Effects of pH on the structure and function of neuronal nitric oxide synthase

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We investigated how pH affects rat brain neuronal nitric oxide synthase (nNOS) with regard to spin-state equilibrium and the thiolate ligand bond of the haem group, catalytic activity, and monomer ↔ dimer equilibrium. At neutral pH, nNOS containing 1 equiv. of (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH₄) per dimer was mostly high-spin (λ_{max} at 398 nm), whereas the BH₄-free enzyme consisted of a mixture of the high-spin and two low-spin forms (λ_{max} at 418 nm, and at 376 and 456 nm respectively). With BH₄-free nNOS, an appreciable high-spin fraction was only observed between pH 7 and 8; at pH 6 and 9, the 418 and 376/456 nm low-spin forms predominated respectively. With nNOS containing 1 equiv. of BH₄ per dimer, similar observations were made, but these involved only half of the enzyme; the other half, presumably the BH₄-containing subunits, remained high-

spin. Since the spin state in the BH₄-free subunit appeared little affected by the state of the other subunit, we conclude that, in dimeric nNOS, the two haem groups function independently. Low pH destabilized thiolate binding and the interaction between NOS subunits, as indicated by CO-binding studies and gel electrophoresis respectively. Formation of L-citrulline was optimal between pH 7.0 and 7.5; the decrease in NOS activity at lower pH proved to be due to uncoupling of NADPH oxidation, resulting in increased formation of H₂O₂. At high pH strict coupling of L-arginine and NADPH oxidation was maintained, even in the absence of exogenous BH₄. The possible pathophysiological implications of the uncoupling at low pH are discussed.

INTRODUCTION

Nitric oxide synthase (EC 1.14.13.39; NOS) produces NO from L-arginine [1,2]. The oxidation of L-arginine and the reduction of O₂ take place at a haem moiety. The required electrons are provided by NADPH and are shuttled to the haem by FAD and FMN. This internal electron transfer occurs only when the enzyme has bound Ca²⁺/calmodulin; in addition, L-Arg oxidation requires the binding of (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH₄), at a site presumably located near the haem. The purified neuronal enzyme (nNOS) usually contains approx. 2 haems and 1 BH₄ per dimer [3], but the activity of such dimers is stimulated 2-fold in the presence of exogenous BH₄, indicating that the enzyme is able to bind two BH₄ molecules per dimer. NOS catalyses the formation of NO only when both L-Arg and BH₄ are present. In the absence of either of these compounds, nNOS oxidizes NADPH at the expense of O₂, which is, under those conditions, reduced to superoxide anion, O₂^{•-} [4,5]. This uncoupling of O₂ reduction from NO formation is down-regulated in the endothelial and inducible isoforms [6–8].

NO synthases are fusion proteins of a cytochrome *P*-450 reductase [9] and an oxygenase domain that spectroscopically [10–12] and functionally [13] resembles cytochrome *P*-450. The spectroscopic similarities between NOS and cytochrome *P*-450 derive from the identity of the axial haem ligand, which is a cysteinyl sulphur atom in both cases [14]. Like many cytochrome *P*-450s, NOS exhibits a blue shift of the Soret maximum upon addition of the substrate, L-Arg, indicative of a low-to-high-spin transition [15]. However, NOS is already mostly high-spin in the absence of substrate, since BH₄ also induces a transition to the high-spin state [16,17]. Sulphur-ligated haemoproteins also form complexes with thiol-containing compounds that are charac-

terized by absorbance spectra with a double Soret band with maxima at 370–390 and 450–470 nm [18–20].

We studied the effect of pH on the absorbance of BH₄-free and BH₄-containing (1 equiv./dimer) nNOS. The results provide further support for our suggestion [21] that the haems in NOS dimers function independently from each other. We also determined the largely unexplored effects of pH on some of the catalytic properties of nNOS. Uncoupling strongly depended on pH, with no uncoupling occurring at high pH, even in the absence of exogenous BH₄, whereas at low pH uncoupling was observed at saturating L-Arg and BH₄ concentrations.

EXPERIMENTAL

Purification of nNOS

Recombinant rat brain nNOS was purified from baculovirus-infected insect cells as described previously [22]. BH₄-free nNOS was obtained from Sf9 cells treated with 2,4-diamino-6-hydroxypyrimidine according to a published procedure [3]. L-[2,3,4,5-³H]Arg hydrochloride (57 Ci/mmol) was purchased from MedPro (Amersham, Vienna, Austria). Enzyme concentrations are expressed as the concentration of the monomer, assuming a molecular mass of 160 kDa. Throughout the present paper the terms nNOS(BH₄–) and nNOS(BH₄+) are used to refer to the enzyme with 0 and 1 equiv. of BH₄ per NOS dimer respectively.

Enzyme activity determination

NOS activity was determined as the formation of L-[2,3,4,5-³H]citrulline from L-[2,3,4,5-³H]Arg [23]. Unless indicated otherwise, the enzyme (10–15 nM) was incubated at 37 °C for 10 min in 50 mM buffer (see below) containing 0.1 mM [³H]L-Arg (≈ 50 000 c.p.m.), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN,

Abbreviations used: NOS, nitric oxide synthase; nNOS, eNOS, neuronal and endothelial isoforms of NOS; nNOS(BH₄–), BH₄-free nNOS; nNOS(BH₄ +), nNOS as isolated, containing 1 equiv. of BH₄ per dimer; BH₄, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin); CaM, calmodulin; Ches, 2-(*N*-cyclohexylamino)ethanesulphonic acid; Bis-tris propane, 1,3-bis[tris(hydroxymethyl)methylamine]propane; DTT, dithiothreitol; LT-PAGE, low-temperature SDS/PAGE.

