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Iron environment in soybean lipoxygenase-1

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The iron coordination in native, Fe(II), lipoxygenase has been studied by Extended X-Ray Absorption Fine Structure (EXAFS). The ligands are 6 ± 1 nitrogen and/or oxygen ligands at 2.05–2.09 Å, with a maximum variance of 0.09 Å. The number of imidazole ligands is estimated at 4 ± 1 using multiple scattering simulations. The remaining ligands are proposed to be carboxylate oxygens.

Introduction

Lipoxygenase (linoleate : oxygen oxidoreductase, EC 1.13.11.12) catalyses the dioxygenation of fatty acids containing a 1*Z*,4*Z*-pentadiene system to form fatty acid hydroperoxides (cf. Ref. 1). In mammalian tissues, lipoxygenases are involved in the initial steps of the biosynthesis from arachidonic acid of the physiologically active compounds, the leukotrienes and lipoxins [2]. In the plant kingdom, lipoxygenase has been suggested to play a role in germination [3], and it has been implicated in the development of flavours and off-flavours [4].

Soybean lipoxygenase-1 (M_r 94038 [5]) contains 1 gatom of iron per mole of enzyme. EPR and other spectroscopic studies have shown that the iron is in an environment with a ligand field of axial symmetry, but not in a porphyrin cofactor [6]. It must be directly ligated by amino acid side-chains in the protein. The absence of absorption bands in the visible spectrum of the native and yellow (Fe(III)) forms of the enzyme that could be attributed to iron phenolate charge-transfer bands [7] argues against coordination by tyrosine residues, while coordination by tryptophan residues, although shown to be present in a large hydrophobic cleft of the protein together with iron [8,9], is considered unlikely. Recent magnetic susceptibility studies have established that the iron in the native enzyme is high-spin Fe(II), and that it does not coordinate dioxygen [10]. Iron in lipoxygenase shuttles between the Fe(II) and Fe(III) states during catalysis [10,11], and linoleyl radicals are formed [12]. In the current mechanism, the Fe(III) enzyme abstracts a hydrogen atom from linoleic acid, forming Fe(II) enzyme, a proton and a linoleyl radical. This radical reacts with dioxygen, yielding a peroxy radical, which takes up an electron and a proton to form the product hydroperoxide and reconstitute the Fe(III) en-

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Abbreviation: TIEOH, 1,1,2-tris(*N*-methylimidazol-2-yl)-1-hydroxyethane.

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zyme [11,13]. The functional role of the iron atom in the catalysis by the enzyme is an important feature in the design of inhibitors [14,15].

The EXAFS (Extended X-ray Absorption Fine Structure) of metals in metalloproteins can be Fourier transformed to reveal, after phase correction, the radial distribution of atoms around the metal till approx. 3 Å or further, depending on the local order. It can be analysed to determine metal-ligand distances with high accuracy (± 0.02 Å) and first shell coordination numbers to within 20%. Sulphur ligands are readily distinguished from carbon, nitrogen and oxygen ligands, but it is difficult to discriminate between the latter three (cf. Ref. 16). Pioneering work includes studies on iron-porphyrin proteins, viz. hemoglobin [17,18] and chloroperoxidase [19]. More recently, EXAFS has been applied to non-heme iron proteins, e.g. hemerythrin [20,21], the reaction centre of photosynthetic bacteria [22,23], protocatechuate 3,4-dioxygenase [24], transferrin [25] and ribonucleotide reductase [26]. We report here the results of an EXAFS study of native soybean lipoxygenase-1, showing that Fe(II) is coordinated to 6 ± 1 oxygen and/or nitrogen ligands, of which 4 ± 1 are imidazole ligands.

Materials and Methods

Lipoxygenase 1 was isolated from soybeans [27,28], dialysed against 0.1 M sodium borate buffer (pH 9.0) and concentrated in a collodion bag SM 13200 (Sartorius Membranfilter, Göttingen, F.R.G.) to a concentration of approx. 2.5 mM.

