Biochem. J. (1988) 255, 353–356 (Printed in Great Britain)

The binding characteristics of the cytochrome *c* iron

Abel SCHEJTER* and Batya PLOTKIN

The Lady Davis Chair of Biochemistry, Sackler Institute of Molecular Medicine, Sackler Medical School, Tel Aviv University, 69978 Tel Aviv, Israel

A comparison of the binding properties of myoglobin and cytochrome *c* shows that the latter, in the reduced state, has an unusually large affinity for ligands, including thioethers. This explains the outstanding stability of the methionine–iron bond of ferrous cytochrome *c*, and results from the intrinsic ability of the cytochrome *c* iron to delocalize its electrons into orbitals of the sixth axial ligand.

INTRODUCTION

In all eukaryotic and prokaryotic cytochromes of the *c*-type, the haem iron is axially co-ordinated by atoms of two protein side chains: an imidazole nitrogen atom and a methionine thioether sulphur atom [1,2]. Although the ability of imidazole to form complexes with iron is well known [3], the widespread role of methionine as iron ligand in cytochromes seems to contradict the poor ligand behaviour of thioether sulphur towards iron [4] and iron porphyrins [5]. Stable thioether–metal bonds occur mainly under favourable stereospecific conditions, especially in environments with low water activity [6]. In cytochrome *c* these two conditions are satisfied: the bond occurs within a hydrophobic crevice, and the conformation of the protein places the methionine-80 sulphur atom in a position advantageous for binding to the metal atom [1]. This argument, however, does not explain why, in spite of the minimal conformational differences between oxidized and reduced cytochrome *c* [7,8], the strength of the bond is significantly greater in the ferrous than in the ferric state of the metal [9]. In ferric cytochrome *c*, cyanide [10,11] and imidazole [12] displace the methionine, but this is reversed when the metal is reduced [12,13]. This suggests that the reduced state of the cytochrome *c* iron has an intrinsic large affinity for bivalent sulphur, a hypothesis that could be tested by comparing the affinities of the ferric and ferrous states of the molecule for a series of ligands. However, the ferrous iron–sulphur bond is so strong that even CO fails to break it, for thermodynamic rather than kinetic reasons [13].

In order to make possible the binding of ligands to ferrocytochrome *c*, so that proposed comparison can be made, the methionine-80 sulphur atom must be alkylated, a modification that results in the displacement of the ligand from the sixth co-ordination position [14,15]. We have studied the formation of complexes between the product of this modification, di-CM-c, and a series of ligands. Our results demonstrate the persistence of the enhanced affinity of the reduced metal in the modified haemoprotein, not only for thioether sulphur but also for other ligands, such as cyanide and imidazole. In order to show that this is a specific property of cytochrome *c*, similar measurements were performed on another haemoprotein, myoglobin. We show here that the affinities of ferric myoglobin for cyanide and imidazole are much larger than those of the ferrous state, whereas both oxidation states bind dimethyl sulphide with equal strength.

MATERIALS AND METHODS

Horse heart cytochrome *c* (type III) and sperm-whale myoglobin, from Sigma Chemical Co., St. Louis, MO, U.S.A., were purified on Amberlite CG-50 [16] and DEAE-Sephadex [17] respectively. Dimethyl sulphide, from Aldrich Chemical Co., Milwaukee, WI, U.S.A., was redistilled; all other reagents were of analytical grade. Di-CM-c was prepared as described elsewhere [14].

Spectrophotometric observations were performed on Cary 14 and Cary 219 instruments. Titrations were carried out in thermostatically controlled cuvettes; dimethyl sulphide was added in ethanolic solution. After stabilization of the spectroscopic readings, whole spectra were recorded in order to ensure that isosbesticity was maintained.

RESULTS AND DISCUSSION

Spectroscopic observations

A characteristic spectroscopic feature of low-spin ferric haem co-ordinated by imidazole and methionine is a near-i.r. absorption [18] represented by the 695 nm band of ferricytochrome *c* [19,20]. A similar band should be expected in thioether complexes of ferric myoglobin or di-CM-c, whose iron ligands are essentially identical with those of native ferric cytochrome *c*. The band exists, indeed, in the complex of ferrimyoglobin with dimethyl sulphide, where it is centred at 790 nm (Fig. 1). In the dimethyl sulphide complex of ferric di-CM-c the band is weaker and centred at 740 nm (Fig. 1). In the visible region both ferric complexes have typical low-spin spectra. In the ferrous state the dimethyl sulphide complex of di-CM-c is low-spin, but that of ferromyoglobin remains in the high-spin state (Fig. 2).

