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Ca^{2+}/Calmodulin-Dependent NO Synthase Type I: A Biopteroflavoprotein with Ca^{2+}/Calmodulin-Independent Diaphorase and Reductase Activities†

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ABSTRACT: NO synthase (NOS; EC 1.14.23) catalyzes the conversion of L-arginine into L-citrulline and a guanylyl cyclase-activating factor (GAF) that is chemically identical with nitric oxide or a nitric oxide-releasing compound (NO). Similar to the other isozymes of NOS that have been characterized to date, the soluble and Ca^{2+}/calmodulin-regulated type I from rat cerebellum (homodimer of 160-kDa subunits) is dependent on NADPH for catalytic activity. The enzyme also possesses NADPH diaphorase activity in the presence of the electron acceptor nitroblue tetrazolium (NBT). We investigated the requirements of NOS and its content of the proposed additional cofactors tetrahydrobiopterin (H_4biopterin) and flavins, further characterized the NADPH diaphorase activity, and quantified the NADPH binding site(s). Purified NOS type I Ca^{2+}/calmodulin-independently bound the [^{32}P]2′,3′-dialdehyde analogue of NADPH (dNADPH), which, at near K_m concentrations during 3-min incubations was utilized as a substrate and at higher concentrations or after prolonged incubations and cross-linking inhibited NOS activity. The NADPH diaphorase activity was Ca^{2+}/calmodulin-independent, required higher NADPH concentrations than NOS activity, and was affected by dNADPH to a lesser degree. Divalent cations interfered with the diaphorase assay. Per dimer, native NOS contained about 1 mol each of H_4biopterin, FAD, and FMN, classifying it as a biopteroflavoprotein, and incorporated 1 mol of dNADPH. No dihydrobiopterin (H_2biopterin), biopterin, or riboflavin was detected. These findings suggest that NOS may share cofactors between two identical subunits via high-affinity binding sites. They also explain why different preparations of NOS have different requirements for exogenous flavins and H_4biopterin for maximal catalytic activity. Furthermore, H_4biopterin alone reduced NBT, and purified dihydropteridine reductase was found to have diaphorase activity. The absence of enzyme-bound biopterins other than H_4biopterin, the reported lack of effect of methotrexate on enzyme activity, and the potent and noncompetitive inhibition of NOS activity by NBT suggest also that NOS type I may have a novel methotrexate-insensitive quinoid-H_2biopterin reductase activity.

In various mammalian cells and tissues a terminal guanidino nitrogen of L-arginine (Iyengar et al., 1987; Schmidt et al., 1988) is oxidized to yield a factor with physicochemical and pharmacological properties similar to nitric oxide (NO;[1] Ignarro et al., 1987; Palmer et al., 1987; Forray et al., 1990; Greenberg et al., 1990; Mordvintsev et al., 1990; Myers et al., 1990). Both in the NO-producing cell and in specific NO-

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[1] Abbreviations: biopterin, 6-(L-*erythro*-1′,2′-dihydroxypropyl)pterin; dNADPH, 2′,3′-dialdehyde NADPH; GAF, guanylyl cyclase-activating factor; H_2biopterin, (6*R*)-7,8-dihydrobiopterin; H_4biopterin, (6*R*)-5,6,7,8-tetrahydrobiopterin; IBMX, 3-isobutyl-1-methylxanthine; Me_2SO, dimethyl sulfoxide; NBT(F), nitroblue tetrazolium (formazan); NO, nitric oxide or nitric oxide-releasing compound; NOS, NO synthase; q-H_2biopterin, (6*R*)-6,7-dihydrobiopterin; SDS, sodium dodecyl sulfate.

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target cells, NO activates soluble guanylyl cyclase (Arnold et al., 1977; GTP pyrophosphate-lyase (cyclizing), EC 4.6.1.2) acting as an endogenous nitrovasodilator (Murad, 1986) and guanylyl cyclase-activating factor (GAF; Schmidt et al., 1991). The formation of GAF/NO from L-arginine is catalyzed by distinct NADPH-dependent (Bredt & Snyder, 1989; Knowles et al., 1989; Kwon et al., 1989; Schmidt et al., 1989; Tayeh & Marletta, 1989) NOS isozymes, which we have suggested to classify as types I–III (Schmidt et al., 1991) on the basis of their regulation by Ca^{2+} and their subcellular localization; NOS-I, soluble and Ca^{2+}/calmodulin-dependent; NOS-II, predominantly soluble and Ca^{2+}/calmodulin-independent; NOS-III, predominantly particulate and Ca^{2+}/calmodulin-dependent. Furthermore, at least the neural NOS-I, which is the subject of the present report, is a cytochrome *c* or cytochrome P_{450} reductase (Mayer et al., 1992) and appears to be identical with the neural NADPH diaphorase (Hope et al., 1991) which reduces NBT to NBT formazan (NBTF) in the presence of NADPH. We investigated the role of Ca^{2+}/calmodulin and other factors in the regulation of NADPH binding and NADPH diaphorase activity of purified rat cerebellar NOS-I and the effects on NOS and NADPH diaphorase activities of cross-linking with the 2′,3′-dialdehyde derivative of NADPH (dNADPH). Besides NADPH, NOS-II requires exogenous H_4biopterin (Kwon et al., 1989; Tayeh & Marletta, 1989), while for NOS-I the absolute requirement for this compound and its mechanism of action (Giovanelli et al., 1991) are unclear. For instance, no (Bredt & Snyder, 1990), partial (Schmidt et al., 1991), or absolute (Mayer et al., 1990) dependence of NOS-I on exogenous H_4biopterin has been reported. We, therefore, also investigated the cofactor content and requirements of purified NOS-I with respect to different pterins and flavins for rat brain NOS-I.