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10 μ M BH_4 , 0.5 mM CaCl_2 , 10 μ g/ml calmodulin (CaM), 2.4 mM 2-mercaptoethanol, and 0.2 mM CHAPS, followed by separation and detection of [^3H]citrulline.

NADPH oxidation was determined spectrophotometrically at 340 nm and 37 °C as described previously [24]. The measuring cuvette contained, depending on the type of experiment, 50–250 nM nNOS, 50 mM buffer (see below), 0.5 mM CaCl_2 , 10 μ g/ml CaM, 2.4 mM 2-mercaptoethanol, 0.2 mM CHAPS, and, when indicated, 0.1–0.2 mM L-Arg and 10–50 μ M BH_4 . The reaction was started by the addition of 0.2 mM NADPH and monitored for 10 min. The observed rates were corrected by subtraction of blank rates obtained in the absence of calmodulin.

H_2O_2 production was determined as published [5]. To correct for non-enzymic H_2O_2 formation, the rates in the absence of CaM were subtracted from those in its presence. Under some conditions this will yield negative values. In the presence of exogenous BH_4 at pH > 7, CaM-independent O_2^- formation will result from BH_4 autooxidation, whereas nNOS will only produce NO, CaM-dependently. Scavenging of O_2^- by NO will result in lower CaM(+) than CaM(–) values in the range of highest NO production, and thus in negative corrected values (see the Results section, Figure 4, inset).

Gel electrophoresis

Low-temperature SDS/PAGE (LT-PAGE) was performed according to a published procedure [25]. In all experiments nNOS ($\approx 2 \mu\text{M}$) was preincubated for 5 min at 37 °C at the indicated pH before electrophoresis. In the cross-linking experiments the preincubated samples were subsequently incubated for 1 min at 20 °C at a concentration of $\approx 0.8 \mu\text{M}$ in 50 mM sodium phosphate in the presence of 1% glutaraldehyde, before standard SDS/PAGE. Relative amounts of monomers and dimers were estimated with a vds 800 video system equipped with the H1D software (Analysentechnik Hirschmann, Taufkirchen, Germany).

Absorbance spectroscopy

Absorbance spectra were measured with a Hewlett–Packard 8452A diode-array spectrophotometer. The final nNOS concentration was approx. 3 μM . These samples contained 2–4 mM 2-mercaptoethanol derived from the enzyme stock solutions [22]. CO binding was measured in a Thunberg cuvette. NOS samples were thoroughly deoxygenated by cycles of evacuation and CO admission; the samples were kept on ice during this procedure to prevent excessive evaporation. CO was then added to about 90% of atmospheric pressure, after which the absorbance spectra were measured. Subsequently, a few grains of sodium dithionite, already present in one of the side bulbs of the cuvette, were added, and spectra were measured immediately afterwards, as well as after distinct incubation periods.

Buffers

For the optical experiments we routinely used 0.1 M concentrations of sodium acetate (pH 4.0–6.0), potassium phosphate (pH 5.5–8.0) and Tris/HCl (pH 7.5–10.0). We did not observe any buffer-specific effects when, at pH 5.5–6.0 and pH 7.5–8.0, the spectra in phosphate buffer were compared with those in acetate or Tris buffer, or when this buffer system was replaced by the series of Good buffers (at 0.1 M) described below. For the activity assays we applied 50 mM sodium acetate (pH 4.5–5.5), Mes (pH 5.5–6.5), Bis-tris propane (pH 6.5–9.0) and 2-(*N*-cyclohexylamino)ethanesulphonic acid (Ches, pH 9.0–10.0), and in some cases Bis-tris (pH 6.0–7.0), triethanolamine/HCl (pH 6.5–

8.0) or Tris/HCl (pH 7.5–8.5). All buffers were interchangeable, except that in Mes we consistently observed slightly lower activities at pH 6.5 (compared with Bis-tris propane) and pH 6.0 (compared with Bis-tris) and very low activities at pH 5.5. The latter values were ignored.

Data analysis

Values for the various parameters were derived by non-linear least-squares fitting (Levenberg–Marquardt algorithm) to the appropriate equations.

Fractions of NOS in the 398 nm high-spin, and the 418 and 376/456 nm low-spin states were estimated from the absorbances at 398, 418, 456, and, as a control, at 380 nm, with a set of four equations of the type:

$$A_\lambda = f_{398} \times C_{398} + f_{418} \times C_{418} + f_{456} \times C_{456} \quad (1)$$

in which A_λ is the measured absorbance, and each term represents the fraction of one of the three enzyme forms multiplied by appropriate proportionality constants.

