Nitric-oxide synthases (NOS)1 constitute a family of heme-thiolate-ligated proteins that catalyze the conversion of L-arginine to NO, which then stimulates many physiological processes. In the active form, each NOS is a dimer; each strand has both a heme-binding oxygenase domain and a reductase domain. In neuronal NOS (nNOS), there is a conserved cysteine motif (CX,C) that participates in a ZnS₄ center, which stabilizes the dimer interface and/or the flavoprotein-heme domain interface. Previously, the Cys₃₃₁ → Ala mutant was produced, and it proved to be inactive in catalysis and to have structural defects that disrupt the binding of L-Arg and tetrahydrobiopterin (BH₄). Because binding L-Arg and BH₄ to wild type nNOS profoundly affects CO binding with little effect on NO binding, ligand binding to the mutant was characterized as follows. 1) The mutant initially has behavior different from native protein but reminiscent of isolated heme domain subchains. 2) Adding L-Arg and BH₄ has little effect immediately but substantial effect after extended incubation. 3) Incubation for 12 h restores behavior similar but not quite identical to that of wild type nNOS. Such incubation was shown previously to restore most but not all catalytic activity. These kinetic studies substantiate the hypothesis that zinc content is related to a structural rather than a catalytic role in maintaining active nNOS.

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1 The abbreviations used are: NOS, nitric-oxide synthase(s); NO, nitric oxide; CO, carbon monoxide; nNOS, neuronal nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; BH₄, tetrahydrobiopterin; WT, wild type.
Fig. 1. Typical data for the rebinding of CO to C331A nNOS. In this case, CO (at 1 atm) is combining, after photolysis, with protein incubated overnight with 10 mM L-Arg and no BH$_4$. The data illustrate the presence of two kinetic phases and show that the slow phase is not a perfect single exponential. In practice, the fast phase, shown as a steep drop in the main figure and expanded in the inset, would be recorded at a much higher time resolution to prove that it also is not a single exponential. In this figure, the fractional amplitudes of the two phases are nearly equal.

flow measurements were carried out at 20 °C following protocols described earlier (16). Carbon monoxide dissociation rates were determined by replacing CO with NO (17). After mixing, samples contained 0.5 μM protein, 25 μM sodium dithionate, 500 μM CO, and 1000 μM NO. (For the ferrous hemes, which alone bind CO, affinity for NO is much larger than for CO.) Absorption changes were monitored at 443 nm.

RESULTS

CO Ligation—UV-visible spectra for the C331A mutant itself and its association with CO were reported previously (9). The kinetics of CO binding for both the C331A mutant and WT nNOS display two phases for concentration-dependent bimolecular combination that differ in rate by a factor of 100. A typical example of absorption changes following flash photolysis is shown in Fig. 1. Mutant and WT nNOS are similar in that fast and slow phases are not perfect single exponentials but require two or more rate constants distributed over a range of a factor of 3 or 4, as may be seen in Fig. 1. This was discussed at some length for WT (14). Once the heterogeneity is noted, it is sufficient for most purposes to consider an effective mean rate.

Mutant and WT proteins are also similar but not identical with respect to the mean value of the fast and slow rate constants for CO addition, as shown in Table I. The slow phase has a rate constant that is almost the same in all circumstances. The fast phase, however, has a rate constant in the mutant that is about twice as fast as in the WT holoenzyme and heme domain. It is, however, the relative proportion of the two phases that is of the most interest. Whatever the interpretation of the two kinetic phases may be, the changes in their relative proportions as conditions are varied offer a critical test for comparing the behavior between WT and mutant preparations. Fig. 2 shows the fraction of the slow phase in two circumstances for C331A. It also includes, for comparison, data on the WT holoenzyme and data for a heme-binding domain (residues 1–714), both of which were reported previously (14). The fraction of the slow phase is at its maximum when both L-Arg and BH$_4$ are bound. It is intermediate when just one or the other is present, and it is at its minimum when both are absent. In the WT holoenzyme (back row), the appearance of the slow phase requires much less than 1 mM L-Arg, and the association of L-Arg with protein occurs faster than we can carry out flash photolysis measurements, which require several minutes of signal averaging. In the C331A mutant, the effects associated with binding L-Arg and BH$_4$ appeared only gradually after extended incubation. The mutant C331A could be forced to display 100% slow phase but only after more than 12 h of incubation with both BH$_4$ and a large, 10 mM concentration of L-Arg (third row, left side). Lesser concentrations of L-Arg were not as effective. Either BH$_4$ or L-Arg alone was not nearly as effective as is either alone in the WT protein. A 2-h incubation (second row) had little effect.

