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THE INTERACTION BETWEEN HEME AND PROTEIN IN CYTOCHROME c_1

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The optical spectrum of reduced bovine cytochrome c_1 at 77 K shows a fine splitting of the β-band, which is indicative of the native conformation of the protein. At room temperature, this conformation is reflected in an absorbance band at 530 nm. The exposure of the heme of ferrocytochrome c_1, investigated by means of solvent-perturbation spectroscopy, appears to be extremely sensitive to temperature and SH reagents bound to the oxidized protein. Addition of combinations of potential ligands to the isolated tryptic heme peptide of cytochrome c_1 reveals that only a mixture of methionine and cysteine (or their equivalents) generates a β-band at 77 K which is identical in shape to that of native cytochrome c_1. In the EPR spectrum of a complex of ferrocytochrome c_1 and nitric oxide at pH 10.5, no hyperfine splitting derived from a second ligated nitrogen atom could be detected. The results indicate that methionine and cysteine are the axial ligands of heme in cytochrome c_1. The EPR spectrum of isolated ferricytochrome c_1 is that of a low-spin heme iron compound with a g_z value of 3.36 and a g_y value of 2.04.

Introduction

Cytochrome c_1 is a part of the ubiquinol-cytochrome *c* oxidoreductase complex (Complex III) of the mitochondrial respiratory chain and is involved in the transfer of electrons from the Rieske iron-sulfur center to cytochrome *c*. Isolation procedures for cytochrome c_1 from bakers' yeast [1], *Neurospora crassa* [2] and bovine heart [3,4] have been described. In this laboratory a monomeric cytochrome c_1 preparation, consisting of only one polypeptide chain, was isolated from bovine heart [4]. In the spectrum of this protein, a small absorbance band was observed, the presence of which appeared to be sensitive to SH reagents and which was found to be closely related to the conformation of cytochrome c_1 [5].

On the basis of similarity in the near-infrared region of the optical spectra of cytochrome *c* and cytochrome c_1, Kaminsky et al. [6] postulated that in cytochrome c_1 a methionine residue is bound to the heme as a ligand. Wakabayashi et al. [7] have proposed that histidine and methionine, in homology with cytochrome *c*, are the heme-ligating amino acids in cytochrome c_1. This was based on the finding that these two amino acids occur in two similar stretches in the primary structure of both cytochrome *c* and cytochrome c_1.

In this paper, a study of the relationship between conformation and spectral properties of the β-band of cytochrome c_1 is presented in order to probe the environment surrounding the heme in cytochrome c_1. It appeared that the amino acids methionine and cysteine occupy the fifth and sixth ligand position of the heme in cytochrome c_1.

Materials and Methods

Cytochrome c_1 was prepared from bovine heart according to the method of König et al. [4]. The

Abbreviation: PCMB, *p*-chloromercuribenzoic acid.

concentration of the enzyme was determined spectrophotometrically, using an absorbance coefficient of 19.2 $mM^{-1} \cdot cm^{-1}$ at 552.4 nm for the difference in absorbance between the reduced and the oxidized form of the protein [5]. Ferricytochrome c_1 was obtained after oxidation by potassium ferricyanide, followed by gel filtration. Unless specified, cytochrome c_1 was diluted in 50 mM potassium phosphate (pH 7.4) and 1% Tween 20. The tryptic heme peptide of cytochrome c_1 was isolated as described by Yu et al. [8].

Nitrogen monoxide (Baker Chemicals) was purified by leading the gas through a cold trap (−20°C). PCMB and *N*-formyl-DL-methionine were products of Sigma, β-mercaptoethanol was from Pierce. Other chemicals were of analar grade, mainly obtained from British Drug Houses.

Optical spectra at room temperature were recorded on a Cary 17 or a Cary 219 recording spectrophotometer. Absorbance spectroscopy at 77 K was carried out on a Perkin-Elmer 356 spectrophotometer, essentially according to the method described by Estabrook [9]. Solvent-perturbation spectroscopy was carried out according to the method of Herskovits [10].

EPR spectra were obtained with an E-9 spectrometer. Temperature, magnetic field and microwave frequency were measured as described in Ref. 11. Anaerobic addition of nitric oxide to EPR tubes was performed using the method described by Boelens and Wever [12].

