signals induced by the paramagnetism of the Cu(II) species would yield information about the rate of electron self exchange of Az. A thorough analysis of the experimental data showed that, for a few NMR signals, the broadening could be analyzed according to the 'strong pulse limit'. In this limit the broadening is completely determined by the lifetime of the protein in the diamagnetic state and this leads directly to a value of the rate of electron self exchange, k. Although not very accurate (estimated accuracy ±50%) the value of 2×10^6 M^{-1} s^{-1} found at 50 °C for k in this way [10] clearly is in agreement with the self exchange rate inferred by Wherland and Pecht from the rate of electron transfer between Az and a variety of other redox proteins [6], though not with the data calculated by Gray and coworkers on the basis of a Marcus treatment of the heterogeneous electron transfer between Az and a series of inorganic transition metal compounds [11]. The high rate of self exchange of Az as well as the relatively fast electron exchange between Az and other redox proteins seems to indicate that non-specific hydrophobic interactions govern the reaction of Az with its reaction partners. This is consistent with the findings from Cr titration experiments [12] and with conclusions from the crystallographic work [2], that there are no pronounced charged patches on the Az surface.

1 P. M. Colman, H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris, J. A. M. Ramshaw and M. P. Venkatappa, *Nature, 272,* 319 (1978).
2 E. T. Adman, R. E. Stenkamp, L. C. Sieker and L. H. Jensen, *J. Mol. Biol., 123,* 35 (1978).
3 E. T. Adman and L. H. Jensen, *Isr. J. Chem., 21,* 8 (1981).
4 T. G. Spiro, Ed., 'Copper Proteins', Wiley, Chichester (1981).
5 S. Niwa, H. Ishikawa, S. Nikai and T. Takabe, *J. Biochem., 88,* 1177 (1980).
6 S. Wherland and I. Pecht, *Biochemistry, 17,* 2582 (1976).
7 J. K. Beattie, D. J. Fensom, H. C. Freeman, E.Woodcock, H. A. O. Hill and A. M. Stokes, *Biochim. Biophys. Acta, 405,* 109 (1975).
8 M. C. Silvestrini, M. Brunori, M. T. Wilson and V. M. Darley-Usmar, *J. Inorgan. Biochem., 14,* 327 (1981).
9 E. T. Adman, G. W. Canters, H. A. O. Hill and N. A. Kitchen, *FEBS Letters, 143,* 287 (1982).
10 G. W. Canters, H. A. O. Hill, N. A. Kitchen and E. T. Adman, to be submitted.
11 D. Cummins and H. B. Gray, *J. Am. Chem. Soc., 99,* 5158 (1977).
12 E. T. Adman, G. W. Canters, H. A. O. Hill and N. A. Kitchen, in preparation.

O17

Specific Labeling of Iron–Sulfur Cluster Subsites

I. MOURA, J. J. G. MOURA and A. V. XAVIER

Centro de Química Estrutural, Complexo I, IST, Av. Rovisco Pais, 1000 Lisbon, Portugal

Desulfovibrio gigas ferredoxin is isolated in different oligomeric forms [1]. These ferredoxins, termed FdI and FdII, are built from one type of monomeric unit of which the sequence of 57 amino acids is known [2, 3]. FdII is a tetramer of molecular weight 24,000. Mössbauer and EPR studies [4] have shown that each monomer of FdII contains one [3Fe-xS] center. The other oligomeric form, the trimeric FdI [5] was shown to contain as a majority species a [4Fe–4S] cluster. However the FdI trimer can accomodate a [3Fe–xS] cluster as a minority species of variable proportions. Evidence was accumulated that both clusters can be accomodated by the polypeptide chain of this ferredoxin:

(a) Controlled reconstitution experiments of the active center of *D. gigas* FdII (containing only [3Fe–xS] cores) were performed in such a way that a reconstituted protein, Fd_R, loaded only with [4Fe–4S] cores was obtained [6].

(b) Mössbauer and EPR spectroscopies have been used to extensively study the process of cluster interconversion in *D. gigas* FdII. The [3Fe–xS] centres could be converted into [4Fe–4S] clusters after incubation with Fe^{2+} in the presence of dithiothreitol [6].

Based on these observations incubations of FdII were performed using 95% enriched ^{57}Fe in the presence of sulfide and dithiothreitol. Conversion from a [3Fe–xS] into a structure with a [4Fe–4S] core occurred; the latter seems to be structurally identical to the cluster of Fd_R. The ^{57}Fe Mössbauer

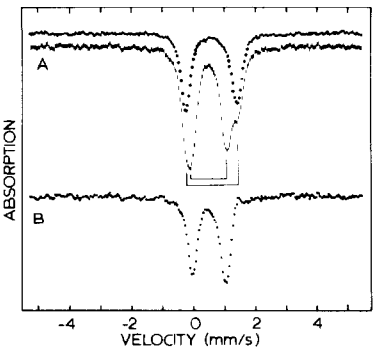

Fig. 1. (A) Mössbauer spectra of a reduced sample of FdII incubated with ^{57}Fe, sulfide and dithiothreitol (full circles) and of reduced reconstituted Fd (hatch marks). The spectra were recorded at 90 K in zero magnetic field. (B) Difference spectrum obtained by subtracting the spectrum of the incubated sample (full circles) from that of reduced Fd_R.

spectra of the newly formed [4Fe–4S] cluster [6] correspond to those obtained for the subsites of the Fd_R cluster (Fig. 1). The data show that the externally added iron occupies either one subsite or at most two structurally equivalent sites of the [4Fe–4S] cluster [6, 7]. This isotopic labeling of specific cluster subsites allows us to study in considerable detail the hyperfine interactions of the [4Fe-4S] core structures. Moreover, this technique will allow us to correlate spectra of subsites in different oxidation states. Application of this technique of isotopic labeling to individual clusters of enzymes with multiple clusters (*e.g.*, hydrogenase) will greatly simplify the spectral assignments.

