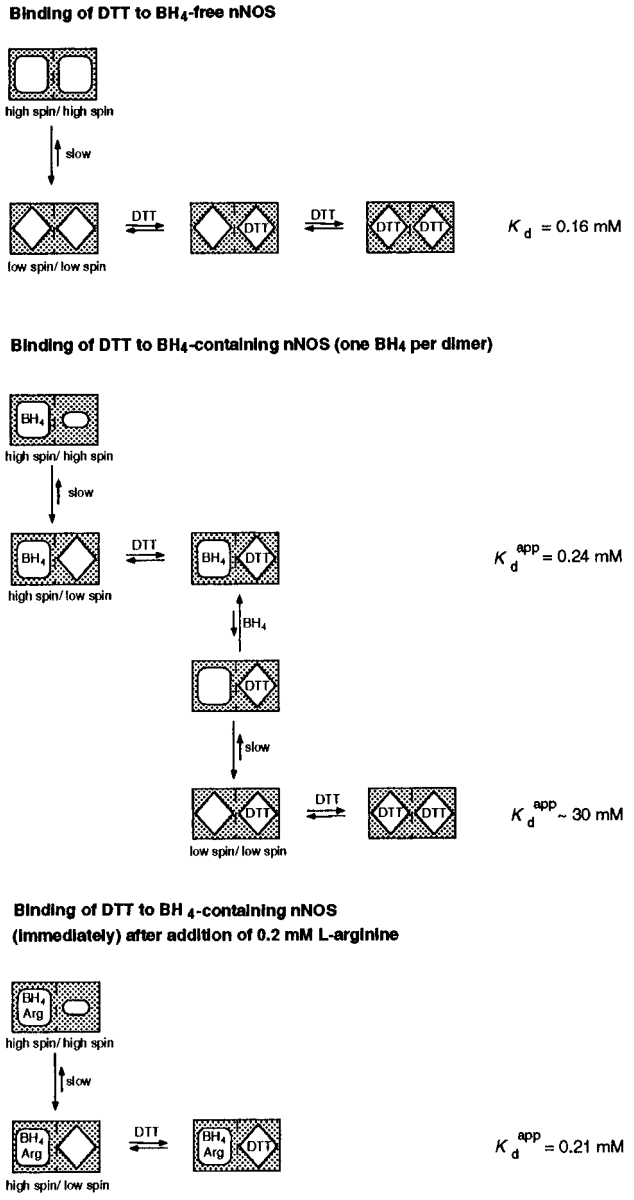
FIGURE 4: Titration of BH_4-containing nNOS with DTT. Absorbance spectra of BH_4-containing nNOS (approximately 1 equiv of BH_4 per dimer) were measured at various DTT concentrations. Plotted are the changes in the peak-to-trough absorbance difference of the Soret band (468 nm − 420 nm) as a function of the DTT concentration both in the absence (closed circles) and presence (open circles) of 0.2 mM L-arginine. The line drawn through the data points in the presence of L-arginine was fitted to the function $\Delta\Delta A = \Delta\Delta A_{max}[DTT]/(K_d + [DTT])$; the data points in the absence of L-arginine were fitted to the sum of two such functions. For the fitting parameters see Results and Discussion. Experimental conditions: 3.6 μM nNOS; 50 mM triethanolamine/HCl (pH 7.0); 25 °C.

The fact that thiols other than DTT, such as 2-mercaptoethanol and D- or L-cysteine, bound less tightly, explains why these compounds do not inhibit NOS activity at concentrations up to 10 mM (results not shown). One prior paper reported complete inhibition by L-cysteine at 4 mM and no inhibiton by D-cysteine, DTT or 2-mercaptoethanol at up to 10 mM (Komori et al., 1995). Another study found no appreciable inhibition by any thiols at up to 10 mM (Hofmann & Schmidt, 1995). Whereas no explanation can be given for the inhibition by L-cysteine in the former study, the presence of BH_4 at micromolar concentrations in both studies can account for the apparent lack of inhibition by DTT.

