The Unusual Reactivities of *Amphitrite ornata* Dehaloperoxidase and *Notomastus lobatus* Chloroperoxidase Do Not Arise from a Histidine Imidazolate Proximal Heme Iron Ligand

Stefan Franzen,[†,‡] Mark P. Roach,[§,⊥] Yung-Pin Chen,[§] R. Brian Dyer,[†] William H. Woodruff,*[,†] and John H. Dawson*[,§,∥]

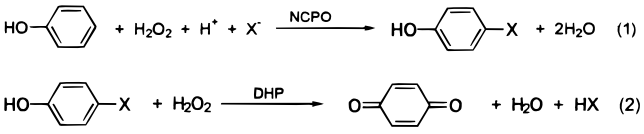
Contribution from the Bioscience and Biotechnology Group, Los Alamos National Laboratory, Los Alamos, New Mexico 87545, and Department of Chemistry and Biochemistry and The School of Medicine, University of South Carolina, Columbia, South Carolina 29208

Received September 12, 1997

Abstract: *Notomastus lobatus* chloroperoxidase (NCPO) and *Amphitrite ornata* dehaloperoxidase (DHP) catalyze the halogenation of phenols and dehalogenation of halophenols, respectively. Both enzymes require peroxide for activity and have recently been shown to contain histidine (His) as their proximal heme iron ligand. DHP is the only heme enzyme known to catalyze peroxide-dependent defluorination reactions, and NCPO is the first His-ligated heme-containing peroxidase capable of chlorinating halogen acceptors using chloride as the halogen donor. To probe the roles of the proximal His ligands in the mechanism of action of these two novel peroxidases, they have been examined by resonance Raman and infrared spectroscopy to determine the vibrational properties of their deoxyferrous and ferrous−CO derivatives. Previous studies of His-ligated heme proteins have revealed characteristic distinctions between dioxygen-binding globins and peroxidases. In the latter, the spectral properties are indicative of strong hydrogen bonding of the His imidazole N-1 hydrogen, giving histidinate character to the His, whereas the globins are thought to contain a predominately neutral His. The partially anionic peroxidase proximal His is thought to facilitate O−O bond cleavage of bound peroxide en route to the key oxo−ferryl compound I peroxidase intermediate. Surprisingly, the vibrational data reported herein for DHP and NCPO are similar to those previously reported for neutral His/imidazole-ligated globins and heme complexes. The vibrational data indicate that these enzymes do not activate bound peroxide through a mechanism dependent on a push effect imparted by a partially ionized proximal histidine as proposed for typical heme peroxidases.

Introduction

Two novel heme-containing peroxidases have been recently discovered in marine worms. *Notomastus lobatus* chloroperoxidase (NCPO) halogenates phenols (reaction 1),[1] and *Amphitrite ornata* dehaloperoxidase (DHP) dehalogenates halophenols (reaction 2).[2] Both enzymes require hydrogen peroxide



for activity,[1,2] and magnetic circular dichroism studies have shown that both contain histidine (His) as their proximal heme iron ligand.[3,4] DHP is the only heme enzyme known to catalyze peroxide-dependent defluorinations,[2] and NCPO is the first His-ligated heme peroxidase to chlorinate halogen acceptors with chloride as the halogen donor.[1] Intriguingly, both enzymes are isolated in their oxyferrous states in the absence of exogenous reductants.[4] Because heme oxo−ferryl catalytic states are crucial to the reaction mechanisms of His-ligated heme peroxidases,[5,6] oxo−ferryl species are likely to also be important in the reactions of these two novel heme enzymes.

Several structural factors are believed to play critical roles in the mechanisms of His-ligated peroxidases.[5−8] The formation of the oxo−ferryl catalytic species via heterolytic cleavage of iron-bound peroxide is thought to depend on properly positioned distal catalytic groups[5−8] and on strong hydrogen bonding of the proximal His imidazole N-1 hydrogen to yield an imidazole anion (imidazolate) ligand.[9,10] Vibrational spectroscopy is the best physical method to study the latter effect because the iron−histidine out-of-plane vibrational mode frequency, ν[Fe−His],

[†] Los Alamos National Laboratory.
[‡] Current address: Department of Chemistry, North Carolina State University, Raleigh, NC 27695-8204.
[§] Department of Chemistry and Biochemistry, University of South Carolina.
[⊥] Submitted by M.P.R. in partial fulfillment of the Ph.D. requirements at the University of South Carolina. Current address: Department of Applied Molecular Science, Institute of Molecular Science, Okazaki 444, Japan.
[∥] School of Medicine, University of South Carolina.

