Inorganica Chimica Acta, 153 (1988) 235–245

Model Complexes for the High Spin Iron(II) State in the Catalytic Cycle of Cytochrome P450

JACK SILVER* and JEHAD A. TAIES

Department of Chemistry and Biological Chemistry, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, U.K.

(Received May 2, 1988)

Abstract

Studies using ^{57}Fe Mössbauer spectroscopy on frozen concentrated solutions of two iron(II) porphyrins in the presence of a large excess of thiophenol or 2-mercaptoethanol are reported. The iron(II) porphyrins used were protoporphyrin IX iron(II) (PPIXFe(II)) and tetra(*p*-sulphophenyl)porphinatoiron(II) (TTPPSFe(II)). Evidence for high-spin five-coordinate iron(II) complexes were found for both iron(II) porphyrins with thiophenol, but no reaction was found to occur with 2-mercaptoethanol. In contrast to these findings in solutions dilute in TPPSFe(II) evidence was found (from electronic absorption spectroscopy and spectrophotometric titrations) for both thiophenol and 2-mercaptoethanol acting as axial ligands in high-spin five-coordinate TPPSFe(II) species. Mössbauer data for frozen solutions containing TPPSFe(II), carbon monoxide (CO) and either thiol, are consistent with the presence of only low-spin six-coordinate iron(II) complexes. These latter complexes are deduced to contain both a thiol and a CO molecule as axial ligands. These results are discussed in relation to earlier work on PPIXFe(II)–thiol solutions and also in relation to the high-spin iron(II) state in the catalytic cycle of cytochrome P450.

The reactions of TPPSFe(II) with excess of either 2-mercaptoethanol or ethyl 2-mercaptoacetate in air are also reported and their complex nature discussed.

Introduction

The cytochrome P450 enzymes belong to the monooxygenase (mixed-function oxidase) class of enzymes. These catalyze the incorporation of one atom of dioxygen into a substrate whilst the other is reduced to water. The hydroxylation of a wide variety of organic compounds through this activation of molecular oxygen is thereby achieved [1, 2]. Their role includes the hydroxylation of CH bonds in metabolism, hormone regulation and oxidative degradation of many toxic agents [3–5]. These proteins are present in mammalian microsomes and mitochondria in plants and in bacteria.

This class of cytochromes, when reduced in the presence of carbon monoxide, exhibits Soret bands at 360 and 450 nm [6, 7], compared to other haem proteins such as myoglobin and haemoglobin which have Soret bands around 420 nm. Mason *et al.* [8, 9] demonstrated the effect of axial sulphur coordination based on evidence that microsomal P450 cytochromes were inactivated by sulphhydryl reagents. The four states in the accepted reaction cycle of cytochrome P450 are: (a) a substrate free low-spin ferric resting state, (b) a substrate bound high-spin ferric stage, (c) a high-spin ferrous (deoxy stage) and (d) a diamagnetic oxygenated ferrous state.

Many groups have produced well characterized iron porphyrin thiolato complexes for states B and C [10–20] and more recently D [21, 22]. Fewer studies have been carried out on naturally occurring porphyrins. Amongst the latter are studies on protoporphyrin IX iron(III) solutions containing various mercaptans [23–25].

As part of our programme aimed at understanding the chemical and physical properties of the protoporphyrin IX iron moiety (PPIXFe) [27–37] we prepared PPIXFe(II) solutions containing sulphur ligands and studied these using Mössbauer and electronic absorption spectroscopies [26]. Our findings of high-spin PPIXFe(II) complexes in the presence of the sulphur ligands were in keeping with the Mössbauer studies on cytochrome $P450_{cam}$ (grown in media enriched with $^{57}FeCl_3$) carried out by Sharrock *et al.* [38]. The latter workers investigated cytochrome P450 in the four states of the reaction cycle and in the ferrous carbon monoxide adduct [39]. In addition, our data were similar to those of Schappacher *et al.* [21, 22]. The structure of $[Fe(TP_{piv}P)SC_6HF_4][NaC_{12}H_{24}O_6]$ is known [22]. It contains a five-coordinate high-spin iron(II) atom with the thiolato ligand bound to the opposite side of the porphyrin plane as the picket fence. The iron is 0.42 Å out of the porphyrin plane. It seems rea-

*Author to whom correspondence should be addressed.

0020-1693/88/$3.50

TABLE I. Frozen Solution ^{57}Fe Mössbauer Data for PPIXFe(II)[a] and TPPSFe(II)[b] with Various Thiol Ligands at 78 K

	Ligand(s) present	δ (mm s^{-1})	Δ (mm s^{-1})	Γ (mm s^{-1})[e]	*A* (%)
1	Thiophenol[a]	0.40(2)	1.63(2)	0.29(2)	87(6)
		0.82(3)	2.54(3)	0.16(2)	12(5)
2	Ethyl 2-mercaptoacetate[a]	0.61(1)	1.53(1)	0.21(2)	100(4)
3	2-Mercaptoethanol[a,c]	0.62(2)	1.62(3)	0.31(4)	66(5)
		0.31(1)	0.61(3)	0.32(12)	33(5)[d]
4	Thiophenol[b]	0.85(1)	2.36(1)	0.22(2)	100(7)
5	2-Mercaptoethanol[b]	0.45(1)	1.07(1)	0.14(1)	100(8)
6	Glycine ethyl ether[b]	0.40(1)	0.56(2)	0.12(3)	28(6)[d]
		0.46(1)	1.13(2)	0.18(1)	71(8)
7	Glycine ethyl ester + CO[b]	0.28(1)	0.37(1)	0.19(1)	100(10)
8	2-Mercaptoethanol + CO[b]	0.27(2)	0.46(3)	0.21(2)	100(16)
9	Thiophenol + CO[b]	0.33(2)	0.48(1)	0.19(1)	100(4)

[a]PPIXFe(II) solution. [b]TPPSFe(II) solution. [c]100 mg of PPIXFe(II) was used. [d]These sites are assigned to μ-oxo-dimer. [e]Half width at half height.

sonable to conclude that the PPIXFe(II) solutions containing sulphur ligands [26] were made up of molecules of similar structure.

Recently we have found differences in the chemistry of tetramethine substituted water soluble iron porphyrins [40–44] and PPIXFe solutions that are dependent on the nature of the substituents on the methine carbons. Because of these differences between water soluble pyrrole substituted iron porphyrins (such as PPIXFe) and methine substituted iron porphyrins (such as tetra(*p*-sulphophenyl)porphinato iron (TPPSFe) [40–42], tetra(*p*-sulphonaphthyl)porphinato iron (TNPSFe) [42, 43] and tetra(*p*-carboxyphenyl)porphinato iron (TCPP) [44]) we have begun an investigation of other reactions of these non naturally occurring iron porphyrins.

We report here studies on TPPSFe(II) solutions containing thiophenol, 2-mercaptoethanol or glycine ethyl ester as models for state C of cytochrome P450.

In addition we have found that concentrated solutions of PPIXFe(II) [37] behave differently (because of porphyrin aggregation) from dilute solutions [30, 32]. We also report studies on concentrated PPIXFe(II) solutions.