Results

Fig. 1 shows the spectrum of purified ferrocytochrome c_1 in the visible region and that of ferricytochrome c_1 in the near-infrared region, as well as the spectrum of the heat-treated (5 min at 50°C) species. The presence of two bands in the spectrum of the untreated protein appears to be indicative of the native conformation. Upon heating cytochrome c_1, the absorbance bands at 695 nm of ferricytochrome c_1 and at 530 nm of ferrocytochrome c_1 disappear. Along with this, the absorbance maximum of ferricytochrome c_1 at 525 nm is shifted to 528 nm (not shown); the α-maximum in the spectrum of ferrocytochrome c_1 shows a blue shift of 2–3 nm. The protein becomes autooxidizable and cannot be reduced any more

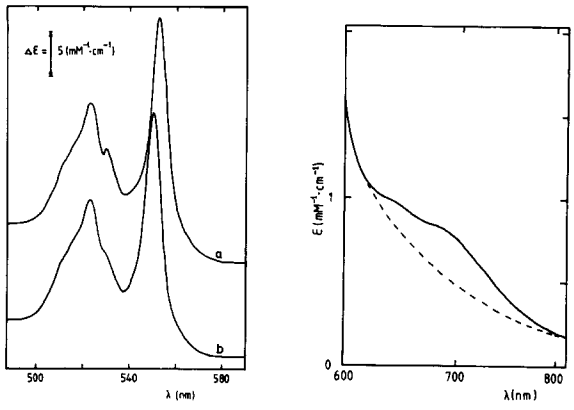

Fig. 1. The effect of heat treatment on the absorbance spectrum of cytochrome c_1. (Left) Ferrocytochrome c_1 (visible region): (a) native cytochrome c_1, (b) heat-treated cytochrome c_1 (5 min at 50°C). (Right) Ferricytochrome c_1 (infrared region): (———) native cytochrome c_1, (- - - - -) heat-treated cytochrome c_1.

by ascorbate, β-mercaptoethanol or ferrocytochrome *c*. It is, however, reducible by sodium dithionite.

The absorbance spectrum of ferrocytochrome c_1 in the visible region at 77 K is shown in Fig. 2. The spectrum has a remarkable splitting of the β-region and is similar to that published by Yu et al. [3], except for the absence of splitting of the α-band. The shoulder at 530 nm in the spectrum at room temperature narrows at low temperature and has a clear maximum at 528.5 nm. Incubation of cytochrome c_1 at 50°C, followed by reduction by sodium dithionite reveals a spectrum at low temperature (Fig. 2) without fine structure in the α,β-region. The same spectrum was obtained upon reduction of either the isolated tryptic heme peptide of cytochrome c_1 [8], or cytochrome c_1 in the presence of ethanol [13], or ferricytochrome c_1 treated with the SH reagent PCMB. However, the effect induced by PCMB can be reversed by addition of an amount of β-mercaptoethanol, substoichiometric to the concentration of PCMB. No effect of PCMB was observed with reduced cytochrome c_1, indicating that the reagent only reacts with a group in the vicinity of the heme when the enzyme is oxidized.

Fig. 3 gives the effect of sucrose on the spectrum of ferrocytochrome c_1 in the Soret region at 4°C and at room temperature. It is clear that at

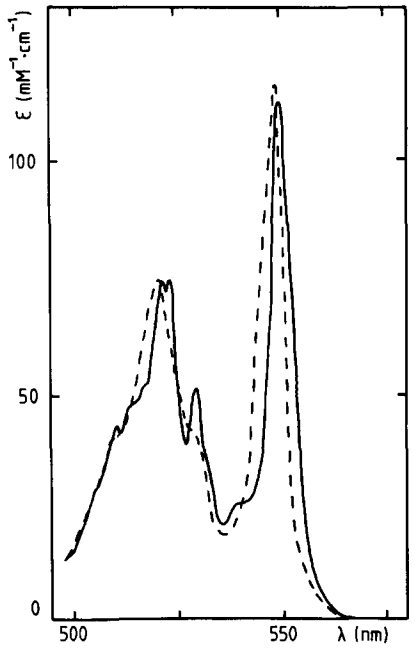

Fig. 2. Absorbance spectrum of ferrocytochrome c_1 at 77 K. (———) Native cytochrome c_1. (- - - - -) Cytochrome c_1 after 5 min at 50°C or tryptic heme peptide of cytochrome c_1 or cytochrome c_1 (5 μM) in the presence of 1 mM PCMB.

