Multiple conformations at functional site of hemerythrin: Evidence from resonance Raman spectra

(oxo bridge/iron protein/oxygen isotope exchange/photochemical conversion)

LORI L. DUFF*, GERALD L. KLIPPENSTEIN†, DUWARD F. SHRIVER*, AND IRVING M. KLOTZ*

*Department of Chemistry, Northwestern University, Evanston, Illinois 60201; and †Department of Biochemistry, University of New Hampshire, Durham, New Hampshire 03824

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ABSTRACT Resonance Raman spectra were obtained for monomeric oxymyohemerythrin and for the azide, thiocyanate, cyanate, cyanide, and fluoride adducts of metmyohemerythrin. The internal ligand vibrations in these complexes appear at essentially the same frequencies as those in the corresponding complexes of octameric hemerythrin. Likewise the Fe—O frequencies in H_2^{16}O do not depend on quaternary structure of the protein. The anionic adducts fall into two classes in regard to isotope exchange behavior in H_2^{18}O . They also manifest a novel photochemical transformation from one class of exchange behavior to the other. It seems evident that the functional site in hemerythrin exists in at least two different conformational states and that irradiation can stimulate isotope exchange in the exchange-resistant form.

Vibrational spectra from resonance Raman scattering have revealed electronic and structural details of the active site of hemerythrin, the nonheme oxygen carrier of several phyla of marine invertebrates (1, 2). In addition to binding dioxygen reversibly, hemerythrin in its met, Fe(III) oxidation state can also form complexes with a wide range of anionic ligands (3, 4). Essentially all of these complexes of octameric hemerythrin from *Phascolopsis gouldii* show a Raman band near 510 cm^{-1} , which has been assigned to an iron-oxygen stretching vibration (4). Curiously, however, there are marked differences among these complexes with respect to their exchange behavior in H_2^{18}O , as followed by shifts in the 510 cm^{-1} vibration (4). The structural basis of these differences is still uncertain although some reasonable rationalizations have been suggested (4).

In the blood of most organisms carrying hemerythrin, the protein exists as an octamer of eight identical subunits, each containing a pair of nonheme iron atoms (1). A monomeric hemerythrin, myohemerythrin, has been isolated, however, from muscles of *Themiste zostericola* (5). The availability of this material makes it possible to determine whether the different shifts in Raman spectra of octameric hemerythrin are a reflection of subunit interactions in the oligomer or whether they are a manifestation of local differences in structure at the functional site of each monomer. This report describes the resonance Raman spectra of monomeric myohemerythrins and the insights they give into these structural problems.

MATERIALS AND METHODS

Myohemerythrin was isolated from the retractor muscle of *T. zostericola*, as described (5), but the azide was deleted and Tris acetate buffer (pH 8) was used to give the methydroxo form. Anionic adducts of metmyohemerythrin were prepared at 4°C

by addition of protein to a solution of the sodium or potassium salt of the ligand in 0.5 M Tris acetate buffer at pH 8.0.

To produce deoxyhemerythrin, metmyohemerythrin was placed in cellulose casing and dialyzed at 25°C against argon-saturated Tris acetate, pH 8.0/0.05 M sodium dithionite. After reduction, dialysis was continued against several successive fresh solutions of argon-saturated buffer. The deoxyhemerythrin was then transferred, with a gas-tight syringe preflushed with argon, to an argon-flushed stoppered test tube. The protein was concentrated by passage of argon over the solution to remove water vapor.

In other experiments starting with deoxymyohemerythrin, the concentrated protein was diluted with an equal volume of H_2^{18}O , and a sodium or potassium salt of the ligand was added. Equilibration was allowed to take place overnight in air at 4°C . Conversion to the methemerythrin adduct by this route was achieved within 10 hr after addition of the salt. Alternatively, metmyohemerythrin solutions in H_2^{18}O were prepared by dilution of concentrated protein with H_2^{18}O and reconcentration, twice in succession.

Resonance Raman spectra were obtained by excitation with a Spectra Physics 164 Ar^+ laser and detection by a computer-controlled Spex 1401 double monochromator equipped with a cooled RCA C31034A photomultiplier tube. Backscattering geometry was used to collect the spectra (6). Incident laser intensity was usually 80 mW. Samples were spun and kept near 3°C in temperature during illumination. In the photochemical experiments, the volume of the sample was small enough (about $25\text{ }\mu\text{l}$) to be totally bathed by the radiation from a line-focused laser source.