Experimental Procedures

Materials. Cerebella from Sprague-Dawley rats (200–300 g) were used for the experiments. Purified sheep liver dihydropteridine reductase was from Sigma (St. Louis, MO). Media and chemicals for the cell culture were from Gibco BRL-Life Technologies (Grand Island, NY) except fetal calf serum (HyClone, Logan, UT). H_4biopterin was from Dr. B. Schircks Laboratories (Jona, Switzerland). Stock solutions (10 mM) were stored at −70 °C under argon in bidistilled argon-degassed water and diluted in argon-degassed water immediately before use. Glycine, Tris (both from Bio-Rad, Richmond, CA), and SDS (Boehringer Mannheim, Mannheim, Germany) were of electrophoresis grade. Prestained high-molecular mass protein markers for SDS–PAGE were from Sigma. All other chemicals were from Sigma and were of the highest analytical or HPLC grade available.

Purified NOS. Rat cerebellar NOS was purified as previously described (Schmidt & Murad, 1991; Schmidt et al., 1991) with the modification that elution from the calmodulin affinity column was performed in the presence of 10 μM calmidazolium and 10 mM EGTA and in the absence of NaCl. In one experiment, purified NOS was precipitated with $(NH_4)_2SO_4$ (242 mg·mL^{-1}, 40% saturation) and resuspended in water for analysis of biopterin content.

NOS Activity. NOS activity was determined as described either by stimulation of soluble guanylyl cyclase in RFL-6 cell monolayers (Ishii et al., 1991; Schmidt et al., 1991) or by citrulline formation (Bredt & Snyder, 1989; Schmidt et al., 1991) in the presence of up to 1 μM (usually 0.3 μM) H_4biopterin, up to 100 μM NADPH or dNADPH, up to 100 μM L-arginine, 20 units·mL^{-1} calmodulin, 20 units·mL^{-1} superoxide dismutase, up to 1 μM FAD, up to 1 μM FMN, and in a final volume of 1.0 or 0.1 mL, respectively. In some experiments, NOS was assayed in the presence of up to 1 mM NBT.

Determination of Diaphorase Activity. Diaphorase activity of NOS or dihydropteridine reductase (NADH:quinoid, 6,7-dihydropteridine oxidoreductase, EC 1.6.99.7) was assayed as described (Hope et al., 1991) with some modifications. The respective purified enzyme preparation was incubated at 37 °C in the presence of up to 1 mM (usually 0.5 mM) nitroblue tetrazolium (NBT) and up to 1 mM NADPH or NADH (usually 1 mM NADPH) in a final volume of 100 μL. In some experiments, Ca^{2+}, Mg^{2+}, calmodulin, L-arginine, and H_4biopterin were included, or NOS was pretreated with dNADPH, or the assay was performed in the absence of enzyme and NADPH and in the presence of up to 100 μM H_4biopterin. The reaction was stopped by the addition of 10 μL of 1.5 N H_2SO_4 and 100 μL of Me_2SO. The NBT formazan product (NBTF) was determined spectrophotometrically at 540 and 595 nm after transfer to microtiter plates (150 μL, light path d = 0.8 cm; 96-well half-area; Costar) in a Bio-Tek EL-311 microplate reader or at 585 nm (4 mL) in a spectrophotometer (light path d = 1 cm; Beckman DU-64). Calculations were based on a molar extinction coefficient for NBTF of ϵ_{540} = 12 600 M^{-1}·cm^{-1}. In some experiments, UV/vis spectra of NBTF (3 μM) were recorded in buffer containing 0.3 μM H_4biopterin, 20 units·mL^{-1} calmodulin, 1 μM free Ca^{2+}, 1 mM NADPH, and/or 100 μM L-arginine or 10 nmol of NO_{aq}.

Synthesis of 2′,3′-Dialdehyde NADPH. The 2′,3′-dialdehyde derivative of NADPH (dNADPH) was synthesized as previously described (Smith et al., 1989a). dNADPH incorporating ^{32}P (90% purity) was prepared following the method of Umei et al. (1991). At all steps, the reactants were purified over a Mono Q anion-exchange column. Throughout the synthesis, products were identified by a combination of scintillation counting, visual and fluorescence spectroscopy, and thin-layer chromatography (Smith et al., 1989a; Umei et al., 1991).