Disregarding the high-spin 398 nm fraction, the low-spin fractions were separately fitted to one pK_a value according to eqn. (2):

$$\frac{[\text{NOS}_{456}]}{[\text{NOS}_{456}] + [\text{NOS}_{418}]} = \frac{f_{\max}}{1 + 10^{\text{pK}_a - \text{pH}}} + \frac{f_{\min}}{1 + 10^{\text{pH} - \text{pK}_a}} \quad (2)$$

in which $f_{\max}$ and $f_{\min}$ represent the fractions at high and low pH, respectively. Since, for nNOS(BH_4^-), $f_{\max}$ and $f_{\min}$ were 1 and 0, the 376/456 nm fraction could be fitted to eqn. (3):

$$\frac{[\text{NOS}_{456}]}{[\text{NOS}_{456}] + [\text{NOS}_{418}]} = \frac{[\text{2-ME}]}{[\text{2-ME}] + K_d(1 + 10^{\text{pK}_a - \text{pH}})} \quad (3)$$

which was derived assuming one K_d for thiolate binding to low-spin nNOS, and one pK_a for the thiol $\leftrightarrow$ thiolate equilibrium (see the Discussion); [2-ME] designates the concentration of 2-mercaptoethanol. For nNOS(BH_4^-) all fractions were then fitted to a set of equations derived from Scheme 1 (below) with the assumption that the binding of the thiolate ligand (RS^-) to low-spin haem is not affected by the protonation state of NOS (eqns. 4–6):

$$f_{\text{NOS}_{398}} = \frac{K_{s2} + K_{s1} \cdot 10^{\text{pK}_{a1} - \text{pH}} + K_{s3} \cdot 10^{\text{pH} - \text{pK}_{a2}}}{(C_L + K_{s2}) + (C_L + K_{s1}) \cdot 10^{\text{pK}_{a1} - \text{pH}} + (C_L + K_{s3}) \cdot 10^{\text{pH} - \text{pK}_{a2}}} \quad (4)$$

$$f_{\text{NOS}_{418}} = \frac{1 + 10^{\text{pK}_{a1} - \text{pH}} + 10^{\text{pH} - \text{pK}_{a2}}}{(C_L + K_{s2}) + (C_L + K_{s1}) \cdot 10^{\text{pK}_{a1} - \text{pH}} + (C_L + K_{s3}) \cdot 10^{\text{pH} - \text{pK}_{a2}}} \quad (5)$$

$$f_{\text{NOS}_{456}} = \frac{(C_L - 1) \cdot (1 + 10^{\text{pK}_{a1} - \text{pH}} + 10^{\text{pH} - \text{pK}_{a2}})}{(C_L + K_{s2}) + (C_L + K_{s1}) \cdot 10^{\text{pK}_{a1} - \text{pH}} + (C_L + K_{s3}) \cdot 10^{\text{pH} - \text{pK}_{a2}}} \quad (6)$$

$$\text{with } C_L = \frac{[\text{2-ME}]/K_d}{1 + 10^{\text{pK}_{a3} - \text{pH}}} + 1.$$

pK_{a1} , pK_{a2} , pK_{a3} , K_{s1} , K_{s2} , K_{s3} and $K_d \equiv K_{d1} = K_{d2} = K_{d3}$ are defined as indicated in Scheme 1 (below).

RESULTS

pH-dependent absorbance spectra

As reported previously, the absorbance spectrum of nNOS(BH_4^-) at neutral pH is due to a mixture of the native

Figure 1 Dependence on pH of the absorbance spectra of $\text{nNOS}(\text{BH}_4^-)$ and $\text{nNOS}(\text{BH}_4^+)$

(A) Absorbance spectra of $\text{nNOS}(\text{BH}_4^-)$ between pH 6.0 (○) and pH 8.9 (●). The intermediate spectra were measured at pH 7.0, 7.6 and 8.3. (B) Absorbance spectra of $\text{nNOS}(\text{BH}_4^+)$ at pH 5.0 (○), pH 7.0 (—) and pH 10.0 (●). Experimental conditions were: $3 \mu\text{M}$ $\text{nNOS}(\text{BH}_4^-)$ or $\text{nNOS}(\text{BH}_4^+)$; 100 mM buffer (sodium acetate at pH 5.0, potassium phosphate from pH 6.0 to 7.6, Tris/HCl from pH 8.3 to 10.0); 25 °C.

high-spin form absorbing at 398 nm, and two low-spin compounds, one absorbing at 418 nm, the other at 376 and 456 nm [17]. The spectra of $\text{nNOS}(\text{BH}_4^-)$ at mildly basic and acidic pH were quite different (Figure 1A): at pH 6.0 [$\text{nNOS}(\text{BH}_4^-)$ was unstable at pH 5.0] the enzyme consisted almost exclusively of the 418 nm compound, whereas at pH 9.0 the 376/456 nm compound predominated. Similar, but less striking effects were obtained with $\text{nNOS}(\text{BH}_4^+)$, which is mainly in the 398 nm high-spin form at pH 7.0. At decreasing pH a band at ≈ 415 nm appeared (Figure 1B), which became more intense than the 398 nm band at pH 5.0; at pH 4 the enzyme was unstable. At high pH, transitions at 416 nm, as well as at 390 and 454 nm became evident. L-Arg (500 μM) or BH_4 (100 μM) caused a shift towards the high-spin form at all pH values with both $\text{nNOS}(\text{BH}_4^-)$ and $\text{nNOS}(\text{BH}_4^+)$ (results not shown).