Fig. 2 (two front rows) shows that CO binding to C331A initially has some similarity to that in the heme domain of WT nNOS. Both BH$_4$ and L-Arg must be present to achieve much slow phase. In both cases, the fraction of slow phase increases for the first few hours (data not shown). Incubation of the heme domain could not be extended to 12 h because of the excessive formation of the “P-420 analogue” (13, 18). Some formation of the P-420 feature also occurred in C331A, especially at low CO concentration and in the absence of BH$_4$, consistent with previous reports of the factors involved in P-420 conversion (13, 18).

As with the WT holoenzyme and heme domain (14), flash photolysis of the CO-ligated C331A mutant displays, in addition to bimolecular association, nanosecond transient absorbance changes that are plausibly attributed to geminate recombination. In all cases, these nanosecond transients are larger whenever the fast phase is larger.

Ligand dissociation of CO was also characterized. Illustrative cases are shown in Table I. Dissociation in extensively incubated C331A is about a factor of 2 faster than in either the WT holoenzyme or heme domain. For both the WT holoenzyme and C331A with BH$_4$ but without L-Arg, somewhat better fits were obtained by using two exponentials (for C331A + BH$_4$, 33% at 0.92 s$^{-1}$ plus 67% at 0.21 s$^{-1}$, and for WT + BH$_4$, 17% at 15 s$^{-1}$ plus 83% at 0.16 s$^{-1}$).

NO Ligation—The association of nitric oxide with C331A was investigated briefly. Unlike CO, which binds only to ferrous iron, NO binds to both ferrous and ferric forms. Association of NO was virtually identical for ferric and ferrous C331A, as was true for WT protein (15). Fresh solutions of C331A displayed association rate constants of 1–2 × 10$^7$ M$^{-1}$ s$^{-1}$ with the faster rate in the absence of L-Arg and BH$_4$ and the slower rate in the presence of both. This is identical to values reported previously for the WT heme domain (15). Table I shows the results for C331A samples incubated overnight with L-Arg. After incubation, the NO association constants match values measured previously for the WT holoenzyme (15), which are reduced by about a factor of 2 from the value for fresh C331A solutions and the heme domain. (These rate constants for NO are all mean rates; detailed fits showed heterogeneity, requiring comparable amplitudes of at least two exponentials differing by a small factor, around 5.) Photolysis of NO-ligated nNOS has only a small amplitude. Presumably much of the photolysed NO undergoes geminate recombination before it can escape to the solvent. Because no geminate recombination was measured with the nanosecond spectrometer, it was determined that most geminate recombination must occur during time scales under a few nanoseconds.

Although it is unwise to overinterpret rate constants that vary by only a factor of 2, the essential finding for NO associ-
Table I

| System | $k_{on,fast}$ | $k_{on,slow}$ | $k_d$ |
|--------|---------------|---------------|-------|
| C331A mutant + Arg + BH₄ + CO | $-2\times10^4$ | $-2\times10^4$ | 0.73 ± 0.04 |
| C331A mutant + BH₄ + CO | $-2\times10^4$ | $-2\times10^4$ | 0.29 ± 0.03 |
| WT holoenzyme + Arg + BH₄ + CO | $-2\times10^4$ | $-2\times10^4$ | 0.33 ± 0.03 |
| WT holoenzyme + BH₄ + CO | $-2\times10^4$ | $-2\times10^4$ | 0.19 ± 0.03 |
| WT heme domain + Arg + BH₄ + CO | $-4\times10^4$ | $-2\times10^4$ | 0.34 ± 0.03 |
| WT heme domain + BH₄ + CO | $-4\times10^4$ | $-2\times10^4$ | 0.16 ± 0.02 |
| C331A + Arg + BH₄ + NO | $0.5\times10^6$ | $2\times10^6$ | 0.02 |
| C331A + NO | $1.0\times10^7$ | $6\times10^6$ | 0.03 |
| WT holoenzyme + Arg + BH₄ + NO | $0.5\times10^6$ | $2\times10^6$ | 0.03 |
| WT holoenzyme + NO | $1.0\times10^7$ | $6\times10^6$ | 0.03 |
| WT heme domain + Arg + BH₄ + NO | $1.0\times10^7$ | $6\times10^6$ | 0.03 |
| WT heme domain | $2.0\times10^7$ | $6\times10^6$ | 0.04 |

* Better when fit to a double exponential; see text.

FIG. 2. Percentage of amplitude in the slow phase for CO association with various nNOS preparations. Front row, results for the heme domain (HmDom) of the WT enzyme, incubated approximately 2 h. Second row, C331A incubated approximately 2 h. Third row, C331A incubated approximately 12 h. Back row, data for the WT holoenzyme, incubated approximately 2 h. Along each row, data labeled with B have 250 μM BH₄, those labeled with A have 10, 1, or 0.1 mM L-Arg as indicated.