X-ray fluorescence excitation spectra at the Fe K-edge (approx. 7100 eV) were recorded on EXAFS station 7.1 [29] of the SERC Synchrotron Radiation Source at the Daresbury Laboratory, operating at an energy of 2 GeV with an average current of 150 mA, using a Si 111 monochromator, and focussing the beam at the sample position using a toroidal mirror, which also minimized harmonic contamination. Data were collected at 200 K using an Oxford Instruments X-ray fluorescence cryostat. Mn filters of 10–15 μm thickness were placed in front of the NaI scintillation counter detectors in order to reduce scattered radiation [29]. A total of 18 scans were collected for the enzyme and each spectrum was checked individually before summing and averaging.

Data analysis and simulations employed the Daresbury Laboratory EXAFS analysis package, including the background subtraction programme EXBACKI, the programme MUFPOT for the ab initio calculation of phaseshifts and backscattering factors, and the fast curved-wave EXAFS simulation and fitting programme EXCURVE [30], allowing calculations of multiple scattering up to third order [31]. It is well established that the theoretical phaseshifts and backscattering factors calculated with MUFPOT are transferable from model compound to model compound [18,25], and this was confirmed in our analysis of the iron imidazole compound, poly[μ-hexakis(2-methylimidazolato-N,N')triiron(II)] [50]. Fits were obtained by simulating the EXAFS on the basis of models, varying occupancies (usually only integer values) and atom types, and iteratively refining the shell radii and Debye-Waller-type factors to a minimum in the fit index.

Results and Discussion

The EXAFS of native lipoxygenase at 200 K is shown in Fig. 1a (near-edge region included) and Fig. 1b (EXAFS range only), together with the phase-corrected Fourier transforms. In the initial steps of the analysis, the EXAFS was Fourier-transformed in the k-range 2.5–10.5 $Å^{-1}$, and the main shell filtered and back-transformed. It could be fitted with 6 nitrogens, at 2.09 Å, with a value for the Debye-Waller type factor, describing effects of static and thermal disorder, quoted as $2\sigma^2$, of 0.017 $Å^2$, or with 6 oxygens, at 2.05 Å (Debye-Waller factor 0.019 $Å^2$), or with combinations of nitrogens and oxygens, for example two oxygens at 2.00 and 4 nitrogens at 2.15 Å. The observation of six-coordination is in line with results of a magnetic circular dichroism study on Fe(II) lipoxygenase frozen in glycol [32], and of Mössbauer studies [33], indicating octahedral ligand symmetry. The low intensity of the pre-edge feature in our X-ray Absorption Edge data (not shown) also points to six-coordination [34].

Single scattering analysis for the small shells identified 3–4 carbons at 3.06 Å and 5–6 carbons at 3.94 Å. The observation of backscattering by