From plots of the absorbances at a few selected wavelengths as a function of pH, apparent pK_a values of 6.9 ± 0.4 and 8.3 ± 0.4 were obtained for $\text{nNOS}(\text{BH}_4^-)$; the data for $\text{nNOS}(\text{BH}_4^+)$ could be fitted to the same values. It should be noted that these pK_a^{app} values have no direct physical significance, since they are affected by spin-state equilibria in addition to (de)protonations. From the pH-dependent absorbance changes we estimated the relative concentrations of the 398, 418, and 376/456 nm compounds in $\text{nNOS}(\text{BH}_4^-)$ and $\text{nNOS}(\text{BH}_4^+)$ as a function of pH (see the Discussion).

Figure 2 Dependence on pH of the formation of L-citrulline by nNOS

The activity ('Act.') of $\text{nNOS}(\text{BH}_4^+)$ was measured (see the Experimental section) between pH 4.5 and 10.0 in the absence (●) and presence (○) of 10 μM BH_4 . The broken lines (—) are best fits to a model assuming two apparent pK_a values. All data points are the averages of three independent determinations, and are presented as means $\pm$ S.E.M. The pH was varied with sodium acetate (pH 4.5–5.5), Mes (pH 5.5–6.5), Bis-tris propane (pH 6.5–9.0) and Ches (pH 9.0–10.0). Experimental conditions were: 12.5 nM $\text{nNOS}(\text{BH}_4^+)$, 50 mM buffer, 0.1 mM [^3H]L-Arg (≈ 50000 c.p.m.), 0.2 mM NADPH, 5 μM FAD, 5 μM FMN, 0.5 mM CaCl_2 , 10 $\mu\text{g/ml}$ CaM, 0.2 mM CHAPS and 2.4 mM 2-mercaptoethanol; 37 °C.

Integrity of the haem–thiolate bond

The transformation into the 418 nm compound could, in principle, be due to replacement of the proximal cysteinyl sulphur haem ligand by a neighbouring amino acid residue. Since the absorbance maximum of the complex of ferrous haem with CO is highly sensitive to the identity of the axial ligand, we studied the CO-binding properties of $\text{nNOS}(\text{BH}_4^+)$ at pH 5.5, 7.5 and 9.5. At pH 7.5 and 9.5 we observed an increase at 444 nm, as expected of a P-450-type haem (results not shown). In both cases the absorbance increase was biphasic, with a fast initial phase (complete within 10 s), followed by a slower phase ($t_{1/2} \approx 4 \pm 1$ min). The contribution of the fast phase to the total absorbance change was 16 and 45 % at pH 9.5 and 7.5 respectively. At pH 5.5 the P-450 spectrum was formed immediately, but was subsequently transformed ($t_{1/2} \approx 6 \pm 1$ min) into a P-420 spectrum with the maximum at 422 nm (not shown), indicating that the cysteinyl sulphur ligation is still intact, but labile, at low pH. At pH 5.5 we also observed a slow increase in turbidity, indicative of protein denaturation.

pH-dependence of L-citrulline formation

Determination of NOS activity at pH 4.5–10.0 showed a pH optimum between 7.0 and 7.5, with sharp decreases in activity below pH 6.5 and above pH 8.0 (Figure 2). Apparent pK_a values for this phenomenon were 6.57 ± 0.06 and 8.00 ± 0.06 . In the absence of exogenous BH_4 , similar apparent pK_a values (6.79 ± 0.06 and 7.97 ± 0.06) were observed, but the maximal activity was about half as high ($49 \pm 5\%$). In addition, exogenous BH_4 induced a small, but reproducible, shift of the optimum towards lower pH, arising from a downward shift (0.2 unit) of the lower apparent pK_a value.

The results of Figure 2 were obtained under routine assay conditions in the presence of 2.4 mM 2-mercaptoethanol. To

Table 1 Enzyme kinetic parameters of nNOS at various pH values

Parameters were determined from standard L-citrulline determination assays assuming simple Michaelis–Menten kinetics. L-Arg concentrations ranged from 0.5 to 200 μM . The buffers used were 50 mM Bis-tris, 50 mM triethanolamine, and 50 mM Bis-tris propane at pH 6.0, 7.0 and 8.5 respectively. All values are the averages of three independent determinations and are given as means $\pm$ S.E.M. See the Experimental section for further details.

pH	No added BH_4		+ 10 μM BH_4	
	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)
6.0	26 ± 3	16 ± 2	58 ± 1	5.9 ± 0.2
7.0	67 ± 5	9 ± 1	105 ± 2	4.2 ± 0.2
8.5	31 ± 1	26 ± 1	39.9 ± 0.4	5.3 ± 0.1

Figure 3 Correlation between nNOS-catalysed NADPH oxidation and L-citrulline production

NADPH oxidation and L-citrulline production were monitored in single samples at different pH values. NADPH oxidation of a 0.5 ml reaction mixture was continuously monitored at 340 nm ($\circ$); 10 μl samples were taken at regular intervals from the cuvette in duplicate for L-citrulline determination ($\bullet$). See the Experimental section for further details. Shown are the results obtained at pH 6.5 in the presence of exogenous BH_4 . The inset shows the NADPH/L-citrulline stoichiometries calculated from such experiments with ($\circ$) and without ($\bullet$) exogenous BH_4 . To obtain the CaM-dependent NADPH oxidation rates CaM(–) rates were subtracted from CaM(+) rates. Experimental conditions were: 0.94 μM nNOS(BH_4 +), 50 mM buffer (sodium acetate at pH 5.5, Bis-tris propane at pH 6.5, 7.5 and 8.5), 0.2 mM NADPH, 0.5 mM CaCl_2 , $\pm 10 \mu\text{g/ml}$ CaM, 0.2 mM [^3H]L-Arg (≈ 1700000 c.p.m.), $\pm 50 \mu\text{M}$ BH_4 , 0.2 mM CHAPS and 2.4 mM 2-mercaptoethanol; 37 $^\circ\text{C}$.