Utilization of dNADPH by NOS and ^{32}P Incorporation. In order to allow covalent incorporation of the radioligand, aliquots of purified NOS were incubated in triethanolamine hydrochloride buffer (50 mM, pH 8.0) with 20 μM [^{32}P]-dNADPH and 0.5 mM $NaBH_3CN$ for 12 h at 4 °C (Umei et al., 1991). Samples were then subjected to SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), and the resulting gels were stained with Coomassie brillant blue, dried, and exposed to Kodak XAR-5 film at −70 °C. The ability of dNADPH to function as a substrate for NOS was examined using a standard assay for NOS activity in which dNADPH was substituted for NADPH (see above). The ability of dNADPH to occupy and inhibit a catalytic nucleotide binding site was examined by incubating NOS with dNADPH (20 μM) at 4 °C in triethanolamine hydrochloride buffer (50 mM) containing $NaBH_3CN$ (0.5 mM) for up to 8 h. Quantitative labeling was accomplished using [^{32}P]dNADPH with a specific activity of 1 Ci·mol^{-1} as determined by spectrophotometry (ϵ_{340} = 6.22 mM^{-1}) and β-counting. Aliquots of NOS (2 μg) were incubated as described above, and the labeling reactions were allowed to proceed for 24 h to ensure maximal incorporation of dNADPH. At the conclusion of the incubation, samples were dialyzed exhaustively prior to β-counting to establish the extent of incorporation of [^{32}P]dNADPH.

Determination of Protein-Associated Flavins. Purified NOS (3 μg) was boiled for 8 min to release protein-associated flavins. The low-molecular mass fraction containing the flavins was

Table I: Cofactor Content of NOS Type I Based on a Dimeric Structure of the Native Protein[a]

cofactor	mol of cofactor/dimer	n
NADPH[b]	0.96 ± 0.09	2
H_4biopterin	0.96 ± 0.02	3
H_2biopterin	0.02 ± 0.01	3
FAD	0.9 ± 0.1	4
FMN	0.8 ± 0.1	4
riboflavin	<0.1	4

[a] Data are reported as mean of (n) separate determinations, each in duplicate. [b] Quantified on the basis of incorporation of [^{32}P]dNADPH.

separated by centrifugation through filters with a 10-kDa molecular mass cutoff (Millipore). Samples were analyzed for flavins by reverse-phase HPLC with fluorometric detection as described (Batey & Eckhert, 1990; Stuehr et al., 1991a) with several modifications to allow shorter separation times. The apparatus consisted of a Rainin HPXL pump, a Hewlett-Packard HP 1046A fluorescence detector, a Rainin Microsorb C_8 column (5 μm, 4.6 mm × 25 cm), an ICI LC 1600 autosampler, and the Dynamax HPLC Method Manager program (Rainin). The column was equilibrated in 5 mM ammonium acetate, pH 6.0, and was eluted with acetonitrile at a flow rate of 1 mL·min^{-1}. The minimum detectable amounts were 2 pmol of FMN, 5 pmol of FAD, and 1 pmol of riboflavin. Flavins were determined in control experiments with >95% accuracy.

Determination of Protein-Associated Biopterins. Purified NOS (0.4 μg) was oxidized with iodine under acidic and basic conditions by a modification of the procedure of Fukushima and Nixon (1980). The former condition gives a quantitative yield of biopterin from both H_2biopterin or H_4biopterin, the latter results in a specific destruction of H_4biopterin and gives quantitative yield of biopterin from H_2biopterin. The difference in measured biopterin between the acidic and the basic oxidation is therefore a measure of H_4biopterin. Samples were oxidized with 1% iodine and 2% KI in the presence of either 0.25 M H_3PO_4 (acidic oxidation) or 0.1 N NaOH (alkaline oxidation) in a final total volume of 100 μL for 1 h and in the dark. To the alkaline oxidation was added 20 μL of 2 M H_3PO_4, followed immediately by crystalline ascorbic acid; to the alkaline oxidation was added only crystalline ascorbic acid. Samples were analyzed for biopterin by reverse-phase HPLC with fluorometric detection. The apparatus consisted of a Waters M6000 pump, a Waters WISP autosampler, a Perkin-Elmer LS-40 fluorescence detector, a Perkin-Elmer Nelson PC integrateor, and a Spherisorb ODS-1 column (5 μm, 4.6 mm × 25 cm). The solvent was 5% methanol/95% water at a flow rate of 1 mL·min^{-1}. The minimum detectable amount of biopterin was 20 fmol per injection. H_4biopterin was determined in control experiments with >95% accuracy.

Determination of Protein. Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

Results

Biopterin and Flavin Content and Requirements of NOS. Purified NOS was found to contain about 1 mol of biopterin (Figure 1A) per dimer of enzyme (see also Table I). Since biopterin was recovered only after acidic but not basic oxidation, it was entirely derived from H_4biopterin (Fukushima & Nixon, 1980), and NOS did not contain H_2biopterin or biopterin. Precipitation of the enzyme with $(NH_4)_2SO_4$ resulted in a remarkable reduction of the H_4biopterin content of the enzyme (0.05 mol per dimer, n = 1) and its NOS activity in the absence of H_4biopterin (data not shown). A

