Hydrazine and hydroxylamine as probes for O₂-reduction site of mitochondrial cytochrome *c* oxidase

Tomomi KUBOTA*† and Shinya YOSHIKAWA†

*Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan,

and †Department of Life Science, Faculty of Science, Himeji Institute of Technology, Harima Science Park, Kamigori, Ako-gun, Hyogo 678-12, Japan

Reactions of hydrazine and hydroxylamine with bovine heart cytochrome *c* oxidase in the fully reduced state were investigated under anaerobic conditions following the visible–Soret spectral change. Hydrazine gave a sharp band at 575 nm with 20% decrease in the α band at 603 nm, and hydroxylamine induced a 2 nm blue-shift for the α band without any clear splitting. The Soret band at 443 nm was decreased significantly in intensity, with the concomitant appearance of a shoulder with hydrazine or a peak with hydroxylamine, both near 430 nm. The dependence on pH of the affinity of these reagents for the enzyme indicates that only the deprotonated forms of these reagents bind

to the enzyme, suggesting a highly hydrophobic environment of the haem ligand-binding site. These spectral changes were largely removed by addition of cyanide or CO. However, detailed analysis of these spectral changes indicates that hydrazine perturbs the shape of the spectral change induced by cyanide and hydroxylamine perturbs that induced by CO. These results suggest that these aldehyde reagents bind to haem *a*₃ iron as well as to a second site which is most likely to be the formyl group on the haem periphery, and that these two sites bind these reagents anti-cooperatively with each other.

INTRODUCTION

Cytochrome *c* oxidase is the terminal enzyme of cell respiration which reduces O₂ to water, and pumps protons across the mitochondrial inner membrane [1]. This enzyme contains two molecules of haem A and three Cu ions as well as a Mg ion and a Zn ion [2,3]. The formyl and farnesylethyl groups at the periphery of haem A, which are not attached to the protohaem, have attracted much attention in terms of their role in the enzymic function [4–6]. The reactivity of various kinds of formyl reagents with the enzyme was examined, but the results were rather qualitative, and were interpreted without realizing that these reagents can act as strong electron donors to transition metals [7–11]. Thus it should be noted that no conclusive evidence for the reactivity of the formyl group with any external reagents has been obtained. Therefore we re-examined the reaction of these reagents with the enzyme in the fully reduced state, and the effect of ferrous haem iron ligands, CO and cyanide, on these reactions. The present results indicate that hydroxylamine and hydrazine in their deprotonated (non-charged) states bind to Fe²⁺ haem *a*₃ as well as to a second site to perturb the absorption spectral change induced by cyanide or CO liganded to haem *a*₃.

MATERIALS AND METHODS

Crystalline cytochrome *c* oxidase preparation was obtained from bovine heart muscle by the method of Yoshikawa et al. [12]. The fully reduced enzyme (14.2 μ M in haem A) was prepared by anaerobic addition of a slight excess of dithionite in 0.1 M pyrophosphate buffer, pH 9.0, to the crystalline enzyme dissolved in an appropriate buffer solution of 0.1 M. The buffer species used were sodium pyrophosphate at pH 9.0, Tris/HCl in the region of pH 8.5–7.7, and sodium phosphate below pH 7.4. All the anaerobic experiments in this study were carried out by the method of Burleigh et al. [13]. The dithionite solution was standardized anaerobically by using ferricyanide as a standard, and kept anaerobic for at least 2 weeks without any change in the

concentration. The dithionite concentration was 3–4 mM in electron equivalents. Aqueous solutions of hydrochloride salts of hydrazine, hydroxylamine and semicarbazide were adjusted to an appropriate pH and kept anaerobic after addition of a minimal amount of solid dithionite to remove dissolved O₂. The fully reduced enzyme under strictly anaerobic conditions in a Thumberg-type cuvette was titrated with these reagents introduced through a port of the cuvette with a Hamilton gas-tight syringe. The syringe is tight enough to keep the enzyme solution anaerobic during the titration. CO was added to the enzyme solution by exchanging N₂ in the gas phase of the Thumberg cuvette with pure CO gas at 1 atm. Consequently, CO concentration in the enzyme solution is estimated to be about 1 mM. The absorption spectrum was measured with a Hitachi-228 recording spectrophotometer at room temperature (22 °C). The enzyme concentration was determined by using $\Delta\epsilon_{605-630}^{\text{red}}$ of 23.3 mM⁻¹·cm⁻¹ in haem A (H. Aoyama, K. Shimzawa-Itoh and S. Yoshikawa, unpublished work).

