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The reactivity of cytochrome *c* with soft ligands

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The spectral changes caused by binding soft ligands to the cytochrome *c* iron and their correlation to ligand affinities support the hypothesis that the iron–methionine sulfur bond of this heme protein is enhanced by delocalization of the metal t_{2g} electrons into the empty 3d orbitals of the ligand atom. These findings also explain the unique spectrum of cytochrome *c* in the far red.

Cytochrome *c*; Soft ligands; Iron–sulfur bond

1. INTRODUCTION

An outstanding and hitherto unexplained characteristic of cytochrome *c* structure, is the ligation of its iron by the sulfur of a methionine side-chain, a situation that is common to all the known eukaryotic and prokaryotic *c*-type cytochromes [1]. Although thioether sulphur is a very poor ligand of heme iron [2], the sulfur-iron bond of cytochrome *c* is very strong, most especially in the reduced state of the metal [3]. A possible explanation of this apparent anomaly [4], based on the theory of hard and soft acids and bases [5], is that the cytochrome *c* low spin-iron 3d electrons delocalize into the orbitals of π-acid or soft ligands, of which thioether sulfur is an example. To test this hypothesis, the binding of the cytochrome *c* iron with non-ionic ligands of various donor and acceptor abilities – thioether, phosphines, phosphite, isocyanide and imidazole – was studied. This was facilitated by alkylation of the methionine-80 sulfur, which displaces it from iron coordination [6] and leaves the metal accessible to exogenous ligands. Here we show that striking spectroscopic similarities exist between the native enzyme and its complexes with soft ligands, and that the stabilities of the reduced complexes are correlated to spectroscopic parameters indicative of the extent of back-bonding in these complexes.

2. MATERIALS AND METHODS

Horse heart cytochrome *c*, type III (Sigma Chemical Co., St. Louis), was purified [7] and modified into dicarboxymethyl cytochrome *c* (di-CM-c) [6]. Tributyl phosphine (TBP), dimethylphenyl phosphine (DMPP), trimethyl phosphite (TMP), dimethyl sulfide (DMS), imidazole (Im) and butyl isocyanide (BIC) were from Aldrich Chemical Co., Milwaukee. Optical spectra were recorded on Cary 14 and Cary 219 instruments.

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3. RESULTS

Complexes of reduced and oxidized di-CM-c were obtained by adding TBP in ethanolic solution. For ferrous di-CM-c, the visible bands shifted from 550 nm and 520 nm, to new peaks at 557 nm and 528 nm (Fig. 1). The α band lost intensity relative to the β band (Table I). In the far red, a weak but sharp band at 688 nm and two additional broad bands centered at 760 and 810 nm, were observed (Fig. 2). Similar bands were present in the complexes of ferrous di-CM-c with DMPP, TMP, Im, BIC and DMS, whose spectroscopic features in the visible and Soret regions are listed in Table I. Except for imidazole, all the ferric complexes had weak bands in the far red, where cytochrome *c* has its characteristic 695 nm band [8,9]. The spectra with best resolved bands are presented in Fig. 3.

Titrations of the binding of ligands by ferrous di-CM-c at various pHs showed Hill plots with slopes of 0.9–1.1; binding constants with the unprotonated ligands, are shown in Table I.

4. DISCUSSION

Trivalent phosphorus compounds are very good ligands because they are strong σ-donors [10]; they are also good π-acceptors because of their empty, low-lying 3d orbitals, a property that they share with divalent sulfur ligands; BIC has molecular orbitals with strong acceptor properties; imidazole is a far stronger base than DMS, BIC or TMP, but a much poorer π-acid. If these ligands are arranged in terms of their decreasing

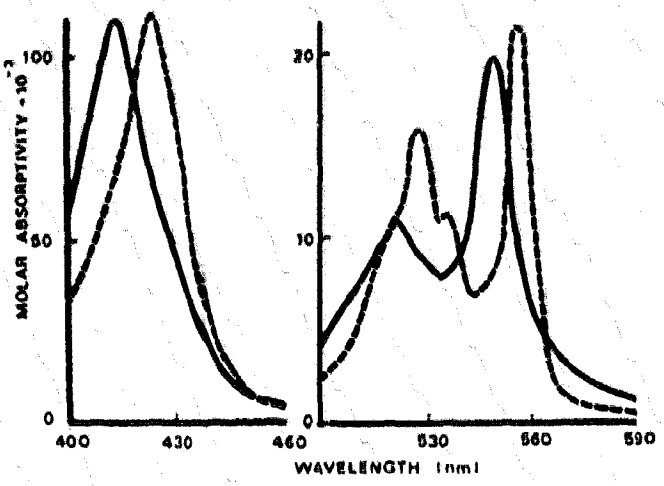

Fig. 1. Optical absorption spectra of ferrous di-CM-c (—) and its complex with tributyl phosphine (·····).

