The Stimulatory Effects of Hofmeister Ions on the Activities of Neuronal Nitric-oxide Synthase

APPARENT SUBSTRATE INHIBITION BY L-ARGININE IS OVERCOME IN THE PRESENCE OF PROTEIN-DESTABILIZING AGENTS*

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A variety of monovalent anions and cations were effective in stimulating both calcium ion-calmodulin (Ca\(^{2+}/CaM\))-independent NADPH-cytochrome c reductase activity of, and Ca\(^{2+}/CaM\)-dependent nitric oxide (NO) synthesis by, neuronal nitric oxide synthase (nNOS). The efficacy of the ions in stimulating both activities could be correlated, in general, with their efficacy in precipitating or stabilizing certain proteins, an order referred to as the Hofmeister ion series. In the hemoglobin capture assay, used for measurement of NO production, apparent substrate inhibition by l-arginine was almost completely reversed by the addition of sodium perchlorate (NaClO\(_4\)), one of the more effective protein-destabilizing agents tested. Examination of this phenomenon by the assay of l-arginine conversion to L-citrulline revealed that the stimulatory effect of NaClO\(_4\) on the reaction was observed only in the presence of oxyhemoglobin or superoxide anion (generated by xanthine and xanthine oxidase), both scavengers of NO. Spectrophotometric examination of nNOS revealed that the addition of NaClO\(_4\) and a superoxide-generating system, but neither alone, prevented the increase of heme absorption at 436 nm, which has been attributed to the nitrosyl complex. The data are consistent with the release of autoinhibitory NO directed to the prothetile group of nNOS, which, in conjunction with an NO scavenger, causes stimulation of the reaction.

The nitric-oxide synthases (NOSs) comprise a family of calmodulin (CaM)-dependent flavoheme enzymes that catalyze the NADPH-dependent oxidation by molecular oxygen of l-arginine to L-citrulline and nitric oxide (NO). The isoforms of NOS are grouped into three categories, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The first two constitutive isoforms are collectively referred to as cNOS. nNOS is a homodimer, which contains one molecule each of heme, FAD, FMN, and tetrahydrobiopterin (BH\(_4\)) per subunit (1–5). The binding sites for NADPH, FAD, and FMN are located in the carboxyl-terminal half of nNOS, which exhibits sequence homology to NADPH-cytochrome P-450 reductase and also contains FMN and FAD (6). The amino-terminal half contains the binding sites for heme, l-arginine, and BH\(_4\) (7). Electron transfer from NADPH via the flavins is facilitated by the binding of Ca\(^{2+}/\)calmodulin (Ca\(^{2+}/CaM\) and -l-arginine (8, 9). Electron transfer to the artificial electron acceptor cytochrome c is stimulated by, but is not totally dependent on, Ca\(^{2+}/CaM\) (10, 11) and represents the NADPH-cytochrome c reductase activity of nNOS.

A primary area of interest regarding the NOSs is the control of their activities. cNOS requires Ca\(^{2+}/CaM\) and is therefore sensitive to levels of Ca\(^{2+}\) in the cell. On the other hand, iNOS contains tightly bound Ca\(^{2+}/CaM\) and is not sensitive to cellular levels of Ca\(^{2+}\). cNOS contain what appears to be an auto-inhibitory element, whereas iNOS does not (12). There is also considerable evidence that cNOS and iNOS are feedback-inhibited by NO (13–18). Wang et al. (19) obtained optical and resonance Raman scattering spectroscopic evidence for a ferrous NO complex of nNOS. These investigators found that arginine stabilized the complex. Abu-Soud et al. (20) showed that the nitrosyl complex was formed rapidly and experienced a relatively slow O\(_2\)-dependent turnover in which nitrate was a product. In the steady state, most of the enzyme appeared to be complexed in this fashion, dissociating upon the depletion of either NADPH or l-arginine. An estimated Km\(_O2\) of about 350 M was determined for NADPH oxidation in the presence of l-arginine. In the presence of l-arginine, a value of K\(_mO2\) of about 350 M was reported (21). It has been suggested that the formation of nitrosyl NO and its slow recycling to free NO enable the enzyme to synthesize NO over a broad range of O\(_2\) concentrations. Confirmation of hexacoordinated NO\(^{−}\) with the heme prosthetic group of nNOS by EPR spectroscopy was reported by Migitia et al. (22), who also showed that l-arginine is bound near the distal side of the heme prosthetic group in close proximity to the bound NO\(^{−}\). The NO-heme complex was stabilized by l-arginine and analogs of l-arginine. Recently, Abu-Soud et al. (23) have reported the results of studies in which the rates of formation and decomposition of the nitrosyl iNOS oxygenase domain dimer were measured. However, no conclusions were drawn regarding the extent of autoinhibition of the full-length isoform by NO\(^{−}\).