RESULTS

Hydrazine, hydroxylamine and other formyl reagents

Figure 1 shows changes in the spectrum of fully reduced cytochrome *c* oxidase induced by hydrazine at pH 8.5 as the difference spectrum compared with fully reduced enzyme, showing a typical blue-shift pattern with maxima at 575 nm and 425 nm and minima at 600 nm and at 444 nm. Arrows in Figure 1 indicate direction of the spectral change with increase in hydrazine concentration. The absolute spectra of the fully reduced form and that in the presence of 182 mM hydrazine are given in the inset (A). The plot of the spectral change induced versus the concentration of hydrazine shows a rectangular-hyperbolic relationship, as typically given by $\Delta(\Delta A_{575-600})$, the change in the difference between the absorbances at 575 nm and 600 nm (Figure 1, inset B). This relationship indicates a reversible reaction between the enzyme (E) and the formyl reagent (I):

† To whom correspondence should be addressed.

Figure 1 Titration of fully reduced cytochrome *c* oxidase with hydrazine

Hydrazine solution (2 M) was added to 2.0 ml of 14.2 μ M fully reduced cytochrome *c* oxidase at pH 8.5, and placed in a Thumberg-type cuvette. The volumes of hydrazine solution added were 0, 10, 30, 60, 100, 150 and 200 μ l for the difference spectra against the fully reduced cytochrome *c* oxidase given. The arrows denote the direction of the spectral changes with increasing concentration of the reagent. Normalization for the volume changes was done for these spectra. Inset (A) shows the absolute spectra of the fully reduced form and that in the presence of 182 mM hydrazine. Inset (B) is a titration curve of this spectral change in which the change in the difference between absorbance at 575 nm and 600 nm induced by hydrazine was plotted against hydrazine concentration. Continuous lines are the best-fit curves assuming a rectangular-hyperbolic relation.

Table 1 Effect of pH on the parameters of the rectangular-hyperbolic relation for binding of hydrazine to fully reduced cytochrome *c* oxidase

Parameters (means $\pm$ S.D.) were determined from the best-fit curves for titration of fully reduced cytochrome *c* oxidase with hydrazine at various pH values as given in Figure 2. The numerical values for $\Delta(\Delta A_{575-600})$ were normalized to 14.2 μ M in haem A.

pH	K_d (mM)	$\Delta(\Delta A_{575-600})_{\max}$
9.0	130 ± 15	0.20 ± 0.012
8.5	120 ± 20	0.19 ± 0.017
8.0	230 ± 20	0.21 ± 0.008
7.7	390 ± 34	0.28 ± 0.018
7.4	1900 ± 280	0.30 ± 0.055

$E + I = EI$. The continuous curve in the inset shows a best-fit curve calculated by a non-linear least-squares regression by using the following equation:

$$\Delta(\Delta A_{575-600}) = \Delta(\Delta A_{575-600})_{\max} \cdot [I] / (K_d + [I]) \quad (1)$$

where K_d , $\Delta(\Delta A_{575-600})_{\max}$, and $[I]$ denote the dissociation constant of the reversible reaction, the maximum change in the absorbance difference and the concentration of hydrazine respectively. No systematic deviation from the continuous curve is detectable. Estimated values for the two parameters in the equation are given in Table 1. The rectangular-hyperbolic relation was observed also in the absorbance change in the other wavelength region. If the enzyme-hydrazine system involves only two

spectrally distinguishable species, E and EI, the spectra of the enzyme at various levels of saturation with hydrazine as given in Figure 1 would form an isosbestic point anywhere that the spectral curves intersect with each other. However, the absence of an isosbestic point is obvious in the intersecting regions near 585 nm and 435 nm. These results indicate the presence of a spectrally distinguishable enzyme species containing two molecules of hydrazine. However, most of the large spectral changes, such as in the α and the Soret region, seem to be due to the formation of EI, whereas the contribution from the second enzyme-reagent complex, EI_2 , is too small to detect at these wavelength regions. Thus the spectral change induced by hydrazine, such as $\Delta(\Delta A_{575-600})$, shows the rectangular-hyperbolic relation as represented by eqn. (1). No attempt at such a quantitative analysis as that given in the inset of Figure 1 has been made to the absorbance changes near the intersecting points, because of the low signal-to-noise ratio.