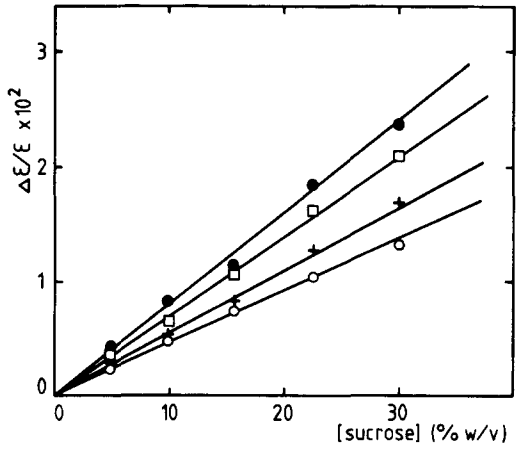

Fig. 3. The effect of sucrose on the optical spectrum of cytochrome c_1 in the Soret region. (○———○) Ferricytochrome c_1 at 415 nm (4°C), (+———+) ferrocytochrome c_1 at 419 nm (4°C), (□———□) ferricytochrome c_1 plus 1 mM PCMB at 416 nm (4°C), (●———●) ferrocytochrome c_1 at 418 nm (room temperature). The cytochrome c_1 concentration was 10 μM.

20°C the perturbation is 30% larger than at 4°C, indicating a more open structure of the protein around the heme group. This effect is completely reversible. Such a temperature dependence of the structure of the heme crevice has not been observed in the case of ferrocytochrome *c* [14]. The perturbing effect of sucrose on the spectrum of ferricytochrome c_1 is also given in Fig. 3. There appears to be little difference between the perturbation of the oxidized and reduced forms of the protein. In contrast to ferrocytochrome c_1 and ferricytochrome *c* [14], the perturbing effect of sucrose on ferricytochrome c_1 is independent of temperature in the range from 4 to 20°C. Treatment with PCMB, however, results in a widening of the heme crevice, giving support to the idea that the reagent is bound close to the heme. Again, this effect can be overcome by adding β-mercaptoethanol.

In order to mimic the environment surrounding the heme of cytochrome c_1, series of components in various combinations were added to the isolated tryptic heme peptide of the protein. This peptide is covalently bound to the heme and contains 15 amino acids [7,8]. The effects of the addition of ligands on the β-region of the low-temperature spectrum of the reduced heme peptide are summarized in Table I.

Only a combination of *N*-formylmethionine and β-mercaptoethanol produces a spectrum with identical peak positions as well as the same multiplicity of splitting of the β-band as native cytochrome c_1, whereas the separate compounds do not produce this effect, indicating that ligands can bind to either side of the heme. A mixture of 1 M *N*-formylmethionine and 200 mM β-mercaptoethanol was found to yield maximal intensity of the peaks in the β-band (65% of that in native cytochrome c_1). The ratio of the concentration of the components in this mixture indicates that β-mercaptoethanol is 5-times more strongly bound to the heme than is *N*-formylmethionine.

The mixture of *N*-formylmethionine and cysteine, also in a ratio of 5 : 1, had the same effect but the intensity is less due to limitations in the solubility of cysteine. Imidazole (10 mM) plus *N*-formylmethionine (1 M), however, gives a spectrum with a fine splitting of the β-band comparable to that of cytochrome *c*. The ratio of the

TABLE I

EFFECTS OF THE ADDITION OF LIGANDS ON THE LOW-TEMPERATURE SPECTRUM OF THE REDUCED ISOLATED TRYPTIC HEME PEPTIDE OF CYTOCHROME c_1 AT 77 K

Intensity is defined as the absorbance difference between the second largest peak in the β-band and the nearest trough, expressed in percent of the value for native cytochrome c_1.

	Multiplicity of splitting	Major peak positions (nm)	Intensity (%)
Native cytochrome c_1 (5 μM)	7	528.5, 522.5, 520.5	100
Native cytochrome c (5 μM)	4	524, 518	23
Tryptic heme-peptide of cytochrome c_1 (5 μM)	1	521	–
Additions to heme peptide			
1.4 M β-mercaptoethanol	1	521	–
1 M *N*-formylmethionine	1	521	–
1 M imidazole	1	521	–
0.2 M methionine + 100 mM β-mercaptoethanol	2	528, 521	15
1 M *N*-formylmethionine + 0.2 M β-mercaptoethanol	7	528.5, 522.5, 520.5	65
1 M *N*-formylmethionine + 10 mM imidazole	2	525.5, 520	6
0.5 M *N*-formylmethionine + 50 mM cysteine	4	528.5, 522.5	20

binding strength of *N*-formylmethionine and imidazole to the heme of cytochrome c_1 proved to be 1 : 100. This is considerably lower than the ratio of 1 : 700 that can be calculated for the binding of *N*-acetylmethionine and imidazole to the reduced heme of cytochrome *c* from the data of Wilgus et al. [15] and Harbury and Loach [16]. Other ligands like sulfide, lysine, azide and cyanide, or their combinations, added to the heme peptide fail to show the characteristic splitting of the β-band of native cytochrome c_1.