Previously, we had shown that 3–4 M urea simulated the stimulatory effect of Ca\(^{2+}/CaM\) on NADPH-cytochrome c reductase activity of nNOS (24). Guanidinium chloride (GmCl),...
which like urea is a protein denaturant, at 0.4—0.5 M, stimulated the reductase activity to essentially the same extent. It was, therefore, of interest to determine whether other reagents of lesser protein denaturing capability would elicit similar changes in reductase activity and whether any of these reagents could promote NO\(^{\cdot}\) synthesis in the absence of Ca\(^{2+}\)/CaM in this work, we have found that neither urea nor GmCl is capable of mimicking the stimulatory effect of Ca\(^{2+}\)/CaM on NO\(^{\cdot}\) synthesis by nNOS and that both reagents are actually inhibitory to the reaction when added in the presence of Ca\(^{2+}\)/CaM. However, we have found that a variety of salts stimulated both the NADPH-cytochrome c reductase and Ca\(^{2+}\)/CaM-dependent NO\(^{\cdot}\) synthase activities of nNOS. Their efficacy in stimulating these activities could be correlated with the ability of these salts to induce structural changes in collagen and cold gelatin in dilute solution and unfolding or destabilization of native ribonuclease. The background of these investigations has been reviewed (25). Concentrations of salts required to stimulate reductase activity were somewhat higher than those required for optimal stimulation of NO\(^{\cdot}\) synthesis. Using the hemoglobin capture assay of the NO\(^{\cdot}\)-producing activity of NOS, it was found that sodium perchlorate (NaClO\(_4\)), at 50 mM, brought about a 5-fold increase in NO\(^{\cdot}\) synthesis at 100 \(\mu\)M L-arginine, the assay concentration of this substrate. At very low arginine concentrations (below 1 \(\mu\)M), NaClO\(_4\) had no effect. However, at higher arginine concentrations, reversal of the substrate inhibition by NaClO\(_4\) was apparent. Use of the NOS assay in which conversion of arginine to citrulline is measured revealed that oxyhemoglobin (oxyHb) or superoxide anion (generated by the oxidation of xanthine in the presence of xanthine oxidase) was required to obtain optimal stimulation with NaClO\(_4\). Comparable results were obtained when sodium chloride (NaCl) was used in place of NaClO\(_4\). Spectrophotometric observation of the nitrosyl nNOS complex, which is characterized by an absorption maximum at 436 nm, revealed that its formation was completely inhibited by the addition of 50 mM NaClO\(_4\) and a superoxide-generating system. Thus, it would appear that NaClO\(_4\), as well as other salts, prevent the formation of, or destabilize, the nitrosyl derivative in conjunction with reagents that react with NO\(^{\cdot}\). CD measurements of nNOS-Ca\(^{2+}\)/CaM indicated that only small changes in the secondary structure of the enzyme occurred in the presence of 25–75 mM NaClO\(_4\).

EXPERIMENTAL PROCEDURES

Materials—GmCl was obtained from Whitaker Corp. (Delaware Water Gap, PA). Guanidinium thiocyanate was a product of Eastman Kodak. NaClO\(_4\) was purchased from Aldrich. Horse heart cytochrome c (type VI), HEPES, BisTris, NADPH, sodium nitrate, and bovine brain CaM were obtained from Sigma. BH\(_4\) was a product of Research Biological (type VI), HEPES, BisTris, NADPH, sodium nitrate, and bovine brain CaM. Other compounds were of reagent grade and were purchased from reputable commercial sources.

Enzymes—nNOS was overexpressed in Escherichia coli and purified according to Roman et al. (26). Highly purified CuZn superoxide dismutase (3,400 units/mg) was a generous gift from Dr. John Crow (University of Alabama at Birmingham). Catalase (25,000 units/mg) and buttermilk xanthine oxidase (1.1 unit/mg) were purchased from Sigma.