The haemochrome spectrum of the reduced di-CM-c derivative could be due to the formation of an intramolecular complex with a nitrogenous group, brought about by partial denaturation. However, the presence of ethanol at a concentration of 0.05 M, similar to that

Abbreviations used: di-CM-c, dicarboxymethyl-cytochrome *c*.

* To whom correspondence should be addressed.

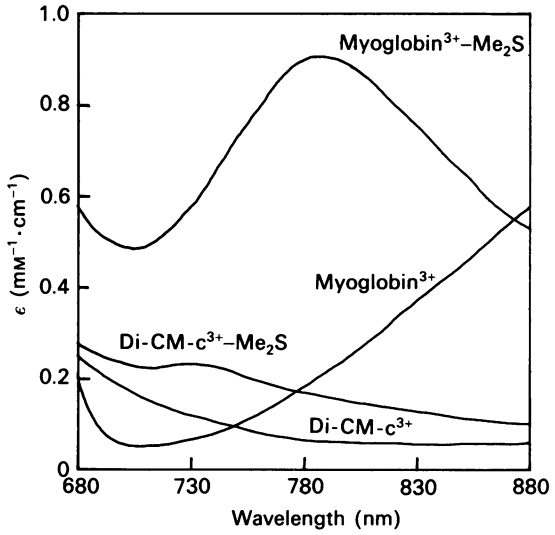

Fig. 1. Spectra of dimethyl sulphide complexes of ferric di-CM-c and myoglobin in the far red

Dimethyl sulphide (Me_2S) dissolved in ethanol and added to a final concentration of 0.35 M. Solutions were made in 0.1 M-phosphate buffer, pH 7.4, and spectra were recorded at 25 °C.

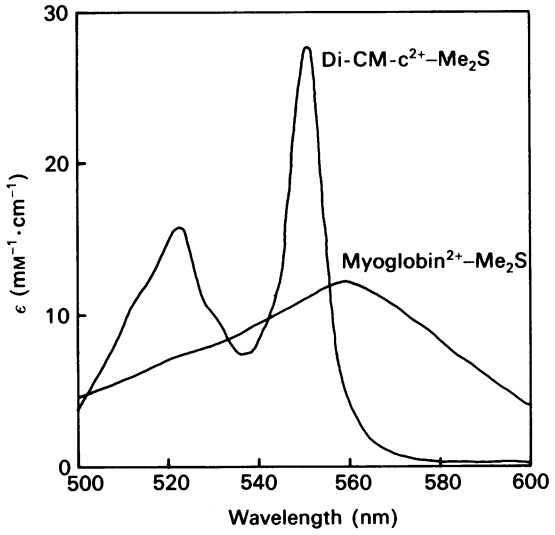

Fig. 2. Spectra of dimethyl sulphide complexes of ferrous di-CM-c and myoglobin in the visible region

Conditions for myoglobin were as indicated in Fig. 1 legend. For di-CM-c the final concentration of ligand was 0.05 M.

present in the solution used to record the spectrum of the ferrous di-CM-c complex with dimethyl sulphide, had no effect on the spectrum of reduced di-CM-c, indicating that the changes observed were due to the formation of a true complex.

Binding constants

Table 1 lists the binding constants of ferric and ferrous myoglobin and di-CM-c with dimethyl sulphide, cyanide and imidazole. The titrations were analysed assuming that binding occurs with the unprotonated forms of the ligands. Hill plots of the titration curves had slopes varying between 0.9 and 1.1, except for the titration of ferrimyoglobin with dimethyl sulphide, for which the Hill-plot slope was 1.5. Dimerization of dimethyl sulphide could explain this deviation from the expected behaviour, but if such were the case the same anomaly should occur in the titrations of ferromyoglobin and ferric di-CM-c, which have similar equilibrium constants with this ligand. It is also possible that, in addition to binding the iron, dimethyl sulphide binds the ferrimyoglobin protein in a non-specific manner. The values for ferrimyoglobin complexes with cyanide and imidazole and for ferromyoglobin complex with cyanide were similar to those found in the earlier literature [21].

As the data show, reduction of the haemoproteins results in a decreased affinity for imidazole and cyanide, but this effect is much less pronounced for di-CM-c than for myoglobin; for dimethyl sulphide the affinity for myoglobin is not affected by the oxidation state of the iron, but that of di-CM-c is two orders of magnitude larger in the ferrous form. We conclude, therefore, that the ability to form a strong bond with a thioether sulphur atom is an intrinsic property of the cytochrome *c* iron that the metal preserves even after the chemical modification of the native methionine sulphur ligand.