This reaction at other pH values gave essentially the same spectral change, but K_d was increased with increase in acidity of the medium below pH 8.5 (Figure 2 and Table 1). But $\Delta(\Delta A_{575-600})_{\max}$ was not changed with pH within the experimental accuracy. The K_d values obtained suggest that the half-maximal value for $1/K_d$ is attained near pH 8.1, which coincides well with the pK_a value, 8.2, for the second deprotonation step for hydrazine ($NH_2NH_3^+ \rightleftharpoons NH_2NH_2 + H^+$). This result indicates that only the non-ionic form of hydrazine is reactive.

Hydroxylamine also induced a significant spectral change at pH 8.5, a slight blue-shift (2 nm) in the α band without any splitting, as well as a significant decrease in the Soret band, with

Figure 2 Effect of pH on the binding of hydrazine to the fully reduced cytochrome *c* oxidase

Fully reduced cytochrome *c* oxidase was titrated with hydrazine solution (2 M) at various pH values. The change in the difference between absorbance at 575 nm and 600 nm induced by hydrazine was plotted against hydrazine concentration as follows: $\square$, pH 9.0; $\triangle$, pH 8.5; $\blacktriangle$, pH 8.0; $\blacksquare$, pH 7.7; $+$, pH 7.4. The data were normalized to 14.2 μ M haem *a* for dilution effect due to addition of the hydrazine solution. Continuous lines are the best-fit curves assuming a rectangular-hyperbolic relation between the absorbance change and the hydrazine concentration. The curve for the data at pH 8.5 is identical with that given in inset (B) of Figure 1.

a clear blue-shift to 432 nm (Figure 3). The difference spectrum of the hydroxylamine-bound form versus the fully reduced form (not shown) indicates that the largest absorbance increase due to hydroxylamine binding in the visible region is centred at 592 nm. The plot (Figure 3, inset) for the change in the absorbance difference between 592 nm and 610 nm against the concentration of hydroxylamine shows a rectangular-hyperbolic relationship, giving K_d of 650 μ M and $\Delta(\Delta A_{592-610})_{\max.}$ of 0.096. The affinity ($K_d \approx 0.5$ mM) was not significantly affected by acidity of the

Figure 4 Effect of hydrazine on the shape of the cyanide-induced spectral change of the fully reduced cytochrome *c* oxidase

(a) Difference spectrum of the fully reduced enzyme saturated with cyanide (3.8 mM) in the presence of hydrazine (150 mM) minus the cyanide spectrum with no hydrazine present. (b) Difference spectrum between the hydrazine-bound form (150 mM hydrazine) and the cyanide-bound (3.8 mM cyanide) without any other ligands. Experimental conditions for sample preparations were as described in Figure 1. The pH of the solution was 8.5.

medium between pH 8.5 and 7.4, but decreased with proton concentration below pH 7.4. The pH-dependency (not shown) was essentially the same as that of the protonation equilibrium of hydroxylamine ($\text{NH}_3^+\text{OH} \rightleftharpoons \text{NH}_2\text{OH} + \text{H}^+$), with $\text{p}K_a \approx 6.0$. This result indicates that hydroxylamine is also active only in the deprotonated form. The absence of isosbestic point, which is

Figure 3 Titration of the fully reduced cytochrome *c* oxidase with hydroxylamine

The experimental procedure was the same as in Figure 1, except that the added volumes of 0.2 M hydroxylamine solution were 0, 1, 3, 6, 10, 20 and 40 μ l to 2.8 ml of the fully reduced enzyme (14.2 μ M haem *a*) as shown by the arrows. The inset is the titration plot for the change in the difference between absorbance at 592 nm and 610 nm against the concentration of hydroxylamine. The continuous curve is the best-fit curve, assuming a rectangular-hyperbolic relationship. No correction for the spectral recordings and the titration curves was required for such small volume change due to addition of the hydroxylamine solution.

