Active Site Chemistry of Hemerythrin

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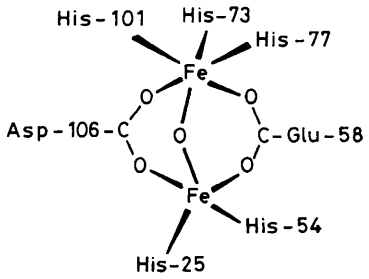
Details of the stepwise reduction of the $Fe^{III,III}$ active site of methemerythrin to the $Fe^{II,II}$ deoxy form by one-equivalent reductants $[Co(nane)_2]^{2+}$, $[Co(sep)]^{2+}$, $[Cr(bipy)_3]^{2+}$, and $[Co(sarCl_2)]^{2+}$ are reported.†

Hemerythrin, in this case from the marine worm *Themiste zostericola*, is one of three naturally occurring O_2 carriers.[1] It consists of an octamer (M.W. 108 000) of eight identical sub-units each of which has a binuclear Fe non-heme active site.[2,3] Recent *X*-ray crystallographic studies on the met form of *Themiste dyseritum* have indicated a structure, shown as (**1**), in which one of the Fe^{III} atoms is octahedral and the other is trigonal bipyramidal.[4] Addition of azide gives two octahedral Fe^{III}s, and it has been suggested that OH^- can similarly co-ordinate (pK_a 7.8)[3] to the five-co-ordinate Fe^{III}. A similar structure is believed to hold for the met form of *Themiste zostericola* although at this stage co-ordination of an H_2O cannot be excluded. EXAFS,[5,6] Mössbauer,[7] and resonance Raman[8] studies support a μ-oxo bridged structure for both met and oxy forms, and structural features determined include bond lengths Fe–O(oxo) (*ca.* 1.75 Å), Fe–Fe (*ca.* 3.3 Å), and an Fe–O–Fe angle of 165°. In the deoxy form the two Fe^{II}s within each dimer are not coupled

† Abbreviations: nane = 1,4,7-triazacyclononane; sep is the sepulchrate cage ligand 1,3,6,8,10,13,16,19-octa-azabicyclo[6.6.6]icosane; bipy = 2,2′-bipyridine; $sarCl_2$ is the cage ligand 1,8-dichloro-3,6,10,13,16,19-hexa-azabicyclo[6.6.6]icosane; phen = 1,10-phenanthroline. Mes = 2-(*N*-morpholino)ethanesulphonic acid.



(1)

antiferromagnetically,[7,9] and EXAFS studies have confirmed that there is no μ-oxo bridge.[5,6] However since no exchange with $H_2{}^{18}O$ is observed (and there is no uptake of H^+ on reduction), it is thought that the μ-oxo atom is retained as a monodentate ligand in the deoxy form, possibly with H-bonding to the Tyr-109 residue.[2]

In order to understand the chemistry of the hemerythrin active site, which is now known to function also in phosphatases[10] and ribonucleotases,[11] Wilkins and colleagues[12] have studied the dithionite reduction of the met $Fe^{III,III}$ state to give the deoxy $Fe^{II,II}$ form and successfully characterised a semi-met $Fe^{II,III}$ intermediate.[13] Three kinetic stages have been reported.[3] The first has been assigned to a bimolecular reduction in which each octamer is converted into the semi-met form $Fe^{II,III}{}_8$. The second stage has been assigned to intramolecular disproportionation steps yielding $Fe^{II,II}$ and $Fe^{III,III}$, the latter being rapidly re-reduced to $Fe^{II,III}$ until $Fe^{II,II}{}_7Fe^{II,III}$ is obtained. The third stage has been presumed to involve intermolecular reduction of the remaining $Fe^{II,III}$ centre.