Exposure of both sets of solutions to CO allowed the preparation of models for state E of cytochrome P450. This state is produced by reacting state C with CO. We also report studies on the CO solutions.

Results and Discussion

Mössbauer Data

Mössbauer spectra of PPIXFe(II) and TPPSFe(II) complexes in concentrated frozen solutions containing mercaptans show three types of iron(II) sites present (Table I). The first type of site is that of a high-spin iron(II) porphyrin-thiol, as in complexes no. **1** and **4**, Table I, with isomer shifts and quadrupole splittings in the range of 0.82–0.85 mm s^{-1} and 2.36–2.53 mm s^{-1} respectively. These Mössbauer parameters are typical of high-spin iron(II) porphyrin complexes, being similar to those reported (a) for the PPIXFe(II)–thiol complexes in dilute solutions (in these ^{57}Fe 90% enriched was used) [26], (b) deoxy cytochrome P450 [38] and (c) other model complexes of this enzyme [27] (see Table II). It can be seen from Table I that the thiophenol shows high-spin states with both iron(II) porphyrins. 2-Mercaptoethanol appears not to form complexes in the presence of high concentrations of these iron(II) porphyrins, only starting material is identified in the Mössbauer spectra (*cf.* ref. 37). This is surprising as previous Mössbauer data [26] on dilute solutions of PPIXFe(II) in solution with 2-mercaptoethanol and 2-mercaptoacetate clearly show high-spin iron(II) species [26]. This may be due to differences of interactions of steric effects between the thiols and these porphyrins in concentrated solutions. There is little evidence of aggregation in the TPPSFe(II) complexes (Table I), but there is in the PPIXFe(II) solutions [37]. It is possible that the thiophenol was able to break the aggregation of the porphyrin and thus approach nearer to the iron centre than the alaphatic thiols in concentrated solutions. This possibly indicates either that it binds strongly or that it first π-bonds to the porphyrin rings as has been found or suggested in other iron porphyrin systems [42, 45, 46].

The second type of site is that of an intermediate spin iron(II) ($S = 1$), this occurs when an aliphatic thiol is used as in complexes no. **2** and **3** of Table I.

TABLE II. Mössbauer Data for Cytochrome P450 Complexes and Other Models for This Enzyme

	Compound	δ (mm s^{-1})	Δ (mm s^{-1})	Γ (mm s^{-1})	T (K)
1	$Fe(TP_{piv}P)[K222][SC_6HF_4]$ [a]	0.82(2)	2.37(2)	0.27(2)	85
2	$Fe(TP_{piv}P)[Na222][SC_6HF_4]$ [a]	0.80	2.36	0.32	77
3	$Fe(TP_{piv})[NaC_{12}H_{24}O_6][SC_6HF_4]CO$ [a]	0.30(2) 0.83(2)	0.56(2) 2.45(2)	0.29(2) 0.33(2)	4.2 4.2
4	$P450_{cam}$ + camphor, reduced [b]	0.77(2)	2.39(2)	0.22	173
5	$P450_{cam}$ + camphor, reduced + CO [b]	0.29(2) 0.25(2)	0.32(2) 0.34(2)	0.30 0.33	4.2 200
6	PPIXFe(II) + thiophenol [c,d]	0.88(2)	2.43(2)	0.36(2)	80
7	PPIXFe(II) + thiophenol + CO [c,d]	0.26(2)	0.94(2)	0.45(2)	80
8	PPIXFe(II) + mercaptoethanol [c,d]	0.86(2)	2.39(2)	0.32(2)	80
9	PPIXFe(II) + mercaptoethanol + CO [c,d]	0.21(2)	0.80(2)	0.54(2)	80

[a] Data from ref. 22. [b] Data from ref. 38. [c] Data from ref. 26. [d] Frozen solution at high pH and PPIX^{57}Fe(II) 90% enriched was used.

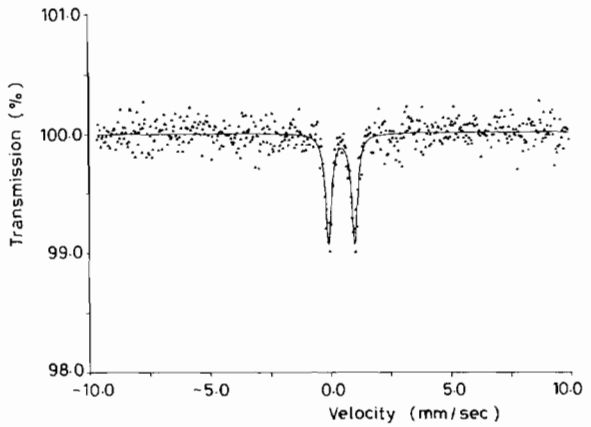

Fig. 1. Mössbauer spectrum of a frozen solution of TPPSFe(II) with 2-mercaptoethanol at 78 K.

This site shows that the PPIXFe(II) has not reacted with the thiol due to self-aggregation [37].

The third iron site is assigned to a TPPSFe(II) low-spin complex (sample no. **5**, Table I), with quadrupole splitting of 1.07 mm s^{-1}, this value is very similar to those [47] reported for low-spin iron(II) porphyrin with two nitrogen ligands in the axial positions and this work (no. **6**, Table I), when glycine ethyl ester is used. Similar δs were found for this site in the TPPSFe(II) and TNPSFe(II) systems in the absence of thiols or amines [41, 43]. A representative spectrum is shown in Fig. 1 for this site. Thus here for sample no. **5** we suggest that the TPPSFe(II) has not reacted with the thiol.

Sample no. **6** (Table I) is a low-spin iron(II) site, in which the nitrogen atoms are bound to the iron(II) centre; it is very similar to those reported for PPIXFe(II) with the same ligand [22].

On adding CO to the solutions **4**–**6**, Table I, solutions **7**–**9** resulted. The Mössbauer data for these solutions are consistent with the presence of low-spin iron(II) porphyrin complexes. These spectra are sharp, well resolved quadrupole split doublets at 78 K. Therefore, addition of CO to the solutions containing high concentrations of TPPSFe(II) (in the presence of mercaptans) forms complexes of the type TPPSFe(II)(SL)(CO) or TPPSFe(II)$(CO)_2$, where SL and CO are both axial ligands (SL = mercaptan). As it is unlikely that TPPSFe(II)(SL) (where SL = thiophenol) would convert to TPPSFe(II)$(CO)_2$ on addition of CO, then the most probable formulation of the low-spin complexes is TPPSFe(II)(SL)(CO). Thus, addition of CO facilitates the binding of the alaphatic mercaptan (clearly proven from the Mössbauer spectra). Representative spectra are shown in Figs. 2 and 3. The isomer shifts and quadrupole splitting for these complexes are similar to those reported by

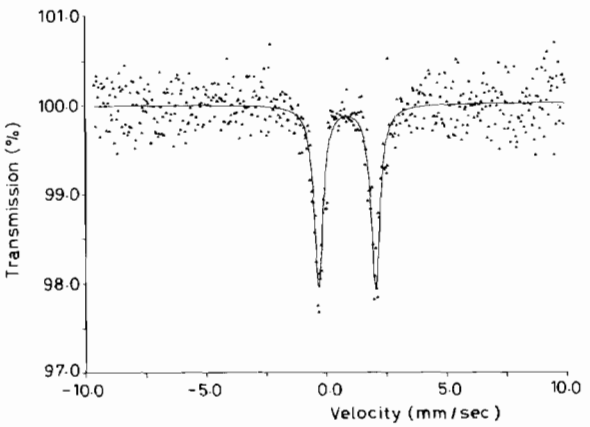

Fig. 2. Mössbauer spectrum of a frozen solution of TPPSFe(II) with thiophenol at 78 K.

