Mössbauer Studies on Protoporphyrin IX Iron(II) Solutions Containing Sulphur Ligands and their Carbonyl Adducts. Models for the Active Site of Cytochromes P-450

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Received August 6, 1983

A number of sulphur ligands in solution with protoporphyrin IX iron(II) give Mössbauer data similar to that recorded for cytochromes P-450 in the deoxy-high-spin ferrous stage. Addition of carbonyl to the solution results in Mössbauer spectra that have wider quadrupole splittings than that of P-450 CO. This suggests that the sulphur to iron bond in the cytochromes P-450 is unusually strong, probably due to other bonding forces in these proteins.

Introduction

As a continuation of a programme of studies aimed at understanding the chemical and physical properties of the protoporphyrin IX iron moiety [1–6] we have investigated solutions containing this entity and sulphur containing ligands.

Other porphyrin iron complexes with sulphur ligands have been suggested as analogues for the active site of cytochrome P-450 [7–19].

The cytochrome P-450 enzymes are members of the monooxygenase (mixed-function oxidase) class of enzymes which catalyze the incorporation of one atom of dioxygen into a substrate whilst the other is reduced to water. These enzymes also catalyze the hydroxylation of C-H bonds in metabolism, hormone regulation, and oxidative degredation of many toxic agents [20–23]. These proteins are found in mammaliam microsomes and mitochondria and in bacteria.

These cytochromes upon reduction in the presence of carbon monoxide exhibit Soret bands at 360 and 450 nm [24–25], compared with haem proteins such as haemoglobin and myoglobin with CO which have Soret bands near 420 nm. Mason *et al.* [26–27] were the first to prepare axial sulphur coordination based on evidence that microsomal P-450 cytochromes were inactivated by sulphydryl reagents.

There are four states in the accepted reaction cycle of cytochrome P-450. These are (A) a substrate free low-spin ferric resting stage, (B) a substrate bound high-spin ferric stage, (C) a high-spin ferrous (deoxy-stage), (D) a dimagnetic oxygenated ferrous state.

Many workers have produced well characterised iron porphyrin thiolato complexes for states B and C [7–17] and more recently, D [18, 19].

Except for studies on hemin solutions containing various mercaptans [28–30] all the work has been carried out on non naturally occurring porphyrins.

Gunsalus and co-workers [31–32] have described the role of a bacterial cytochrome P-450 (designated P-450 cam) in the methylene hydroxylation of D(+) camphor in *Pseudomonas putida*.

Mössbauer studies on cytochrome P-450 cam grown in media enriched $^{57}FeCl_3$ have been carried out by Sharrock *et al.* [33]. They investigated cytochrome P-450 in the four states of the proposed reaction mechanism [34] and in the ferrous carbon monoxide adduct. The Mössbauer spectra of the latter complex are very similar to those of haemoglobin carbon monoxide [33].

We report here Mössbauer and electronic spectral studies that show that protoporphyrin IX iron(II) solutions containing the sulphur containing ligands such as ethyl mercapto acetate, mercaptoethanol, ethanediol, thiophenol and thiocreosol are models for species C. Our studies demonstrate that these solutions on exposure to CO show the presence of adducts that display the usual hyperporphyrin electronic spectra. These adducts show distinctive Mössbauer parameters similar to those reported by other workers.

We also report studies on protoporphyrin IX iron(II) cysteine solutions. The results differ from those of the other sulphur containing ligands and are discussed with reference to similar results for glycine ethyl ester.

Results and Discussion

The Mössbauer data obtained in this work are presented in Table I. Table II contains the Mössbauer data for cytochrome P-450 cam by Sharrock *et al.* [33] and model data by Schappacher *et al.* [18, 19] are included for comparison purposes.

0020-1693/84/$3.00

TABLE I. Frozen Solution Mössbauer Data for Protoporphyrin IX Iron(II) with Various Sulphonated Ligands at 80 K.