Figure 5 Effect of cyanide on the binding of hydrazine to the fully reduced cytochrome *c* oxidase

The fully reduced cytochrome *c* oxidase previously treated with various amounts of cyanide was titrated with hydrazine solution (2 M) at pH 8.5. The difference in absorbance between the two wavelengths at 575 nm and 600 nm was plotted against hydrazine concentration at various concentrations of cyanide as follows: ■, 0 mM; □, 0.44 mM; ▲, 1.26 mM; △, 2.49 mM. The data were normalized to 14.2 μ M haem A for the dilution effect due to addition of the hydrazine solution. Each continuous line is the best-fit curve, assuming a rectangular-hyperbolic relationship.

most obvious near the α peak, indicates the second enzyme-hydroxylamine complex, EI₂, as in the reaction with hydrazine.

Other formyl reagents, methylamine and semicarbazide, did not affect the spectrum of cytochrome *c* oxidase under the conditions examined, i.e. pH 9.0–7.4 and the reagent concentration below 0.2 M.

Reactions of formyl reagents in the presence of haem ligands, CN[−] and CO

When cyanide was added to the enzyme solution containing 150 mM hydrazine, both the peak at 575 nm and the trough around 585 nm disappeared, to give a single peak at 602 nm with a shoulder around 590 nm (not shown). The resulting spectrum with excess cyanide concentration looks very much like that of the cyanide-bound form in the absence of hydrazine. However, the difference between the spectrum of the fully reduced enzyme solution containing 3.8 mM cyanide and 150 mM hydrazine and that of the same solution from which hydrazine was omitted gives a curve as shown in Figure 4(a). This spectral difference is not due to an insufficient amount of cyanide for complete removal of hydrazine on the haem *a*₃ iron, since the shape of the spectrum given in Figure 4(a) is clearly different from that of the difference spectrum of the hydrazine-bound enzyme versus the cyanide-bound enzyme in the absence of hydrazine (Figure 4b), in particular in the α -band region. This result indicates formation of, at least, a cyanide-enzyme-hydrazine complex.

The effect of cyanide on the reactivity of hydrazine with the fully reduced enzyme was examined as shown in Figure 5. The spectral change induced by hydrazine at any constant level of cyanide showed a rectangular-hyperbolic relation. The estimated values for K_d and $\Delta(A_{575-600})_{\max}$ are given in Table 2. The latter parameter seems to decrease with the concentration of cyanide; and the standard deviations indicate statistically that the value of 2.49 mM cyanide is significantly smaller than the other three. This result indicates that cyanide at 2.49 mM perturbs the shape of the spectral change induced by infinite amount of hydrazine. In other words, hydrazine cannot remove cyanide completely from the enzyme-cyanide complex. This result, together with the preceding one, indicates two binding sites for cyanide, one of which is different from any of the hydrazine-binding sites.

Table 2 Effect of cyanide on the parameters for the reaction of hydrazine with fully reduced cytochrome *c* oxidase

Parameters (means $\pm$ S.D.) were determined from the best-fit curves for titration of fully reduced cytochrome *c* oxidase with hydrazine at various concentrations of cyanide, given in Figure 5. The maximum absorbance changes, $\Delta(A_{575-600})_{\max}$, are the values normalized to 14.2 μ M haem A.

Concn. of CN [−] (mM)	K_d (mM)	$\Delta(A_{575-600})_{\max}$
0	120 $\pm$ 15	0.180 $\pm$ 0.0120
0.44	200 $\pm$ 17	0.152 $\pm$ 0.0056
1.26	330 $\pm$ 27	0.157 $\pm$ 0.0058
2.49	350 $\pm$ 53	0.122 $\pm$ 0.0082

An excess of CO ($\sim$ 1 mM) removed hydrazine bound to the fully reduced enzyme completely to give a CO spectrum identical with that in the absence of hydrazine. No detectable spectral change was observed upon addition of hydrazine up to 0.2 M to CO-saturated enzyme solution. This result indicates that CO removes both hydrazine molecules at the two sites.

Cyanide had no effect on the spectrum of hydroxylamine-bound enzyme even at a cyanide concentration 10 times that of hydroxylamine, at pH 7.4, consistently with an earlier observation [7]. Strangely, the K_d values of fully reduced enzyme for these reagents are similar, several hundred micromolar. In contrast with cyanide, CO affected the spectrum of the enzyme saturated with hydroxylamine, and vice versa. Figure 6 shows the difference in the spectra of the hydroxylamine-bound species after versus before addition of CO (continuous line) and that of the CO-bound species after versus before addition of 1.5 mM hydroxylamine (broken line). If hydroxylamine reacts only with haem *a*₃ iron competitively with CO, these two difference spectra should be symmetrical with respect to the abscissa. But obviously this is not the case, in particular near 610 nm, 560 nm and in the Soret-band region. This result also suggests the existence of a complex consisting of hydroxylamine, CO and the enzyme.