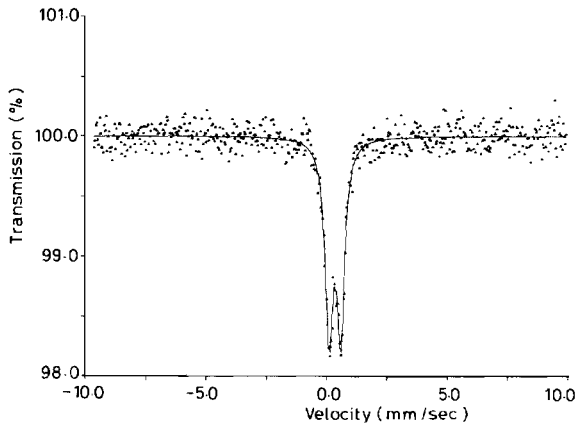

Fig. 3. Mössbauer spectrum of a frozen solution of TPPSFe(II) with thiophenol and CO present at 78 K.

Connor and Straub [48] for carbonyl haemochromes. The cytochrome $P450_{cam}$ + camphor (reduced) + CO shows a quadrupole splitting smaller than those of this work [38]. This suggests that the sulphur is more strongly bound to the iron in cytochrome P450, and this is probably due to the binding forces within the protein itself. The large quadrupole splittings of PPIXFe(II)(SL)(CO) complexes were explained by us [26] as follows: '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 as it does with amines'. The TPPSFe(II)(SL)(CO) complexes reported here exhibit quadrupole splitting data much closer to that of cytochrome P450. This may suggest that it is the porphyrin (PPIX) structure that causes the larger quadrupole splittings possibly by steric interactions of the substituent groups (probably the propionate groups) with the thiols in solution. Similar interactions would be inhibited by the protein structure of cytochrome P450 in the normal enzyme.

Chang *et al.* [18] have found evidence that the carbonyl stretching frequency of [RS–haem–CO] in DMA is 1923 cm^{-1}, the lowest νCO reported for CO–haem complexes [15, 49, 50]. Since νCO is supposed to reflect the extent of π-back-bonding to CO, then this in turn must reflect the π-electron density of the iron atom. This is evidenced by the fact that the very strong bonding of the [RS–haem–CO] causes a small quadrupole splitting.

Thus for TPPSFe(II) the quadrupole splitting of the complexes in this work are in the same range as those reported for models of cytochrome P450 [38], and the S–Fe–CO bonding is strong. It is thus necessary to re-examine our previous argument and look for another reason to explain the large quadrupole splittings observed [26]. Either the haems of Chang *et al.* [18] and TPPSFe(II) have very different electron densities on the pyrrole nitrogens compared to PPIXFe(II), or the side chain (propionic and vinyl groups) sterically interact (either by bulk effects or charge effects or both) with the thiols, prohibiting their close approach. As both PPIX and its ester have large side chains, a combination of charge and steric effects seems more likely.

Electronic Absorption Spectra

It is convenient to first briefly describe the spectra of TPPSFe(II) glycine ethyl ester in aqueous solution at high pH.

The visible absorption spectrum of this complex is very similar to those of other Fe(II) porphyrins with amines as axial ligands and is a typical spectrum of a haemochrome. It has a Soret band at 423 nm and two visible bands at 532 and 563 nm. These bands changed in intensity and position when CO was bubbled into the solution (Table III). The final position of the bands for the CO complex being at 420.7 nm and 540.8 nm.

The electronic absorption spectra of TPPSFe(II)–thiols (taken in dilute solutions) at high pH have Soret bands around 410 nm (see Table III) identical to those of reduced cytochrome P450 [17, 51] (in the absence of CO). It has been suggested [17, 51] that the former complex is a penta-coordinate haem complex, and thus the solutions reported here must

TABLE III. Electronic Absorption Spectral Data for TPPSFe(II) Aqueous Solutions Containing Sulphur Ligands and their Carbonyl Adducts

	Ligand	λ_1 (nm)	λ_2 (nm)	λ_3 (nm)
1	Glycine ethyl ester	423	532	563
2	Glycine ethyl ester + CO	420.7	540.8	
3	2-Mercaptoethanol	409.6, 438.5[a]		
4	2-Mercaptoethanol + CO	415.1, 439	538.3	
5	Ethyl 2-mercaptoacetate	410,.438[a]		
6	Ethyl 2-mercaptoacetate + CO	415, 436.5	536.2	
7	Thiophenol	410, 440[a]		
8	TPPFe(II)–thiol[b]	408, 440[a]		

[a]These bands appear as shoulders in the spectra. [b]The visible absorption spectra of this complex was taken in DMSO solvent [14].

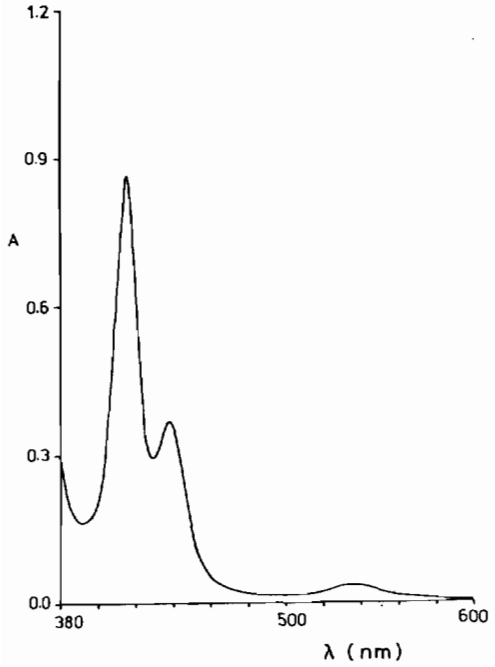

Fig. 4. Electronic absorption of TPPSFe(II) solution in the presence of ethyl 2-mercaptoacetate and CO.

also contain such high-spin five-coordinate iron(II) complexes. Similar Soret bands were found by Chang *et al.* [18] for PPDMEFe(II)–thiol complexes in toluene at 23 °C around 408 nm and by Silver *et al.* [26] for PPIXFe(II)–thiol at 405 nm (as a shoulder). Additionally to the above, there is a shoulder in this present system at 438 nm, this may be assigned to TPPSFe(II) [41] itself at high pH (some unreacted starting complex is present). Moreover, it is worth noting that although in dilute solutions there is evidence for these complexes, in concentrated solutions (Mössbauer spectroscopic studies) such TPPSFe(II)(SL) (SL = 2-mercaptoethanol, ethyl 2-mercaptoacetate) were not established.