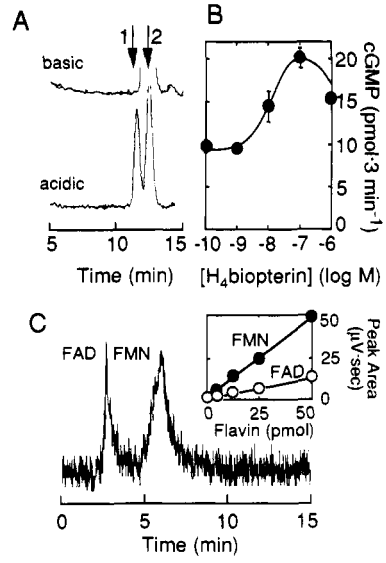

FIGURE 1: Biopterin/flavin content and dependence of NOS. Panel A: NOS (0.4 μg) was analyzed for its biopterin content by acidic (lower trace) and basic (upper trace) oxidation and HPLC and fluorescence detection of biopterin as described (see Experimental Procedures). Peak 1 was only present after acid oxidation and is therefore identical with H_4biopterin-derived biopterin; peak 2 is unrelated to biopterin and cochromatographed with flavins. Panel B: Purified NOS (10 ng) was assayed for guanylyl cyclase activation (cGMP increase in RFL-6 detector cells) as described (see Experimental Procedures) in the presence of different concentrations (log M) of H_4biopterin. Panel C: NOS (4 μg) was analyzed for its flavin content by HPLC and fluorescence detection as described (see Experimental Procedures). The two eluted fluorophores were identified as FAD and FMN, respectively. No riboflavin was detected. The insert shows the calibration (picomoles of flavin versus peak area in microvolt seconds) for FAD (○, r^2 = 0.992) and FMN (●, r^2 = 0.998).

second fluorescent peak that eluted after H_4biopterin-derived biopterin coeluted with flavins. Purified NOS was found to contain about 1 mol each of FAD and FMN (Figure 1C) per dimer. No riboflavin was detected. However, the same NOS preparations showed significant NOS activity in the absence of exogenous H_4biopterin, FAD, or FMN. Upon addition of 0.1 μM H_4biopterin (K_m 20 nM), NOS activity increased up to 190% on the basis of the RFL-6 cell bioassay (Figure 1B) and up to 160% on the basis of citrulline formation without affecting the apparent K_m value for L-arginine (3.3 μM). The V_{max} value of 172 nmol of citrulline min^{-1}·mg^{-1} corresponds to a turnover number of 65 min^{-1}. FAD and FMN, in the presence of saturating concentrations of all other cofactors, did not significantly stimulate (110% and 105% of control, respectively) the specific activity of NOS during 3-min incubations. However, the requirements for exogenous flavins may vary between different enzyme preparations, incubation times, and initial specific activity.

NADPH Binding sites of NOS. dNADPH up to a concentration of 10 μM and in the absence of BH_3CN^- was also able to substitute for NADPH as a cofactor of NOS (Figure 2A). The apparent K_m (2.0 and 0.8 μM) and V_{max} values for NADPH and dNADPH were similar. At a concentration 40 times higher than the apparent K_m, dNADPH caused a marked inhibition of GAF/NO formation. When NOS was preincubated with near K_m concentrations of dNADPH and in the presence of the Schiff's base reductant BH_3CN^- for up to 8 h, both enzyme activities, NOS and NADPH diaphorase, were subsequently decreased even when assayed in the presence of saturating concentrations of NADPH (Figure 3B). The inhibition of diaphorase activity by dNADPH was, however, less complete. When purified NOS was incubated with

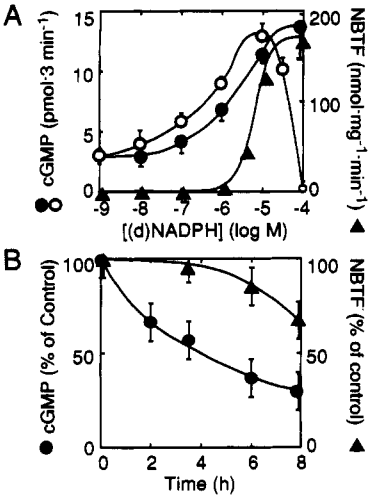

FIGURE 2: Effects of NADPH and dNADPH on NOS and its diaphorase activity. Purified NOS was assayed for guanylyl cyclase activation (10 ng of NOS, ● and ○; cGMP increase in RFL-6 detector cells) or NADPH diaphorase activity (1 μg of NOS, ▲; NBTF formation) as described (see Experimental Procedures). In panel A, NOS was assayed in the presence of different concentrations (log *M*) of NADPH (●, ▲) or dNADPH (○). In panel B, NOS was first incubated for the times indicated with 20 μM dNADPH and 0.5 mM $NaBH_3CN$ and then assayed in the presence of 1 mM NADPH.

