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STUDIES ON THE REACTION OF IMIDAZOLE WITH CYTOCHROME c_3 FROM *DESULFOVIBRIO VULGARIS*

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Summary

1. Cytochrome c_3, a unique hemoprotein with a negative redox potential and four heme groups bound to a single polypeptide chain, reacts with imidazole in the reduced state to form a low-spin ferro · imidazole complex which is spectrally characterized by a 3.1 nm blue shift in the α-peak (from 550.5 to 547.4 nm). The spectral imidazole · cytochrome c_3 complex is detectable at 77 but not at 298 K.

2. Mammalian ferrocytochrome *c* did not undergo a spectral interaction with imidazole at either 77 or 298 K, indicating that the imidazole · cytochrome c_3 complex reflects a unique event for cytochrome c_3.

3. Formation of the imidazole · cytochrome c_3 complex is strongly dependent on imidazole concentration (apparent K_d of approx. 50 mM), and is abolished in the presence of 100 mM phosphate. This latter effect is attributable to formation of an imidazole · phosphate complex. A pH titration of the imidazole · cytochrome c_3 spectral complex implicates ionization of an imidazole function (pK = 8.5).

4. EPR studies at 8.5 K of ferricytochrome c_3 before and after one reduction-oxidation cycle indicate that at least two of the hemes undergo reaction with imidazole forming two different low-spin ferric heme · imidazole complexes, with significant shifts in the *g* values of two heme signals.

5. The spectral and EPR results are consistent with formation as the primary event of a low-spin ferrocytochrome c_3 · imidazole complex in which increased hydrophobicity and protonation-deprotonation effects are contributary to the consequent lability of cytochrome c_3.

Introduction

Cytochrome c_3 (M_r = 13 000) from *Desulfovibrio vulgaris* contains four heme groups per molecule (a four-electron acceptor), is autooxidizable (with

a mid-point redox potential near −210 mV) and is remarkably stable in the ferric state as judged by reactivity towards exogenous ligands [1]. EPR [1—4] and NMR studies [5,6] have established that the heme groups are liganded with histidine at the 5th and 6th coordinative positions of each heme. EPR studies [1,4] have indicated that ferricytochrome c_3 shows four distinct and non-equivalent ferric low-spin resonances. Ferricytochrome c_3 appears to be only slightly reactive towards imidazole. Reduction of cytochrome c_3 in the presence of imidazole to EPR-inactive low-spin diamagnetic ferrocytochrome c_3, followed by reoxidation to the ferric state and prolonged standing, resulted in drastic changes to the ferric low-spin resonances. These changes consisted of the appearance of highly distorted high-spin ferric heme resonance with g values of 7.38 and 4.47 [3] and apparently distorted minor low-spin ferric heme resonances at $g = 3.52$ and 3.78 [1] and a decrease in intensity of both light and EPR absorptions. These latter signals were similar in g values to cytochromes b-562 and b-566 found in phosphorylation membranes from mammalian [7,8] and a bacterial system [9]. The detection of these unusual resonances suggested that replacement of histidine bound to heme-iron by the more hydrophobic imidazole resulted in distortion and lability of the hemoprotein structure of cytochrome c_3.

In view of these unusual observations on the reaction of ferrocytochrome c_3 with imidazole, this investigation was undertaken on light-absorption spectral changes for this reaction both at 298 and 77 K in order to correlate the low-temperature EPR results with low-temperature light absorption spectra. The latter studies carried out at 77 K would also result in a much greater resolution and sensitivity than similar studies carried out at 298 K. The EPR spectrum of ferricytochrome c_3 in the presence of imidazole was measured at liquid helium temperatures before and after one oxidation-reduction cycle for additional information on the nature of the reaction with imidazole. A preliminary report on these studies has been presented elsewhere [10].

Methods

Growth of *Des. vulgaris* and preparation of cytochrome c_3 were performed as previously described [2,3]. Light-absorption spectra were recorded on an Aminco DW-2 spectrophotometer. For studies at 298 K, cuvettes of 1-cm light path and 1-ml capacity were used. The Aminco low-temperature attachment (4-9603) was used with a 2 mm light-path cell. Cytochrome c_3 was dissolved in the appropriate buffer system reduced with sodium dithionite and quickly frozen in liquid nitrogen. To enhance the intensity of peaks, the cell was thawed to 223 K and refrozen at 77 K. Spectra were recorded at a slit of 0.2 nm, time constant of 100 ms and scanning rate of 0.5 nm/s.