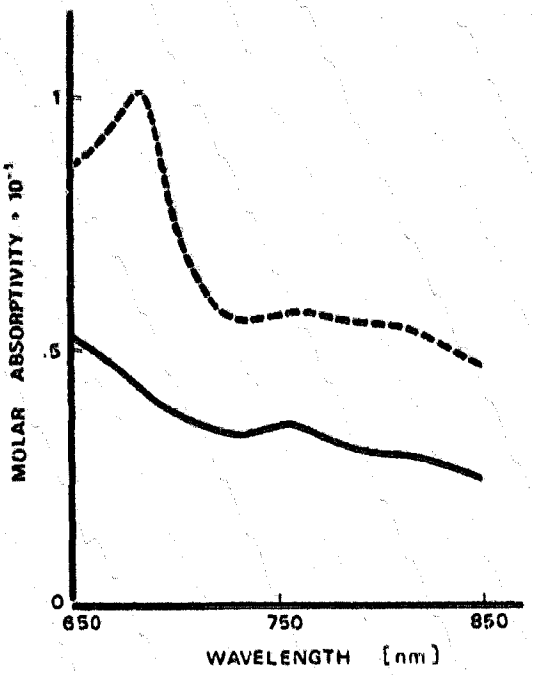

Fig. 2. Optical absorption spectra of ferrous di-CM-c (—) and its complex with tributyl phosphine (·····) in the far red.

affinities for ferrous di-CM-c, the following series is obtained: TMP >DMPP >BIC, TBP >DMS >Im. The strongest affinities are those of the strongest π-acceptors, indicating that the softness of the ligands [5] plays an important role in determining their affinities for the di-CM-c iron. The spectral effects of the ligands on the energies and intensities of the visible bands are roughly correlated: ligands that shift the bands to the red, decrease the ratio of intensities of the α and β bands. Aligning the ligands according to the decreasing energies of the baricenters of their o and Soret bands [11], the following order is obtained: TBP < DMPP < TMP < BIC >DMS < Im. Thus, soft ligands have the bathochromic effects on the porphyrin bands of di-CM-c that should be expected from metal electron

Table I

Optical spectra and binding constants of various complexes of ferrous di-CM-c; phosphate buffer, 0.1 M, pH 7.4; 25°C

Ligand	Absorption maxima (nm) (Absorbancies ($mM^{-1} \cdot c^{-1}$)) [Oscillator strengths]			Binding constants ($1\ M^{-1}$)
None	414 (117) [0.76]	520 (11.8) [0.075]	549 (22.3) [0.048]	
BIC	423 (151) [0.67]	522 (14.9) [0.075]	552 (18.9) [0.034]	2.5×10^4
Im	414 (112) [0.66]	520 (13.5) [0.064]	549 (28.1) [0.042]	8.7×10^2
TBP	426 (118) [0.65]	528 (15.8) [0.081]	557 (21.3) [0.032]	2.2×10^4
DMPP	425 (97) [0.71]	528 (12.6) [0.072]	557 (14.6) [0.019]	5.0×10^4
TMP	424 (109) [0.70]	523 (13.2) [0.067]	552 (17.1) [0.034]	8.3×10^4
DMS	416 (123) [0.62]	522 (14.8) [0.068]	551 (25.8) [0.043]	2.5×10^3

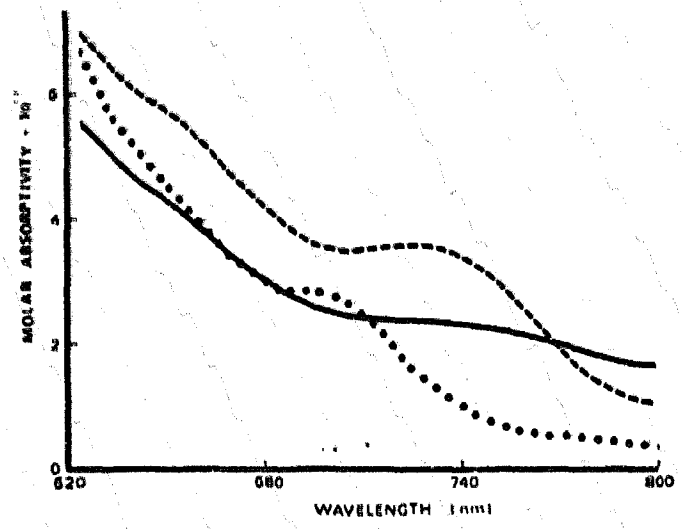

Fig. 3. Optical absorption spectra of ferric di-CM-c complexes with butyl isocyanide (—), dimethyl sulfide (.....) and trimethyl phosphite (------) in the far red.

delocalization into their orbitals [12,13]. A similar series, DMPP < BIC < TMP < TBP < DMS < Im, is obtained when the ligands are ordered according to the decreasing ratios of the intensities of the α and β bands, $\epsilon_\alpha/\epsilon_\beta$, as is typically found in low-spin ferrous heme complexes with ligands of increasing π-accepting power [11]; or when they are ordered according to the ratios of the oscillator strengths of the same bands (Table I). (Oscillator strength = $4.6 \times 10^{-9} \cdot \epsilon_{max} \cdot \Delta_{1/2}$, where ϵ_{max} is the molar absorptivity of the band, and $\Delta_{1/2}$ its width in c^{-1} where its absorptivity has half of its maximal value [14].) The similarities between the three series of ligands, two defined by spectroscopic correlates of back-bonding and the other by affinities to ferrous di-CM-c, is in keeping with the hypothesis that the cytochrome *c* iron has strong covalent tendencies [4]. The hypothesis is also supported by the spectral similarities between the complexes of di-CM-c with soft ligands and native cytochrome *c* in the far red, the spectral region in which the d-d and porphyrin π-d transitions are observed. These charge transfer and d-d bands, uniquely characteristic of ferrous cytochrome *c*, where they appear between 600 and 900 nm [15], are reproduced by the ferrous di-CM-c complexes with TBP. In the ferric state the spectrum of cytochrome *c* is outstanding because of the presence of a well defined band at 695 nm [7,8]. The observation of corresponding bands in the complexes of di-CM-c with soft ligands (Fig. 3) suggests that the electronic configurations of the iron and its ligand field in these complexes are very similar to that of native cytochrome *c*, and explains the unique observation of these bands in cytochrome *c* and some of its complexes as due to a combination of two factors in the sixth iron ligand: a weak or moderate ligand field, and a strong or moderate π-accepting power.

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