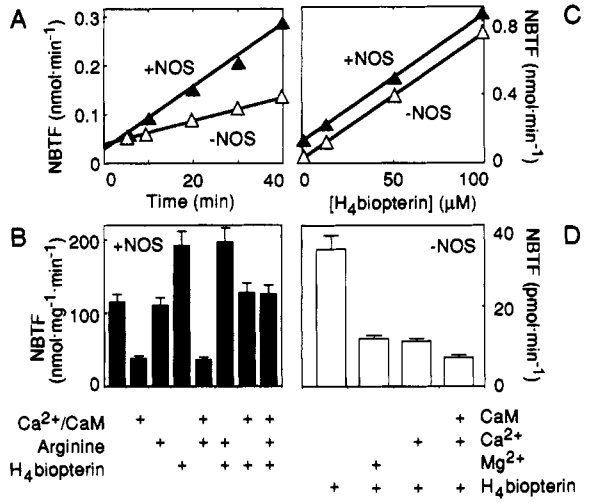

FIGURE 3: Effects of Ca^{2+} and H_4biopterin on NBT, NBTF, and diaphorase activity of NOS. Diaphorase activity (NBTF formation) was assayed as described (see Experimental Procedures) in the absence (−NOS; △, open bars) or the presence of 1 μg of NOS (+NOS; ▲, closed bars) and in the absence (panel D) or presence (panels A–C) of 1 mM NADPH. In panel A, incubations were performed for different times and in the absence of H_4biopterin; in panel B, incubations were performed in the absence or presence of 1 μM free Ca^{2+}, 0.3 mM L-arginine, and 1 μM H_4 biopterin as indicated (+); in panel C, incubations were performed in the presence of different concentrations (micromolar) of H_4biopterin; in panel D, incubations were performed in the absence or presence of 20 units·mL^{-1} calmodulin (CaM), 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 1 μM H_4biopterin as indicated (+).

[^{32}P]dNADPH in the presence of BH_3CN^-, radioactivity cochromatographed with enzyme protein in gel electrophoresis. Thus, under these conditions, the radiolabeled dNADPH was incorporated into NOS and cross-linked. Incorporation of dNADPH was independent of the presence of Ca^{2+}/calmodulin, H_4biopterin, or L-arginine and was specific in that it was blocked by the addition of native NADPH to the incubation mixture (data not shown). Quantitative labeling of NOS with [^{32}P]dNADPH (Table I) revealed that about 1 mol of dNADPH was incorporated into each mol of holoenzyme (n = 2, performed in duplicate).

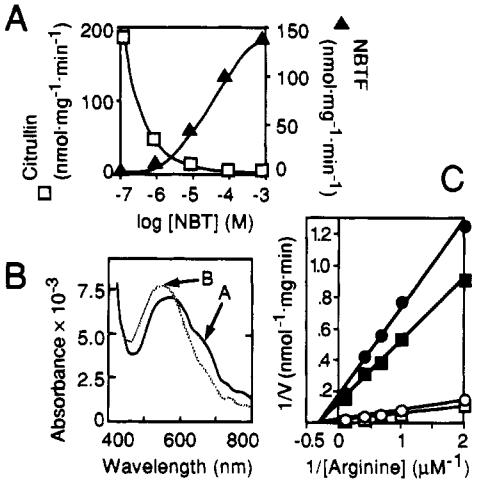

FIGURE 4: Effects of NBT on NOS and diaphorase activities. Purified NOS was assayed for citrulline formation (0.2 μg of NOS, ○, □, ●, and ■; panels A and C) or diaphorase activity (1 μg NOS, ▲; panels A and B) as described (see Experimental Procedures). In panel A, incubations were in the presence of different concentrations (log *M*) of NBT; in panel B, UV/vis spectra were recorded after incubation (3 μM NBT, 30 min) in the absence (trace A) or presence (trace B; identical to traces obtained with 10 nmol of NO_{aq}) of 0.3 mM L-arginine, 1 μM free Ca^{2+}, 20 units·mL^{-1} calmodulin, 1 μM H_4biopterin; in panel C (Lineweaver–Burk double-reciprocal plot), incubations were in the absence (○, □) or presence (●, ■) of 3 μM NBT and in the absence (●, ○) or presence (■, □) of 3 μM H_4biopterin.

Diaphorase Activity of NOS. In the presence of NADPH, NOS time-dependently (Figure 3A) converted NBT to NBTF (Hope et al., 1991). The apparent K_m value of NADPH for the NADPH diaphorase activity of NOS was 35 μM, which is about 6 times that for NOS activity; the V_{max} value was 122.9 nmol·mg^{-1}·min^{-1}, which is equivalent to a turnover number of 39 min^{-1}. Control experiments without enzyme revealed a linear background NBT formation equivalent to about 100 pmol·min^{-1}. NADPH diaphorase activity appeared initially to be inhibited by Ca^{2+} (Figure 3B), which effect was subsequently identified as an interference of divalent cations (Ca^{2+}, Mg^{2+}) with NBTF formation (see below) rather than a Ca^{2+}/calmodulin-mediated effect on the enzyme. Addition of H_4biopterin appeared to prevent this inhibition of NADPH diaphorase activity. However, H_4biopterin (≥1 μM) showed artificial NBTF formation in the absence of NADPH and enzyme (Figure 3C) with a molar activity of 0.5 min^{-1} at 1 μM H_4biopterin. The H_4biopterin-catalyzed NBTF formation was also inhibited by divalent cations, Ca^{2+} or Mg^{2+}, and independent of calmodulin (Figure 3D). When the diaphorase activity of NOS was monitored in the presence of L-arginine and all identified cofactors necessary for GAF/NO formation (0.3 μM H_4biopterin, 1 μM free Ca^{2+}, and 20 units·mL^{-1} calmodulin), the maximum of the NBTF (3 μM) UV/vis absorption spectrum was shifted from 580 to 520 nm (Figure 4, isosbestic point 585 nm). The same spectral shift of NBTF was observed with 10 nmol of NO_{aq} or by bubbling the incubation mixture with pure NO_{gas} for 15 s and is therefore likely to result from a nitrosation of the NBTF product by NO or an intermediate. Under no condition did L-arginine affect the total amount of NBTF formed as determined by the absorbance at 585 nm. NBT potently (Figure 4A) inhibited NOS activity (citrulline formation) in a noncompetitive fashion and with an apparent K_i of 0.1 μM (Figure 4C). Addition of 3 μM H_4biopterin partially reversed the inhibition by 3 μM NBT.

