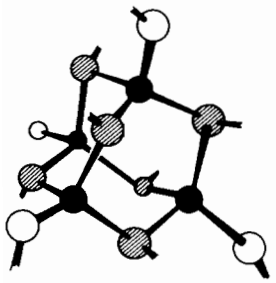

Fig. 1. Adamantane-type of metal–thiolate cluster proposed for metallothionein. The filled circles represent the metal, the empty circles terminal sulfur ligands, and the hatched circles the bridging sulfur ligands (from Ref. 3).

measurements using MT which contains the short-lived excited isomer ^{111m}Cd(II) [7].

The involvement of four sulfur ligands in the coordination of each metal ion is reconcilable with the overall $Me(II)_7(Cys^-)_{20}$ stoichiometry of the molecule only if the metal complexes are sharing some thiolate ligands, thereby forming metal–thiolate clusters. This conjecture is now also confirmed experimentally. Thus, the partitioning of the metal-coordinating ligands into bridging and non-bridging (terminal) thiolate ligands is manifested in a substantial broadening of the sulfur core electron binding energy profile in the X-ray photoelectron spectrum (ESCA) of MT as compared to those of elemental sulfur or monothiols [3]. Direct proof for clustering of the metal centers comes from the demonstration of metal–metal interactions by magnetic resonance spectroscopy. Thus, in ^{113}Cd-enriched MT ^{113}Cd NMR resonances are split into multiplets by ^{113}Cd–^{113}Cd scalar coupling [8] and in fully substituted Co(II)–MT the ESR-resonances are largely suppressed due to antiferromagnetic spin-canceling. This latter effect is also confirmed by magnetic susceptibility measurements [9]. By monitoring the changes in ESR amplitude and in magnetic susceptibility attending the stepwise incorporation of Co(II) into the protein, it is moreover possible to follow the building-up of the cluster structure. This process is biphasic. Up to binding of 4 equivalents of Co(II) the paramagnetic signals increase as expected for magnetically noninteracting high-spin complexes. However, upon further addition of Co(II), these features are lost again, signalling the transition to the magnetically interacting oligonuclear structures [9].

This two-step mode of metal-binding in which separate Co(II) tetrathiolate complexes are formed first is compatible with the model of two separate metal–thiolate clusters in MT deduced from ^{113}Cd NMR homonuclear decoupling experiments [10] and from proteolytic cleavage studies [11] as well as from a ^{113}Cd NMR study on cluster formation in progress in this laboratory. However, the actual steric organization of these clusters remains to be determined. An attractive structure consistent with all spectroscopic information at hand is the cage-like adamantane decahedron built up of thiolate units of tetrahedral symmetry (Fig. 1). The remarkable simplicity of the spectra and the unique capacity of MT to accommodate stoichiometrically different paramagnetic and diamagnetic metal ions, *i.e.*, Ni(II), Co(II), Zn(II), Cd(II), Hg(II), Pb(II) and Bi(III), and to force them into environments of tetrahedral microsymmetry could be optimally accounted for by such a model or a variation of it. The need for these regular bioinorganic structures in MT would explain the remarkable preservation of the positions of all cysteine residues throughout mammalian evolution.

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Second Coordination Sphere Influences on Heme Electronic Structure and Reactivity in Hemoproteins

GERD N. LA MAR

Department of Chemistry, University of California, Davis, Calif. 95616, U.S.A.

A large variety of oxygen-binding hemoproteins with quite different functions share the common iron protoporphyrin IX prosthetic group axially ligated by a histidyl imidazole. To the degree that the members of this class of proteins differ in reactivities and electronic/magnetic properties, they

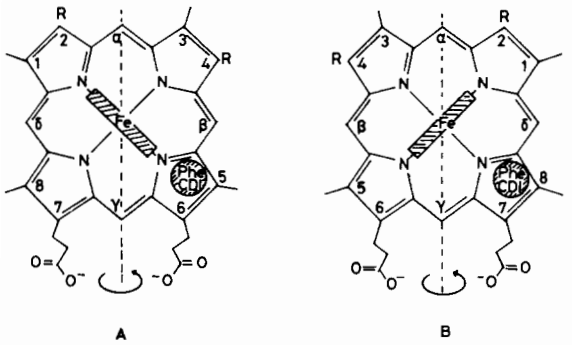

Fig. 1. Heme orientation as viewed from proximal side of heme; the plane of the proximal histidyl imidazole is shown as the shaded rectangle. The orientation as found in single crystals of: A, *Chironomus* hemoglobin [1], and B, sperm whale myoglobin [2].

must therefore reflect wholly influences of the second coordination sphere, *i.e.* the highly folded polypeptide chain. These influences can exert themselves by varying the axial imidazole bonding or by modulating the porphyrin π electronic cloud *via* Van der Waals interactions. Perhaps the most subtle protein effect is to differentiate between the two isomers which result from axial coordination of the imidazole or the two sides of the heme (Fig. 1). Changing the location of the axial imidazole interchanges the position of methyl-1 with vinyl-4 and vinyl-2 with methyl-3.

While all X-ray crystallographic analyses have proposed a single heme orientation for each protein studied to date [1–3], proton NMR evidence [4–8] from our laboratory has demonstrated that a large number of b-type hemoproteins, in fact, exist in solution as a mixture of two species, differing by the rotation of the heme about the α-γ-meso axis. This heme orientational or rotational disorder has been characterized for the monomeric *Chironomus* hemoglobin [5], cytochrome b_5 [7], both native and reconstituted myoglobin [4, 8], and reconstituted horseradish peroxidase [6]. In other cases, heme rotational disorder can be induced by reconstitution of the protein [9]. Thus the initial reaction between apo-myoglobin and hemin yields a 1:1 mixture of the two heme orientations depicted in Fig. 1, and equilibration to the native protein is very long ($\geqslant$30 min) at physiological pH. It is therefore important to assess the influence of the heme orientation (or the altered second coordination sphere) on the properties of the individual proteins.

We have studied two properties of such disordered proteins: the position of the high-spin, low-spin equilibrium in the met-azide forms of native disordered *Chironomus* hemoglobins, and the oxygen affinity of the kinetically trapped disordered sperm whale myoglobin. Using the average heme methyl hyperfine shift as an index of the position of the high-spin, low-spin equilibrium [10], we find that the *Chironomus* met HbN_3 exhibits a significantly larger high-spin contribution for the heme orientation in A of Fig. 1, which dictates that in this orientation the iron experiences a weaker axial ligand field than in orientation B. In sperm whale metMbN_3, heme orientation A also yields a weaker axial ligand field, but by a much reduced margin.

Qualitative estimates of the difference in oxygen affinity for the two heme orientations for a given polypeptide chain have been made for sperm whale myoglobin. Reduction of the 1:1 mixture of heme orientations resulting from an *in situ* reconstitution yields an average oxygen half-saturation pressure a factor of 3 smaller than for the native protein or the 1:1 mixture after equilibration of the heme orientation [11]. Thus the protein with the 'wrong' orientation appears to have an oxygen affinity greater by an order of magnitude than for the X-ray determined orientation [2]. Preliminary evidence indicates that myoglobin with the 'wrong' heme orientation may, in fact, be physiologically functional.

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