exclude the possibility that the thiol acted as a competitive inhibitor [21], we replaced 2-mercaptoethanol by 2.5 mM dithiothreitol (DTT), which has a much higher affinity for nNOS at pH 7.5, or with 2.5 mM glutathione, which does not bind to nNOS. In both cases essentially identical pH profiles were obtained (not shown).

To study the effect of pH on enzyme kinetic parameters, we determined the activity of nNOS(BH_4 +) with varying L-Arg concentrations (0.5–200 μM) at pH 6.0, 7.0 and 8.5 with and without exogenous BH_4 (10 μM). At pH 7.0, added BH_4 increased k_{cat} from 67 ± 5 to $105 \pm 2 \text{ min}^{-1}$ and decreased K_m (L-Arg) from 9 ± 1 to $4.2 \pm 0.2 \mu\text{M}$ (Table 1). Qualitatively similar results were obtained at pH 6.0 and 8.5. The increase of k_{cat} in the presence of exogenous BH_4 was markedly decreased at pH 8.5, but statistically significant ($P < 0.05$).

Figure 4 Uncoupling of NADPH oxidation and L-citrulline ('Citr.') production by nNOS at low pH

The amounts of NADP $^+$, L-citrulline, and H_2O_2 , produced by nNOS in 10 min, were determined simultaneously from single samples. NADPH oxidation of a 0.25 ml reaction mixture was continuously monitored at 340 nm. After 10 min incubation two 100 μl samples were taken for duplicate H_2O_2 and L-citrulline determinations (see the Experimental section). In the main Figure the pH-dependence of the [NADP $^+$]/[L-citrulline] stoichiometries, observed in the presence ($\circ$) and absence ($\bullet$) of 50 μM BH_4 , is plotted. The inset shows the amounts of H_2O_2 produced in the same reactions. The pH was varied with sodium acetate (pH 5.0–5.5), Mes (pH 6.0–6.5), Bis-tris propane (pH 6.5–9.0) and Ches (pH 9.0–9.5). To obtain the CaM-dependent NADPH and H_2O_2 oxidation rates CaM(–) rates were subtracted from CaM(+) rates. Experimental conditions were: 0.38 μM nNOS(BH_4 +), 50 mM buffer, 0.2 mM NADPH, 0.5 mM CaCl_2 , $\pm 40 \mu\text{g/ml}$ CaM, 0.2 mM [^3H]L-Arg (≈ 330000 c.p.m.), $\pm 50 \mu\text{M}$ BH_4 , 0.2 mM CHAPS and 2.4 mM 2-mercaptoethanol; 37 $^\circ\text{C}$.

Correlation of L-citrulline formation and NADPH oxidation

The rate of NADPH oxidation, both in the presence and absence of 200 μM L-Arg and 50 μM BH_4 , were optimal between pH 5.5 and 6.5 (results not shown), suggesting that NADPH oxidation has a pH profile different from L-citrulline production. However, the differences between the assay conditions do not allow direct comparison. Therefore we performed two series of experiments. In one series we took aliquots out of the cuvette during the NADPH assay at specific time intervals for L-citrulline determination. The time course of NADPH oxidation corresponded well with that of L-citrulline formation at all pH values studied [pH 5.5, 6.5 (Figure 3), 7.5 and 8.5]. From these experiments, which were performed in the presence of L-Arg and exogenous BH_4 , we calculated NADP $^+$ /L-citrulline stoichiometries (Figure 3, inset). The stoichiometry increased from the totally coupled value of 1.5 to a considerably uncoupled value of 3.0 when going from high to low pH. A similar pattern, but with slightly higher stoichiometries, was observed without exogenous BH_4 (Figure 3, inset).

These observations were confirmed in a second series of experiments in which NADPH oxidation, L-citrulline formation and H_2O_2 production were determined after 10 min incubation over a wide pH range (Figure 4). We found that the NADP $^+$ /L-citrulline stoichiometry was close to 1.5 at pH ≥ 7.0 in the presence of exogenous BH_4 , but increased at lower pH. Without exogenous BH_4 the stoichiometry already deviated from 1.5 at pH ≤ 8 , and reached values well above 4 at low pH. The increase in stoichiometry was accompanied by H_2O_2 formation, which was negligible above pH 7 (with exogenous BH_4) and maximal at pH 5.5 (Figure 4, inset).