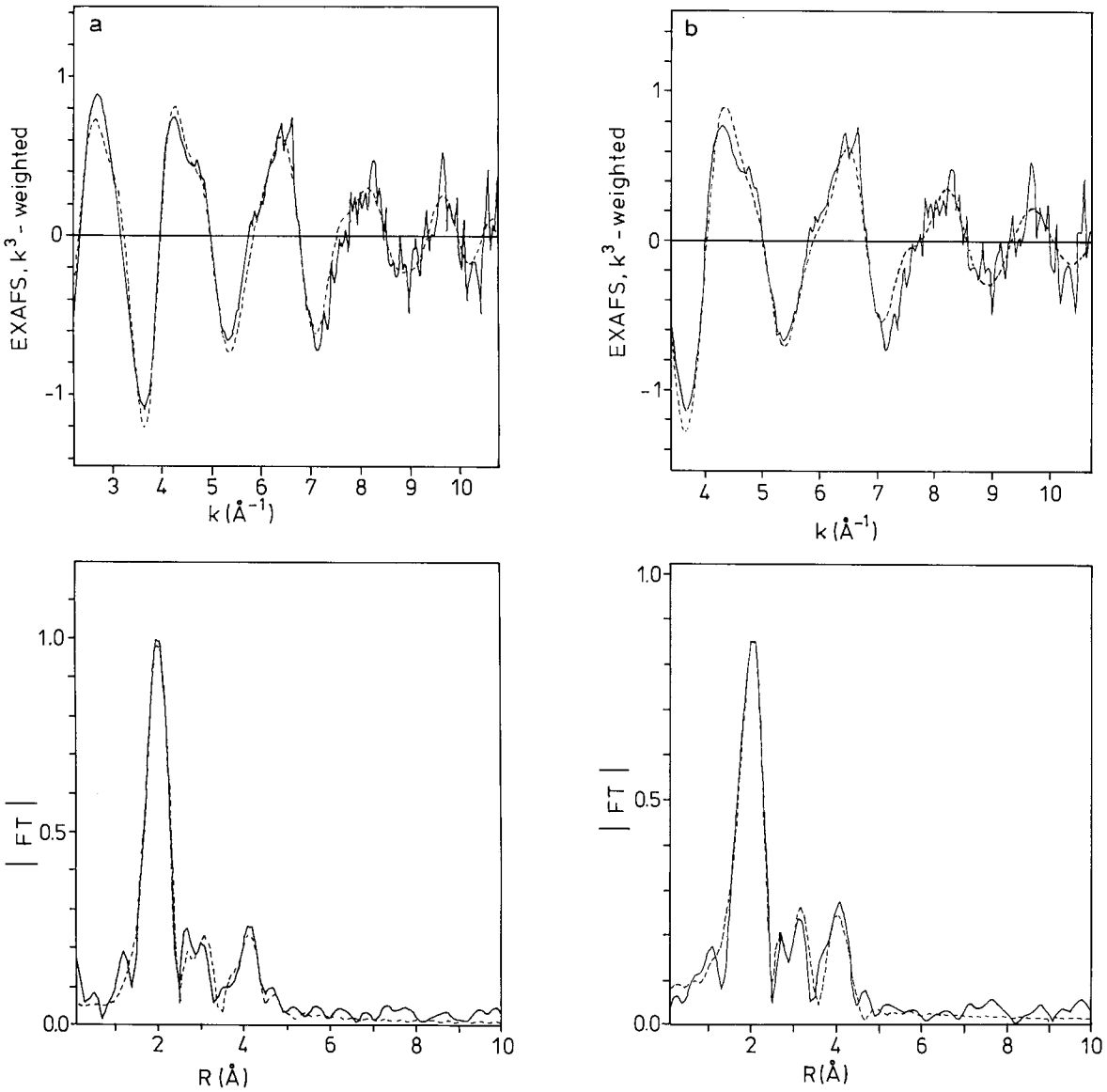

Fig. 1a. Fe K-edge k^3-weighted EXAFS with the data range expanded into the near-edge region (upper panel) and its phase-corrected Fourier transform (lower panel) of native soybean lipoxygenase 1. Solid line, experimental, dashed line, single scattering simulation including multiple scattering with the parameters given in Table I, left half. Threshold energy, ΔE_0, 19.05 eV, fit index, 1.50118. Fig. 1b. As Fig. 1a, but with EXAFS data range only, and the simulation including multiple scattering with the parameters given in Table I, right half. ΔE_0, 20.40 eV, fit index, 1.85303. For both simulations, imaginary potential, −1 eV, amplitude reduction factor, 0.6.

other atoms than the ligand donor atoms is not common in EXAFS and usually points to coordination by a rigid ligand system, like the imidazole group of histidine or the pyrrole moiety of a porphyrin cofactor. In view of the evidence against the presence of the latter in lipoxygenase [6], we propose that the small shells represent backscattering by atoms of imidazole rings, with some of the atoms found representing nitrogens. Coordination by tryptophan residues is expected to lead to the occurrence of more small shells in the Fourier transform than are observed. The ratio of

TABLE I

PARAMETERS USED FOR THE SIMULATIONS OF THE EXAFS (Fig. 1)

R (distance to Fe) in Å; Debye-Waller-type factor, given as $2\sigma^2$, in Å^2. Bond angle values, in degrees, for the distant atoms refer to the angle Fe–N (at 2.14 Å)-distant atom in the unit set up for the multiple scattering calculations.