The electronic absorption spectra of TPPSFe(II)–thiol in the presence of CO (see Fig. 4), shows two Soret bands at 415 nm (intense) and a less intense band around 439 nm. The latter is due to the TPPSFe(II)(SL)(CO) species, similar bands were observed for CO cytochrome P450 [15, 18, 39], its models and other model complexes [26]. The band at 415 nm in this work most likely arises from the TPPSFe(II)$(CO)_2$ complex. Indeed, adding CO to a TPPSFe(II) solution at high pH shows the Soret band at 416 nm and another visible band at 538 nm. The third band visible in this system (TPPSFe(II)(CO)-thiol), around 536–538 nm, is very similar to those found in TPPSFe(II)(CO)(amine) and TPPSFe(II)-$(CO)_2$ haemochrome complexes [52].

The ratio of the absorbance of the Soret peaks (A439:A415) is around 0.4. This compares with those for PPDMEFe(III) reduced by CO in the presence of thiols which were found in the range 0.2–0.8 (A450:A413) [53] (dependent both on base concentration and the thiol present).

Soret band absorption maxima of cytochrome P450–CO adducts and model complexes range from 439 to 462 nm. As the polarity of the solvent is increased, the peak shifts to longer wavelength. Since the peak in the protein itself is at about 450 nm, this may indicate that its active site is non-polar [53].

Table IV shows the spectral characteristics of some CO complexes, details of solvent [16], source of the protein [53] and the substitution on the porphyrins, all of which may effect the absorption peak position, are given where relevant.

Chang *et al.* [18] have pointed out that the polarity of the haem environment appears to play a significant role in the aborption spectrum of the [RS–haem–CO] complex. Splitting of a single Soret band into two bands observed in their model and in the CO–P450 complex has been interpreted as charge transfer from the mercaptide sulphur lone pair orbital to the porphyrin $e_g(\pi^*)$ coupled to the normal porphyrin $\pi \rightarrow \pi^*$ transition [54].

The structure of a model complex $[(Fe(TP_{piv}P)S_6C_6HF_4][NaC_{12}H_{24}O_6]$ [22] has been reported recently. The similarity of the Mössbauer and the electronic absorption data (Tables II and III) suggest that

TABLE IV. Electronic Absorption Spectral Data for Cytochrome P450 and Some Model Complexes

Compound	λ (nm)	Reference
Bacterial P450 in solution	447	49
Single crystal	446	49
[RS–haem–CO] in DMA	460	48
[RS–haem–CO] in toluene	451	48
[RS–PPIXFe(II)–CO] in H_2O	442.5	26
[RS–TPPSFe(II)–CO] in H_2O	438.3	this work
T_{piv}PPFe(II)–CO + (NA) SCH_3 in benzene[a]	449	16
PPIXDEEFe(II)–CO + (NA) SCH_3 in benzene[a]	450	16

[a] (NA) = dibenzo 18-crown-6 containing Na^+.

theTPPSFe(II)(SL) species in this work are penta-coordinate high-spin iron(II) complexes.

When a PPDMEFe(III)-DMSO solution was mixed with an equal volume of a solution of n-BuSH and (1–2 M) $N(CH_3)_4OH$ in ethanol, under CO, the visible spectrum exhibited two Soret peaks at 450 and 413 nm [53]. The ratio of the two peaks is dependent on the concentration of thiol and the base as seen by the work of others [17, 53]. Similar peaks were found in the TPPSFe(II) water solution with thiol and under CO at high pH using NaOH as a base.

Chang *et al.* [17] have observed 100% conversion to the long wavelength Soret band with PPDMEFe(II) and 2,4 diacetyl deutero porphyrin iron(II) and 95% with TPPFe(II). They [17] have suggested the Soret band between 449 to 458 nm should be assigned to the $[RS–Fe(II)–CO]^-$ species, whereas the peak at position 408 nm they assigned to $[RS–Fe(II)–SR]^{2-}$ or $[RS–Fe(II)SRK]^-$. These complexes are hexa-coordinate low-spin Fe(II) species. The disulphur Fe(II) porphyrin was suggested by the same workers [17] using evidence from CO titration experiments.

Collman *et al.* [55] have found that in the presence of any Lewis base the ferric complex TPPFe(III)-(SC_6H_5) is rapidly reduced to the ferrous complex $TPPFe(II)(SC_6H_5)$. They [55] have pointed out that although this reaction is very slow in solution at low temperature, ESR spectra characteristic of low-spin $P450_{cam}$ can be obtained. To explain this they suggested the formation of a six-coordinate complex that is unstable in solution at 25 °C.

Mössbauer parameters of such an aliphatic thiol with TPPSFe(II) at high pH are in agreement with the presence of low-spin Fe(II) six-coordinate complexes at 78 K. However, in this case it is more likely that this is due to unreacted $TPPSFe(II)(H_2O)_2$. Therefore we have no evidence for low-spin TPPSFe(II)-$(SL)_2$ species being present in our solutions.

TPPSFe(II) in the Presence of a Large Excess of Thiol Ligands

Addition of a large excess of either 2-mercapto-ethanol or ethyl 2-mercaptoacetate to TPPSFe(II) solutions (1 ml of 5×10^{-5} M of TPPSFe(II) + 2 ml neat thiols) in the presence of excess NaOH initiates some interesting reactions. For 2-mercaptoethanol the formation of two new Soret bands at 418 nm and 445 nm with a ratio (A445:A418) around 0.75 and five visible bands at 540, 576, 595, 621 and 744 nm