To investigate whether the uncoupling originates from dramatic changes in the BH_4 affinity, we determined the dependence

Table 2 Quantification of NOS dimers at various pH values

Dimer fractions were estimated by densitometric analysis of the gels after SDS/PAGE. All values are the averages for three experiments and are given as means $\pm$ S.E.M. For the cross-linking experiments, nNOS(BH₄+) was cross-linked as described in the Experimental section before electrophoresis, and after preincubation for 10 min at 20 °C at the indicated pH. LT-PAGE was performed as described in the Experimental section after preincubation of nNOS(BH₄+) in the presence or absence of 200 μ M BH₄ and 1 mM Arg for 10 min at 20 °C at the indicated pH.

pH	Dimers (%)		
	SDS/PAGE after cross-linking		LT-PAGE
	—BH ₄ /Arg	—BH ₄ /Arg	+BH ₄ /Arg
4.5	36 $\pm$ 2	0	0
5.0	48 $\pm$ 3		
5.5	55 $\pm$ 2	13 $\pm$ 1	42 $\pm$ 3
6.5	61 $\pm$ 2	25 $\pm$ 1	46 $\pm$ 2
7.5	64 $\pm$ 1	29 $\pm$ 1	48 $\pm$ 1
8.5	58 $\pm$ 2	28 $\pm$ 2	48 $\pm$ 1
9.5	59 $\pm$ 1	19 $\pm$ 3	49 $\pm$ 3

Table 3 Reversibility of the effect of pH on activity

The activity of nNOS(BH₄+) was measured under standard assay conditions (see the Experimental section) with or without 10 min preincubation and are presented as % of control. The activity at pH 7.5 without preincubation was taken as 100%. The applied buffers were sodium acetate at pH 5.5, Bis-tris propane at pH 7.5 and Ches at pH 9.0. Preincubation conditions were: 1.9 μ M nNOS(BH₄), 0.17 M buffer, 0.2 mM CHAPS, 2.4 mM 2-mercaptoethanol; 21 °C. Assay conditions were: 12.5 nM nNOS(BH₄), 50 mM buffer, 0.1 mM [³H]Arg ($\approx$ 50 000 c.p.m.), 0.2 mM NADPH, 5 μ M FAD, 5 μ M FMN, 0.5 mM CaCl₂, 10 μ g/ml CaM, 0.2 mM CHAPS, 2.4 mM 2-mercaptoethanol; 37 °C. All values are the averages of three independent determinations and are presented as mean values $\pm$ S.E.M.

pH of assay	pH of preincubation	Relative activity
7.5	—	100 $\pm$ 5.6
7.5	7.5	99.5 $\pm$ 5.7
5.5	—	20.8 $\pm$ 1.1
5.5	5.5	13.0 $\pm$ 0.4
7.5	5.5	83.3 $\pm$ 6.0
9.0	—	12.8 $\pm$ 0.4
9.0	9.0	10.7 $\pm$ 0.6
7.5	9.0	62.6 $\pm$ 6.8

of L-citrulline production on the BH₄ concentration at pH 5.5, and found that the EC₅₀ for BH₄ was not affected much by the low pH (0.14 $\pm$ 0.08 μ M; results not shown).

Monomer $\leftrightarrow$ dimer equilibria

To establish whether the changes in activity and haem spin state were accompanied by changes in the quaternary structure of the enzyme, we performed LT-PAGE after incubation at various pH values (Table 2). In the absence of L-Arg and exogenous BH₄ most of the enzyme was monomeric. Dimerization was maximal at pH 7.5, decreased slightly at high pH, decreased sharply at low pH and was absent at pH 4.5. The presence of BH₄ and L-Arg during preincubation substantially stabilized the dimer, yielding equal fractions over the whole pH range, except at pH 4.5, where no dimers were present.

It was recently found in our laboratory that a rapid and robust estimation of the dimer fraction can be made by cross-linking the subunits prior to conventional SDS/PAGE (B. Hemmens and B.

Mayer, unpublished work). By this method we found virtually constant levels of dimer for nNOS(BH₄+) above pH 5.5, with decreasing values at lower pH (Table 2).

Reversibility of pH effects

To establish whether the activity decrease at the pH extremes is caused by irreversible inactivation, we determined the effect of preincubation at various pH values on the activity of nNOS in a standard L-citrulline assay (Table 3). Preincubation of $\approx$ 2 μ M nNOS at pH 7.5 and 21 °C for 10 min did not significantly decrease the activity. Likewise, the much lower activities at pH 5.5 and 9.0 only slightly decreased after preincubation. Moreover, when the activity was determined at pH 7.5, instead of at the pH of preincubation, the enzyme regained most (60–80 %) of its activity. These results demonstrate that, between pH 5.5 and 9.0, the pH effects are largely reversible.

DISCUSSION

Effects of pH on the spectral properties of nNOS

All published spectroscopic studies to date were performed at pH values between 7.4 and 7.9. As shown here, the effect of pH on the absorbance of nNOS(BH₄+) in general, and over the pH range from 7 to 8 in particular, is modest. By contrast we saw large effects on the absorbance spectrum of nNOS(BH₄–), which illustrates the importance of BH₄ in maintaining NOS in its active high-spin form. The spectral forms absorbing at 398, 418 and 376/456 nm have been respectively assigned to high-spin 5-co-ordinate haem [10,11], low-spin 6-co-ordinate haem with water as the sixth ligand [26], and low-spin 6-co-ordinate haem with 2-mercaptoethanol-derived thiolate as the sixth ligand [17]. The present data demonstrate that the 418 nm compound is the dominating species at low pH; at high pH almost all of the enzyme is converted into the 376/456 nm compound.