Ligand	δ	Δ	Γ
1. Ethylmercaptoacetate	0.85(2)	2.41(2)	0.32(2)
2. Mercaptoethanol	0.86(2)	2.39(2)	0.32(2)
3. Ethandiol	0.88(2)	2.31(2)	0.37(2)
4. Thiophenol	0.88(2)	2.43(2)	0.36(2)
5. Thiocreosol	0.90(2)	2.45(2)	0.36(3)
6. Cysteine methyl ester	0.51(2)	1.22(2)	0.28(2)
7. Glycine ethyl ester	0.49(2)	1.15(2)	0.28(2)
8. Ethylmercaptoacetate + CO	0.22(2)	0.60(2)	0.48(2)
9. Mercaptoethanol + CO	0.21(2)	0.80(2)	0.54(2)
10. Ethandiol + CO	0.28(2)	0.85(2)	0.45(2)
11. Thiophenol + CO	0.26(2)	0.94(2)	0.45(2)
12. Thiocresol + CO	0.25(2)	0.73(2)	0.52(2)
13. Cysteine methyl ester + CO	0.26(2)	0.49(2)	0.30(2)
14. Glycine ethyl ester + CO	0.27(2)	0.42(2)	0.28(2)

TABLE II. Reference Data.

	δ	Δ	Γ	T K
1. $Fe(TP_{piv}P)[KC222][SC_6HF_4]$ [a]	0.82(2)	2.37(2)	0.27(2)	85
2. $Fe(TP_{piv}P)[NaC222][SC_6HF_4]$ [a]	0.80	2.36	0.32	77
3. $Fe(TP_{piv}P)[NaC_{12}H_{24}O_6][SC_6HF_4]CO$ [a]	0.30(2)	0.56(2)	0.29(2)	4.2
	0.83(2)	2.45(2)	0.33	4.2
4. $P\text{-}450_{cam}$ + camphor, reduced [b]	0.77(2)	2.39(2)	0.22	173
5. $P\text{-}450_{cam}$ + camphor, reduced + CO [b]	0.29(2)	0.32(2)	0.30	4.2
	0.25(2)	0.34(2)	0.33	200

[a] Data from reference 19. [b] Data from reference 33.

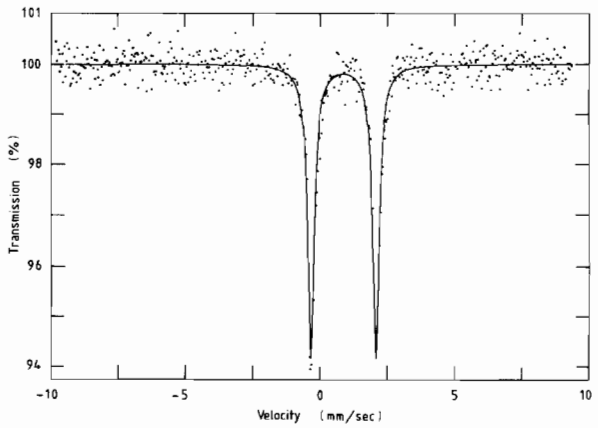

Fig. 1. Mössbauer spectrum of a frozen solution of protoporphyrin IX iron(II) with ethylmercapto acetate.

In Table I, data 1–5 (and Fig. 1) our frozen solution Mössbauer data for the protoporphyrin IX iron-(II) plus sulphur ligands all show the presence of high spin iron(II) with similar Mössbauer parameters to those reported in Table II compounds 1, 2, and 4 [18, 19, 33]. The structure of $[Fe(TPpivP)SC_6HF_4]$-$[NaC_{12}H_{24}O_6]$ is known [19]; it contains a five coordinate iron with the thiolato ligand bound to iron on the opposite side of the porphyrin plane to that containing the picket fences. The iron is 0.42 Å out of the porphyrin plane.

From the similarity of our data 1–5 to those of the known structure [19] it is likely that our species are also 5-coordinate and it may be that the propionate groups are on the opposite side of the porphyrin to the sulphur ligands forming a partial picket fence [6].

The similarity of all these Mössbauer data to that of P-450 (Table II, no. 4) shows that in the reduced stage C (high spin ferrous) a sulphur ligand is a good model for this stage confirming results of other workers [7–19].