The EPR spectrum of isolated ferricytochrome c_1 is depicted in Fig. 4. The spectrum shows a small signal at g 4.3, which is attributed to the nonheme iron present in the preparation [5]. The g_z signal at g 3.36 is typical for a low-spin heme iron, indicating the presence of strong field ligands as suggested by Kaminsky et al. [6]. The value of 3.36 is in close agreement with that reported by Leigh and Erecińska [17] for cytochrome c_1 in succinate-cytochrome *c* oxidoreductase from pigeon heart mitochondria. The g_y signal in the spectrum is rather broad, with a central g value estimated to equal 2.04. Superimposed on this signal a sharp resonance at g 1.97 is observed. This resonance can probably be attributed to a radical. No g_x value of ferricytochrome c_1 was observed due to the large line-width of the corresponding signal in the EPR spectrum.

Ferrocytochrome c_1 rapidly reacts with NO under anaerobic conditions at pH values above 10, resulting in a change in the optical spectrum (not shown). In the EPR spectrum of the cytochrome c_1-NO complex (Fig. 5), the 3-fold splitting of the central resonance signal is caused by the hyperfine interaction with the nitrogen nucleus of the bound NO. The g value of the signal is 2.03 and the coupling constant of the splitting is 1.63 mT. No

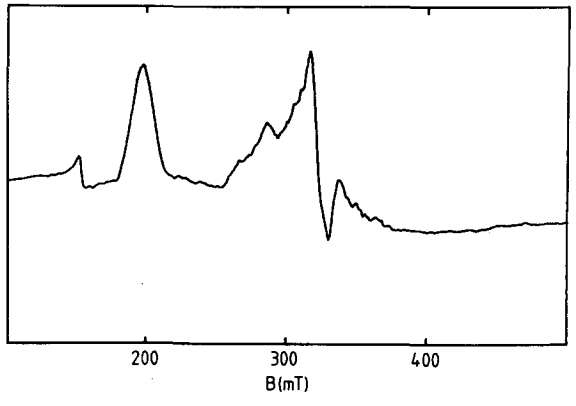

Fig. 4. EPR spectrum of ferricytochrome c_1. Conditions: 0.30 mM cytochrome c_1; temperature, 17 K; frequency, 9.2282 GHz; microwave power, 50 mW, modulation amplitude, 1.6 mT.

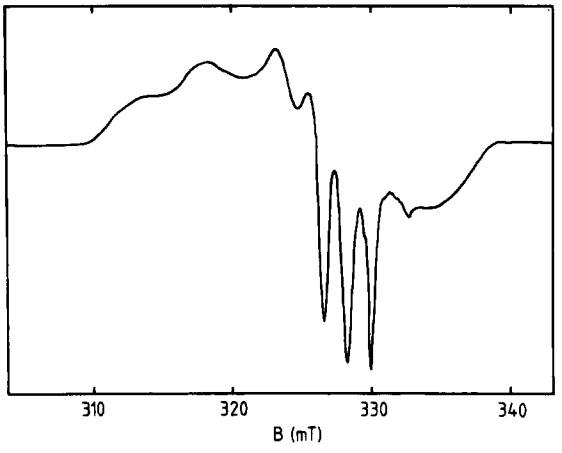

Fig. 5. EPR spectrum of ferrocytochrome c_1 in the presence of NO. Conditions: 0.20 mM cytochrome c_1 in 250 mM potassium phosphate (pH 10.5); temperature, 34 K; NO pressure, 12.7 kPa; frequency, 9.2555 GHz; microwave power, 2 mW; microwave amplitude, 0.32 mT.

hyperfine splitting induced by a second ligated nitrogen atom, as found for cytochrome *c* [18], cytochrome *c* oxidase [19] and hemoglobin [20], was observed. This finding is consistent with the results of the other experiments described in this section, i.e., that sulfur is ligated to both sides of the heme.

Discussion

We showed that a modest elevation of temperature decreased the reducibility of purified cytochrome c_1 and changed the optical spectrum at both room temperature and 77 K. Also, sucrose was found to have a temperature-dependent perturbing effect on the spectrum of ferrocytochrome c_1, again indicative of a conformational change of the protein around the heme group. As described for cytochrome *c*, such a conformational change can result in a drop in midpoint potential, making the protein less reducible [15,21–23]. The splitting of the β-band in the low-temperature spectrum of ferrocytochrome c_1 appears to be caused by the interaction between heme and protein. The absorbance band at 530 nm in the optical spectrum of reduced cytochrome c_1 at room temperature can be used as a marker for the native conformation of the protein, just as the 690 nm absorbance band of the oxidized protein is used as a marker for the ligation of methionine to the heme iron [6].