(1) Chen, Y. P.; Lincoln, D. E.; Woodin, S. A.; Lovell, C. R. *J. Biol. Chem.* **1991**, *266*, 23909−23915.
(2) Chen, Y. P.; Woodin, S. A.; Lincoln, D. E.; Lovell, C. R. *J. Biol. Chem.* **1996**, *271*, 4609−4612.
(3) Zhang, E.; Chen, Y.-P.; Roach, M. P.; Lincoln, D. E.; Lovell, C. R.; Woodin, S. A.; Dawson, J. H.; Lebioda, L. *Acta Crystallogr., Sect. D* **1996**, *52*, 1191−1193.
(4) Roach, M. P.; Chen, Y.-P.; Lincoln, D. E.; Woodin, S. A.; Lovell, C. R.; Dawson, J. H. *Biochemistry* **1997**, *36*, 2197−2202.
(5) Dawson, J. H. *Science* **1988**, *240*, 433−439.
(6) Ortiz de Montellano, P. R. *Annu. Rev. Pharmacol. Toxicol.* **1992**, *32*, 89−107.
(7) Goodin, D. B. *J. Biol. Inorg. Chem.* **1996**, *1*, 360−363.
(8) Poulos, T. L. *J. Biol. Inorg. Chem.* **1996**, *1*, 356−359.
(9) Kitagawa, T.; Nagai, K.; Tsubaki, M. *FEBS Lett.* **1979**, *104*, 376−378.

S0002-7863(97)03212-5 CCC: $15.00
Published on Web 05/05/1998

in the deoxyferrous state monitors the Fe−N(His) bond strength.[10] The imidazolate nature of the proximal His is thought to facilitate peroxide O−O bond cleavage ("push effect").[5,8] A distal "pull effect" results from hydrogen bonding from the distal histidine to the bound peroxide. The resulting electrostatic environment favors heterolytic O−O bond cleavage.[5,8] Spiro and co-workers have shown that the Fe−C and C−O stretching frequencies in ferrous−CO peroxidases are both lowered due to these push and pull effects.[11,12] Thus, the distinctive vibrational properties of deoxyferrous and ferrous−CO peroxidases relative to the parallel globin derivatives appear to be indicators of an ability to catalyze heterolytic cleavage of bound peroxide.

The weak hydrogen bonding of the myoglobin proximal His to a serine and a backbone carbonyl[13] results in iron−neutral imidazole out-of-plane frequencies for various deoxyferrous myoglobins in the range of 218−221 cm^{-1}.[14] In contrast, iron−imidazolate out-of-plane frequencies for heme peroxidases appear at >240 cm^{-1}.[9,15−18] In cytochrome *c* peroxidase, the structural origin of the anionic character of the proximal His-175 results from the strong hydrogen bond to Asp-235. Cytochrome *c* oxidase, on the other hand, has a weak iron−histidine axial out-of-plane mode at 215 cm^{-1} due to a single weak hydrogen bond between the proximal His and an amide carbonyl.[19−21] The hydrogen bonding interactions explain the ordering of the frequencies and ligation strengths of these His-ligated heme proteins. The imidazolate character of the proximal His in peroxidases stabilizes both the ferric resting state and the oxo−ferryl intermediate states by lowering their Fe^{3+}/Fe^{2+} reduction potentials relative to the globins (cytochrome *c* peroxidase, −174 mV; myoglobin, +50 mV).[10] The observed stability of the oxyferrous states of DHP and NCPO indicates a radically different redox couple for these enzymes than for peroxidases and suggests a novel mechanism of action.[4] The vibrational spectral data reported herein for the deoxyferrous and ferrous−CO forms of these two novel peroxidases suggest that their mechanism of activation of peroxide cleavage must involve some modification of the classical peroxidase push/pull mechanism.