Results now obtained add considerably to this interpretation. Kinetic detail for the first stage has been substantially extended, and interpretation of the second and third stage modified. Four reductants† $[Co(nane)_2]^{2+}$ ($E^\circ = -0.40$ V),[14] $[Co(sep)]^{2+}$ ($E^\circ = -0.30$ V),[15] $[Cr(bipy)_3]^{2+}$ with a 3-fold excess of bipyridine ($E^\circ = -0.25$ V),[16] and $[Co(sarCl_2)]^{2+}$ ($E^\circ = -0.13$ V)[15] chosen for their inertness were used at pH 6.3 (Mes–H_2SO_4), $I = 0.15$ M (Na_2SO_4). The inorganic complexes were in ≥10-fold excess of the protein (*ca.* 5×10^{-5} M in terms of monomer). Protein samples were purified by oxidation to give the met form with $[Fe(CN)_6]^{3-}$ and Sephadex G 100 column chromatography. Stopped-flow rate constants (25 °C) for the first stage were $[Co(nane)_2]^{2+}$ (122 M^{-1} s^{-1}),‡ $[Co(sep)]^{2+}$ (260 M^{-1} s^{-1}), $[Cr(bipy)_3]^{2+}$ (2.5×10^5 M^{-1} s^{-1}), and $[Co(sarCl_2)]^{2+}$ (113 M^{-1} s^{-1}). The second (2.0×10^{-3} s^{-1}) and third stages (1.2×10^{-4} s^{-1}), monitored by conventional spectrophotometry (320—400 nm), were independent of the concentration and identity of the reductant. After correction for absorbance by the inorganic complexes, changes in spectra corresponding to the three stages are as indicated in Figure 1. We note the large spectrophotometric change for the third stage which seems unlikely to correspond to reduction of the last of sixteen Fe^{III}s.

The stoicheiometry of the first and second stages has been determined using a 5:1 excess of the reducing agent, $[Cr(phen)_3]^{2+}$,[16] which gives a large absorbance change ($\Delta\varepsilon$ 3650 M^{-1} cm^{-1} at 850 nm) at wavelengths at which the protein (4×10^{-5} M) does not absorb, making it possible to monitor

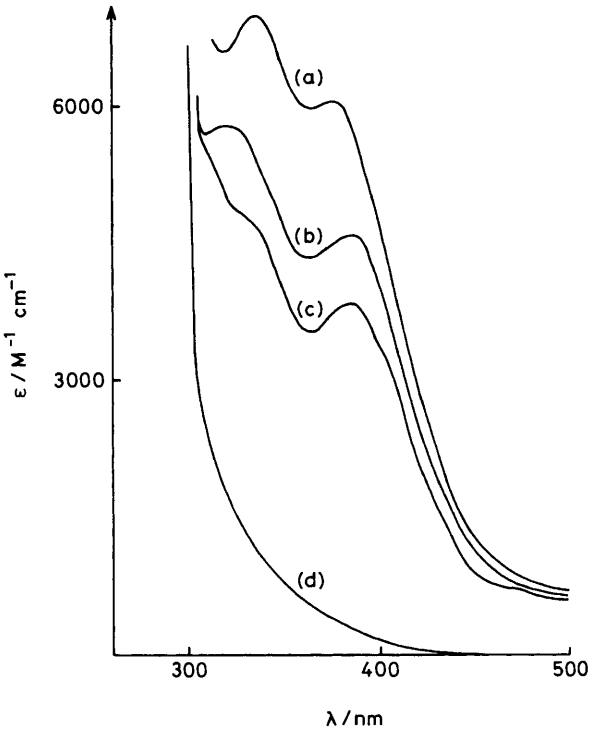


Figure 1. Absorbance spectra obtained for the $[Co(sep)]^{2+}$ reduction of methemerythrin (5.6×10^{-5} M) at 25 °C, pH 6.3 (Mes), $I = 0.15$ M (Na_2SO_4). These correspond to (a) methemerythrin, (b) semi-met protein at the end of the first stage, (c) product obtained at the end of the second stage, and (d) deoxyhemerythrin.

reduction of the protein quantitatively. For the first stage eight equivalents of $[Cr(phen)_3]^{2+}$ are consumed. However after the second stage it was found that precisely four moles of $[Cr(phen)_3]^{2+}$ are consumed, leaving a further four to be accounted for in the final stage. In all experiments quantitative formation of the deoxy protein was observed. The length of the third stage (*ca.* 12 h) made it more difficult to monitor its stoicheiometry. In accordance with these observations the semi-met product at the end of this first stage does not bind O_2, and the quarter-met product at the end of the second stage consumes 4 moles of O_2 (determined as oxyhemerythrin $\lambda_{max.}$ 500 nm; ε 2200 M^{-1} cm^{-1}).