Diaphorase Activity of Dihydropteridine Reductase. Purified dihydropteridine reductase from sheep liver (Sigma) was

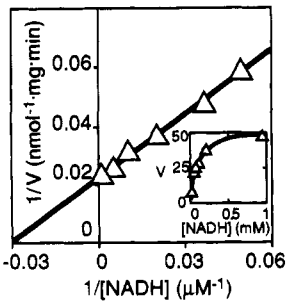

FIGURE 5: Diaphorase activity of dihydropteridine reductase. Dihydropteridine reductase (5 μg) was assayed for diaphorase activity (Δ; V given in nanomoles per milligram per minute) in the presence of different concentrations (micromolar) of NADH (Lineweaver–Burk double-reciprocal plot) as described (see Experimental Procedures).

found to have diaphorase activity. The apparent K_m value for NADH and V_{max} value for NADH diaphorase activity were 33.3 μM and 45.7 nmol of NBTF·mg^{-1}·min^{-1}, respectively (Figure 5). NADPH was also utilized as a cofactor but its K_m value was >0.3 mM and the resulting specific activity was lower (8.0 nmol of NBTF·mg^{-1}·min^{-1} at 1 mM NADPH). Methotrexate (L-amethopterin; up to 100 μM) did not affect the diaphorase activity of dihydropteridine reductase (99.5% of control, data not shown).

Discussion

NOS-I contained high-affinity binding sites for FAD, FMN, and NADPH (each 1 mol per dimer), which is in agreement with recent studies showing that purified NOS-II contains FAD and FMN (Stuehr et al., 1991a), although others could not verify this observation (Yui et al., 1991). Also, the predicted amino acid sequence of cloned rat cerebellar NOS-I suggests recognition sites for NADPH, FAD, and FMN similar to that of NADPH cytochrome P_{450} reductase (Bredt et al., 1991). The amounts of FAD and FMN that have been reported or can be predicted from the primary structure of NOS differ, i.e., 1–2 mol of each flavin/dimer (Bredt et al., 1991; Stuehr et al., 1991a). Protein-bound flavins may have been partially removed during purification and underestimated in the present and other studies analyzing purified enzyme preparations (Stuehr et al., 1991a). Native NOS is a dimer (Schmidt et al., 1991). Cofactor binding sites may be shared between subunits or, as is probably the case in NOS-II, where subunits with different molecular masses (Stuehr et al., 1991a) contain unequal amounts of bound FAD (2.2 mol/dimer) and FMN (1.1 mol/dimer), binding sites may differ in the two subunits, e.g., one high-affinity and one low-affinity site. Interestingly, the latter preparation of NOS-II is inactive as a monomer, and dimerization of the enzyme requires FAD, L-arginine, and H_4biopterin (Stuehr et al., 1991a). A similar cofactor-dependent binary enzyme complex has been described for dihydropteridine reductase and NADH (Hasegawa, 1977). Novel is the finding that purified NOS-I contained a high-affinity binding site for H_4biopterin (1 mol/dimer), while another NOS-I (Mayer et al., 1991) and NOS-III (Pollock et al., unpublished observation) were reported to contain only traces of this cofactor bound. Exogenous H_4biopterin stimulated the activity of different preparations of purified rat cerebellar NOS up to 160% on the basis of L-citrulline formation [confer also to Mayer et al. (1990) and Schmidt et al. (1991)], suggesting that NOS may contain an additional low-affinity binding site for H_4biopterin. In all known H_4biopterin-utilizing enzymes, quinoid-H_2biopterin (q-H_2biopterin) is the product of normal catalysis and is regenerated to H_4biopterin by a q-H_2biopterin reductase (dihydropteridine reductase). The fact that biopterin was bound to NOS only in its fully reduced form (H_4biopterin) but not as (q-)H_2biopterin or biopterin may indicate either that NOS does not metabolize H_4biopterin (Giovanelli et al., 1991) or that it contains q-H_2biopterin reductase activity. Clearly, final evidence for this proposal will have to come from studies showing that H_4biopterin undergoes redox changes during normal catalysis. However, it is known that H_2biopterin partially substitutes for H_4biopterin while biopterin inhibits NOS activity (Giovanelli et al., 1991). Furthermore, the putative reductase site of NOS may also be the site of NADPH diaphorase activity (Figure 6) and cytochrome reduction: dihydropteridine reductase had diaphorase activity with a K_m value for NADH to support NBT reduction (33 μM) that was similar to the reported K_m for NADH to support q-H_2biopterin reduction (4–39 μM; Nielsen et al., 1969; Craine et al., 1972; Firgaira et al., 1981); in addition, a cytochrome *c* reductase was reported to utilize a H_4biopterin analogue for cytochrome *c* reduction as well as for NADPH diaphorase activity and to have dihydropteridine reductase-like activity (Harano, 1972). For several reasons, the putative q-H_2biopterin reductase site of NOS may, however, be different from the one in dihydropteridine reductase: NOS has a lower apparent K_m value for NADPH than for NADH; even micromolar concentrations of methotrexate, which will inhibit both dihydropteridin and dihydrofolate reductase (Craine et al., 1972), do not affect NOS activity (Giovanelli et al., 1991) or NADPH diaphorase activity (this report); the K_m value for H_4biopterin is lower in NOS (20 nM) than in dihydropteridine reductase (1–17 μM; Craine et al., 1972; Firgaira et al., 1981); and, finally, NOS shows a unique specificity for H_4biopterin in that it does not utilize closely related analogues of H_4biopterin which are utilized by other H_4biopterin-dependent enzymes (Kwon et al., 1989; Tayeh & Marletta, 1989; Giovanelli et al., 1991). NOS released H_4biopterin upon precipitation in 40% saturated $(NH_4)_2SO_4$. The latter observation could explain why different purification schemes for NOS-I, e.g., one including protein precipitation at 30% saturated $(NH_4)_2SO_4$ (Mayer et al., 1990), result in enzyme preparations