Although the optical spectrum of ferrocytochrome *c* at low temperatures shows little splitting of the β-band compared to cytochrome c_1, the β-band in the circular dichroism spectrum of the former protein shows five distinct peaks [15]. This fine splitting also appeared to be correlated to the activity and the midpoint potential of the protein. Splitting of the α-band, which was found in the optical spectra at room temperature of a particular class of bacterial cytochromes, has also been proposed to be related to the heme environment [24,25].

The perturbation of ferrocytochrome c_1 at 4°C as reflected in the spectrum ($\Delta\varepsilon/\varepsilon = 11.2$ at 20% sucrose) is considerably less than the values reported for other cytochromes *c* [14,26], indicating a more closed structure around the heme of cytochrome c_1, as proposed by Kaminsky et al. [6]. The shielding of the heme group from the solvent, also reflected in the fact that cytochrome c_1 in contrast to cytochrome *c* is not oxidizable by oxygen, may be connected with the fact that the protein is embedded in the membrane in the mitochondria.

The redox state of cytochrome c_1 seems to have little effect on the exposure of the heme group, in contrast to other hemoproteins [27,28]. Yet, the finding that only oxidized cytochrome c_1 can bind PCMB close to the heme indicates that a conformational change takes place upon reduction, during which a sulfhydryl group moves into the core of the protein. When a bulky group, like PCMB, is attached to this SH group, the conformation around the heme becomes less tight, giving rise to a low-temperature spectrum which is like that obtained after heat treatment.

Compounds which were ligated to the heme peptide of cytochrome c_1 were found to affect the splitting of the β-band in the optical spectrum at 77 K of this protein. The peptide could serve as a model for studying the ligation of amino acid side chains to the heme of cytochrome c_1. As only the combination of cysteine and methionine (or their equivalents) yields a low-temperature spectrum which is identical to that of native cytochrome c_1, we propose that these amino acids are the axial heme ligands in cytochrome c_1. This suggestion is supported by the NO-binding studies. The binding

of NO to cytochrome c_1 and the quenching of the 690 nm band as reported by Kaminsky et al. [6] show the same pH dependence. This suggests a displacement of methionine as a ligand. The absence of hyperfine splitting in the EPR spectrum of the cytochrome c_1-NO complex indicates that no nitrogen atom is bound opposite to methionine in cytochrome c_1.

The complete accessibility of the iron in the heme peptide of cytochrome c_1 also implies that His-41, the amino acid adjacent to the cysteines covalently bound to the heme, is not involved in ligation. This is in contrast to the binding of His-18 (which is at a similar position) to the heme in the octapeptide of cytochrome *c* [16].

On the basis of sequence homology with cytochrome *c*, Wakabayashi et al. [7] proposed that Met-208 is the sixth ligand in cytochrome c_1. Because of the proximity of Cys-55 to the heme binding region (Cys-37, Cys-40), this amino acid is the most likely candidate for occupying the fifth ligand position. On the carboxyterminal side of Cys-55 the first hydrophobic stretch (Tyr-126, Leu-143) which may enter the phospholipid bilayer is encountered in the amino acid sequence [7]. A large series of cytochromes *c* has been demonstrated to possess histidine and methionine as heme-chelating amino acids [29]. Displacement of histidine by a thiol group does not seem to have an important effect on the electrochemical properties of the heme, as the values of the midpoint potential of the cytochromes *c* and c_1 are similar [17,30].

Due to broadening of the signal, no g_x value for cytochrome c_1 could be deduced from the EPR spectrum. This value can be estimated, however, assuming that the sum of the squares of the *g* values equals 16 [31]. The value of g_z was thus calculated to be 0.74. The set of *g* values (3.36, 2.04 and 0.74) was used to determine the crystal field parameters of ferricytochrome c_1, using the method of Taylor [32] with the choice of the geometrical axes according to Peisach et al. [33]. The rhombicity then equals 0.993 and tetragonality is 1.819. These values do not fall into any area in the 'truth diagram' in which Peisach et al. [33] classified a series of heme compounds [33]. Proteins which contain thiol groups as ligands show considerably smaller values for g_z and for the rhombicity.

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