EPR spectroscopy was carried out as previously described [1—4]. Other experimental conditions are described in the legend for Fig. 4. Cytochrome c (type VI) was obtained from Sigma. All other reagents used were of the highest purity available.

Results

Light absorption studies at 298 K

When cytochrome c_3 was reduced with sodium dithionite and spectra were

recorded between 500 and 600 nm, it was found (not shown) that the same spectrum of ferrocytochrome c_3 was obtained whether cytochrome c_3 was taken up in 100 mM histidine (pH 7.5) or in 100 mM imidazole (pH 7.5). The α- and β-peaks were found at 552.2 and 523.0 nm, respectively. When cytochrome c_3 at a constant protein concentration was reduced with sodium dithionite at different pH values (in different cuvettes) in the pH range of 7.5—10.3 in 100 mM histidine or pH 5.9—10 in 100 mM imidazole, the α- and β-peaks did not change in positions. For comparative purposes mammalian cytochrome *c* was reduced in the presence of imidazole or histidine at 100 mM final concentration (pH 7.5) and measured in the same spectral range. No deviations were noted from the usual α-peak position at 550.0 nm and β-peak position at 520.2 nm in the presence of either compound.

Light absorption studies at 77 K

When cytochrome c_3 was reduced in the presence of 100 mM histidine (pH 7.5) or 100 mM Tris buffer (pH 7.5), the α- and β-peaks were found (Fig. 1, solid line) to be at 550.5 and 522.3 nm, respectively, when measured at 77 K.

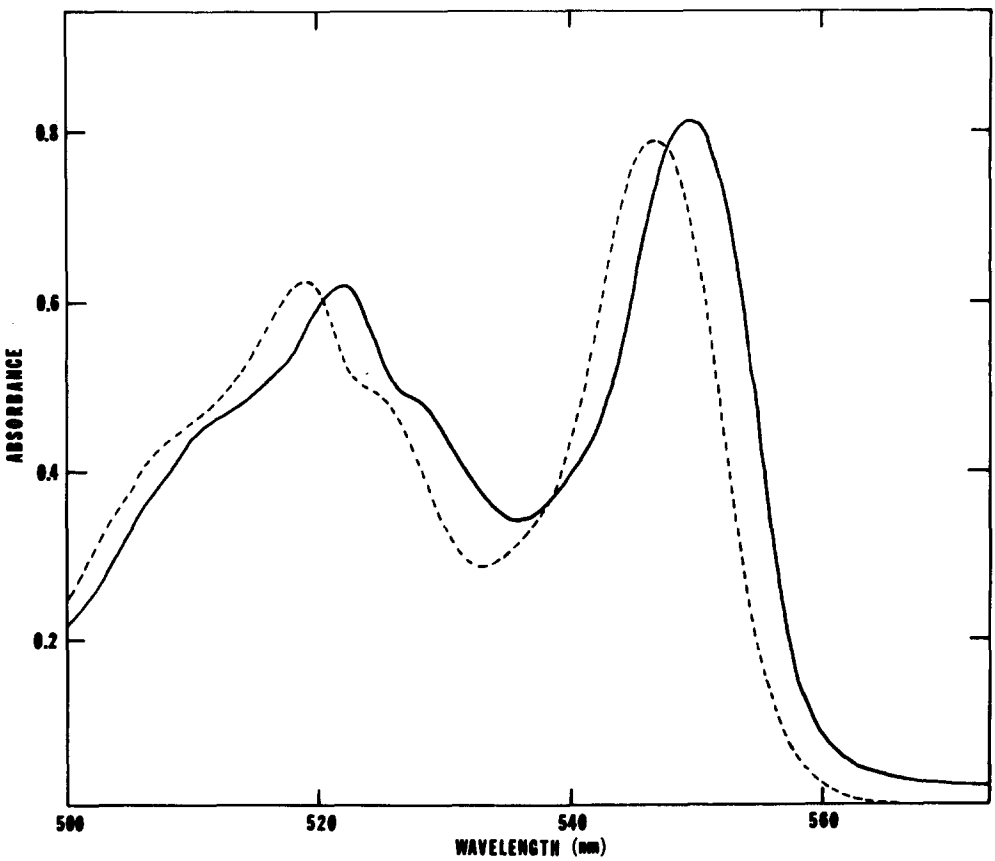


Fig. 1. Light absorption spectra at 77 K of ferrocytochrome c_3 (16 μM in protein), reduced with sodium dithionite; in 100 mM histidine, pH 7.5 (———) and in 100 mM imidazole, pH 7.5 (- - - - -).