The question arises as to what causes the ferrous cytochrome *c* iron to bind comparatively so strongly to ligands in general, and to bivalent sulphur in particular. Ferrous iron is a 'borderline' metal, capable of binding both 'hard' [22] ligands that donate electrons to the metal, and 'soft' [22] or π-acid ligands [23] that can accept metal electrons in their orbitals. Imidazole and cyanide, a weak and a moderate base respectively, are good donor ligands. Cyanide, in addition, is a strong π-acid ligand. Dimethyl sulphide is a very weak base, pK −6.4 [24], but at the same time is a good π-acid ligand because of the overlap between its low-lying empty 3*d* orbitals and the 3*d* orbitals of the metal. We are thus led to attribute the enhanced affinity of the reduced iron of cytochrome *c* for thioethers to the 'soft' or π-acid nature of the bivalent sulphur atom. Since the iron is in the low-spin state, delocalization of its electrons into ligand orbitals is favoured by the accompanying release of pairing energy. Although some extent of back-bonding may also occur in the ferric state, this is certainly less than in the reduced molecule, because ferric iron is a 'hard' metal [22].

The unusual strength of the ferrous cytochrome *c* crevice is thus the outcome of two separate contributions: one is the conformation of the protein fold, which places the methionine close to the iron, and the other is the metal-to-ligand back-bonding. Independent evidence for electron delocalization in cytochrome *c* can be found in the analysis of the e.p.r. spectrum of the ferric state [25] and the Mossbauer spectrum of the ferrous form [26,27].

The reasons for the different behaviours of iron in myoglobin and di-CM-c may be traced to differences in *trans* and *cis* effects [28,29] arising from the dissimilar bonding of the haem group in these proteins. In both molecules imidazole is one of the axial ligands, but its spatial orientation with respect to the haem is quite different. In myoglobin [30] the plane containing the imidazole ring lies close to the line formed by the porphyrin nitrogen atoms $N_{(1)}$–$N_{(3)}$. This results in a non-bonded contact between an imidazole carbon atom and a porphyrin nitrogen atom, which forces the imidazole

Table 1. Binding constants of myoglobin and di-CM-c with various ligands at 25 °C at pH 7.0

The constants were calculated assuming binding with the unprotonated forms of the ligands.

	Binding constant (M^{-1})			
	Myoglobin		di-CM-c	
Ligand	+2	+3	+2	+3
Imidazole	1.4×10^0	2.8×10^2	8.7×10^2	1.3×10^3
Cyanide	3.1×10^0	1.4×10^7	8.3×10^5	1.6×10^7
Dimethyl sulphide	3.0×10^1	3.1×10^1	2.6×10^3	2.1×10^1

ring to move away from the porphyrin plane [31], pulling with it the iron atom and causing its *d*-electrons to acquire a high-spin configuration; both facts act against back-donation to the *trans* ligand. When dimethyl sulphide binds ferrimyoglobin, part of the free energy released by the formation of the new bond must be spent to reorient the imidazole so that the iron can move back into the porphyrin plane. In ferromyoglobin the free energy released by dimethyl sulphide binding appears to be insufficient for this purpose, perhaps because of the larger ionic radius of the metal, so that the latter remains outside of the porphinato ligand centre, in the high-spin state.

In cytochrome *c* the imidazole plane projection almost exactly bisects two of the N–Fe–N angles [7]. In the absence of non-bonded contacts the iron remains in the porphyrin plane, in the low-spin state, favouring back-donation to the *trans* ligand. As a consequence the free energy gained by the binding of ligands to di-CM-c is not spent in reorientations of the protein conformation, and appears expressed in the strength of the metal–ligand bond. The orientation of the imidazole plane also minimizes the overlap between the imidazole ring π-orbital and the iron d_{xz} and d_{yz} orbitals ([32], but see also ref. [33] for a different view). This, in turn, minimizes the extent of back-donation from the iron to the imidazole, which could compete with back-donation to the other axial ligand.

A further contribution to the π-donor quality of the cytochrome *c* iron stems from the *cis* effect of its porphyrin. The vinyl groups of the myoglobin protohaem withdraw electronic charge, decreasing the basicity of the porphyrin nitrogen atoms [29]. In cytochrome *c* the vinyl groups are saturated by formation of thioether bonds to the protein [9]. The increased electronic charge of the porphyrin nitrogen atoms is transmitted to the metal, increasing its electron-donating power [28,29]. Furthermore, through hyperconjugation [34] the lone pairs of electrons of the thioether sulphur atoms of cytochrome *c* may also contribute to the electron density of the porphyrin ring, increasing the *cis* effect.