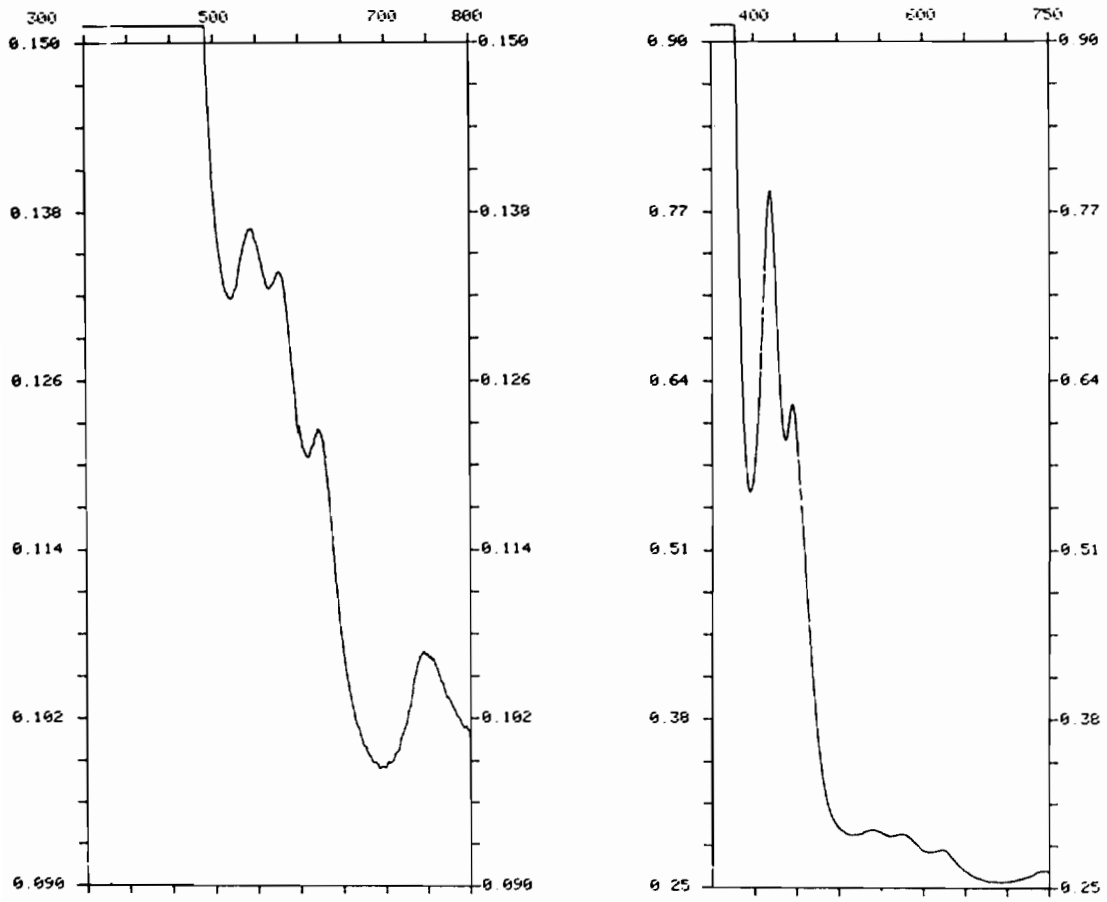

Fig. 5. Electronic absorption spectra of TPPSFe(II) solution in the presence of a large excess of 2-mercaptoethanol.

have been found. For ethyl 2-mercaptoacetate only small indications of the Soret bands were found (these are two shoulders, the more prominent is at 441 nm and the other is around 410 nm), in addition visible bands were found at 536, 576, 616 and 740 nm. Unlike the earlier, more dilute (in ligand) work, these experiments were carried out in the air. The colour of these solutions changed from green TPPSFe(II) to pale yellow. The electronic absorption spectra are shown in Figs. 5 and 6.

Sakurai *et al.* [56] have found that the visible absorption spectrum of TPPFe(II) and TGE (thioglycolic acid ethyl ester), in acetone in the presence of tetramethylammonium hydroxide (present as a base) exhibits six peaks which are 375, 428, 460 nm (the Soret bands, those at 375 and 460 are split hyper-porphyrin type) and three broad bands at 562, 576 and 626 nm, the half-life of this complex was around 5 minutes in air. They [56] suggested that the peaks at 428 and 576 nm are due to formation of an oxygen adduct complex and that the bands at 562 and 626 nm are due to the formation of a ferric complex in the high-spin state, although its Soret band is not mentioned [56].

As similar peak positions were found in the TPPSFe(II) excess thiol systems (except in this work the presence of a split Soret band was not confirmed as the sodium dithionite masked this region), it is possible to suggest a similar story to fit the observed spectra, thereby assigning the peaks 418 and 576 nm to an oxygen adduct of the TPPSFe(II)–thiol. Similar assignments were established in the spectrum of P450 + S [2, 38] with oxygen and for haemoglobin and myoglobin which contains no sulphur ligands. In our system the peak at 540 nm is similar to that found for P450 + S [27] and in the earlier part of this paper (for lower thiol concentrations) in the absence of O_2 or CO. This is assigned to a high-spin five-coordinate TPPSFe(II)(SL) complex.

The peaks at 441 nm and 740 nm (for ethyl 2-mercaptoacetate) and 445 nm and 744 nm (for 2-mercaptoethanol) are not assignable to any known complex and probably result from either a degradation product or are a part of a hyperporphyrin spectrum.

Stern and Peisach [53] have found that addition of either 2-mercaptoethanol or n-propane thiol in greater than 1000-fold molar excess to haemin in

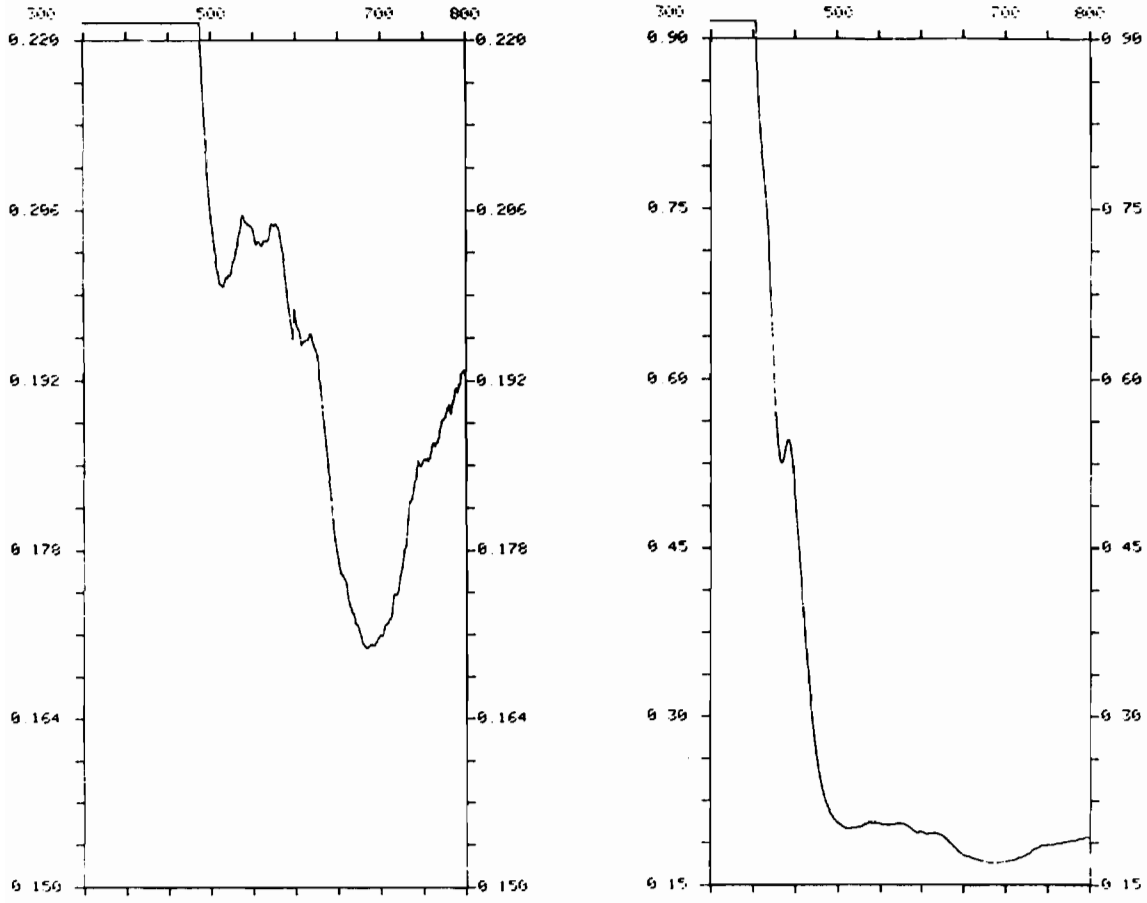

Fig. 6. Electronic absorption spectra of TPPSFe(II) solution in the presence of a large excess of ethyl 2-mercaptoacetate.