It has been suggested that a cysteinyl thiol may be involved in cytochrome P-450 [28, 30]. We have examined a frozen solution of cysteine methyl ester with protoporphyrin IX iron(II) (Table I, no. 6, Fig. 2) but the Mössbauer parameters obtained are dissimilar to that of P-450 cam, and in fact are similar to those found for low spin iron(II) porphyrins containing two nitrogen ligands [35]. This interpretation

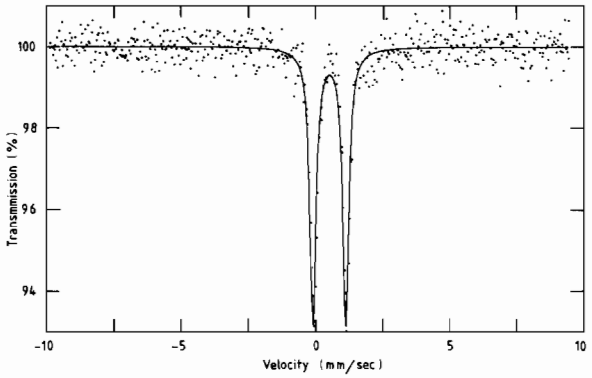

Fig. 2. Mössbauer spectrum of a frozen solution of protoporphyrin IX iron(II) with cysteine methyl ester.

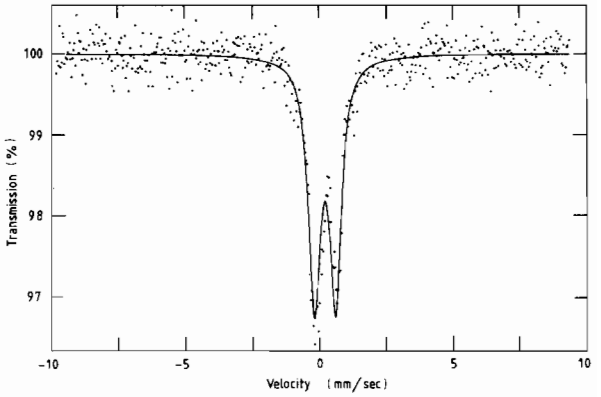

Fig. 3. Mössbauer spectrum of a frozen solution of protoporphyrin IX iron(II) with mercaptoethanol and CO present.

is borne out by the result for glycine ethyl ester (Table I, no. 7) which shows similar Mössbauer parameters and contains no sulphur atom.

It is interesting for cysteine methyl ester (the ester was used instead of straight cysteine so the acid group would not interfere) that from our data the nitrogen is a stronger ligand to iron in porphyrins than the sulphur. Of course, in a protein there is no free nitrogen ligand on cysteine. Munck [36] lists the arguments for the presence of sulphydryl group coordinated to the iron(II) porphyrin in cytochrome P-450 and points out that for the similar cytochrome, chloroperoxidase, spectroscopic and chemical evidence disagree as to the presence of a thiolate ligand.

On adding CO to the solutions 1–7, Table I, solutions 8–14 resulted. The Mössbauer data for solutions 8–12 all show low spin iron(II).

All of the carbonyls gave sharp, well resolved quadrupole split doublets at 80 K. A representative spectrum is shown in Fig. 3; the isomer shifts are in the range 0.21–0.28 mm s^{-1}. These isomer shifts are similar to those reported by Connor and Straub for carbonyl hemochromes [37], however, the quadrupole splittings are larger. In the presence of sulphur bonding to the iron, the electric fields provided by the axial ligands are weaker and so the iron does not approach cubic symmetry to the same extent with amines and the quadrupole splittings are larger.

Connor and Straub [37] point out that CO provides a much stronger electric field than do amines and significantly reduces the >1.0 mm s^{-1} splittings found for the bis(amine) hemochromes [35]. Our solutions 13 and 14, Table I, show similar data to those of Connor and Straub [37] and are explained as being carbonyl hemochromes.

The fact that solutions 8–12, Table I, show wider quadrupole splittings than complex 5 of Table II, is interesting. Obviously this shows that when CO is bound to P-450 then the sulphur is more strongly bound to iron than in our model complexes and this is probably due to binding forces within the protein.

TABLE III. Electronic Spectral Data for Iron(II) Protoporphyrin IX Aqueous Solutions Containing Sulphur Ligands and Their Carbonyl Complexes.