Enzyme Assays—Superoxide dismutase and xanthine oxidase were assayed as described by McCord and Fridovich (27). NADPH-cytochrome c reductase assays were performed according to the procedure of Masters et al. (28). Nitric oxide formation was measured by the hemoglobin capture assay at 25 °C following the procedures of Kelm et al. (29) and Stuehr et al. (30). Measurements were made in a Shimadzu UV-2101 PC (dual beam) scanning spectrophotometer. Assay samples were read against blanks containing all assay components except nNOS. L-Citrulline formation from L-arginine was measured after separation of the amino acids using cation exchange chromatography by the method of Bredt and Snyder (31), except that 5 \(\mu\)M BH\(_4\) was added to the assay solution, and 0.5 \(\mu\)M/ml L-[\(^{14}\)C]arginine was used in place of L-[\(^{14}\)H]arginine. Dilutions of nNOS were made with 50 mM Tris-HCl (pH 7.5) containing 0.1 mM sodium EDTA, 100 mM NaCl, 0.1 mM 2-mercaptoethanol, and 10% glycerol.

Salt Solutions—Salts were usually prepared as 2 M solutions in 50 mM HEPES/KOH (pH 7.6). When necessary, the pH was adjusted with small volumes of 6 M HCl or 10 M NaOH.

RESULTS

To understand better the effects of urea and GmCl on the NADPH-cytochrome c reductase activity of nNOS, the effects of other protein denaturants and non-denaturants were examined. The results are shown in Fig. 1. As with urea and GmCl (24), in most of the cases there was a concentration dependence of activity, attaining an optimal level followed by a decline. As expected (Fig. 1A), GmSCN was the most effective on a molar basis, undoubtedly because of the destabilizing effects of both guanidinium and thiocyanate ions (25). It is notable that the stimulation at optimal GmSCN was equal to that of Ca\(^{2+}\)/CaM. As reported earlier (24), GmCl stimulation of NADPH-cytochrome c reductase activity at 0.4–0.5 M equaled that of Ca\(^{2+}\)/CaM. The remaining anions described in the figure were added as their sodium salts. In Fig. 1A, the order of effectiveness of the anions, as indicated by lower concentrations required to reach optimal activity, was SCN\(^{-}\), ClO\(_4\)\(^{-}\), NO\(_3\)\(^{-}\), and 1/2 SO\(_4\)\(^{2-}\) = CH\(_3\)COO\(^{-}\). Although the magnitudes of the stimulations by the halides were significantly lower than those of many of the other anions (Fig. 1B), there was a clear order of effectiveness: I\(^{-}\), Br\(^{-}\), Cl\(^{-}\) and F\(^{-}\). Taking these data together, the order of molar effectiveness of the anions in stimulating NADPH-cytochrome c reductase activity of nNOS was SCN\(^{-}\) > I\(^{-}\) > ClO\(_4\)\(^{-}\) > NO\(_3\)\(^{-}\) > Br\(^{-}\) > F\(^{-}\).
Br\(^-\) > NO\(_3^-\) > Cl\(^-\) > SO\(_4^{2-}\) = F\(^-\) = CH\(_3\)COO\(^-\). This order is in general agreement with the efficacy of these compounds as denaturants of other proteins (25). Thus, for native collagen and ribonuclease, the order observed for these anions was SCN\(^-\) > I\(^-\) > ClO\(_4^-\) > NO\(_3^-\) > Br\(^-\) > Cl\(^-\) > CH\(_3\)COO\(^-\) > SO\(_4^{2-}\). Monovalent cations, added as their chloride salts, gave less definitive stimulations than the anions tested, as shown in Fig. 2. For the most part, optimal stimulations required concentrations close to 1 M. NH\(_4^+\) was the most stimulatory, an unexpected observation in view of its reported effect as a protein-stabilizing cation (25). Over a broad range of concentrations, Cs\(^+\), Na\(^+\), K\(^+\), and Rb\(^+\), in that order, gave stimulations of 5–3-fold, whereas Li\(^+\), at the same concentrations, was only weakly stimulatory. It is of interest that Li\(^+\) was more effective than the other four alkali metal cations in stabilizing collagen (25). In an overall sense, these results probably indicate that various salts exert their stimulatory effect by influencing the conformation of nNOS and that mere changes in ionic strength cannot explain the striking differences in the data.