Assuming that the spectrum of nNOS(BH₄+) at pH 7.4 in the

Figure 5 Dependence on pH of the relative concentrations of the 398, 418, and 376/456 nm compounds in nNOS(BH₄–) and nNOS(BH₄+) between pH 5.0 and 10.0

(A) Calculated fractions between pH 5.0 and 10.0 for nNOS(BH₄–) of the 398 nm compound (■), the 418 nm compound (○) and the 376/456 nm compound (●). The lines through the data points are best fits to eqns. (4)–(6) (see the Experimental section) and yielded the fitting parameters as indicated in the Discussion. (B) Calculated fractions between pH 5.0 and 10.0 for nNOS(BH₄+) of the 398 nm compound (■), the 418 nm compound (○) and the 376/456 nm compound (●). The continuous line through the data points of the 398 nm compound was fitted assuming that one half of the enzyme remains high-spin throughout the pH range, and the other half obeys eqn. (4) (see the Discussion). The broken lines (---) are smooth curves through the low-spin fraction data. They were drawn for clarity and have no physical significance. (C) Dependence on pH of the fraction of low-spin haem NOS that is in the 376/456 nm form for nNOS(BH₄–) (●, continuous line) and nNOS(BH₄+) (○, broken line). The lines through the data points were fitted to eqn. (2) (see the Experimental section), and yielded the following parameters: for nNOS(BH₄–) $pK_a = 7.53 \pm 0.04$, $f_{max} = 1.02 \pm 0.02$, and $f_{min} = -0.02 \pm 0.02$; for nNOS(BH₄+) $pK_a = 7.4 \pm 0.2$, $f_{max} = 0.50 \pm 0.04$, and $f_{min} = 0.03 \pm 0.05$.

Scheme 1 Interdependence between spin-state equilibrium and protonation state of nNOS(BH₄[−]), and the BH₄-free subunit of nNOS(BH₄⁺)

The three protonation states are indicated as NOS, NOS H⁺ and NOS 2H⁺. The suffices h.s. and l.s. designate high- and low-spin haem. RSH and RS[−] represent 2-mercaptoethanol in the thiol and thiolate form. In nNOS(BH₄[−]) the population of enzyme states NOS_{h.s.} and NOS_{h.s.}2H⁺ is negligible. The spin-state equilibrium constants (K_s) are defined as $[NOS_{h.s.}]/[NOS_{l.s.}]$.

presence of L-Arg represents the pure 398 nm compound, and that the nNOS(BH₄[−]) spectra at low and high pH (obtained by extrapolation of the pH-dependent absorbances) represent the pure 418 and 376/456 nm compounds respectively, we estimated the fraction of each compound as a function of pH (see the Experimental section). The results, shown in Figure 5, indicate that nNOS(BH₄⁺) is for the most part (60–80%) high spin between pH 5 and 10, whereas nNOS(BH₄[−]), contains only 40–45% high-spin haem between pH 7 and 8, and very little outside that range.

A model describing the pH-dependence of the NOS haem spin states: nNOS dimers contain two independently functioning haems

A description of the pH profile requires three protonation states of the enzyme and must take into account the pK_a of 2-mercaptoethanol as well. The corresponding model is presented in Scheme 1. We decreased the number of parameters by first considering the equilibrium between the two low-spin forms separately (see the Experimental section). The pH-dependence of this equilibrium (Figure 5C) is well fitted to one apparent pK_a of 7.53 ± 0.04 , which can be ascribed to the (de)protonation of 2-mercaptoethanol. With eqn. (3) (see the Experimental section), $[2\text{-ME}] = (2.5 \pm 0.5) \times 10^{-3}$ M, and $pK_{a3} = 9.5$ [27], one arrives at a dissociation constant for the 2-mercaptoethanol anion of $(2.6 \pm 0.5) \times 10^{-5}$ M. From a previously obtained apparent dissociation constant of 15 mM at pH 7.0 [21], one can calculate a value for the true K_d of 2×10^{-5} M, in excellent agreement with the value derived from our model.

The observation that eqn. (3) describes the low-spin equilibrium implies that the affinity of the thiolate for nNOS does not depend strongly on the protonation state of the enzyme ($K_{d1} = K_{d2} = K_{d3} \equiv K_d$). This allowed us to derive a simplified set of equations (eqns. 4–6; see the Experimental section) with which good fits to the pH profiles for all three nNOS(BH₄[−]) fractions were obtained (Figure 5A), with parameters $pK_{a1} = 7.9 \pm 0.3$, $pK_{a2} = 8.5 \pm 0.5$, $K_{s1} = 0.06 \pm 0.04$, $K_{s2} = 6 \pm 2$, and $K_{s3} = 0 \pm 1$; pK_{a1} and pK_{a2} may represent two protonatable enzyme groups.