Experimental Section

Materials. DHP was purified from *A. ornata* according to the procedure of Chen *et al.*[2] NCPO was purified from *N. lobatus* by the method of Chen *et al.*[1] Activity measurements on both enzymes carried out at various stages of purification were comparable to the values previously published by Chen *et al.*[1,2]

Methods. Continuous wave resonance Raman spectra were obtained using an argon ion pumped Coherent dye laser with Stilbene 420 dye. Typical laser powers at the sample ranged from 5 to 20 mW focused to an approximately 100 μm beam waist. A 135° backscattering geometry was employed. Scattered light was collected by a *f*/1 lens with a 5 cm focal length. A polarization scrambler was placed in front of the entrance slit to the monochromator. A SPEX triplemate 0.6 m monochromator was used to disperse the collected light onto a Photometrics CCD with a resolution of 2.5 cm^{-1} using slit widths of 60 μm. The concentration of protein samples in Raman experiments was ∼100 μM in 100 mM potassium phosphate buffer, pH 5.0. Deoxyferrous DHP and NCPO samples were prepared by addition of a minimum volume of 1 M dithionite. The ferrous−CO form was prepared by equilibrating these samples with CO. The spectra of deoxyferrous DHP and NCPO were obtained with λ = 431.7 nm excitation. The DHP−CO and NCPO−CO excitation data were obtained with λ = 422.2 nm excitation. A spinning sample cell was used for the measurement of DHP−CO and NCPO−CO resonance Raman spectra. The sample cell consisted of an NMR tube in a Teflon holder attached to a shaft driven by an electric motor at 30 Hz using a large O-ring as the belt. Comparison of the ferrous−CO forms of the enzymes using ^{13}CO and ^{12}CO was performed by alternation of the samples with 2 min intervals. Typical integration times were 10−20 min in total. The quantities of sample available did not permit direct observation of the C−O stretching frequency by FTIR spectroscopy. To circumvent this problem, the samples were photolyzed and the C−O bands were observed as difference IR bands by step-scan FTIR. The concentration of protein samples in FTIR experiments was ∼100 μM in 100 mM potassium phosphate buffer, pH 5.0. The protein solution was reduced with 1 M dithionite, equilibrated with CO, and loaded into a purged, sealed solution cell composed of CaF_2 windows separated by a 50 μm spacer. Fourier transform infrared (FTIR) spectra were obtained on a BioRad FTS 60. Step-scan spectra were obtained with 5 μs time resolution using an external cavity with focusing optics as previously described.[22] The sample was photoexcited by a Spectra-Physics DCR-11 YAG laser with doubled output at 532 nm and with a 10 ns pulse duration. The timing of the laser Q-switch with respect to displacements of the mirror in the interferometer was controlled by a DG535 digital delay generator. The laser repetition rate was 10 Hz with pulse energies of 1 mJ. A delay of ∼40 ms for each mirror position was applied to ensure that the mirror has settled prior to digitization of the signal.

Results and Discussion

The low-frequency resonance Raman spectra for deoxyferrous DHP and NCPO are shown in Figure 1. Resonance Raman bands in this frequency range are observed for iron−histidine out-of-plane modes in a wide range of heme enzymes and globins.[10,14,19,23−26] Consistent with data reported for other heme proteins, this vibrational band is absent in six-coordinate ferrous−CO and ferrous−O_2 adducts of DHP and NCPO (data not shown). While the frequency of 233 ± 2 cm^{-1} for the ν-[Fe−His] mode of deoxyferrous DHP is higher than seen for the globins, it is significantly lower than the 240−246 cm^{-1}