Complex	λ_{max} (nm)		
	δ soret		
Fe(PPIX) cysteine methyl ester	419.0	524.0	555.5
Fe(PPIX) cysteine methyl ester + CO	416.5	536.5	567.0
Fe(PPIX) glycine ethyl ester	421.0	525.0	555.5
Fe(PPIX) glycine ethyl ester + CO	416.0	536.0	566.5
Fe(PPIX) ethyl mercapto acetate	407.0*		556.0
Fe(PPIX) ethyl mercapto acetate + CO	439.5		554.0
Fe(PPIX) ethandiol	404.0*		546.5
Fe(PPIX) ethandiol + CO	442.5		554.5
Fe(PPIX) mercapto ethanol	400.5*		551.0
Fe(PPIX) mercapto ethanol + CO	442.5		553.5

*These band positions were estimated. They appear as shoulders in the spectra.

It is interesting to point out that Mössbauer data for sulphur bridged binuclear iron(II) complexes containing nitrogen [38] and iron(II) glutathione complexes containing sulphur and nitrogen ligands with CO [39] show Mössbauer parameters similar to those discussed in this work.

The electronic spectra of some of the complexes appear in Table III. The visible spectra of the carbonyl adducts (with sulphur ligands) display the expected hyperporphyrin spectra. Those that contain no sulphur ligand do not display the hyperporphyrin type spectra in their carbonyl adducts, though they do show alteration confirming that CO complexes have been found.

Experimental

Preparation of ^{57}Iron–PPIX Complexes in Solution for Mössbauer Experiments

5 mg of enriched ^{57}Fe–PPIX prepared according to the method of Caughey [40] was dissolved in 3 ml 25% $(CH_3)_4NOH$ aqueous solution.

The solution was then centrifuged to remove the insoluble particles. This solution was then transferred into a two-necked flask and put under N_2 atmosphere. A few drops of concentrated sodium dithionite were then added to the solution. Finally 1 ml of ligand was added with continuous stirring, to the solùtion.

For the solid ligands, thiocresol. This was first dissolved in acetone, before being added to the haem solution. The other ligands, cysteine, methyl ester and glycine ethyl ester, were added as solids.

Electronic Absorption Experiments

2 mg hematin (Sigma) were dissolved in 2.5 ml 0.5 N $(CH_3)_4NOH$ and then diluted to 50 ml with 25% $(CH_3)_4NOH$. A few drops of concentrated sodium dithionite solution were added. The spectra were taken after the ligands were added to the solution at high pH.

Instrumentation

Electronic spectra were recorded on a Beckmann DU 7 spectrophotometer in 1 cm cells. The Mössbauer spectra were recorded on an instrument previously described [9]. The source was ^{57}Co (10 mCi) in rhodium (Radiochemical Centre, Amersham), at 20 °C. The spectrometer was operated in a saw tooth mode and the spectra computer fitted. The spectrometer was calibrated with a 25 μM thick natural ion reference absorber. All isomer shifts are referred to this as zero shift.