Fitting the high-spin fraction in nNOS(BH₄⁺) to eqn. (4) yielded $pK_{a1} = 7.6 \pm 0.2$ and $pK_{a2} = 9.2 \pm 0.7$ (Figure 5B), which demonstrates that BH₄ (1 equiv./dimer) has no major effect on the pK_a values of the enzyme. However, the low-spin haem contribution at the pH extremes appeared to level off at about 50%. In recent studies on the binding of DTT to nNOS [21] we found that DTT binds with much lower affinity to the BH₄-containing subunit of nNOS(BH₄⁺) than to the BH₄-free subunit. Likewise, we postulate that the pH effects involve the BH₄-free subunit exclusively, and we arrive at values for the remaining parameters ($K_{s1} = 0.3 \pm 0.1$, $K_{s2} = 9 \pm 2$ and $K_{s3} = 4 \pm 4$), which are not very different from those obtained for nNOS(BH₄[−]). We conclude that the BH₄-containing subunit remains high-spin between pH 5 and 10, and that the spin equilibrium of the BH₄-free subunit is only weakly affected by the BH₄-containing subunit. This suggests two independently functioning subunits within the NOS dimer with relatively little cross-talk, a conclusion we previously reached by a completely different route [21].

Effects of pH on nNOS activities

The pH profile of the catalytic activity of nNOS over a narrower pH range was reported previously [5,28,29]. In those studies pH optima between 6.5 and 7.0 were observed, whereas the optimum we obtained was about half a unit higher, closer to the published optima for the two other NOS isoforms [7,30,31]. There must be multiple causes for the divergent results, since none of the differences between those and our studies regarding enzyme source [5,29], choice of assay [28] and experimental conditions [5,28,29], apply to all three prior studies.

The pH profiles of the haem spin states (Figure 5) and L-citrulline formation (Figure 2) were remarkably similar. However, the pH profile of enzyme activity did not change when 2-mercaptoethanol was replaced with DTT or glutathione. Moreover, pH only affected the spin state of one haem per NOS(BH₄⁺) dimer, whereas the activity at the two pH extremes approached zero. We therefore conclude that there is no direct causal relationship between the pH-dependence of the activity and the haem spin-state.

Excess BH₄ decreased $K_m(\text{L-Arg})$, in line with prior observations that the binding of BH₄ and L-Arg is co-operative [32,33], and increased k_{cat} , in agreement with the binding of a second equivalent of BH₄ per dimer.

NADP⁺/L-citrulline stoichiometry: uncoupling at low pH

One of the most surprising results is the direct demonstration that, even in the presence of high concentrations of L-Arg and BH₄, the oxidation of NADPH was no longer strictly coupled to L-citrulline formation at low pH. Very low affinities of nNOS for L-Arg or BH₄ would present a rather trivial explanation for the phenomenon. However, these affinities were not drastically decreased at acidic pH (see the Results section), which demonstrates that the uncoupling occurred at saturating concentrations of L-Arg and BH₄.

The observation that the stoichiometry exceeds the theoretical value of 1.5 at pH ≤ 7.0 may explain why values of 2 were reported in some studies [34–36]. The strong correlation between NADPH oxidation and L-citrulline formation above pH 7 re-establishes 1.5 as the true stoichiometry for NOS catalysis. This value was recently challenged when it was suggested that the stoichiometry could be overestimated in the absence of thiols because of oxidation of NADPH by the putative NOS product peroxynitrite [34]. The present experiments were performed with

millimolar concentrations of thiols and the 1.5 stoichiometry pertained at high pH, conditions under which peroxynitrite is relatively unreactive and which favour its reaction with thiol groups [37].

Structural effects

The low rate of haem reduction by dithionite, the less than two-fold increase of k_{cat} in the presence of exogenous BH_4 , and the strict coupling, may be indicative of restricted accessibility of the haem at high pH. The decreased stability of the dimer and of the haem-cysteiny l thiolate bond, and the increased tendency to precipitate, suggest that nNOS is labile at very low pH. Other than that, pH seems to have no major impact on the structure of nNOS. There is no connection between the pH-dependence of the spectroscopic and catalytic properties and the nNOS quaternary structure.

Physiological implications

The increased uncoupling at low pH suggests that the intracellular pH regulates NOS, not only by determining the yield, but also the identity of the reaction products. This may be particularly relevant for nNOS, which has a greater tendency to produce O_2^- than the other isoforms [6–8]. It has been suggested that nNOS will switch to combined production of NO and O_2^- , and thus of ONOO^- (peroxynitrite) under conditions of limited availability of L-Arg and BH_4 [2]. Our results suggest that the intracellular pH may play a decisive part in this process. Neuronal NOS has been implicated as a major culprit in brain injury during ischaemia-reperfusion [2]. Since ischaemia is accompanied by a large decrease in intracellular pH, pH-dependent uncoupling may explain how nNOS shifts from the production of NO to the cytotoxic compounds ONOO^- and O_2^- . Although uncoupling is less pronounced in eNOS, it has been shown that, under conditions of limited L-Arg availability, eNOS also produces peroxynitrite, resulting in cell damage [38]. Recently, impaired vascular relaxation during myocardial ischaemia was suggested to be due to acidosis-induced loss of eNOS activity [39]. We propose that intracellular acidification may cause endothelial dysfunction not just by reducing NO production, but also by increasing the production of $\text{ONOO}^-/\text{O}_2^-$ by eNOS.

In summary, the confirmation of our earlier proposal that the two haems in NOS dimers act independently has important consequences for the catalytic mechanism. Similarly, re-establishing 1.5 as the true $\text{NADP}^+:\text{L-citrulline}$ stoichiometry, a value that recently came under fire, is crucial to our understanding of NOS catalysis. Finally, the observation of uncoupling at low pH may have great significance for the role of NOS under pathological physiological conditions.

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