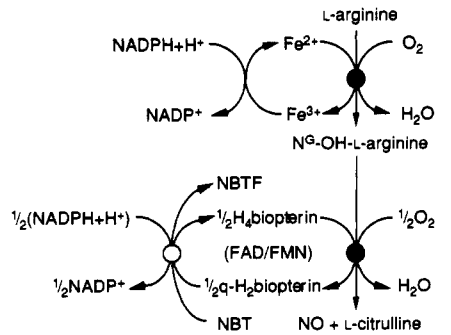

FIGURE 6: Proposed reaction mechanisms for L-arginine deoxygenase, NADPH diaphorase, and q-H_2biopterin reductase activity of NOS. Molecular oxygen (dioxygenase) is incorporated into both NO and L-citrulline (Leone et al., 1991). Oxygen consumption by NOS-I is Ca^{2+}/CaM-dependent (●; Mayer et al., 1991) and non-heme iron may be involved in the electron transfer to oxygen (Mayer et al., 1991). Non-heme iron and diaphorase/flavins are suggested to be part of different enzyme domains. Metabolism of L-arginine and its intermediate, N^G-hydroxy-L-arginine (Stuehr et al., 1991b) requires 1.5 and 0.5 mol of NADPH, respectively, per mol of L-citrulline formed (Stuehr et al., 1991b) and is equally dependent on H_4biopterin and FAD, suggesting the latter cofactors (and possibly also FMN) are not essential to form N^G-hydroxy-L-arginine but to further oxidize it (Stuehr et al., 1991b). NOS contains stoichometric amounts of H_4biopterin but not q-H_2biopterin. We suggest that NOS-I contains a Ca^{2+}/CaM-independent (○) q-H_2biopterin reductase activity which is related to the NADPH diaphorase activity of NOS and the noncompetitive inhibition of NOS activity (L-arginine turnover) by NBT.

that largely differ in basal activity and their dependence on H_4biopterin (Knowles et al., 1989; Mayer et al., 1990; Schmidt et al., 1991). The fact that the dependency of NOS on exogenous H_4biopterin inversely correlates to the amount of enzyme-bound cofactor may explain apparently contradictory reports on the role of H_4biopterin in NOS catalysis (Knowles et al., 1989; Mayer et al., 1990; Giovanelli et al., 1991; Schmidt et al., 1991; Snyder & Bredt, 1991). On the basis of its unique cofactor contents, NOS-I belongs to a novel class of biopteroflavoproteins. In fact, no other H_4biopterin-metabolizing and/or binding protein has been isolated with this cofactor tightly bound. It will now be of interest to identify the H_4biopterin binding site(s) of NOS.

The NADPH analogue d-NADPH was used as an alternative cosubstrate. Additionally, in the presence of the selective Schiff's base reductant $NaBH_3CN$, dNADPH could be cross-linked to NOS resulting in the incorporation of 1 mol of radiolabeled dNADPH per dimer and a marked inhibition of NOS activity. d-NADPH will therefore be a useful tool to radiolabel and verify the predicted NADPH binding site(s) of NOS (Bredt et al., 1991) as has been recently described for the human neutrophil NADPH oxidase (Smith et al., 1989a,b).