DMSO–ethanol solvent leads to the formation of a broad Soret absorption with maxima at 395 and 415 nm. They [53] state that 'the peak intensities decrease with time until eventually (after 30 minutes) the solution bleaches and no optical absorption is seen'. Moreover, if the thiol (100-fold molar excess over haem) was added aerobically to the tetramethylammonium hydroxide–ethanol solution, then mixed with haemin in DMSO, two Soret bands are seen at 404 nm and 435 nm (a shoulder). The 404 nm peak disappears after 5 minutes and is replaced by an absorption at 435 nm having a shoulder near 420 nm. If 1000-fold thiol excess was added to the haemin, the most intense of the Soret bands was seen at 422 nm, with shoulders at 404 and 435 nm.

Silver and Lukas [57] have found that a green–blue colour forms when excess thiol was added to the PPIXFe(II), but the visible absorption was unclear. From the above discussion it is suggested that excess thiol quickly destroys the metal porphyrins and the results are a mixture of $[LS–TPPSFe(II)–O_2]^{-5}$, $[LS–TPPSFe(II)]^{5-}$, $[LS–TPPSFe(III)]^{4-}$ and $[Fe(SL)_4]^-$ complexes and their relative proportions are time-dependent (where SL = LS = mercaptan).

Spectrophotometric Titrations

The spectrophotometric titration data of TPPSFe(II) in aqueous solution at high pH ~ 13 with thiols and glycine ethyl ester shows evidence that only one molecule of mercaptide per TPPSFe(II) and two molecules of glycine ethyl ester per haem bind.

The reaction between TPPSFe(II) and ligand is described in eqn. (1) after the method of Nardo and Dawson [58] and noted in brief here:

$$\text{TPPSFe(II)(L)}_n \overset{K_d}{\rightleftharpoons} \text{TPPSFe(II)} + n\text{L} \qquad (1)$$

where $n = 1$ for thiol, $n = 2$ for amines.

$$K_d = \frac{[\text{TPPSFe(II)}]\,[\text{L}]^n}{[\text{TPPSFe(II)(L)}_n]}$$

It is then possible to define the relationship.

$$\frac{1}{\Delta A} = \frac{K_d}{\Delta A_\infty} \cdot \frac{1}{[\text{L}]} + \frac{1}{\Delta A_\infty}$$

Thus in a titration, the reciprocal of the change in the absorbance observed after addition of ligand to the TPPSFe(II) solution, $1/\Delta A$, is plotted *versus* the reciprocal of the added ligand concentration, 1/L. The reciprocal of the Y-intercept of this plot yields the absorbance change observed at infinite ligand concentration, ΔA_∞.

$$Y = \frac{\Delta A}{\Delta A_\infty} \qquad (2)$$

and the Hill eqn. (3) is used to calculate the binding constants.

$$\log[Y/1 - Y] = \log[\text{L}] - \log[K_d] \qquad (3)$$

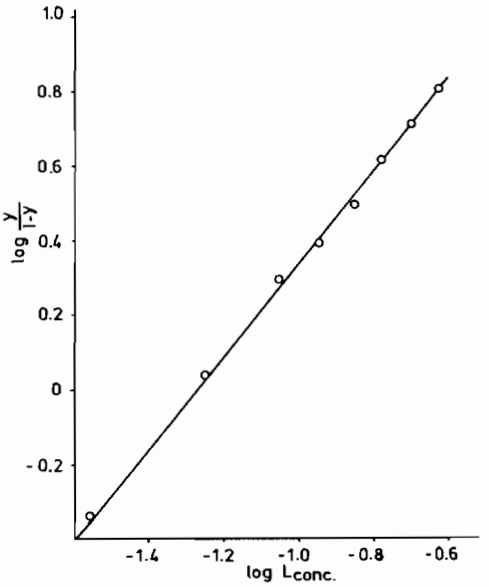

Fig. 7. Hill plot for TPPSFe(II) with 2-mercaptoethanol.

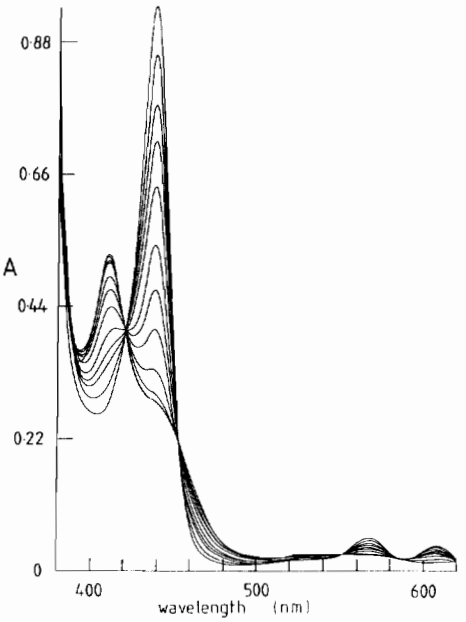

Fig. 8. Spectral changes occurring upon titration of a TPPSFe(II) solution with ethyl 2-mercaptoacetate.

The binding stoichiometry is determined from eqn. (3) by plotting $\log[Y/1 - Y]$ *versus* $\log[\text{L}]$ (Fig. 7); from the slope, formation of 1:1 or 1:2 complexes can be established.

On addition of the sulphur ligands to the TPPSFe(II) solution, the visible absorption spectra changes, the Soret band at 439 nm and other visible bands at 567 and 608 nm decrease in intensity, and a new band occurs in the Soret band region at 410 nm, which is assigned to the TPPSFe(II)(SL) complex [51]. As the visible bands disappear due to the formation of the 1:1 complex, there is little evidence of new bands replacing them (Fig. 8).

TABLE V. Dissociation and Stability Constants for TPPSFe(II) Complexes Containing Thiols or Glycine Ethyl Ester

	Ligand[a]	K_d (mM)[b] (±5)	log K_{eq} (M^{-1}) (±0.05)	Slope[c] (±0.05)	50% Saturation (mM)
1	Ethyl 2-mercaptoacetate	120	0.88	1.39	120
2	2-Mercaptoethanol	52.5	1.28	1.20	52.5
3	Glycine ethyl	18	3.50	1.93	18

[a]All titrations were carried out in aqueous solution at 15 °C, TPPSFe(II) concentration 3.5×10^{-5} M. [b]K_d values were determined from eqn. (3) and/or from the Hill plot (see Fig. 7), log L = log K_d or from a plot Y *vs.* free ligand concentration at half saturation = K_d (Fig. 9). [c]Slope calculated from eqn. (3) using Hill plots.