Atom type	No.	Multiple scattering (Fig. 1a)			Single scattering (Fig. 1b)	
		R	$2\sigma^2$	bond angle	R	$2\sigma^2$
O	2	1.99	0.011		1.97	0.017
N	4	2.14	0.011		2.12	0.011
C	4	2.92	0.008	127	2.90	0.018
C	4	3.11	0.003	−128	3.12	0.009
C	4	4.28	0.020	161	3.96	0.012
N	4	4.49	0.038	−161	4.40	0.023

apparent occupancies of the small shells, which one would expect to be 1, and the outer shell distance, which should be approx. 4.2 Å (cf. crystal structure of model compound [35], Table I), seem to contradict the proposal that they represent backscattering by imidazole ligands. However, similar effects have been observed in metalloproteins that definitely have imidazole ligands [36–38].

The analysis of the imidazole model compound, poly[μ-hexakis(2-methylimidazolato-N,N')triiron (II)], the crystal structure of which is known [35], is described elsewhere [50]. It was found that, using single scattering only, a good fit in the range 25–445 eV is obtained for 4 methylated imidazoles coordinating to the iron, in excellent agreement with the crystal structure for the first two shells. Beyond these shells, however, the agreement deteriorates, because of the inadequacy of the single scattering theory for the coordinating imidazole ring, due to the prominence, even at relatively high k, of multiple scattering effects in these systems. Typically, some of the atoms expected at approx. 4.1–4.2 Å are found to refine to shorter distances if the single scattering approximation is used, as has been found earlier in such systems [36–38]. However, the EXAFS is in satisfactory agreement with the crystallographic data, if multiple scattering effects are considered, in line with recent studies on copper [39] and zinc [40] imidazole compounds.

Multiple scattering theory was applied to the analysis of the lipoxygenase data. The number of imidazole ligands was varied, assuming that if this number were x, the remaining $6-x$ ligands to iron would be oxygen atoms. This is a reasonable assumption, as the occurrence of carbon donor ligands is unlikely, as is that of nitrogen donor ligands not belonging to an imidazole group, the possible coordination of the ε-NH_2 of lysine to iron in cytochrome c at high pH being the only example reported [41]. The lowest fit index was achieved with 4 imidazole and 2 oxygen ligands, with the latter at the shorter distance (see Fig. 1a, parameters in left half of Table I). For comparison, a single scattering simulation, refined over the EXAFS data range, with the same number of shells and the same occupancies as the best multiple scattering simulation is also shown (Fig. 1b, parameters in right half of Table I). Interestingly, the recently reported crystal structure of a coordination compound of Fe(III) with 1,1,2-tris(N-methylimidazol-2-yl)-1-hydroxyethane (TIEOH) in protonated and deprotonated form, viz. [Fe(TIEO)(HTIEO)] $(ClO_4)_2$, also has 4 imidazoles at the longer, and 2 oxygens at the shorter distance from iron [42]. On the other hand, it should be noted that the positions of the imidazole ring carbons attached to the nitrogen donor atoms, as derived from the single or the multiple scattering simulations (3.06 (see above) or 2.90–3.12 and 2.92–3.11 Å, respectively, cf. Table I) could be taken as an indication that the nitrogen donor ligands rather than the oxygen ligands are at the shorter distance (cf. the crystal structure of poly[μ-hexakis(2-methylimidazolato-N,N')triiron(II)] with nitrogens at 2.03 and carbons at 3.04–5 Å from iron [35]). The spread in the distances to iron of the carbon

atoms at approx. 3.0 Å is smaller, viz. 3.10 and 3.12 Å, if one introduces 2 further carbons at 2.91 Å, which could well be attached to the coordinating oxygens. The best simulations with the 4 imidazoles rather than the oxygen ligands at the shorter distance, and the simulations involving 5 imidazoles/1 oxygen, and 3 imidazoles/3 oxygens, for the lipoxygenase EXAFS had slightly higher fit indices than the simulation presented in Fig. 1a. Our results can be summarized as follows:

(1) There are 6 ± 1 nitrogen and/or oxygen ligands to iron in lipoxygenase, at an average ligand distance between the values 2.05–2.09 Å, as found for oxygen or nitrogen coordination, respectively.

(2) The variance σ in the distances, as derived from the value for the Debye-Waller-type factor, making the simplification that it is totally due to static, and not thermal, disorder, has a maximum value of approx. 0.09 Å.