When, however, cytochrome c_3 was reduced to the ferro form in the presence of 100 mM imidazole (pH 7.5), the α- and β-peaks (Fig. 1, dashed line) were blue-shifted to 547.4 and 519.2 nm, respectively. It should be noted that after a single reduction-reoxidation cycle in the presence of imidazole, cytochrome c_3, on standing, becomes labile in terms of both appearance of highly distorted EPR resonances and a decrease in intensity of both light and EPR absorption. The blue spectral shift is strongly dependent on the imidazole concentration. At 10 mM imidazole, virtually no spectral shift is found, whereas at 100 mM imidazole the maximum blue spectral shift is observed. From titration of cytochrome c_3 at constant protein concentration in different cells and at varying imidazole concentrations, an apparent dissociation constant (K_d) was determined at the half-maximum shift and found to be approx. 50 mM. Since the K_d determined is an arbitrary constant depending on the specific conditions of freezing used, no further significance is attached to the K_d value determined, except to emphasize that the blue spectral shift is a direct function of the imidazole concentration. The latter titration was performed in the presence of 50 mM Tris buffer (pH 7.5) to insure sufficient buffering capacity with regard to the added sodium dithionite. Tris itself in the concentration range used

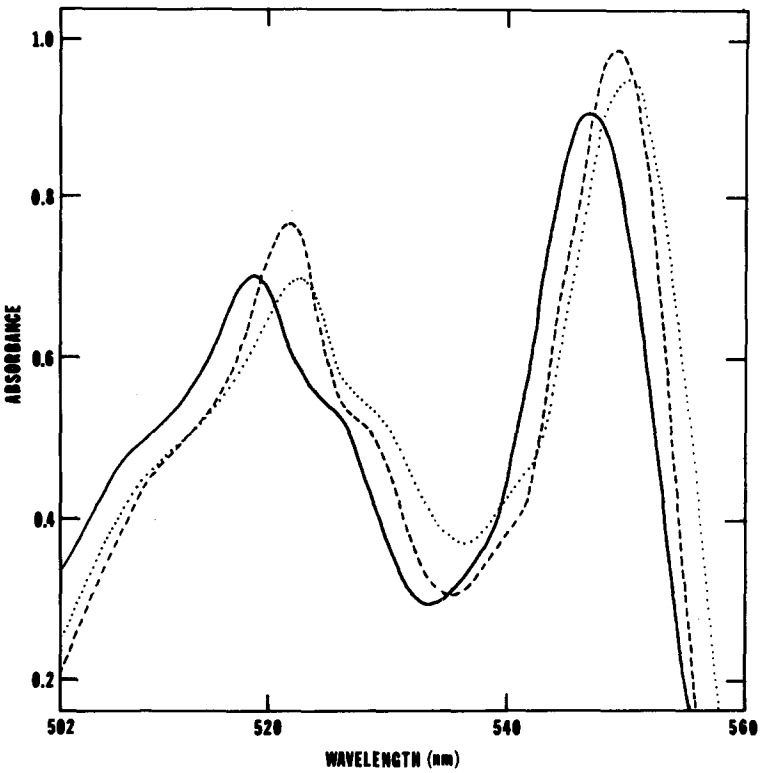


Fig. 2. Light absorption spectra at 77 K of ferrocytochrome c_3 (19 μM in protein), reduced with sodium dithionite: in 100 mM imidazole plus 1 mM potassium phosphate (pH 7.5) (———); in 100 mM imidazole plus 40 mM potassium phosphate (pH 7.5) (- - - - -); in 100 mM imidazole plus 100 mM potassium phosphate (pH 7.5) (· · · · · ·). The three traces have been slightly displaced in the vertical direction for graphical representation.

(50—100 mM, pH 7.5) caused no spectral changes on ferrocytochrome c_3.