Chang and Dolphin [18] have studied the equilibrium constants for Fe(II) protoporphyrin IX dimethyl ester with mercaptide in toluene and dimethyl acetamide (DMA) solvents. They found different values of K_{eq} and suggested that this was due to solvent effects.

Dissociation and binding constants with 50% saturation are listed in Table V. A typical titration curve is shown in Fig. 9. Slopes that are slightly higher than 1.0, result from water solvent effects (polar solvents) [18, 59] though they do not greatly affect the log K (binding constant), since OH^- and H_2O can bind to the haem [29]. The low value of log K_d for thiol ligands in aqueous solution must be due to the polar solvent participating vial solute–solvent interactions. The aggregation and polymerization of the porphyrin in aqueous media will lower the value of K_d [60]. Steric effects are also known to lower the value of K_d.

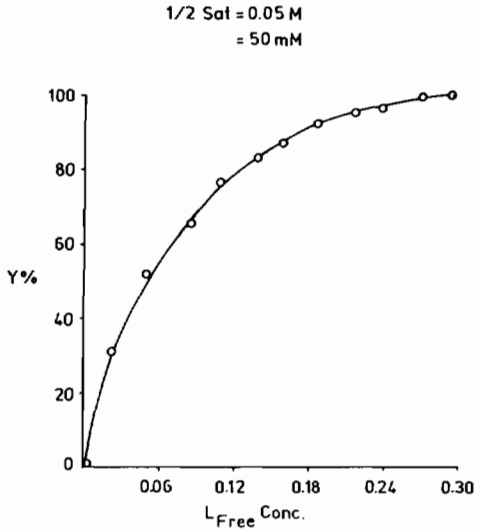

Fig. 9. Plot Y (%) *vs.* ligand concentration of 2-mercaptoethanol.

Conclusions

Mössbauer parameters of concentrated frozen solutions of TPPSFe(II) and PPIXFe(II) with thiophenol at 78 K provide evidence that the Fe(II) complexes are high-spin, whereas alaphatic thiols display no reaction with such solutions of these iron porphyrins.

Electronic absorption spectra on dilute solutions of TPPSFe(II) with thiols all show spectra that can be assigned to high-spin iron(II) at 15 °C.

Spectrophotometric titrations of TPPSFe(II) with thiols show results where $n = 1.2$ to 1.39, these are assigned to one molecule being bound to the Fe(II) atom in dilute solution at 15 °C. This agrees with high-spin five-coordinate complexes. Higher values of n, when thiols were used, are due to the aggregation and stacking of the iron porphyrins. Such effects are known even in the presence of strong ligands such as pyridine and imidazole [61]. When glycine ethyl ester was used as a ligand, the slope was found to be around $n = 1.9$, this is assigned to a low-spin iron(II) six-coordinate complex.

Polar solvents have an effect on the binding constants, and OH^-, H_2O can react as axial ligands [29, 30, 59, 62]. Thus the affinity constants for haem [17, 62, 63] with thiols or amines in benzene and toluene solutions are always higher than these for haem (as in this work) in aqueous solution.

Mössbauer parameters of TPPSFe(II)–thiol complexes in the presence of CO at high pH are very clearly those of straightforward S–Fe–CO complexes (low-spin iron(II)). They have small quadrupole splittings which are very different to those found for PPIXFe(II) but similar to cytochrome P450 and other model complexes [21, 38].

Experimental

Preparation of Fe(II)PPIX and Fe(II)TPPS Complexes in Solution for Mössbauer Spectroscopy

Haematin (from bovine blood) was purchased from Sigma and used without further purification. TPPSFe was prepared according to the method of Fleischer [64].

200 mg of these iron porphyrins were dissolved in 1 ml of 2 M NaOH in a two-necked flask under an N_2

atmosphere. A few drops of concentrated sodium dithionite in water were then added to the solution. Finally, 3 ml of ligand was added with continuous stirring to the solution (glycine ethyl ester was added as solid). The solution was transferred to a nylon cell, frozen and placed in the cryostat for Mössbauer spectroscopy. In the case where CO adducts were prepared (no. 7–9, Table 1), the solutions from no. 4–6 (Table 1) were thawed, CO was bubbled in for about 5 min, and the solutions were refrozen and studied. Mössbauer spectrometer and other experimental details, along with computer fitting (of spectra) have been previously described (for instance see refs. 40–43). All Mössbauer data are referenced to metallic Fe as zero shift.

Electronic Absorption Titration Experiments

Fe(III)TPPS was prepared in buffer solution (0.1 M KCl, 26 ml + 66 ml of 0.1 M NaOH to form buffer pH ~ 13). For electronic absorption spectra cells of 1 cm path length, containing 2.5 ml of solution, were used. The cells were quartz or glass and were fitted with tap tops enabling the solutions to be kept under an N_2 atmosphere. All electronic spectra were recorded at 15 °C. A few drops of concentrated sodium dithionite were added to the cell, then the ligand was injected, using a micro syringe.

The concentration of the ligands were either neat from the bottle for the thiols, but for glycine ethyl ester the concentration was 1 M.

The concentration of TPPSFe(II) used was in the range $3–5 \times 10^{-5}$ M depending upon which absorption band was involved in the study (the ionic strength used was 0.1 M $NaNO_3$).

Acknowledgement

J.S. thanks the Government of Iraq for the support to J.A.T.