(3) 4 ± 1 of the ligands are imidazoles, the remaining ligands are oxygens.

In EXAFS studies on transferrin, it was proposed that 2 resolved short-distance oxygens at 1.83–1.87 Å from the iron were phenolate oxygens [25]. Lipoxygenase is definitely different in this respect and is not a member of the class of iron-tyrosinate proteins. There is magnetic and spectroscopic evidence against the coordination of dioxygen or water [10,13,43,44]. Therefore, the oxygen ligands are most probably carboxylate oxygens. One feasible arrangement of 4 imidazoles and 2 carboxylate oxygens is to have the coordinating imidazole nitrogens in a plane with the oxygens in axial positions, as shown in Fig. 2a. Such an arrangement, if it could be extrapolated to the Fe(III) enzyme, would account nicely for the $g = 6$ signal in the EPR spectrum [6], which indicates axial ligand symmetry, like in aquometmyoglobin [45]. However, it is not excluded that environments with 3-fold instead of 4-fold axial symmetry, or environments of 5 imidazoles/1 oxygen, or even 3 imidazoles/3 oxygens, which gave poorer fits to the EXAFS, could give such an EPR signal. On the other hand, in the crystal structures of the photosynthetic reaction centre of *Rhodopseudomonas viridis* [46] and *Rhodobacter sphaeroides* [47], a slightly different iron ligand arrangement, as shown in Fig. 2b, with the 2 oxygens belonging to one carboxylate residue, has been found. The lipoxygenase EXAFS presented here is quite similar to that of the *Rhodobacter sphaeroides* protein [22,23], as far as number, type and average ligand distance of the ligand donor atoms, as well as the occurrence of small shells are concerned. In various recent EPR studies [48,49], weak $g = 6$ signals have been attributed to the Fe(III) form of these proteins, indicating that there are structural as well as spectroscopic analogies with the lipoxygenase iron site. With the present lipoxygenase EXAFS data, we cannot discriminate between the two possible iron sites shown in Fig. 2.

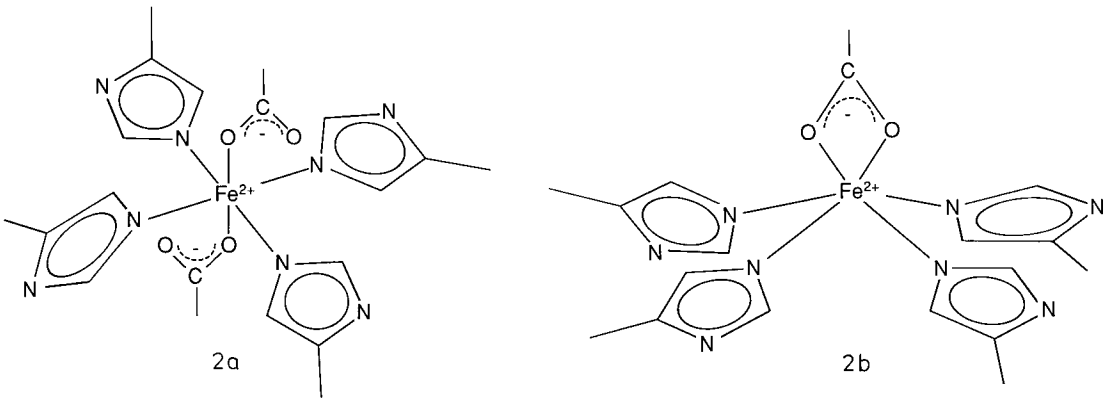

Fig. 2. Models for the lipoxygenase iron site based on the EXAFS results. Fig. 2a. Model with 4 N (His) in a plane, accounting for the axial ligand symmetry observed in EPR [6] when extrapolated to the yellow (Fe(III)) enzyme. Fig. 2b. Model based on the site found in crystallographic studies of the reaction centre in photosynthetic bacteria [46,47].