Acknowledgements. We are indebted to our colleagues who have contributed to the work described here, Drs. B. H. Huynh, T. A. Kent, J. LeGall, J. D. Lipscomb, and E. Münck. This research was supported by grants from INIC and JNICT, Portugal.

1 M. Bruschi, E. C. Hatchikian, J. LeGall, J. J. G. Moura and A. V. Xavier, *Biochim. Biophys. Acta, 449,* 275–284 (1976).
2 J. Travis, D. J. Newman, J. LeGall and H. D. Peck, Jr., *Biochem. Biophys. Res. Commun., 45,* 452–458 (1971).
3 M. Bruschi, *Biochem. Biophys. Res. Commun., 91,* 623–628 (1979).
4 B. H. Huynh, J. J. G. Moura, I. Moura, T. A. Kent, J. LeGall, A. V. Xavier and E. Münck, *J. Biol. Chem., 255,* 3242–3244 (1980).
5 J. J. G. Moura, A. V. Xavier, E. C. Hatchikian and J. LeGall, *FEBS Lett., 89,* 177–179 (1978).
6 J. J. G. Moura, I. Moura, T. A. Kent, J. D. Lipscomb, B. H. Huynh, J. LeGall, A. V. Xavier and E. Münck, *J. of Biol. Chem., 257,* 6259–6267 (1982).
7 T. A. Kent, I. Moura, J. J. G. Moura, J. D. Lipscomb, B. H. Huynh, J. LeGall, A. V. Xavier and E. Münck, *FEBS Lett., 138,* 55–58 (1982).

O18

Spectroscopic Probes of Homotropic and Heterotropic Interactions of the Hemocyanin Active Site

YEONG TSYR HWANG, EDWARD I. SOLOMON*

Dept. of Chemistry, Stanford University, Stanford, Calif. 94305, U.S.A.

and LEONARD J. ANDREWS

GTE Laboratories, Waltham, Mass. 02154, U.S.A.

Under physiological conditions, both arthropod and mollusc hemocyanins are assembled into highly aggregated biopolymers with a molecular architecture that differs dramatically between the two phyla. For arthropods, a single subunit has a molecular weight of ~70,000 and contains one binuclear copper active site. At pH below 8 and in the presence of Ca^{2+} these subunits aggregate, and in the case of *Limulus polyphemus* which is the arthropod discussed here, eight hexamers comprise the resultant biopolymer. For molluscs, the smallest subunit contains eight binuclear copper active sites covalently linked in a single polypeptide chain of ~400,000 daltons. At pH less than 8, *Busycon canaliculatum* being representative, twenty chains aggregate to form a whole molecule. When aggregated, all of the hemocyanins are highly cooperative in their oxygen binding, with Hill coefficients dependent on pH and the presence of divalent cations. The cooperative oxygen binding can be interpreted as the equilibrium between a low oxygen affinity tensed quaternary structure (deoxy) and a high oxygen affinity relaxed quaternary structure (oxy), in which the conformational change is induced by the homotropic effector (O_2) and heterotropic effectors (H^+, Ca^{2+}).

We have been able to develop a direct spectroscopic approach to study these allosteric interactions at the active site level through the preparation of Spectral Probe (SP) derivatives of the hemocyanin biopolymer [1]. These SP derivatives contain a small fraction of EPR-detectable half met [Cu(II)-Cu(I)] sites homogenously dispersed among the EPR nondetectable oxy binuclear copper active sites. Our studies demonstrate that deoxygenation of the dominant oxy site (homotropic effect) induces change in the hemocyanin quaternary structure which results in significant spectral and structural changes in the half met probe sites. The extensively defined and well-understood chemistry and spectroscopy of the half met derivatives [2], suggest that the changes in half met SP sites represent elimination of the exogenous ligand (due to the steric competition of endogenous ligand, OR^-) and rearrangement of the geometric structure at the copper site. Furthermore, *Busycon* hemocyanin SP derivatives show the regulatory role of the heterotropic effectors: where Ca^{2+} can induce cooperativity by stabilizing the tensed quaternary structure, proton increases the oxygen affinity and stabilizes the relaxed quaternary structure. Finally, we use the SP derivatives to study the intersubunit and intrasubunit interactions in hemocyanin.

We have also been able to probe the allosteric relationship between changes in the coupled binuclear copper site and the Ca^{2+} binding site [3]. Here, we substitute Eu^{3+} for Ca^{2+} and study its resonance emitted f–f fluorescence. Direct excitation of Eu^{3+} emission is accomplished by a pulsed laser source. Transition between the nondegenerate ground (^7F_o) and excited (^5D_o) states gives a single unsplit line. Since this transition energy is very sensitive to the environment of Eu(III), we are able to differentiate the bound Eu^{3+} peak from the free Eu^{3+} peak. Excited state life times of bound Eu^{3+} measured in D_2O and H_2O permit a direct estimation of the