Binding of DTT to BH_4-Containing nNOS

Since the activity of nNOS was affected by DTT in the presence of low L-arginine and BH_4 concentrations, DTT binding is expected to be observed optically under the same conditions. However, since optical experiments require an enzyme concentration in the micromolar range, a BH_4 concentration of 3 nM can not be attained with BH_4-containing nNOS. Nevertheless we attempted to measure binding of DTT to 3.6 μM BH_4-containing nNOS (yielding a BH_4 concentration of 1.8 μM), and observed partial formation of the hyperporphyrin spectrum (not shown). The titration curve was clearly biphasic (Figure 4), with about half of the sites ($\Delta\Delta A = 0.034 \pm 0.003$) binding DTT with high affinity ($K_d = 0.24 \pm 0.07$ mM) and the other half ($\Delta\Delta A = 0.030 \pm 0.004$) with low affinity ($K_d = 30 \pm 10$ mM). Remarkably, the low-affinity phase was completely abolished in the presence of 0.2 mM L-arginine (Figure 4), whereas the high-affinity phase was not significantly affected ($K_d = 0.21 \pm 0.08$ mM and $\Delta\Delta A = 0.029 \pm 0.002$). Very similar results, both with respect to K_d and $\Delta\Delta A$, were obtained in the presence of 20 μM or 0.1 mM L-arginine, and in the presence of 0.2 mM L-arginine plus 10 μM BH_4 (not shown).

Scheme 1: Binding of DTT to nNOS Dimers



A somewhat different picture emerged when L-arginine (0.2 mM) was allowed to preincubate for 1 h before the start of the titration with DTT. Although this procedure did not significantly change the amplitude of the absorbance change ($\Delta\Delta A = 0.032 \pm 0.005$), the apparent K_d for DTT was slightly higher (0.84 ± 0.07 mM). Preincubation of the enzyme for 1 h with both 0.2 mM L-arginine and 10 μM BH_4 did not diminish the affinity for DTT any further ($K_d^{app} = 0.7 \pm 0.3$ mM), but decreased the amplitude of the absorbance change by 56% ($\Delta\Delta A = 0.014 \pm 0.001$). When the BH_4 concentration was raised to 50 μM, no bisthiolate complex formation could be discerned. It should be noted, however, that under those conditions optical titration studies were hampered by additional absorbance changes that were caused by the autoxidation of BH_4.

We propose that DTT binds with high affinity to the BH_4-free subunit, whereas low-affinity binding involves the BH_4-containing subunit. Binding of DTT to the BH_4-containing subunit no longer occurs when L-arginine is present, and at sufficiently high concentrations of both L-arginine and BH_4, DTT binding to the enzyme is suppressed completely. Scheme 1 gives a representation of DTT binding to nNOS dimers.

This interpretation of the DTT titration curves is supported by the rates with which equilibrium was attained after each DTT addition. With BH_4-free nNOS, absorbance changes were complete within the mixing time (≤ 5 s), indicating that DTT association is rapid. With BH_4-containing nNOS, absorbance changes were also rapid during the first part of the titration, but a slow phase (0.1 min^{-1}) became ever more prominent during the later stages. From separate plots of the fast and slow phases vs the concentration of DTT (not shown) it was clear that most of the high-affinity binding ($79 \pm 4\%$) was fast, whereas low-affinity binding was largely ($83 \pm 6\%$) slow. This behavior is in agreement with the model in Scheme 1. BH_4-free nNOS and the BH_4-free subunit in BH_4-containing nNOS are for the most part low spin, allowing rapid association of DTT. Since the BH_4-containing subunit is largely high spin, the DTT association rate in that case is limited by the slow equilibration between the spin states (Gorren et al., 1996).

These results and their interpretation may have important and unexpected implications for NOS function. It appears that formation of the bisthiolate complex with the BH_4-free subunit is not affected by the adjacent BH_4-containing subunit, suggesting that each subunit can bind DTT independently. Another important implication follows from the apparent discrepancy between the optical and the enzyme kinetic studies. The optical titrations of BH_4-containing nNOS (at an estimated BH_4 concentration of 1.8 μM) were clearly biphasic with a high-affinity K_d of 0.16−0.24 mM, but the enzyme inhibition studies (at an estimated BH_4 concentration of 3 nM) yielded simple competitive inhibition, with a much higher inhibition constant ($K_i = 11$ mM). This implies that the heme of the BH_4-free subunit, that binds DTT with high-affinity, is not involved in catalysis. Therefore L-arginine must be bound to the BH_4-containing subunit, and models that assume that BH_4 binding to one subunit stimulates L-arginine binding to the other can be discounted, as can models which assume that the two oxygenase domains function as one active site. That the two hemes are part of a single active site was recently proposed as one alternative to explain why two heme sites are required per dimer to sustain NO production (Xie et al., 1996).