On the basis of fluorescence [8] and fluorescence perturbation [9] experiments, it was proposed that soybean lipoxygenase 1 has a large hydrophobic active site, containing the tryptophan residues of the protein and iron. In the recently published amino acid sequence of lipoxygenase [5], 7 of the 13 tryptophan residues occur in a relatively short region, viz. between residues 617 and 683. This region contains two histidines at positions 646 and 656, the former directly adjacent to a tryptophan pair, as well as 7 aspartic and 6 glutamic acid residues. It is tempting to propose that these two histidines are iron ligands, the other histidine ligand(s) being provided by other regions in the polypeptide chain. Further studies are under way in which we address the influence of valence state of iron and the presence of substrates and inhibitors on the iron coordination.

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References

1 Veldink, G.A. and Vliegenthart, J.F.G. (1984) in Advances in Inorganic Biochemistry, Vol. VI (Eichhorn, G.L. and Marzilli, L.G., eds.), pp. 139–162, Elsevier, Amsterdam.
2 Samuelsson, B., Dahlén, S.-E., Lindgren, J.Å., Rouzer, C.A. and Serhan, C.N. (1987) Science 237, 1171–1176.
3 Vernooy-Gerritsen, M., Leunissen, J.L.M., Veldink, G.A. and Vliegenthart, J.F.G. (1984) Plant Physiol. 76, 1070–1079.
4 Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1977) Prog. Chem. Fats Lipids 15, 131–166.
5 Shibata, D., Steczko, J., Dixon, J.E., Hermodson, M., Yazdanparast, R. and Axelrod, B. (1987) J. Biol. Chem. 262, 10080–10085.
6 De Groot, J.J.M.C., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J., Wever, R. and Van Gelder, B.F. (1975) Biochim. Biophys. Acta 377, 71–79.
7 De Groot, J.J.M.C., Garssen, G.J., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J. and Egmond, M.R. (1975) FEBS Lett. 56, 50–54.
8 Finazzi Agró, A., Avigliano, L., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochim. Biophys. Acta 326, 462–470.
9 Finazzi Agró, A., Avigliano, L., Egmond, M.R., Veldink, G.A. and Vliegenthart, J.F.G. (1975) FEBS Lett. 52, 73–76.
10 Petersson, L., Slappendel, S. and Vliegenthart, J.F.G. (1985) Biochim. Biophys. Acta 828, 81–85.
11 Petersson, L., Slappendel, S., Feiters, M.C. and Vliegenthart, J.F.G. (1987) Biochim. Biophys. Acta 913, 228–237.
12 De Groot, J.J.M.C., Garssen, G.J., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochim. Biophys. Acta 326, 279–284.
13 Feiters, M.C., Aasa, R., Malmström, B.G., Slappendel, S., Veldink, G.A. and Vliegenthart, J.F.G. (1985) Biochim. Biophys. Acta 831, 302–305.
14 Galpin, J.R., Tielens, A.G.M., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1976) FEBS Lett. 69, 179–182.
15 Spaapen, L.J.M., Verhagen, J., Veldink, G.A. and Vliegenthart, J.F.G. (1980) Biochim. Biophys. Acta 617, 132–140.
16 Cramer, S.P. and Hodgson, K.O. (1979) Progr. Inorg. Chem. 25, 1–39.
17 Eisenberger, P., Shulman, R.G., Kincaid, B.M., Brown, G.S. and Ogawa, S. (1978) Nature 274, 30–34.
18 Perutz, M.F., Hasnain, S.S., Duke, P.J., Sessler, J.L. and Hahn, J.E. (1982) Nature 295, 535–538.
19 Cramer, S.P., Dawson, J.H., Hodgson, K.O. and Hager, L.P. (1978) J. Am. Chem. Soc. 100, 7282–7290.
20 Hendrickson, W.A., Co, M.S., Smith, J.L., Hodgson, K.O. and Klippenstein, G.L. (1982) Proc. Natl. Acad. Sci. USA 79, 6255–6259.
21 Elam, W.T., Stern, E.A., McCallum, J.D. and Sanders-Loehr, J. (1982) J. Am. Chem. Soc. 104, 6369–6373.
22 Eisenberger, P., Okamura, M.Y. and Feher, G. (1982) Biophys. J. 37, 523–538.
23 Bunker, G., Stern, E.A., Blankenship, R.E. and Parson, W.W. (1982) Biophys. J. 37, 539–551.
24 Felton, R.H., Barrow, W.L., May, S.W., Sowell, A.L., Goel, S., Bunker, G. and Stern, E.A. (1982) J. Am. Chem. Soc. 104, 6132–6134.
25 Garratt, R.C., Evans, R.W., Hasnain, S.S. and Lindley, P.F. (1986) Biochem. J. 233, 479–484.
26 Bunker, G., Petersson, L., Sjöberg, B.-M., Sahlin, M., Chance, M., Chance, B. and Ehrenberg, A. (1987) Biochemistry 26, 4708–4716.
27 Slappendel, S. (1982) Ph. D. Thesis, State University of Utrecht, Utrecht, The Netherlands.
28 Al-Hakim, M. (1986) Ph. D. Thesis, Salford University, Salford, U.K.
29 Hasnain, S.S., Quinn, P.D., Diakun, G.P., Wardell, E.M. and Garner, C.D. (1984) J. Phys. E: Sci. Instrum. 17, 40–43.