The CO reactions are with ferrous heme; the NO data are virtually identical for ferric and ferrous heme. 0.3 s⁻¹, and we detected substantial absorbance changes on nanosecond time scales, attributed to geminate recombination. Subsequently, Sato et al. (20) studied CO association with nNOS (rat). In the course of a study of a variety of substrates and inhibitors, they confirmed again that with both L-Arg and BH₄ present, one observes essentially a single phase with the slow rate constant found earlier. They also reported careful measurements of equilibrium constants from which one may deduce a CO dissociation rate constant equal to 0.3 s⁻¹, which is in agreement with our kinetic determination. Recently, Tetreau et al. (21) confirmed the 100-fold factor in rates between the two phases, the need for a distribution of rate constants within each phase, and the presence of nanosecond absorbance changes. They added information about CO association kinetics at cryogenic temperatures. In an effort to explain the differences between phases, they measured the hydrodynamic volume of the protein under different conditions. When the slow phase dominated, the volumes were reasonable for a globular protein of the mass of NOS. When the fast phase dominated, the hydrodynamic volume was 10-fold larger. They inferred that the fast-reacting form of nNOS involves either substantial unfolding of the protein or aggregation or both.

Native WT protein exists largely in the fast-reacting form in the absence of L-Arg and BH₄, but it converts rapidly to the slow form even at low concentrations of those agents. Modifying the protein either by truncation to the oxygenase (heme) domain or by mutating C331A renders it more difficult to achieve the slow-reacting active form, but sufficiently large concentrations of L-Arg and extended incubation eventually accomplish much the same change that occurs more readily in the native holoenzyme. In the absence of BH₄ and L-Arg, CO association with C331A behaves much like that in the WT heme domain fragment. Extended incubation restores almost normal behavior. The dissociation of CO, however, follows a slightly different pattern. The dissociation from the WT heme domain and WT holoenzyme is very similar, whereas CO dissociation from C331A is faster by a factor of 2 and remains so even after extended incubation with 10 mM L-Arg. This suggests that the restoration of the normal structure by incubation of C331A is not quite perfect.

Association with NO shows similar effects. There is only one phase for NO association under any condition, but there is a slight difference in the rate between the WT heme domain and WT holoenzyme. That difference is replicated in C331A before and after incubation.

Complementing the kinetic studies discussed here may be resonance Raman measurements of vibrational frequencies for ligands bound to heme. Recent publications (22, 23), building on earlier investigations cited therein, infer “open” and “closed”...
conformations for the immediate environment of the iron binding site with the open form correlating with the fast-reacting species observed in kinetic measurements and the closed form correlating with the slow-reacting form. A plausible inference is that both l-Arg and BH$_4$ bind close to the iron, affecting both ligand access to and binding with the iron. It is conceivable that their effect on ligation is attributable to simple steric blockage. However, it is more likely that the effects of l-Arg and BH$_4$ are better described as inducing conformational change either in the distal pocket or at the proximal cysteine bond, or both. The Raman investigation of the immediate neighborhood of the iron explores a very different length scale than do the light-scattering studies that inferred different hydrodynamic volumes. Kinetic data involve both scales. There are presently too many uncertainties for us to propose a model that can explain the data from Raman spectroscopy, light-scattering measurements, and ligation kinetics. (For example, full understanding of kinetics requires data on geminate recombination in the picosecond regime.) However, some ideas may be inspired by crystal structures, which are starting to appear.

In rat brain nNOS, the C331A mutant involves substitution into the generally conserved motif (CXXXXC) of the two (identical) subunits as follows.

\[ \text{T G C T E H I C M G} \]

**Motif 1**

A crystal structure of nNOS is not yet reported, but that of eNOS has been (24). In bovine eNOS the analogous position is Cys$^{301}$, whereas in human eNOS it is Cys$^{399}$. The four cysteines coordinate to a zinc in both eNOS (24) and nNOS. This ZnS$_4$ center stabilizes the dimer interface and/or the flavoprotein-heme domain interface. (We have argued elsewhere (24) that nNOS also requires a similar, tetrahedrally coordinated zinc.) In the C331A mutant of nNOS, there are structural perturbations that make binding both BH$_4$ and l-Arg more difficult, but sufficiently long incubation at large concentrations of L-Arg allows binding to occur. Once binding has occurred, a structure is stabilized that is similar to the native conformation and is kinetically active. Still, the incubated C331A is probably not quite identical to WT. The enzymatic activity of the incubated C331A is 84% of that in WT nNOS, and CO dissociation is twice as fast.