(10) Spiro, T. G.; Smulevich, G.; Su, C. *Biochemistry* **1990**, *29*, 4497−4508.
(11) Li, X.-Y.; Spiro, T. G. *J. Am. Chem. Soc.* **1988**, *110*, 6024−6033.
(12) Ray, G. B.; Li, X.-Y.; Ibers, J. A.; Sessler, J. L.; Spiro, T. G. *J. Am. Chem. Soc.* **1994**, *116*, 162−176.
(13) Takano, T. *J. Mol. Biol.* **1977**, *110*, 569−584.
(14) (a) Argade, P. V.; Sassaroli, M.; Rousseau, D. L.; Inubushi, T.; Ikeda-Saito, M.; Lapidot, A. *J. Am. Chem. Soc.* **1984**, *106*, 6593−6596. (b) Teraoka, J.; Kitagawa, T. *J. Biol. Chem.* **1981**, *256*, 3969−3977.
(15) Goodin, D. B.; McRee, D. E. *Biochemistry* **1993**, *32*, 3313−3324.
(16) Hashimoto, S.; Tatsuno, Y.; Kitagawa, T. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 2417−2421.
(17) (a) Smulevich, G.; Evangelista-Kirkup, R.; English, A. M.; Spiro, T. G. *Biochemistry* **1986**, *25*, 4426−4430. (b) Dasgupta, S.; Rousseau, D.; Anni, H.; Yonetani, T. *J. Biol. Chem.* **1989**, *264*, 654−662.
(18) Iron−histidine axial out-of-plane mode frequencies in horseradish peroxidase and cytochrome *c* peroxidase are greater than 240 cm^{-1} for histidines with anionic imidazoles. In cytochrome *c* peroxidase, this is known to be due to strong hydrogen bonding to neutral Asp-235. A tautomer consisting of anionic Asp-235 and neutral histidine has a lower frequency, 233 cm^{-1}. The data indicate that the high-frequency forms with imidazolate ligands to the heme iron have greater reactivity toward O−O bond scission.
(19) Argade, P. V.; Ching, Y.-C.; Rousseau, D. L. *Science* **1984**, *255*, 329−331.
(20) Tsukihara, T.; Aoyama, H.; Yamashita, E.; Tomizaki, T.; Yamaguchi, H.; Shinzawa-Itoh, K.; Nakashima, R.; Yaono, R.; Yoshikawa, S. *Science* **1995**, *269*, 1069−1074.
(21) Iwata, S.; Ostermeier, C.; Ludwig, B.; Michel, H. *Nature* **1995**, *376*, 660−669.
(22) Omberg, K. M.; Schoonover, J. R.; Treadway, J. A.; Leasure, R. M.; Dyer, R. B.; Meyer, T. J. *J. Am. Chem. Soc.* **1997**, *119*, 7013−7018.
(23) Wells, A. V.; Sage, T. J.; Morikis, D.; Champion, P. M.; Chiu, M. L.; Sligar, S. G. *J. Am. Chem. Soc.* **1991**, *113*, 9655−9660.
(24) Matsukawa, S.; Mawatari, K.; Yoneyama, Y.; Kitagawa, T. *J. Am. Chem. Soc.* **1985**, *107*, 1108−1113.
(25) Friedman, J. M.; Scott, T. W.; Stepnowski, R. A.; Ikeda-Saito, M.; Yonetani, T. *J. Biol. Chem.* **1983**, *258*, 10564−10572.
(26) Shelnutt, J. A.; Alden, R. G.; Ondrias, M. R. *J. Biol. Chem.* **1986** *261*, 1720−1723.

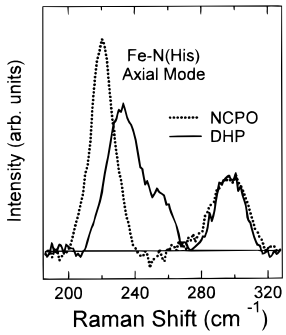


Figure 1. Low-frequency resonance Raman spectra (431.7 nm excitation) of 100 μM deoxyferrous DHP (solid line) and NCPO (dashed line) in 100 mM potassium phosphate, pH 5.0, at 25 °C.

range of strongly polarized histidines in peroxidases.[10,18,26] Polarized histidines in peroxidase enzymes have anionic character and therefore are strongly electron donating ligands to heme iron. In cytochrome *c* peroxidase, the polarization arises from strong hydrogen bonding by a neighboring aspartate (Asp-235) that removes a proton from histidine to produce a histidinate ligand to the heme iron.[10] There is a conformer of cytochrome *c* peroxidase that has a ν[Fe−His] frequency of 233 cm^{-1}, identical to the frequency observed for DHP. This conformer has been assigned to a weakly polarized histidine in which the histidine remains largely protonated (i.e., neutral). Presumably the electrostatic environment of the proximal histidine determines the equilibrium between these two conformations in cytochrome *c* peroxidase. The DHP spectrum indicates that the proximal histidine is neutral, more closely resembling the electronic structure of a globin than a peroxidase. The Fe−N(His) mode for deoxyferrous NCPO is found at 220 ± 2 cm^{-1}, even lower in frequency than is observed for deoxyferrous DHP. The low frequency of the ν[Fe−His] mode in NCPO suggests that the origin of enzyme reactivity lies not in the proximal histidine, but elsewhere in the structure. These results are somewhat surprising in light of the unusual catalytic activities supported by these two peroxide-dependent, His-ligated enzymes.