Because it was clear that many salts were able to mimic the stimulating effect of Ca\(^{2+}\)/CaM on nNOS-catalyzed NADPH-cytochrome c reductase, it was important to determine whether the presence of these reagents could affect NO\(^-\) synthesis by nNOS in the absence of Ca\(^{2+}\)/CaM. When urea was tested in the hemoglobin capture assay or in the citrulline assay, it was found not to substitute for Ca\(^{2+}\)/CaM. In fact, in the presence of Ca\(^{2+}\)/CaM, 2 mM urea completely inhibited the enzyme, as measured by both the hemoglobin capture and citrulline assay methods. Likewise, in the hemoglobin capture assay, GmCl failed to stimulate NO\(^-\) synthesis in the absence of Ca\(^{2+}\)/CaM, and, at 0.1–0.2 mM, it completely inhibited the reaction in the presence of Ca\(^{2+}\)/CaM. This was not surprising because guanidinium ion would be expected to antagonize the binding of L-arginine to the enzyme. Sorrentino et al. (32) have shown that GmCl and other guanidinium compounds are inhibitory to both cNOS and iNOS. In the present study, other compounds, including NaClO\(_4\), NaNO\(_3\), and NaCl, failed to substitute for Ca\(^{2+}\)/CaM in NO\(^-\) synthesis.

In the course of using the hemoglobin capture method in the assay of NO\(^-\) synthesis by nNOS, it was found that several of the compounds used in the study of the NADPH-cytochrome c reductase activity of nNOS actually stimulated Ca\(^{2+}\)/CaM-dependent NO\(^-\) synthesis. In this study, GmSCN was not used because of its inhibitory effect on the hemoglobin capture assay of NO\(^-\) synthesis. NaSCN, as well as GmSCN, brought about a rapid and nonenzymatic change in the absorption spectrum of oxyHb in the presence of assay solution components; it was thus precluded from being used in the assay. However, the order of effectiveness in the stimulation of NO\(^-\) synthesis by nNOS was ClO\(_4^-\) > I\(^-\) = Br\(^-\) > NO\(_3^-\) > CH\(_3\)COO\(^-\) = Cl\(^-\) (Fig. 3). With the exception of the positioning of I\(^-\) and Br\(^-\), the order of efficacy, as anticipated from the protein-stabilizing and -destabilizing properties of this group of anions, was essentially maintained (see above discussion of Fig. 1). The data show that ClO\(_4^-\) brought about a 5-fold stimulation of the reaction at a concentration of 50 mM. The effects of I\(^-\) and Br\(^-\) were practically superimposable, whereas the effect of NO\(_3^-\) was distinguishable from that of either acetate ion or Cl\(^-\). F\(^-\) was not tested because of its likely interaction at high concentrations with Ca\(^{2+}\).

All of the alkali metal cations stimulated the hemoglobin capture assay without demonstrating differences in their effective concentrations, as shown in Fig. 4. In this respect, the results are similar to those obtained with these cations in the NADPH-cytochrome c reductase reaction (Fig. 2). Again, Li\(^+\) was the least stimulatory, which is consistent with its reported protein-stabilizing effects (25).

All of the salts tested did not behave predictably. Thus, as shown in Fig. 5, NaSO\(_4\) produced significant stimulation of NO\(^-\) production over a broad concentration range (0.05–0.5 mM SO\(_4^{2-}\)), in stark contrast to its lack of effect on NADPH-cytochrome c reductase activity (Fig. 1). In addition, NH\(_4\)Cl and (NH\(_4\))\(_2\)SO\(_4\), both stabilizing salts (25), were unexpectedly effective at relatively low ammonium ion concentrations (optimal at 0.1–0.2 mM), although (NH\(_4\))\(_2\)SO\(_4\) was significantly less stimulatory. In the NADPH-cytochrome c reductase assay, NH\(_4^+\) was the most stimulatory cation tested (Fig. 2). It is possible that the stimulatory effect of NH\(_3\)/NH\(_4^+\) may represent a phenomenon unrelated to stabilization or destabilization of the enzyme protein.