From the observation that low-affinity binding to the BH_4-containing subunit was abolished by L-arginine, one can infer strong positive cooperativity between L-arginine and pteridine binding. The differences in the optical titrations that are caused by increasing the incubation time of L-arginine and BH_4, agree well with the observation that for binding of L-arginine and BH_4 to nNOS, the enzyme must be in the high spin state, and that the interconversion between the high- and low-spin states is fairly slow (0.1−0.6 min^{-1}; Gorren et al., 1996). Consequently, when the enzyme is titrated with DTT immediately after L-arginine addition, only the nNOS fraction that was already in the high-spin state before L-arginine addition will bind L-arginine rapidly. Since this fraction consists mainly of the BH_4-containing subunits, the low-affinity DTT-binding phase is abolished, without any effect on the high-affinity phase. Upon longer incubation with L-arginine, the BH_4-free subunit will slowly bind L-arginine as well, which gives rise to the slight increase in the apparent K_d for DTT. The observation that a combination of high L-arginine and BH_4 concentrations prevented DTT

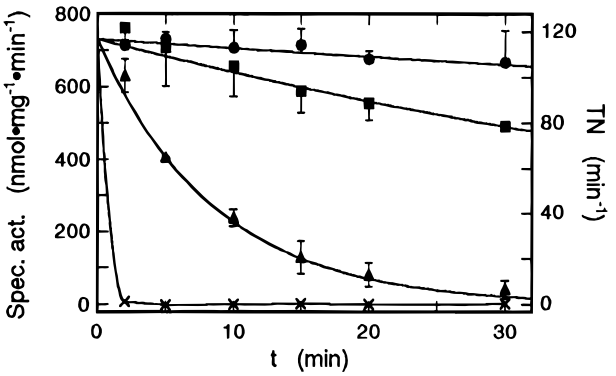


FIGURE 5: Thermolability of nNOS. nNOS was preincubated at a temperature of 20 (circles), 30 (squares), 37 (triangles), or 50 °C (crosses) for 2−30 min. After preincubation the enzyme activity was determined for 5 min at 37 °C under standard assay conditions. Plotted are the specific activities as a function of the preincubation period. The lines drawn through the data points are best fits to the function (Spec.act.) = (Spec.act.$_0$)exp($-kt$), in which k is the observed first-order rate constant of inactivation and (Spec.act.$_0$) the specific activity at zero preincubation time. The latter parameter was set to 730 nmol mg^{-1} min^{-1}. The estimated values for k were 0.003 ± 0.001 min^{-1} at 20 °C, 0.0132 ± 0.0006 min^{-1} at 30 °C, 0.117 ± 0.001 min^{-1} at 37 °C, and 2.4 ± 0.6 min^{-1} at 50 °C. Conditions for preincubation: 90 nM nNOS, 1 mM CHAPS; 12 mM 2-mercaptoethanol; 0.5 mM EDTA; 50 mM triethanolamine/HCl (pH 7.0). Conditions for activity assay: 18 nM nNOS, 0.1 mM [^{3}H]arginine (∼50 000 cpm), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 0.5 mM $CaCl_2$, 10 μg/mL of calmodulin, 10 μM BH_4, 0.2 mM CHAPS, 2.4 mM 2-mercaptoethanol, 0.125 mM EDTA, and 50 mM triethanolamine/HCl (pH 7.0) were incubated at 37 °C for 5 min, followed by separation and detection of [^{3}H]citrulline. Enzyme activity is expressed both as nmol of L-citrulline production per mg of protein per minute (left-hand axis) and as turnover number (right-hand axis).

binding to the second subunit as well, implies that nNOS can bind 2 equiv of L-arginine and BH_4 per dimer.

Thermal Stability of nNOS and Effect of Thiols

In the course of experiments designed to compare the thermostability of BH_4-free and BH_4-containing nNOS, we noticed that, at nanomolar concentrations, BH_4-containing nNOS is rapidly deactivated even at physiological temperatures (37 °C). Figure 5 shows the effect of preincubating BH_4-containing nNOS at several temperatures prior to determination of enzyme activity for 5 min at 37 °C. While the enzyme was essentially stable at 20 °C, inactivation rates increased very rapidly at higher temperatures, yielding nNOS half-lives of 53 and 6 min at 30 and 37 °C, respectively. Inactivation of BH_4-free nNOS was even more rapid; inactivation proceeded approximately 3 times faster at all temperatures (not shown). Inactivation of BH_4-containing nNOS at 37 °C was 10 times slower when the enzyme was incubated at micromolar instead of nanomolar concentrations (not shown).

Since L-citrulline production under standard assay conditions at 37 °C proceeded linearly for more than 15 min, we determined how the ingredients of the assay mixture affect enzyme stability. Table 1 shows the effect of preincubation for 10 min at 30 °C in the complete assay mixture with one or two ingredients missing. Two series of experiments were performed; in one series the reaction was started by addition of NADPH, in the second series by addition of calmodulin. Therefore, NADPH and calmodulin were missing from all preincubations in the first and second series, respectively.