RESULTS AND DISCUSSION

A detailed spectrum of oxymyohemerythrin was difficult to obtain because this oxygenated monomeric protein is much less stable than its octameric counterpart. In the laser beam, even at 0°C , conversion of oxymyohemerythrin to the met form is complete within 15–20 min. Nevertheless, an unequivocal peak at 844 cm^{-1} was visible. This frequency is identical with that observed in oxygenated octameric protein and has been assigned to the peroxide-type O—O vibration.

In contrast to the oxy form, azidometmyohemerythrin and thiocyanatometmyohemerythrin are relatively stable and yielded spectra (Figs. 1 and 2) with relative peak intensities that are comparable to those of the corresponding octameric proteins. Minor differences are evident in peak positions of these adducts of the monomeric hemerythrin as compared to the octameric form (Table 1). These differences caused no problems in assigning peaks to specific bonds. Satisfactory spectra were also obtained for metmyohemerythrin adducts with cyanate, cyanide, and fluoride and the relevant peak frequencies for these complexes are also listed in Table 1.

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FIG. 1. Resonance Raman spectrum of azidometmyohemerythrin in H_2^{16}O . Laser excitation, 457.9 nm, 80 mW; spectral slit, 4 cm^{-1} .

Oxygen isotope exchange experiments were carried out by mixing concentrated deoxyhemerythrin with H_2^{18}O and adding the sodium or potassium salt of a specific ligand in the presence of air. It is noteworthy that the behavior of the anionic adducts fell into two classes: one group showed a shift in the $\nu_{\text{Fe}-\text{O}}$ Raman vibration, and the other set did not (Table 1). The different results can be represented by the equations

FIG. 2. Resonance Raman spectrum of thiocyanatometmyohemerythrin in H_2^{16}O . Laser excitation, 457.9 nm, 80 mW; spectral slit, 4 cm^{-1} .

Table 1. Resonance Raman spectroscopy of metmyohemerythrin complexes

Ligand	Vibrational frequency (ν), cm^{-1}			
	Fe—L	Internal ligand	Fe—O (in H_2^{16}O)	Fe—O (in H_2^{18}O)
N_3^-	376 (376)	2050 (2049)	507 (507)	489 (491)
SCN^-	289 (298)		514 (513)	514* (513)
OCN^-			506 (508)	490 (493)
CN^-			512 (511)	493 (499)
F^-			505 (509)	505* (509)

Numbers in parentheses are corresponding frequencies for *P. gouldii* octameric hemerythrin.

* This frequency loses intensity after exposure to the laser beam (see text).

Such behavior parallels that observed with octameric hemerythrin, in which the same anionic adducts fall into two corresponding classes with respect to H_2^{18}O exchange (4). Clearly these functional features of the iron site are not dependent on intersubunit communication.

With monomeric metmyohemerythrin, however, an unexpected photochemical effect on isotope exchange was encountered that has not been observed with octameric protein. In the unexchanged thiocyanate adduct, continued exposure to the radiation of the laser beam decreased the 510 cm^{-1} peak and simultaneously generated a 495 cm^{-1} vibration (Fig. 3). The sample exposed to laser radiation for 2 hr showed significant

FIG. 3. Shift in resonance Raman peak of thiocyanatometmyohemerythrin (prepared through aquo intermediate) in H_2^{16}O upon illumination for several hours by Ar^+ laser excitation source. Upper trace shows initial peak; lower trace is peak after illumination. Laser excitation, 457.9 nm, 80–100 mW; spectral slit, 4 cm^{-1} .

FIG. 4. Shift in resonance Raman peak of azidometmyohemerythrin (prepared through aquo intermediate) in H_2^{18}O upon illumination for several hours by Ar^+ laser excitation source. Upper trace shows initial peak; lower trace is peak after illumination. Laser excitation, 457.9 nm, 80–100 mW; spectral slit, 4 cm^{-1} .

intensity below 500 cm^{-1} , whereas a spectrum of the same material at an earlier time gave only a peak at 513 cm^{-1} . Similar results were obtained with the F^- complex but the signal-to-noise ratio in these spectra was poorer.

As with octameric hemerythrin, when azidometmyohemerythrin was prepared by converting a solution of oxymyohemerythrin in H_2^{18}O to aquo met through oxidation with ferricyanide followed by addition of azide, oxide exchange was not observed—that is, the major $\nu_{\text{Fe}-\text{O}}$ peak in H_2^{18}O was at 507 cm^{-1} , the same position as in H_2^{16}O . On the other hand, on continued exposure to laser radiation, the peak at 507 cm^{-1} diminished in intensity and a new feature appeared at 491 cm^{-1} , increasing in magnitude with time of irradiation. Exchange did not occur at room temperature or on exposure of the sample to room light. With large sample volumes, the reduced illumination per unit volume results in slower exchange.