References

1 D. Y. Cooper, O. Rosenthal, R. Snyder and C. Witmer, 'Cytochromes P450 and b_5', Plenum, New York, 1975, p. 1.
2 I. C. Gunsalus, J. R. Meek, J. D. Lipscomb, P. Debrunner and E. Munck, in O. Hayashi (ed.), 'Molecular Mechanisms of Oxygen Activation', Academic Press, New York, 1974, p. 559.
3 V. Ullrich, *Angew. Chem., Int. Ed. Eng., 11,* 701 (1972).
4 A. H. Conney, *Pharmacol. Rev., 19,* 317 (1967).
5 I. C. Gunsalus, *Hoppe-Seyler's Z. Physiol. Chem., 349,* 1610 (1968).
6 M. Klingenberg, *Arch. Biochem. Biophys., 75,* 376 (1958).
7 D. Garfinkel, *Arch. Biochem. Biophys., 77,* 493 (1958).
8 H. S. Mason, J. C. North and M. Vanes'e, *Fed. Proc. Fed. Am. Soc. Exp. Biol., 24,* 1172 (1965).
9 K. Murikam and M. S. Mason, *J. Biol. Chem., 242,* 1102 (1967).
10 J. P. Collman, T. N. Sorrell and B. M. Hoffman, *J. Am. Chem. Soc., 97,* 913 (1975).
11 S. Koch, S. C. Tang, R. H. Holm and R. B. Frankel, *J. Am. Chem. Soc., 97,* 914 (1975).
12 S. Koch, S. C. Tang, R. H. Holm, R. B. Frankel and J. A. Ibers, *J. Am. Chem. Soc., 97,* 916 (1975).
13 H. Ogoshi, H. Sugimoto and Z. Yoshida, *Tetrahedron Lett.,* 2289 (1975).
14 J. O. Stern and J. Peisach, *J. Biol. Chem., 249,* 7495 (1974).
15 J. P. Collman and T. N. Sorrell, *J. Am. Chem. Soc., 97,* 4133 (1975).
16 J. P. Collman, T. N. Sorrell, J. H. Dawson, J. R. T. Trudell, E. Brunnenberg and C. Djerassi, *Proc. Natl. Acad. Sci. U.S.A., 73,* 6 (1976).
17 C. K. Chang and D. Dolphin, *J. Am. Chem. Soc., 97,* 5948 (1975).
18 C. K. Chang and D. Dolphin, *Proc. Natl. Acad. Sci. U.S.A., 73,* 3338 (1976).
19 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).
20 J. P. Collman, T. N. Sorrell, K. O. Hodgson, A. Kulshresta and C. E. Strouse, *J. Am. Chem. Soc., 99,* 5180 (1977).
21 M. Schappacher, L. Ricard, R. Weiss, R. Montiel-Montoya, E. Bill, U. Gonser and A. Trautwein, *J. Am. Chem. Soc., 103,* 7646 (1981).
22 M. Schappacher, L. Ricard, R. Weiss, R. Montiel-Montoya, U. Gonser, E. Bill and A. Trautwein, *Inorg. Chim. Acta, 78,* L9 (1983).
23 A. Roader and E. Bayer, *Eur. J. Biochem., 11,* 89 (1969).
24 J. Peisach, W. E. Blumberg and A. Alder, *Ann. N. Y. Acad. Sci., 206,* 310 (1972).
25 H. A. O. Hill, A. Roder and R. J. P. Williams, *Struct. Bonding (Berlin), 8,* 123 (1970).
26 J. Silver and B. Lukas, *Inorg. Chim. Acta, 91,* 279 (1984).
27 B. Lukas, J. R. Miller, J. Silver, M. T. Wilson and I. E. G. Morrison, *J. Chem. Soc., Dalton Trans.,* 1035 (1982).
28 B. Lukas, J. Silver, I. E. G. Morrison and P. W. C. Barnard, *Inorg. Chim. Acta, 78,* 205 (1983).
29 J. Silver and B. Lukas, *Inorg. Chim. Acta, 78,* 219 (1983).
30 J. Silver and B. Lukas, *Inorg. Chim. Acta, 80,* 107 (1983).
31 B. Lukas, J. Peterson, J. Silver and M. T. Wilson, *Inorg. Chim. Acta, 80,* 245 (1983).
32 J. Silver, B. Lukas and G. Al-Jaff, *Inorg. Chim. Acta, 91,* 125 (1984).
33 J. Silver and B. Lukas, *Inorg. Chim. Acta, 106,* 7 (1985).
34 B. Lukas and J. Silver, *Inorg. Chim. Acta, 106,* 219 (1985).
35 B. Lukas and J. Silver, *Inorg. Chim. Acta, 124,* 97 (1986).
36 M. T. Ahmet, K. T. Douglas, J. Silver, A. J. Henson and D. E. V. Wilman, *Anticancer Drug Design,* 189 (1986).
37 J. Silver, G. Al-Jaff and J. A. Taies, *Inorg. Chim. Acta, 135,* 151 (1987).
38 M. Sharrock, E. Münck, P. G. Debrunner, V. Marshall, J. D. Lipscomb and I. C. Gunsalus, *Biochemistry, 12,* 258 (1973).
39 I. C. Gunsalus, C. A. Tyson, R. Tsai and J. D. Lipscomb, *Chem.-Biol. Interact., 4,* 75 (1971).
40 J. Silver and B. Lukas, *Inorg. Chim. Acta, 92,* 259 (1984).
41 J. Silver, B. Lukas and J. A. Taies, *Inorg. Chim. Acta, 136,* 99 (1987).
42 J. R. Miller, J. A. Taies and J. Silver, *Inorg. Chim. Acta, 138,* 205 (1987).
43 J. Silver and J. A. Taies, *Inorg. Chim. Acta, 151,* 69 (1988).

44 H. Abu-Soud, A. Houlton and J. Silver, *Inorg. Chim. Acta, 151,* 77 (1988).
45 C. A. Reed, T. Mashiko, S. P. Bentley, M. E. Kastner, W. R. Scheidt, K. Spartalian and G. Lang, *J. Am. Chem. Soc., 101,* 2948 (1979).
46 P. A. Adams, C. Adams and D. A. Baldwin, *J. Inorg. Biochem., 28,* 441 (1986).
47 L. M. Epstein, D. K. Straub and C. Maricondi, *Inorg. Chem., 6,* 1720 (1967).
48 W. M. Connor and D. K. Straub, *Inorg. Chem., 15,* 2289 (1976).
49 W. S. Caughey, *Ann. N. Y. Acad. Sci., 174,* 148 (1970).
50 W. S. Caughey, J. O. Alben, S. McCoy, S. H. Boyer, S. Charache and P. Hathaway, *Biochemistry, 8,* 59 (1969).
51 C. K. Chang and D. Dolphin, *J. Am. Chem. Soc., 98,* 1607 (1976).
52 J. A. Taies and J. Silver, unpublished results.
53 J. O. Stern and J. Peisach, *J. Biol. Chem., 249,* 7495 (1974).
54 L. K. Hanson, W. A. Eaton, S. G. Sligar, I. C. Gunsalus, M. Gouterman and C. R. Connell, *J. Am. Chem. Soc., 98,* 2672 (1976).
55 J. P. Collman, T. N. Sorrell and B. M. Hoffman, *J. Am. Chem. Soc., 97,* 913 (1975).
56 H. Sakuai, K. Ishizu and K. Okada, *Inorg. Chim. Acta, 91,* L4 (1984).
57 J. Silver and B. Lukas, unpublished results.
58 V. J. Nardo and J. Dawson, *Inorg. Chim. Acta, 123,* 9 (1986).
59 D. B. McIees and S. Winslow-Caughey, *Biochemistry, 7,* 642 (1968).
60 S. B. Brown and R. F. G. J. King, *Biochem. J., 153,* 479 (1976).
61 W. A. Gallagher and W. B. Elliott, *Ann. N. Y. Acad. Sci., 206,* 463 (1971).
62 D. Brault and M. Rougee, *Biochemistry, 13,* 4591 (1974).
63 M. Rougee and D. Brault, *Biochemistry, 14,* 4100 (1975).
64 E. B. Fleischer, J. M. Palmer, T. S. Srivastava and A. Chaterjee, *J. Am. Chem. Soc., 93,* 3162 (1971).