The ferrous−CO adducts of DHP and NCPO have been examined by resonance Raman and FTIR spectroscopy to determine the Fe−C and C−O stretching frequencies shown in Figure 2 and Figure 3, respectively. Figure 2 shows the isotopic substitution data of ^{13}CO, demonstrating the Fe−C stretching frequency is at 499 ± 2 and 501 ± 2 cm^{-1} for DHP and NCPO, respectively. The isotopic shift of ^{13}CO is 5.5 cm^{-1} to lower frequency in DHP and 4.2 cm^{-1} to lower frequency in NCPO on the basis of fits of the Raman bands to a Gaussian model. The full width at half-maximum of the Gaussian bands was 12−13 cm^{-1} in all of the fits. The bending mode is observed at 576 ± 2 cm^{-1} in DHP and 578 ± 2 cm^{-1} in NCPO. Both Fe−C−O bending modes shift by 20 cm^{-1} to lower energy with ^{13}CO substitution. The frequency of the C−O stretch was determined by step-scan FTIR difference spectroscopy as shown in Figure 3. The data shown in Figure 3 represent the average of the data obtained during the first 125 μs following photolysis after subtracting a baseline averaged from the 125 μs preceding the photolysis flash. These data demonstrate an important application of step-scan FTIR spectroscopy to determine spectral features in dilute samples. The frequency of the C−O stretch was observed at 1950 ± 2 and 1963.5 ± 2 cm^{-1} for DHP−CO and NCPO−CO, respectively. The data observed for DHP and NCPO fall in the range found for heme proteins and models with neutral imidazole proximal ligands as evidenced by their positions on the back-bonding correlation diagram (Figure 4).

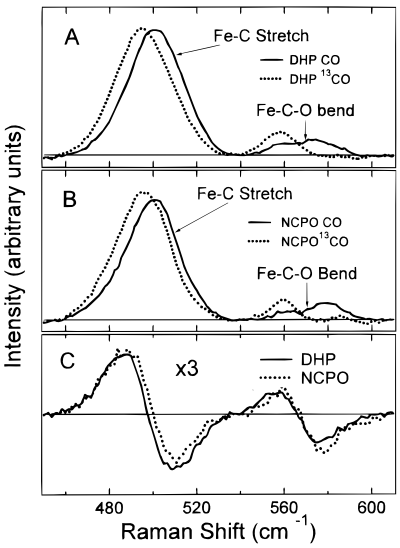


Figure 2. Mid-frequency resonance Raman (422.2 nm excitation) spectra of 100 μM ferrous−CO DHP (A) and NCPO (B) in 100 μM potassium phosphate, pH 5.0, at 25 °C. Raman spectra were obtained as in Figure 1 except for the use of a spinning cell. Spectra of identically prepared samples with ^{13}CO substituted for ^{12}CO are shown (dotted line) for comparison with the natural abundance ferrous−CO form (solid line). The data for DHP−CO shown in panel A indicate a 5 cm^{-1} shift to lower frequency for the Fe−C stretching mode. The data for NCPO−CO in panel B also show a 5 cm^{-1} shift that is consistent with an Fe−CO two-body harmonic oscillator. Difference spectra of the ^{13}CO isotope data minus the natural abundance CO data are shown in panel C.

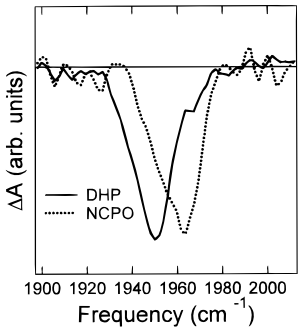


Figure 3. FTIR difference spectra of 100 μM ferrous−CO DHP and NCPO in 100 mM potassium phosphate, pH 5.0, at 25 °C. The C−O stretching region of the IR spectrum is shown.