With azide and thiocyanate adducts prepared from metmyohemerythrin in H_2^{18}O , no exchange was observed initially. After further exposure, however, these complexes exhibited a progressive shift in intensity to the region below 500 cm^{-1} . With exposure to laser illumination, the lower frequency peak continued to grow and, simultaneously, the 510 cm^{-1} region peak lost intensity (Figs. 3 and 4). The conversion occurred only in the laser beam; between exposure periods, the ratio of the peak heights was unchanged. Mass spectral analyses of these samples showed approximately 80% H_2^{18}O in these solutions; the spectra in Figs. 3 and 4 indicate $>50\%$ exchange.

Once the photochemical conversion had taken place, the reverse reaction did not occur. The spectra illustrated in Figs. 3 and 4 remained unchanged over periods of days. However, when a solution of the complex was diluted with an equal volume of H_2^{16}O , the higher frequency peak began to gain intensity at the expense of that in the 495 cm^{-1} region. Again, the conversion occurred only during exposure to the laser beam.

It is apparent from these photochemical experiments that irradiation facilitates the entrance of oxygen from H_2^{18}O into the iron functional site of myohemerythrin in the exchange-resistant form. The radiation may function by breaking an Fe-ligand bond or by stimulating a transition into a metastable conformational form that is labile to exchange. This metastable form may be a state of higher free energy like that of the *cis* form of azobenzenes in photochemical *trans-cis* conversions (7, 8).

Photochemical stimulation of exchange is not seen in octameric hemerythrin. The difference in photolability of oligomer and monomer may arise from one or more of the following factors. (i) The iron sites in myohemerythrin may be more accessible to water and, therefore, more susceptible to photochemical exchange with water. (ii) There may be subtle differences in the absorption spectra or in the deexcitation processes in the monomer and oligomer, which lead to the dissimilarity in photochemistry. (iii) If the exchange occurs through an intermediate that is a metastable labile conformer, this state may not be reached by irradiation of octameric hemerythrin because the aggregate suppresses the necessary conformational rearrangements.

Since the original discovery of anion complexes of methemerythrin (3), differences in effects of specific adducts have been observed. N_3^- and OH^- behave differently in perturbing the reactivity of the sulfhydryl group (cysteine-50) in hemerythrin. Perchlorate ion has been found to alter properties at the functional site, even though it is not bound there (9). Most recently, dichotomies in the oxide exchange reaction have been described (4). All these features have pointed to the possibility of alternative structures at the functional site of different adducts of hemerythrin. Furthermore, the pairs of doublets observed in the Mossbauer spectra (10, 11) of this oxygen carrier can be interpreted in terms of alternative structures (4). Thus, chemical and spectroscopic properties of hemerythrin strongly suggest multiple alternative structural conformations at the functional site of this protein.

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1. Klotz, I. M., Klippenstein, G. L. & Hendrickson, W. A. (1976) *Science* **192**, 335–344.
2. Kurtz, D. M., Jr., Shriver, D. F. & Klotz, I. M. (1977) *Coord. Chem. Rev.* **24**, 145–178.
3. Keresztes-Nagy, S. & Klotz, I. M. (1965) *Biochemistry* **4**, 919–931.
4. Freier, S. M., Duff, L. L., Shriver, D. F. & Klotz, I. M. (1980) *Arch. Biochem. Biophys.* **205**, 449–463.
5. Klippenstein, G. L., Van Riper, D. A. & Oosterom, E. A. (1972) *J. Biol. Chem.* **247**, 5959–5963.
6. Shriver, D. F. & Dunn, J. B. R. (1974) *Appl. Spectrosc.* **28**, 319–323.
7. Hartley, G. S. (1937) *Nature (London)* **140**, 281.
8. Horowitz, M. G. & Klotz, I. M. (1955) *J. Am. Chem. Soc.* **77**, 5011–5012.
9. Garbett, K., Darnell, D. W. & Klotz, I. M. (1971) *Arch. Biochem. Biophys.* **142**, 455–470.
10. Okamura, M. Y., Klotz, I. M., Johnson, C. E., Winter, M. R. C. & Williams, R. J. P. (1969) *Biochemistry* **8**, 1951–1958.
11. York, J. L. & Bearden, A. J. (1970) *Biochemistry* **9**, 4549–4554.