30 Gurman, S.J., Binsted, N. and Ross, I. (1984) J. Phys. C: Solid State Phys. 17, 143–151.
31 Gurman, S.J., Binsted, N. and Ross, I. (1986) J. Phys. C: Solid State Phys. 19, 1845–1861.
32 Whittaker, J.W. and Solomon, E.I. (1986) J. Am. Chem. Soc. 108, 835–836.
33 Funk, M.O. (1987) J. Am. Oil Chem. Soc. 64, 642–643.
34 Roe, A.L., Schneider, D.J., Mayer, R.J., Ryrz, J.W., Widom, J. and Que, L., Jr. (1984) J. Am. Chem. Soc. 106, 1676–1681.
35 Spek, A.L., Duisenberg, A.J.M. and Feiters, M.C. (1983) Acta Cryst. C 39, 1212–1214.
36 Bordas, J., Dodson, G.G., Grewe, H., Koch, M.H.J., Krebs, B. and Randall, J. (1983) Proc. R. Soc. Lond. B 219, 21–39.
37 Hasnain, S.S., Wardell, E.M., Garner, C.D., Schlösser, M. and Beyersmann, D. (1985) Biochem. J. 230, 625–633.
38 Diakun, G.P., Fairall, L. and Klug, A. (1986) nature 324, 698–699.
39 Strange, R.W., Hasnain, S.S., Blackburn, N.J. and Knowles, P.F. (1986) J. Phys. 12-47-C8, 593–596.
40 Pettifer, R.F., Foulis, D.L. and Hermes, C. (1986) J. Phys. 12-47-C8, 545–550.
41 Gupta, R.K. and Koenig, S.H. (1971) Biochem. Biophys. Res. Commun. 45, 1134–1143.
42 Gorun, S.M., Papaefthymiou, G.C., Frankel, R.B. and Lippard, S.J. (1987) J. Am. Chem. Soc. 109, 4244–4255.
43 Slappendel, S., Aasa, R., Malmström, B.G., Verhagen, J., Veldink, G.A. and Vliegenthart, J.F.G. (1982) Biochim. Biophys. Acta 708, 259–265.
44 Nelson, M.J. (1987) J. Biol. Chem. 262, 12137–12142.
45 Feher, G., Isaacson, R.A., Scholes, C.P. and Nagel, R. (1972) Ann. NY Acad. Sci. 222, 86–102.
46 Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) Nature 318, 618–624.
47 Allen, J.P., Feher, G., Yeates, T.O., Komyia, H. and Rees, D.C. (1987) Proc. Natl. Acad. Sci. USA 84, 5730–5734.
48 Petrouleas, V. and Diner, B.A. (1986) Biochim. Biophys. Acta 849, 264–275.
49 Itoh, S., Tang, X.-S. and Satoh, K. (1986) FEBS Lett. 205, 275–281.
50 Feiters, M.C., Navaratnam, S., Al-Hakim, M., Allen, J.C., Spek, A.L., Veldink, G.A. and Vliegenthart, J.F.G. (1988) J. Am. Chem. Soc., in press.