REFERENCES

1. Dickerson, R. E., Takano, T., Eisenberg, D., Kallani, O. B., Samson, L., Cooper, A. & Margoliash, E. (1971) J. Biol. Chem. **246**, 1511–1532
2. Matthews, F. S. (1985) Prog. Biophys. Mol. Biol. **45**, 1–56
3. Sundberg, R. J. & Martin, R. B. (1974) Chem. Rev. **74**, 471–517
4. Murray, S. G. & Hartley, F. R. (1981) Chem. Rev. **81**, 365–414
5. Ellis, P. E., Jones, R. D. & Basolo, F. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 5418–5420
6. Sigel, H., Rheinberger, V. M. & Fischer, B. E. (1979) Inorg. Chem. **18**, 3334–3339
7. Takano, T. & Dickerson, R. E. (1981) J. Mol. Biol. **153**, 79–94
8. Takono, T. & Dickerson, R. E. (1981) J. Mol. Biol. **153**, 95–115
9. Margoliash, E. & Schejter, A. (1966) Adv. Protein Chem. **21**, 113–283
10. George, P. & Tsou, C. L. (1952) Biochem. J. **50**, 443–448
11. George, P., Glausser, S. C. & Schejter, A. (1967) J. Biol. Chem. **242**, 1690–1695
12. Schejter, A. & Aviram, I. (1969) Biochemistry **8**, 149–153
13. George, P. & Schejter, A. (1964) J. Biol. Chem. **239**, 1504–1508
14. Schejter, A. & Aviram, I. (1970) J. Biol. Chem. **295**, 1552–1557
15. Wuthrich, K., Aviram, I. & Schejter, A. (1971) Biochim. Biophys. Acta **253**, 98–103
16. Margoliash, E. & Walassek, O. F. (1967) Methods Enzymol. **10**, 339–348
17. Wittenberg, J. B. & Wittenberg, B. A. (1981) Methods Enzymol. **76**, 29–42
18. Zerner, M., Gouterman, M. & Kobayashi, H. (1966) Theor. Chim. Acta **6**, 363–400
19. Schejter, A. & George, P. (1964) Biochemistry **3**, 1045–1049
20. Eaton, W. A. & Hochstrasser, R. M. (1967) J. Chem. Phys. **46**, 2533–2537
21. Antonini, E. & Brunori, M. (1971) Hemoglobin and Myoglobin in their Reactions with Ligands, p. 30, North-Holland, Amsterdam and London
22. Pearson, R. G. (1963) J. Am. Chem. Soc. **85**, 3533–3539
23. Cotton, F. A. & Wilkinson, G. (1980) Advanced Inorganic Chemistry, p. 1049, John Wiley and Sons, New York
24. Bonvicini, P., Levi, A., Lucchini, V. & Scorrano, G. J. (1972) J. Chem. Soc. Perkin Trans. 2 2267–2269
25. Salmeen, I. & Palmer, G. (1968) J. Chem. Phys. **48**, 2049–2052
26. Cooke, R. & Debrunner, P. (1968) J. Chem. Phys. **48**, 4532–4537
27. Lang, G., Herbert, D. & Yonetani, T. (1968) J. Chem. Phys. **49**, 944–950
28. Caughey, W. S., Barlow, C. H., O'Keeffe, D. H. & O'Toole, M. C. (1973) Ann. N.Y. Acad. Sci. **206**, 296–309
29. Caughey, W. S. (1973) in Inorganic Biochemistry (Eichhorn, G. L., ed.), vol. 2, pp. 797–831, Elsevier, Amsterdam
30. Fermi, G. (1975) J. Mol. Biol. **97**, 237–256

31. Gelin, B. R. & Karplus, M. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 801–805
32. Fergusson, J. E., Robinson, W. T. & Rodley, G. A. (1972) Aust. J. Biol. Sci. **25**, 1365–1371
33. Timkovich, R. (1979) in The Porphyrins (Dolphin, D., ed.), vol. 7, pp. 261–294, Academic Press, New York
34. George, P. & Griffith, J. S. (1963) Enzymes 2nd Ed. **1**, 347–390

Received 18 May 1988/19 July 1988; accepted 27 July 1988