The experiments described raise the question as to whether $Fe^{II,III}$ units generated in the first and second stages have identical structures, and whether intramolecular processes involving cleavage of the μ-oxo bridge are relevant. It has been established that the third stage is first-order in protein concentration, and intramolecular processes similar to but slower than those in the second stage must therefore be relevant. Since the 330 nm band, which has been assigned to the μ-oxo ligand,[17] decreases significantly during the second stage, (Figure 1), it is possible that loss of the μ-oxo ligand gives rise to the slower third stage. We note that μ-oxo is present in the oxy (but not deoxy) protein, and since only a single stage is observed in O_2 uptake (0.75×10^7 M^{-1} s^{-1}) and release (82 s^{-1})[18] rapid formation and cleavage of the μ-oxo bridge is implied.

It has never been clear why, in the absence of co-operativity as in hemoglobin, hemerythrin should exist as an octamer. One possible explanation is that slow deoxy into met interconversion in the octamer in the presence of adventitious

‡ M^{-1} = mol^{-1} dm^3.

oxidant retains at least some protein in the deoxy state with an O_2 binding capability.

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References

1 A. G. Sykes, *Adv. Inorg. Bioinorg. Mech.*, 1982, **1**, 121.
2 I. M. Klotz and D. M. Kurtz, *Acc. Chem. Res.*, 1984, **17**, 16.
3 R. G. Wilkins and P. C. Harrington. *Adv. Inorg. Biochem.*, 1983, **5**, 52.
4 R. E. Stenkamp, L. C. Sieker, and L. H. Jensen, *J. Am. Chem. Soc.*, 1984, **106**, 618.
5 W. A. Hendrickson, M. S. Co, J. L. Smith, K. O. Hodgson, and G. L. Klippenstein, *Proc. Natl. Acad. Sci. USA*, 1982, **79**, 6255.
6 W. T. Elam, E. A. Stern, J. D. McCallum, and J. Sanders-Loeh, *J. Am. Chem. Soc.*, 1982, **104**, 6369; 1983, **105**, 1919.
7 K. Garbett, C. L. Johnson, I. M. Klotz, M. Y. Okamura, and R. J. P. Williams, *Arch. Biochem. Biophys.*, 1971, **142**, 574.
8 S. M. Freier, L. L. Duff, D. F. Shriver, and I. M. Klotz, *Arch. Biochem. Biophys.*, 1980, **205**, 449.
9 I. M. Klotz, G. L. Klippenstein, and W. A. Hendrickson, *Science*, 1976, **198**, 335.
10 J. C. Davis and B. A. Averill, *Proc. Natl. Acad. Sci. USA*, 1982, **79**, 4623.
11 B.-M. Sjöberg, T. M. Loehr, and J. Sanders-Loehr, *Biochemistry*, 1982, **21**, 96.
12 P. C. Harrington, D. J. A. DeWaal, and R. G. Wilkins, *Arch. Biochem. Biophys.*, 1978, **191**, 444.
13 B. B. Muhoberac, D. C. Wharton, L. M. Babcock, P. C. Harrington, and R. G. Wilkins, *Biochim. Biophys. Acta*, 1980, **626**, 337.
14 K. Wieghardt, W. Schmidt, W. Herrmann, and H.-J. Kuppers, *Inorg. Chem.*, 1983, **21**, 2953.
15 I. I. Creaser, R. J. Geue, J. M. Harrowfield, A. J. Herlt, A. M. Sargeson, M. R. Snow, and J. Springborg, *J. Am. Chem. Soc.*, 1982, **104**, 6016.
16 G. Brauer, 'Handbook of Preparative Inorganic Chemistry,' vol. 2, 2nd edn., Academic Press.
17 K. Garbett, D. W. Darnell, I. M. Klotz, and R. J. P. Williams, *Arch. Biochem. Biophys.*, 1969, **135**, 419.
18 A. L. Petrou, F. A. Armstrong, A. G. Sykes, P. C. Harrington, and R. G. Wilkins, *Biochim. Biophys. Acta*, 1981, **670**, 377.