Table 1: Effect of Ingredients of nNOS Activity Assay on Enzyme Stability during Preincubation[a]

	relative specific activity (%)	
	reaction initiated with	
preincubation conditions	NADPH	calmodulin
control[b]	≡ 100 ± 10	≡ 100 ± 10
control without L-arginine	108 ± 5	90 ± 10
control without 2-mercaptoethanol	9 ± 1	40 ± 10
control without CHAPS	86 ± 8	77 ± 6
control without $CaCl_2$/calmodulin	102 ± 5	91 ± 4
control without FMN/FAD	50 ± 10	60 ± 7
control without BH_4	110 ± 20	80 ± 7
control without NADPH		76 ± 1
no preincubation	190 ± 40	120 ± 20

[a] 21−24 nM nNOS was preincubated in the assay mixture with the exception of NADPH or calmodulin and the indicated compound(s) at 30 °C for 10 min. The omitted compound(s) and NADPH or calmodulin were then added and incubated at 37 °C for 5 min, followed by separation and detection of [3H]citrulline. Assay conditions: 19 nM nNOS, 50 mM triethanolamine (pH 7.0), 0.1 mM L-[3H]arginine, 10 μM BH_4, 5 μM FAD, 5 μM FMN, 0.5 mM $CaCl_2$, 10 μg/mL of calmodulin, 0.2 mM CHAPS, 2.4 mM 2-mercaptoethanol, and 0.2 mM NADPH. [b] The control samples were preincubated with all ingredients except for the compound with which the reaction was started (NADPH or calmodulin).

The main difference between the two series lies in the comparison to the activity obtained without preincubation: whereas the activity in the control experiments of the second series, which lacked only calmodulin during preincubation, was not much lower than that of the activity without preincubation (83 ± 13%), the activity in the control experiments of the first series, which lacked NADPH, was considerably lower (53 ± 13%). Nevertheless, the effects of the various ingredients in the two series were comparable. Omission of L-arginine, exogenous BH_4, or Ca^{2+}/calmodulin had no effect on activity. Fairly small decreases in activity were found when CHAPS (20%) or NADPH (25%) was omitted, while leaving out the flavins decreased the activity by 40−50%. When 2-mercaptoethanol was excluded, 60−90% of the activity was lost. In general, it appears that the presence of compounds that provide reducing equivalents stabilizes nNOS to some extent. The spectacular activity decrease after incubation of the enzyme for only 10 min in the absence of 2-mercaptoethanol at the relatively low temperature of 30 °C, underlies the importance of thiols for NOS stability. The stabilizing effect of thiols during preincubation of NOS has been noted previously, and was found to be largely due to protein thiol reduction (Hofmann & Schmidt, 1995; Komori et al., 1995). In line with those reports, thiols protected against, but did not reverse, inactivation (unpublished observations), suggesting that protein thiol oxidation is followed by other irreversible steps.

To look for changes of the heme and the protein structure during thermal inactivation in the absence of reducing compounds, the effect of incubating nNOS at various temperatures on the optical absorbance and fluorescence spectra was monitored. At 30 °C or lower no changes occurred. At 37 °C effects were small; the fluorescence emission maximum shifted from 329 to 334 nm, while the absorbance at 420 nm increased slightly. At 50 °C the fluorescence emission maximum shifted to 336 nm, and the intensity increased by 20%. Comparison of spectra at 20 °C of samples that had been incubated for 30 min at 50 °C to control spectra of nonheated samples showed that these

changes were irreversible. In the absorbance spectrum the disappearance of the 398 nm band was accompanied by the formation of a peak at 420 nm. Subsequently, the heme absorbance disappeared and strong scattering was observed. These results suggest the formation of a P420 state, followed by heme loss and protein denaturation. However, as these changes were much slower than the almost instantaneous loss of activity at 50 °C, we conclude that inactivation does not involve major protein structural changes.

CONCLUSIONS

In summary, we have shown that (1) thiols bind to BH_4-free nNOS with apparent affinities ranging from 10^{-4} to 10^{-2} M; (2) DTT binds to the BH_4-free subunit of BH_4-containing nNOS with the same affinity ($K_d^{app} \approx 0.2$ mM) as to BH_4-free nNOS, but with much lower affinity to the BH_4-containing subunit; (3) L-arginine abolishes thiol binding to the BH_4-containing subunit, but has little effect on thiol binding to the BH_4-free subunit; (4) DTT is a L-arginine-competitive inhibitor ($K_i^{app} \approx 11$ mM) of nNOS; (5) thiols stabilize nNOS by preventing the oxidation of protein thiol groups.

Our results provide support for the previous suggestion of strong anticooperativity between binding of BH_4 to two identical sites per NOS dimer, and of strong cooperativity between BH_4- and L-arginine binding. Moreover, we propose that in nNOS, containing one equiv of BH_4 per dimer, the BH_4-free subunit does not participate in NO production.

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