Spiro and co-workers[11,12] have found that a strong proximal ligand weakens the Fe−C σ bond by competing for the iron d_{z^2} orbital. Therefore, ferrous−CO complexes with anionic proximal ligands fall well below the correlation line for adducts with neutral histidine or imidazole ligands. Ferrous−CO derivatives with weak oxygen-donor ligands such as cytochrome *c* oxidase and five-coordinate ferrous−CO model systems lie well above the same correlation line because of stronger Fe−CO ligation. The data in Figure 4 indicate that DHP and NCPO do not have strongly polarized proximal ligands despite their reactivity toward hydrogen peroxide. This is consistent with the frequencies of the Fe−His out-of-plane mode as discussed above. It has recently been shown that distal pocket polarity can also affect the C−O stretching frequency. For example, the ferrous−CO/imidazole C_2−Cap model heme complex[12] does not conform to the correlation line due largely to the effect of negative distal pocket polarity. Similarly cytochrome *c* oxidase may well be displaced from the line by the presence of the copper B site distal to bound CO. The surprising result that DHP and NCPO

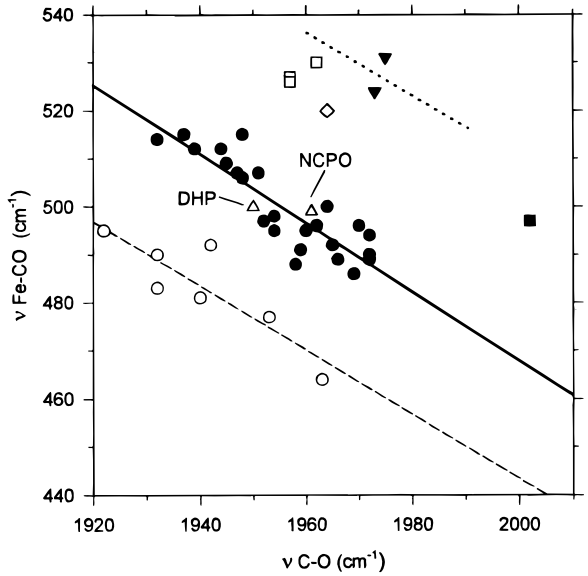


Figure 4. ν(Fe−C) vs ν(C−O) for ferrous−CO adducts of DHP and NCPO (Δ) (present data) and 38 globins, porphyrins, P450s, and cytochrome oxidase (data from refs 11 and 12). The solid line is a least-squares fit to the filled circles. Symbols: ●, globins and models with neutral His/imidazole ligands; □, models with oxygen donor ligands; ▼, five-coordinate models (no trans ligand); ○, cytochrome *c* peroxidase, alkaline horseradish peroxidase, imidazolate- and thiolate-ligated models, and two P450s; ◇, cytochrome *c* oxidase; ■, imidazole adduct of a sterically constrained distal-capped porphyrin (C_2−Cap) with a negative polar distal CO-binding pocket (adapted from ref 12).

fall on the globin correlation line yet possess the reactivity of heme proteins with strongly basic σ-donor ligands leads to the inference that distal pocket effects must be important for reactivity even if they do not affect the vibrational spectroscopic marker bands as observed in other peroxidases.

In summary, the vibrational data for DHP and NCPO (Figures 1−4) are unexpectedly similar to data previously reported for complexes with neutral His or imidazole ligation. The results do not support a special catalytic role for the proximal His ligands of these two heme enzymes unlike the partially ionized histidines in other His-ligated heme-containing peroxidases. DHP and NCPO may rely on an alternative distal mechanism to generate the oxoferryl intermediate, as is the case with cytochrome *c* oxidase. On the other hand, the mechanism of peroxide-catalyzed reactions carried out by these two enzymes may differ from that of other His-ligated peroxidases; catalytic roles for peroxo− and hydroperoxo−ferric intermediates, for example, have recently been suggested for cytochrome P450 aromatase and nitric oxide synthase.[27,28] Finally, we note that the heme-containing subunits of DHP and NCPO have molecular masses below 16 000 Da,[1−3] making these the smallest known heme-containing enzymes. Crystallographic studies of DHP are being carried out by Lebioda and co-workers[3] and should reveal the active site characteristics which are important for catalysis of dehalogenation reactions by this novel His-ligated heme peroxidase.

Acknowledgment. Financial support provided to S.F. by a Director's Postdoctoral Fellowship at Los Alamos, to W.H.W. by a LANL Laboratory-Directed Research and Development Grant (XAM1), and to J.H.D. by the National Science Foundation (Grant MCB 96-04004). We thank D. E. Lincoln, C. R. Lovell, S. A. Woodin, and A. E. Pond for helpful discussions.

JA973212D

(27) Akhtar, M.; Corina, D. L.; Miller, S. L.; Shyadehi, A. Z.; Wright, J. N. *Biochemistry* **1994**, *33*, 4410−4418.
(28) Marletta, M. A. *J. Biol. Chem.* **1993**, *268*, 12231−12234.