References

1 B. Lukas, J. R. Miller, J. Silver, M. T. Wilson and I. E. G. Morrison, *J. Chem. Soc. Dalton Trans.*, 1035 (1982).
2 B. Lukas, J. Silver, I. E. G. Morrison and P. W. C. Bernard, *Inorg. Chim. Acta, 78*, 205 (1983).
3 J. Silver and B. Lukas, *Inorg. Chim. Acta, 78*, 219 (1983).
4 J. Silver and B. Lukas, *Inorg. Chim. Acta, 80*, 107 (1983).
5 B. Lukas, J. Peterson, J. Silver and M. T. Wilson, *Inorg. Chim. Acta, 80*, 245 (1983).
6 J. Silver, B. Lukas and G. Al-Gaff, *Inorg. Chim. Acta, 91*, 125 (1984).
7 J. P. Collman, T. N. Sorrell and B. M. Hoffman, *J. Am. Chem. Soc., 97*, 913 (1975).
8 S. Koch, S. C. Tang, R. H. Holm and R. B. Frankel, *J. Am. Chem. Soc., 97*, 914 (1975).
9 S. Koch, S. C. Tang, R.-H. Holm, R. B. Frankel and J. A. Ibers, *J. Am. Chem. Soc., 97*, 916 (1975).
10 H. Ogoshi, H. Sugimoto and Z. Yoshida, *Tetrahedron Lett.*, 2289 (1975).
11 J. O. Stern and J. Peisach, *J. Biol. Chem., 249*, 7495 (1974).
12 J. P. Collman and T. N. Sorrell, *J. Am. Chem. Soc., 97*, 4133 (1975).
13 J. P. Collman, T. N. Sorrell, J. H. Dawson, J. R. T. Trudell, E. Brunnenberg and C. Djerassi, *Proc. Natl. Acad. Sci., USA, 73*, 6 (1976).
14 C. K. Chang and D. Dolphin, *J. Am. Chem. Soc., 97*, 5948 (1975).
15 C. K. Chang and D. Dolphin, *Proc. Natl. Acad. Sci., USA, 73*, 3338 (1976).
16 S. C. Tang, S. Koch, C. Papaefthymiou, S. Foner, R. B. Frankel, J. A. Ibers and R. H. Holm, *J. Am. Chem. Soc., 98*, 2414 (1976).
17 J. P. Collman, T. N. Sorrell, K. O. Hodgson, A. Kulshresta and C. E. Strouse, *J. Am. Chem. Soc., 99*, 5180 (1977).
18 M. Schappacher, L. Ricard, R. Weiss, R. Montiel-Montoya, E. Bill, U. Gonser and A. Trantwein, *J. Am. Chem. Soc., 103*, 7646 (1981).
19 M. Schappacher, L. Ricard, R. Weiss, R. Montiel-Montoya, U. Gonser, E. Bill and A. Trautwein, *Inorg. Chim. Acta, 78*, L9 (1983).
20 V. Ullrich, *Angew. Chem. Int. Ed. Eng., 11*, 701 (1972).
21 I. C. Gunsalus, J. R. Meeks, J. D. Lipscomb, P. Debrunner and E. Munck in 'Molecular Mechanisms of Oxygen Activation', O. Hayaishi, Ed., Academic Press, New York, N. Y. p. 561 (1974).
22 A. H. Conney, *Pharmacl. Rev., 19*, 317 (1967).
23 I. C. Gunsalus, Hoppe-Sayler's, *Z-Physiol. Chem., 349*, 1610 (1968).
24 M. Klingenberg, *Arch. Biochem. Biophys., 75*, 376 (1958).
25 D. Garfinkel, *Arch. Biochem. Biophys., 77*, 493 (1958).
26 H. S. Mason, J. C. North and M. Vanneste, *Fed. Proc. Fed. Am. Soc. Exp. Biol., 24*, 1172 (1965).
27 K. Murikam and H. S. Mason, *J. Biol. Chem., 242*, 1102 (1967).
28 A. Roder and E. Bayer, *Eur. J. Biochem., 11*, 89 (1969).
29 J. Peisach, W. E. Blumberg and A. Alder, *Ann. N. Y. Acad. Sci., 206*, 310 (1972).
30 H. A. O. Hill, A. Roder and R. J. P. Williams, *Struct. Bonding, (Berlin), 8*, 123 (1970).
31 J. Hedegaard and I. C. Gunsalus, *J. Biol. Chem., 240*, 4038 (1965).
32 M. Katagiri, B. N. Ganguli and I. C. Gunsalus, *J. Biol. Chem., 243*, 3543 (1968).
33 M. Sharrock, E. Münck, P. G. Debrunner, V. Marshall, J. D. Lipscomb and I. C. Gunsalus, *Biochemistry, 12*, 258 (1973).
34 I. C. Gunsalus, C. A. Tyson, R. Tsai and J. D. Lipscomb, *Chem.-Biol. Interactions, 4*, 75 (1971).

35 L. M. Eptein, D. K. Straub and C. Maricondi, *Inorg. Chem., 6,* 1720 (1967).
36 E. Münck in 'The Porphyrins', D. Dolphin, Ed., Acad. Press, New York, N. Y., vol. IV, p. 404 (1979).
37 W. M. Connor and D. K. Straub, *Inorg. Chem., 15,* 2289 (1976).
38 K. D. Karlin and S. J. Lippard, *J. Am. Chem. Soc., 98,* 6951 (1976).
39 M. Y. Hamed, J. Silver and M. T. Wilson, *Inorg. Chim. Acta, 80,* 237 (1983).
40 W. S. Caughey, W. Y. Fujimoto, A. J. Bearden and T. H. Moss, *Biochem., 5,* 1255 (1966).