Two multivalent cations and other multivalent anions were also studied with regard to their stimulatory/inhibitory effects on NO\(^-\) formation. Sodium phosphate (between 150 and 250 mM) stimulated NO\(^-\) formation some 3–4-fold. Sodium tartrate (between 100 and 200 mM) stimulated about 2.5-fold. Sodium citrate was stimulatory at low levels (2-fold at 25 mM) but inhibitory above 50 mM, probably because of its ability to chelate Ca\(^{2+}\). CaCl\(_2\) and MgCl\(_2\) were slightly stimulatory (10–30%) at low concentrations (<4 mM and <1 mM, respectively) but inhibitory at higher concentrations.

Further experiments were performed with NaClO\(_4\) because this reagent was effective at relatively low concentration (see Fig. 3) and did not interfere with either the hemoglobin capture assay or, as will be discussed shortly, the citrulline assay. Km values for L-arginine of 1.5 \(\mu\)M (31), 2.2 \(\mu\)M (33), and 2.8 \(\mu\)M (26) have been reported for nNOS. In the experiment described in Fig. 3, the L-arginine concentration was 100 \(\mu\)M. It was therefore of interest to examine the effect of NaClO\(_4\) over a broad range of L-arginine concentrations. For this purpose, the nNOS preparation was subjected to removal of residual

\(^2\) J. S. Nishimura and B. S. S. Masters, unpublished observation.
L-arginine introduced during the purification procedure by the use of spin columns of Sephadex G-50 (34). The results of this study are described in Fig. 6. Substrate inhibition of NO\textsubscript{z} synthesis by L-arginine is quite apparent in the data (Fig. 6A). The first evidence of inhibition appeared at about 5 μM L-arginine. The addition of 50 mM NaClO\textsubscript{4} substantially increased NOS activity over most of the concentration range studied. At 100 μM, the concentration of L-arginine used routinely in the assay, a stimulation of more than 5-fold was observed. Examination of the kinetics at low L-arginine concentrations (Fig. 6B) reveals stimulation by NaClO\textsubscript{4} at concentrations in the 1–2 μM L-arginine range. It was at concentrations below 1 μM L-arginine that NO\textsubscript{z} synthesis was not stimulated by NaClO\textsubscript{4}. It thus appears that NO\textsubscript{z} synthesis by nNOS was extensively inhibited by its substrate L-arginine and that 50 mM NaClO\textsubscript{4} almost completely reversed the inhibition. This impression is supported by the data in Fig. 6C, a double reciprocal plot of the data in Fig. 6A. In the presence or absence of NaClO\textsubscript{4}, the data can be extrapolated to give the same V\textsubscript{max} and a K\textsubscript{m} value of approximately 5 μM for L-arginine. However, substrate inhibition was obvious in the absence of NaClO\textsubscript{4} but much less apparent in its presence.

Further study of the effect of NaClO\textsubscript{4} was pursued by use of the citrulline assay. This method involves incubation of nNOS in the presence of necessary cofactors with substrate L-[14C]arginine and subsequent ion exchange chromatography to separate substrate and product (31). Up to 300 mM NaClO\textsubscript{4} or NaCl in the incubation solution could be tolerated in the subsequent ion exchange chromatography on Dowex 50-Na\textsuperscript{+} columns. The concentrations of the components in the assay solution were the same as those described for the hemoglobin capture assay except that oxyHb was omitted and the L-arginine concentration was 20 μM instead of 100 μM as employed in the capture assay. As shown in Table I (Experiment 1), 50 mM NaClO\textsubscript{4} alone had no effect on the reaction. However, the addition of oxyHb with NaClO\textsubscript{4} stimulated the reaction some 3–4-fold. Using this assay method, Rogers and Ignarro (13) have shown that the addition of 30 μM oxyHb linearized the kinetics of cerebellar NOS beyond 5 min, ostensibly by trapping inhibitory NO\textsubscript{z}. It thus appeared likely that NaClO\textsubscript{4} brought about release of inhibitory NO\textsuperscript{-} from the heme prosthetic group of nNOS and that the released NO\textsuperscript{-} was then scavenged by the heme group of oxyHb. In this process, NO\textsuperscript{-} is oxidized to nitrate (35), which is not inhibitory to NOS. Because NO\textsuperscript{-} is known to
react with superoxide to produce peroxynitrite (36), xanthine/xanthine oxidase, which generates superoxide anion, was used in place of oxyHb. As shown in Table I (Experiment 3), this resulted in a near 3-fold stimulation in the presence of NaClO₄. This effect was negated by superoxide dismutase in the presence of catalase. Qualitatively comparable data were obtained when superoxide anion generation by the autoxidation of BH₄ (37) at 100 \( \mu \text{M} \) (38), instead of the normal assay concentration of 5 \( \mu \text{M} \), was performed in the presence of 50 mM NaClO₄ (data not shown).