The NADPH diaphorase (Hope et al., 1991) and NADPH oxidase (Mayer et al., 1991) activities of NOS are apparently uncoupled from L-arginine metabolism. However, oxygen consumption and NADPH oxidase activity are dependent on Ca^{2+}/calmodulin (Mayer et al., 1991). On the other hand, we found that the NADPH diaphorase activity is Ca^{2+}/calmodulin-independent. Interferences by higher concentrations of Ca^{2+} and Mg^{2+} with the NBTF formation were identified as artifacts. The NADPH diaphorase activity is also less susceptible than the NOS activity to inhibition by the cross-linking reagent dNADPH (in the presence of BH_3CN^-) suggesting at least one NADPH binding site that is not shared by the diaphorase and NOS activities. In a process that probably involves nitroso-NBTF formation, NO formed in the presence of L-arginine was scavenged by NBT or NBTF resulting in a spectral shift of the NBTF product. NBT has also been reported to competitively inhibit L-arginine at a yet unidentified substrate binding site of NOS by competing for reducing equivalents (Hope et al., 1991). However, not L-arginine but oxygen would be expected to accept electrons from NADPH and/or H_4biopterin. We found similar turnover numbers of NOS for NBT and L-arginine (39 and 65 min^{-1}, respectively) and confirmed that NBT inhibits NOS activity. The mechanisnm of inhibition was, however, clearly noncompetitive and partially reversed by H_4biopterin. Interestingly, H_4biopterin itself, although with lower molar activity than NOS, was able to form NBTF nonenzymatically and in the absence of NADPH, suggesting that the diaphorase and reductase activity of NOS may be related to protein-associated, NADPH-dependently recycled H_4biopterin. By a similar mechanism, cytochrome *c* was earlier reported to be directly reduced by H_4biopterin, which process was augmented by cytochrome *c* reductase (Harano, 1972). Furthermore, dihydrofolate reductase, which reduces H_2biopterin to H_4biopterin, is also a diaphorase (Gunlack et al., 1966; Nixon & Blakley, 1968) and has cytochrome *c* reductase activity that is dependent on catalytic amounts of H_4biopterin (Nagai et al., 1967).

In conclusion, the Ca^{2+}/calmodulin-regulated NOS-I from rat cerebellum was found to be a biopteroflavoprotein containing 1 mol each of H_4biopterin, FAD, and FMN and one binding site for NADPH per mol of enzyme (dimer). The H_4biopterin content depended on the method of enzyme preparation and was inversely correlated with the dependency of the enzyme on exogenous H_4biopterin. The enzyme's NADPH diaphorase activity may at least partially be explained by H_4biopterin-catalyzed NBTF formation and/or the putative q-H_2biopterin reductase site. Specific nucleotide binding sites for NADPH were involved in both catalytic activities, NOS and diaphorase. However, the two activities differed in their extent of inhibition by cross-linking with dNADPH, their apparent K_m for NADPH, and their K_m and K_i (noncompetitive) values, respectively, for NBT and their regulation by Ca^{2+}/calmodulin.

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GroE Prevents the Accumulation of Early Folding Intermediates of Pre-β-lactamase without Changing the Folding Pathway†

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ABSTRACT: In folding studies of pre-β-lactamase in the presence of GroE, we investigated the pH dependence of the folding reaction. Two critical intermediates in the folding pathway were defined kinetically. I_1 is an early folding intermediate recognized by GroE; the misfolding of I_1 leads to aggregation, and this is prevented by GroE. A second intermediate I_2 is released from GroE after ATP hydrolysis. Its pH-dependent misfolding to a nonnative form, which is not an aggregate, is not prevented by GroE. From these results, a model is proposed, in which the crucial role of GroE consists of allowing the change from I_1 to I_2 to take place in the complex. Fluorescence spectra of the pre-β-lactamase complexed to GroE are very similar to those of the native state. The pathway of pre-β-lactamase folding is not changed by GroE as evidenced by the same half-time and pH dependence of the folding reaction. GroE probably recognizes the signal sequence and some portion of the mature protein since mature β-lactamase does not interact with GroE even under conditions of slow folding.

While the central dogma in protein folding of the sequence determining the structure (Anfinsen, 1973; Creighton, 1978; Jaenicke, 1987) stands unchallenged, the involvement of cellular factors in the folding process is now emerging (Pelham, 1986; Ellis, 1987; Rothman, 1989; Fischer & Schmid, 1990; Schmid, 1991; Jaenicke, 1991). A number of such putative protein folding modulators have been proposed, but mechanistic folding experiments on any of those are still sparse.

Some factors have been described, where at least the chemical reaction catalyzed is understood (if not their physiological role), such as peptidyl-prolyl cis–trans isomerase (Lang et al., 1987) or disulfide isomerase (Bulleid & Freedman, 1988; Freedman, 1989). Other proteins, sometimes termed "molecular chaperones" (Ellis, 1987) have been described whose functions involve the noncovalent and transient association with folding intermediates. These include heat-shock proteins and their homologues of the Hsp70, Hsp60, and Hsp10 class, and they have been demonstrated to be involved in protein folding [see, e.g., Laskey et al. (1978), Bochkareva et al. (1988), Chirico et al. (1988), Deshaies et al. (1988), Hemmingsen et al. (1988), Flynn et al. (1989), Goloubinoff et al. (1989a,b), Laminet et al. (1990), Buchner et al. (1991), and Martin et al. (1991)]. The extent of this involvement, the mechanisms of action, and their specificities are largely unknown. Roles in the assembly of oligomeric proteins (Goloubinoff et al., 1989a,b) and in preventing transported proteins from folding before crossing a membrane (Bochkareva

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