DISCUSSION

The present result, that the visible-Soret spectral changes induced by hydrazine and hydroxylamine were removed largely by cyanide and CO, clearly shows that these reagents primarily bind to ferrous haem-*a*₃ iron. Although many nitrogenous bases, including these formyl reagents, have long been known as strong ligands to various model haems [7,8], no such reaction has been reported for other ferrous haemoproteins. It has been shown that phenylhydrazine reacts with metmyoglobin and methaemoglobin in such a way that a phenyl group remains bound to the iron atom, that is, phenylhydrazine is not a simple reversible ligand, but induces a chemical reaction to form a phenyl-liganded haem [14,15]. Reduction of ferric iron by the strong reductant phenylhydrazine seems to trigger the above reaction. Such a reaction is unlikely to occur in the present reaction system, which contains an excess of dithionite. Furthermore, the complete removal of hydrazine by CO strongly suggests the absence of any degradation of hydrazine or irreversible incorporation of any hydrazine adduct to haem or any other site.

This enzyme in the fully reduced state has unusually strong affinity to cyanide, compared with other ferrous haemoproteins, which suggests a functional group near the ligand-binding site to neutralize the negative charge of the cyanide bound to ferrous haem *a*, a neutral compound [16]. Similar mechanism is possible

Figure 6 Effects of CO on the spectra of hydroxylamine-saturated enzyme and of hydroxylamine on the spectrum of CO-saturated enzyme

Continuous line, hydroxylamine-saturated fully reduced enzyme prepared at 1.5 mM hydroxylamine was treated with CO (~ 1 mM). The difference spectrum is between those after and before CO treatment. Broken line, the difference between the spectra of CO-saturated enzyme after and before treatment with an excess of hydroxylamine (1.5 mM). The spectrum of the hydroxylamine-treated sample was normalized to $14.2 \mu\text{M}$ haem A concentration.

for the strong affinity of this enzyme in the fully reduced state to those formyl reagents. Perhaps this group may stabilize the monobasic hydroxylamine in the deprotonated form as the sixth ligand more effectively than does dibasic hydrazine.

As shown in Figure 5, the highest concentration of hydrazine used is higher than 800 mM. However, the following results indicate the absence of any significant irreversible denaturing effect. The enzyme in the presence of such a high concentration of hydrazine was stable for at least several days without any significant spectral change under strictly anaerobic conditions. CO removes bound hydrazine molecules completely to provide a spectrum of enzyme-CO complex identical with that in the absence of hydrazine. Any non-specific binding of hydrazine which induces a spectral change is also unlikely, because a simple rectangular-hyperbolic relation is observed at any cyanide level for the absorbance changes over a wide range of hydroxylamine concentration, as shown in Figure 5. Spectral change due to non-specific binding of hydrazine would induce some deviation from the rectangular-hyperbolic relation for the specific binding of hydrazine. The following discussion will not assume any non-specific binding of, and/or irreversible denaturation by, these ligands. However, in order to prove completely that the above spectral changes are all due to the specific binding of these reagents, the binding sites must be identified by any other method which provides clearer structural information than absorption spectroscopy.

The spectra of the enzyme bound to hydrazine or hydroxylamine at different levels of saturation do not form any clear isosbestic point at some intersecting points in Figures 1 and 3, indicating the presence of the second binding site for hydrazine and hydroxylamine, in addition to the haem iron. The presence of the ternary complex, CO-Enzyme- NH_2OH , as shown in Figure 6, also suggests the second hydroxylamine-binding site, since CO is unlikely to have any binding site other than haem a_3 iron. Considering the structure of these two formyl reagents and the similarity in the spectral changes induced by them, the second sites for these reagents in the enzyme are most likely to be identical. The spectral change induced by a saturating amount of

hydrazine, as typically shown by $\Delta(\Delta A_{575-600})_{\text{max}}$ in Table 2, was affected by cyanide. Thus hydrazine cannot completely displace cyanide. This result suggests that this enzyme in the fully reduced state has a second binding site for cyanide which is not identical with any of the hydrazine-binding sites. These results indicate that there are three ligand-binding sites in the enzyme, as follows: site 1 for CN^- , CO, hydrazine and hydroxylamine, site 2 for hydrazine and hydroxylamine, and site 3 for cyanide. Site 1 must be haem a_3 iron, since, first of all, it is the CO-binding site. Sites 2 and 3 could be either the formyl group of haem a_3 or Cu_B^+ , since cyanide is also a strong formyl reagent, and the formyl reagents hydrazine and hydroxylamine could react with Cu^+ as well as Fe^{2+} .