Cysteine 331 is not the heme-binding amino acid, nor is it involved directly in the binding of either BH$_4$ or l-Arg. The question then is how the mutation C331A affects their binding. An explanation may be offered on the basis of the crystal structure data (24). Those data indicate that l-Arg is a structural mimic of BH$_4$ and that l-Arg and BH cross-talk with each other through heme propionate. In eNOS, the BH$_4$ hydrogen bonds to Ser$^{104}$, which is a part of the chain that is connected to the zinc atom, which is tetrahedrally coordinated to the cysteine residues on the two chains of the dimer. Zinc is one of the few metals capable of readily forming tetrahedral (most often), octahedral, and square planar (occasionally) structures. Thus the structure around the zinc should be able to switch among multiple conformations. Any change in the stereochemistry around the zinc will be transmitted to the binding of BH$_4$ (and in turn to the l-Arg through the heme-propionate bridge) via Ser$^{104}$ or its analog in nNOS. Thus the data from ligand binding kinetics and enzyme reactivity bring out rather dramatically the importance of the tetrahedrally coordinated zinc for functional integrity in the enzyme.

**REFERENCES**

1. Bredt, D. S., and Snyder, S. H. (1990) Proc. Natl. Acad. Sci. U. S. A. **87**, 682–685
2. McMillan, K., Bredt, D. S., Hirsch, D. J., Snyder, S. H., Clark, J. E., and Masters, B. S. S. (1992) Proc. Natl. Acad. Sci. U. S. A. **89**, 11141–11145
3. White, K. A., and Marletta, M. A. (1992) Biochemistry **31**, 6627–6631
4. Stuehr, D. J., and Ikeda-Saito, M. (1992) J. Biol. Chem. **267**, 20547–20550
5. Klatt, P., Schmidt, K., and Mayer, B. (1992) Biochem. J. **286**, 15–17
6. Rayb, M. A., and Marletta, M. A. (1989) J. Biol. Chem. **264**, 19654–19658
7. Ruan, N. S., Nathan, C. F., and Stuehr, D. J. (1989) J. Biol. Chem. **264**, 20566–20571
8. Mayer, B., John, M., and Bohme, E. (1999) FEBS Lett. **277**, 215–219
9. Martásek, P., Miller, R. T., Liu, Q., Roman, C. S., Salerno, J. C., Migita, C. T., Raman, C. S., Gross, S. S., Ikeda-Saito, M., and Masters, B. S. S. (1999) J. Biol. Chem. **274**, 34799–34805
10. Miller, R. T., Martásek, P., Raman, C. S., and Masters, B. S. S. (1999) J. Biol. Chem. **274**, 14537–14540
11. Wang, J., Rousseau, D. L., Abu-Soud, H. M., and Stuehr, D. J. (1994) Proc. Natl. Acad. Sci. U. S. A. **91**, 10512–10516
12. Hershman, A. R., and Marletta, M. A. (1995) Biochemistry **34**, 5627–5634
13. Wang, J., Stuehr, D. J., and Rousseau, D. L. (1995) Biochemistry **34**, 7089–7097
14. Scheele, J. S., Khariotov, V. G., Martásek, P., Roman, L. J., Sharma, V. S., Masters, B. S. S., and Magde, D. (1997) J. Biol. Chem. **272**, 12525–12529
15. Scheele, J. S., Bruner, E., Kharitonov, V., Martásek, P., Roman, L. J., Masters, B. S. S., Sharma, V. S., and Magde, D. (1999) J. Biol. Chem. **274**, 13105–13110
16. Sharma, V. S., Isaacsen, R. A., John, M. E., Waterman, M. R., and Chevion, M. (1983) Biochemistry **22**, 3897–3902
17. Olson, J. S. (1981) in *Hemoglobin: Methods in Enzymology* (Antonini, E., Rossi-Bernardi, L., and Chiancone, E., eds) Vol. 76, pp. 651–651, Academic Press, NY
18. Huang, L., Abu-Soud, H. M., Hille, R., and Stuehr, D. J. (1999) Biochemistry **38**, 1912–1920
19. Matsuzuka, A., Stuehr, D. J., Olsen, J. S., Clark, P., and Ikeda-Saito, M. (1994) J. Biol. Chem. **269**, 20235–20239
20. Sato, H., Nomura, S., Sagami, I., Ito, O., Daff, O., and Shimizu, T. (1998) FEBS Lett. **430**, 377–380
21. Tetereau, C., Tourbez, M., Gorren, A., Mayer, B., and Lavalette, D. (1999) Biochemistry **38**, 7210–7218
22. Wang, J., Stuehr, D. J., and Rousseau, D. L. (1997) Biochemistry **36**, 4585–4596
23. Mogadon, A., Boucher, J. L., Wu, C., Gachhui, R., Sari, M. A., Maney, D., and Stuehr, D. (1998) Biochemistry **37**, 6367–6374
24. Raman, C. S., Li, H., Martásek, P., Král, V., Masters, B. S. S., and Poulos, T. L. (1998) Cell **95**, 939–950