Because Na⁺ and Cl⁻ have been described as close to the "null point" between denaturing and nondenaturing ions (39), the effect of 300 mM NaCl on the citrulline assay was also studied. The results were consistent with those observed with NaClO₄ (Table I, Experiment 2). Thus, NaCl had some effect in the absence of oxyHb, but, when added with the latter, a 2–3-fold stimulation was observed.

The question of whether NaClO₄ or NaCl acted by interfering with nitrosyl nNOS formation or by dissociating nitrosyl nNOS was approached by delaying addition of NaClO₄ or NaCl to the assay solution, as shown in Fig. 7. Whether NaClO₄ was added at zero time or up to 2 min after the complete assay solution was constituted, stimulation of NOS activity was significantly greater than that observed in the absence of the salt. The same result was observed with 300 mM NaCl.

Direct evidence that the presence of perchlorate and a superoxide-generating system prevented formation of the nitrosyl nNOS complex, monitored at 436 nm, is shown in Fig. 8. In agreement with Abu-Soud et al. (20), nitrosyl nNOS was formed rapidly and decayed rapidly as NADPH and L-arginine were consumed (panel A). The addition of 50 mM NaClO₄ alone (panel B) or of a superoxide-generating system alone (panel C) had no effect on the formation of the complex or its disappearance. However, in the presence of both (panel D), nitrosyl nNOS formation was totally inhibited.

The possibility that the effects of the various anions were exerted through conformational changes in the protein was addressed by examination of the nNOS-Ca²⁺/CaM complex by circular dichroism, using a Jasco model J-720 spectropolarimeter (data not shown). In the presence of 25, 50, and 75 mM NaClO₄, only minor changes in the CD spectrum of 1 \( \mu \text{M} \)

![Fig. 5. Effects of ammonium and sulfate salts on the activity of nNOS as measured by the hemoglobin capture assay. Conditions of the assay were the same as those described in the legend of Fig. 3. The (NH₄)₂SO₄ and Na₂SO₄ concentrations were plotted as those of the ammonium ion and sodium ion, respectively. The data are representative of two experiments.](image)

![Fig. 6. Effect of the L-arginine concentration on the activity of nNOS in the hemoglobin capture assay. The experiment was performed with nNOS at a specific activity of 1,000, which had been subjected to removal of L-arginine. 8 mM nNOS was assayed as described in Fig. 2, with various concentrations of L-arginine. Panel A, activity measurements with L-arginine concentrations from 0 to 250 \( \mu \text{M} \). Panel B, activity measurements with L-arginine concentrations from 0 to 10 \( \mu \text{M} \). Panel C, double reciprocal plot of data in panel A. The data are representative of two experiments.](image)
TABLE I

L-Arginine formation by nNOS. Effects of oxyhemoglobin, NaClO₄, NaCl, and a superoxide-generating system on the reaction

| Addition(s) | Specific enzyme activity |
|-------------|--------------------------|
| Expt. 1     |                          |
| Control     | 140                      |
| 50 mM NaClO₄| 150                      |
| 8 μM oxyHb  | 150                      |
| 50 mM NaClO₄plus 8 μM oxyHb | 560 |
| Expt. 2     |                          |
| Control     | 130                      |
| 300 mM NaCl | 240                      |
| 8 μM oxyHb  | 140                      |
| 300 mM NaClO₄plus 8 μM oxyHb | 440 |
| Expt. 3     |                          |
| Control     | 220                      |
| Superoxide dianurate | 200 |
| 50 mM NaClO₄| 220                      |
| Xanthine oxidase | 230 |
| 50 mM NaClO₄, xanthine oxidase | 580 |
| 50 mM NaClO₄, xanthine oxidase, superoxide dianurate | 210 |

nNOS were observed. These results are consistent with the induction of local, rather than global, changes by the perturbing agent.