Complete removal of hydrazine by CO and no reactivity of the hydroxylamine-bound form with cyanide, as described above, suggest a strong repulsive interaction between the Fe of haem a_3 and site 2. Binding of CO to haem- a_3 Fe greatly decreased the affinity of site 2 to hydrazine, whereas hydroxylamine bound to site 2 greatly decreased the affinity of cyanide to haem- a_3 Fe^{2+} and site 3. The weaker ligands hydrazine and cyanide, compared with hydroxylamine and CO respectively, cannot induce such a drastic interaction. Thus the stable quaternary complex, containing a cyanide at site 3, a hydrazine at site 2 and a cyanide or hydrazine at haem- a_3 Fe^{2+} , is detectable. Hydroxylamine, strongly bound to site 2, is not removed completely even under the strongly repulsive interaction from CO at haem- a_3 Fe^{2+} . The interaction between site 2 for the formyl reagents and the O_2 -binding site (haem- a_3 Fe^{2+}) depends on the reactivity of the ligands on both sites. A stronger ligand induces stronger interaction. The repulsive interaction between site 1 and site 2, if site 2 is the formyl group of haem a_3 , could be caused by an electronic interaction between the two sites through the porphyrin ring. However, another possibility is a steric interaction due to a conformational change in protein structure induced by ligand binding to one of the sites. For example, binding of CO at site 1 affects the configuration of amino acid side chains around haem a_3 to decrease the space around the formyl group. Site 2, whatever the entity is, may serve as a binding site for an ϵ -amino

group of a lysine residue to control the O₂ reduction at haem α_3 iron. This control could involve a conformational change triggered by binding of the ϵ -amino group to site 2.

REFERENCES

- 1 Malmström, B. G. (1990) *Chem. Rev.* **90**, 1247–1260
- 2 Einersdottir, O. and Caughey, W. S. (1984) *Biochem. Biophys. Res. Commun.* **124**, 836–842
- 3 Einersdottir, O. and Caughey, W. S. (1984) *Biochem. Biophys. Res. Commun.* **129**, 840–847
- 4 Caughey, W. S., Wallace, W. T., Volpe, J. A. and Yoshikawa, S. (1976) *Enzymes* 3rd Ed. **13**, 299–344
- 5 Puustinen, A. and Wikström, M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6122–6126
- 6 Bobcock, G. T. and Callahan, P. M. (1983) *Biochemistry* **22**, 2314–2319
- 7 Orii, Y. and Okunuki, K. (1964) *J. Biochem. (Tokyo)* **55**, 37–48
- 8 Takemori, S., Sekuzu, I. and Okunuki, K. (1960) *J. Biochem. (Tokyo)* **48**, 569–578
- 9 Yoshikawa, S. and Orii, Y. (1972) *J. Biochem. (Tokyo)* **71**, 859–872
- 10 Bloksil-Hoffman, M. F. and van Gelder, B. F. (1971) *Biochim. Biophys. Acta* **234**, 493–498
- 11 Yoshikawa, S. and Orii, Y. (1970) *J. Biochem. (Tokyo)* **68**, 145–156
- 12 Yoshikawa, S., Tera, T., Takahashi, Y., Tsukihara, T. and Caughey, W. S. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1354–1358
- 13 Burleigh, B. D., Jr., Foust, G. P. and Williams, C. H., Jr. (1969) *Anal. Biochem.* **27**, 536–544
- 14 Kunze, K. L. and Ortiz de Montellano, P. R. (1983) *J. Am. Chem. Soc.* **105**, 1380–1381
- 15 Ringe, D., Petsko, G. A., Kerr, D. E. and Ortiz de Montellano, P. R. (1984) *Biochemistry* **23**, 2–4
- 16 Yoshikawa, S. and Caughey, W. S. (1990) *J. Biol. Chem.* **265**, 7945–7958

Received 22 July 1992/22 December 1992; accepted 13 January 1993