DISCUSSION

The phenomena in which both protein denaturants and non-denaturants stimulate the NADPH-cytochrome c reductase activity of nNOS in the absence of Ca²⁺/CaM and NO synthesis in the presence of Ca²⁺/CaM present interesting questions with regard to the relationship between the structure and function of nNOS. The ability of a given compound to destabilize the native structure of other proteins and the efficacy of that compound in stimulating the reactions catalyzed by nNOS initially suggested that significant conformational changes in the enzyme protein might be involved. The data presented in this manuscript have provided evidence that several of the compounds tested, in addition to urea and GmCl (24), bring about increases in NADPH-cytochrome c reductase activity which are equivalent to those induced by Ca²⁺/CaM. With practically all of the compounds used, there was a peak concentration at which optimal activity was observed. Thus, it was possible to make comparisons of the efficiencies of these compounds on a molar basis. It was of interest that, by this criterion, the stimulatory effectiveness of each ion tested could usually be correlated with its relative position in the Hofmeister ion series, which originally defined the relative efficacy of certain salts/ions to precipitate proteins from whole chicken egg white (39, 40). Theoretical treatments of the Hofmeister effect have been put forth for the solubility in aqueous solution of amino acids, synthetic peptides, and other model compounds, as a function of the concentrations of various salts (39, 41–44). Interestingly, a correlation could be drawn between these anions and cations and their ability to effect transitional changes in the structures of proteins, synthetic polymers, and DNA (25, 41). Thus, with respect to proteins, the more effective a salt was in precipitating a macromolecule, the more it stabilized its “native” structure. In the present study, for the most part, sodium salts of anions of interest and chloride salts of cations of interest were employed. The rationale for this was that, with regard to their stabilizing/destabilizing properties, Na⁺ and Cl⁻ have been found to be relatively neutral ions (39). With regard to nNOS (Figs. 1 and 3), it appears that the destabilizing anions were more effective in stimulating both NADPH-cytochrome c reductase activity and NO synthesis by nNOS. This was clear-cut in the case of the reductase activity (Fig. 1), which was stimulated most effectively by GmSCN and with decreasing molar efficacy by the sodium salts of SCN⁻ = I⁻, ClO₄⁻, Br⁻, NO₃⁻, and Cl⁻, and 1/2 SO₄²⁻ = F⁻ – acetate ion. There was little discrimination on the part of the alkali metal cations, except for Li⁺, which, consistent with its protein-stabilizing properties, stimulated the least strongly (Fig. 2). The stimulation by NH₄⁺ was not expected (see discussion of Fig. 5 below).

With regard to NO synthesis, the field of anions was reduced somewhat becouse GmSCN was an inhibitor of NO synthesis and, like NaSCN, interfered with the hemoglobin capture assay. However, the order of effectiveness in stimulating the NO synthesis reaction of ClO₄⁻, I⁻ = Br⁻, NO₃⁻, and acetate ion = Cl⁻ was consistent with the decreasing destabilizing properties of these ions (Fig. 3). SO₄²⁻ exhibited a broad range of stimulatory capacity (Fig 5). Phosphate, which at pH 7.6 is nearly a di-anion, was more effective in stimulating nNOS activity than SO₄²⁻ (data not shown). The reverse would have been predicted from the precipitating capacity of these ions with respect to bovine ribonuclease (25). As mentioned earlier, both MgCl₂ and CaCl₂ were inhibitory at relatively low concentrations. It is thus clear that a more extensive study would be required to develop insight into the mechanisms whereby divergent cations and anions affect nNOS activity.

It is difficult to discern any distinguishing features regarding
the effects of the alkali metal cations on NO synthesis because their molar efficacies were essentially the same (Fig. 4). However, as in the case of the reductase activity, Li⁺ was the least stimulatory of these ions (Fig. 2). NH₄⁺, in the form of NH₄Cl and (NH₄)₂SO₄, exhibited molar efficacies that were significantly lower than what was expected on the basis of the protein-stabilizing properties of these salts (Fig. 5). Thus, ammonium ion or ammonia may have a specific stimulatory effect on nNOS.

It is of interest that the concentrations required to attain the optimal effect with each compound tested were, in general, greater for the NADPH-cytochrome c reductase reaction than for NO synthesis, e.g. the optimal concentrations of NaClO₄ and NaNO₃ for reductase activity were approximately 7–8 times and approximately 3 times that for NO synthesis, respectively. In the case of reductase activity, the role of the reagent was to replace the requirement for Ca²⁺/CaM. However, with NO synthesis, the effect was only observed in the presence of Ca²⁺/CaM. From the literature and the results presented here, it may be possible to rationalize how the salts affect Ca²⁺/CaM-dependent NO synthesis. It is possible that the salts relieve the autoinhibition of NO by NO' (13–18).

Abu-Soud et al. (20) have presented evidence for the rapid and reversible formation (<2 s) of a six-coordinate ferrous-nitrosyl complex, involving 70–90% of nNOS upon mixing of the enzyme with substrates. Thus, when substrate (L-arginine or NADPH) is depleted, the nitrosyl form of the enzyme can undergo dissociation to release NO' and form free NOS (20). Based primarily on the inhibition of NADPH oxidation in the presence of L-arginine, it was estimated that the enzyme was 90% inhibited during the assay. It has been proposed that, in this state, compared with NADPH oxidation in the absence of L-arginine, the enzyme is able to generate NO' over a broader range of O₂ concentrations (21).

The results presented in this report suggest that NaClO₄, NaCl, and probably other salts act by dissociating NO' from the nitrosyl form of NOS. However, both NaClO₄ and NaCl were without significant stimulating effect in the absence of oxyHb or a superoxide anion-generating system (xanthine/xanthine oxidase), reagents that can be considered as traps of NO'. Therefore, the data are consistent with the ability of the salt to dissociate the ferrous nitrosyl complex of nNOS. Because the equilibrium of NO' binding would appear to favor the binding of NO' to give the inhibitory complex, the perturbation introduced by the appropriate salt could expose free NO' to either oxyHb or superoxide anion produced by the xanthine oxidase reaction or the autoxidation of BH₄. The net effect would be to shift the equilibrium between nitrosyl nNOS and free nNOS, favoring the latter and causing enzyme activity to rise dramatically. The fact that the time of the addition of either NaClO₄ or NaCl is not critical may indicate that either nitrosyl nNOS undergoes turnover once formed and that either reagent prevents reformation of the complex or these reagents act directly on the complex to cause its dissociation. The observation of the quenching of nitrosyl nNOS absorption at 436 nm in the presence of NaClO₄ and a superoxide-generating system (Fig. 8) provides direct evidence for this interpretation. That the quenching appeared to be total would favor the argument that the stimulation by NaClO₄ of nNOS activity in the hemoglobin capture assay reflected a complete reversal of autoinhibition by NO' of nNOS activity.

It would seem paradoxical that denaturing agents would elicit increases in the biological activity of a protein. However, in the circumstances studied here, it is clear that, as the perturbing compound/ion is increased in concentration, there is a decrease, sometimes sharp, following the stimulation, indicating that disruption of the active structure has been attained. It was therefore of interest to determine whether large conformational changes were involved in the stimulatory effects of the salts, particularly those that were very stimulatory. The CD experiment would appear to rule out changes that are global in magnitude and to favor changes that are more localized in nature. In this regard, a possible explanation comes from the observations of Gregor et al. (45). These investigators showed a selectivity of various anions for a quaternary base ammonium anion exchange resin. The order of selectivity involved a ranking that was very similar to the Hofmeister series described earlier. Thus, the more selective anion would displace a less selective anion in an anion-quaternary ammonium resin ion pair. By analogy, in the nitrosyl nNOS complex, an ion pair may be destabilized by perchlorate ion. The labilized nitrosyl nNOS would then be likely to break down, especially in the presence of an NO' trap, such as oxyHb or superoxide anion. It is also conceivable that the binding of a natural, albeit yet to be identified, activator/ligand of nNOS could also act by disrupting an inhibitory ion pair, yielding a significantly more active enzyme molecule.

After this manuscript was submitted for publication, a report...
appeared in which effects of various Hofmeister salts on NO\textsuperscript{-} synthesis by nNOS, eNOS, and iNOS were described (46). Although the results regarding stimulation of nNOS activity through the addition of the salts were similar to those observed by us, the experiments were conducted using the citrulline forming assay, in the absence of hemoglobin. No inference was made concerning the importance of a nitric oxide scavenging system or the prevention of nitrosyl nNOS formation in the stimulation of citrulline synthesis in this publication (46).

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