Fig. 2 demonstrates the effect of varying phosphate concentrations on the imidazole-ferrocytochrome c_3 blue shift. Potassium phosphate buffer (pH 7.5) in the concentration range of 1—100 mM caused no spectral changes on ferrocytochrome c_3. At the low phosphate concentration of 1 mM and in the presence of 100 mM imidazole, the blue spectral shift is readily seen. At the intermediate phosphate concentration of 40 mM, the imidazole spectral shift is intermediate in magnitude with the α-peak at 548.9 nm and β-peak at 520.6 nm. At the high phosphate concentration of 100 mM, the blue spectral shift elicited by imidazole is abolished. Since rather high concentrations of imidazole are necessary to cause the spectral blue shift, the effect of high phosphate concentrations is attributed to a diminished imidazole concentration due to formation of a postulated imidazole · phosphate complex.

It should be noted that under similar conditions neither imidazole nor histidine at a concentration of 100 mM (pH 7.5) had any effect on the peak positions of mammalian ferrocytochrome *c*. The imidazole-induced blue spectral shift in ferrocytochrome c_3 appears to be a specific property of the multi-heme cytochrome c_3 and is associated with the subsequent lability of the hemoprotein.

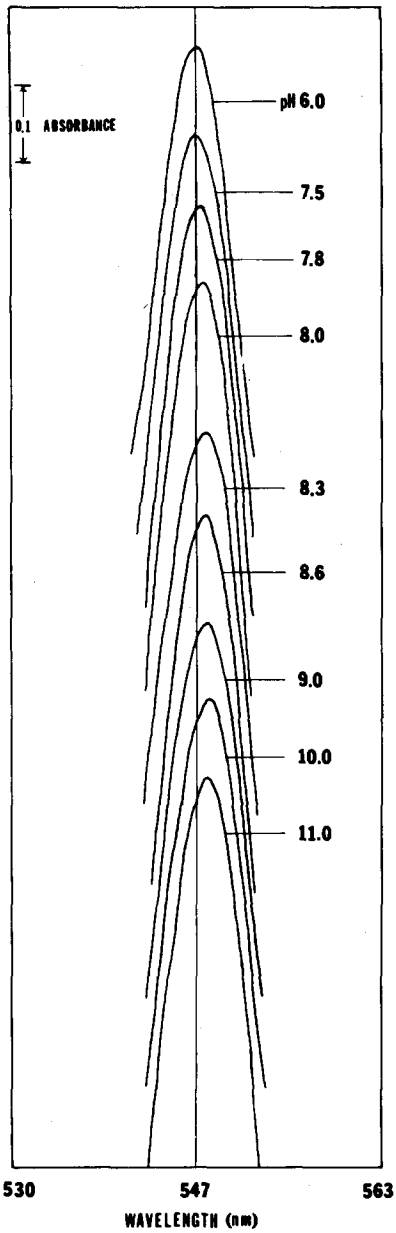
Effect of pH on the imidazole-induced blue spectral shift on ferrocytochrome c_3

Fig. 3 illustrates the effect of pH between 6 and 11 on the imidazole-caused blue spectral shift observed in ferrocytochrome c_3. Each spectral pH determination was carried out in 100 mM imidazole in different low-temperature cells at the same protein concentration. It can be seen that a red spectral shift occurs at the α-peak position with a maximum shift of 1 nm (from 547 to 548 nm), with a determined pK value of 8.5. A similar pH titration of ferrocytochrome c_3 in the presence of 100 mM histidine yields a blue shift in the α-position of 1 nm (from 550.7 to 549.7 nm), with a determined pK value of 9.6. The effects seen are clearly complex but based on the distinct spectral events noted some involvement of protonated versus deprotonated forms of imidazole or of imidazole from histidine in the spectral shifts appears evident.

EPR spectrum of ferricytochrome c_3 before and after one oxidation-reduction cycle

Fig. 4 shows a single oxidation-reduction cycle of ferricytochrome c_3 in 100 mM imidazole (pH 7.5), measured at 8.5 K. Fig. 4A shows that ferricytochrome c_3 prior to anaerobic reduction reveals four non-equivalent low-spin ferric heme resonances ($S = \frac{1}{2}$) with g_z * values at 3.16, 2.95, 2.71 and 2.45 and g_y * values at 2.29 in each case. For simplification of discussion the previous g_z values are arbitrarily termed hemes I, II, III and IV, respectively. Note that this nomenclature bears no relation to the four heme binding sites from amino acid sequence studies [11]. Indeed, it cannot be stated unequivocally that the four different heme signals observed arise from the four hemes of the

* g_z and g_y are defined as the end maximum and mid-point crossing, respectively, of the first derivative absorption.



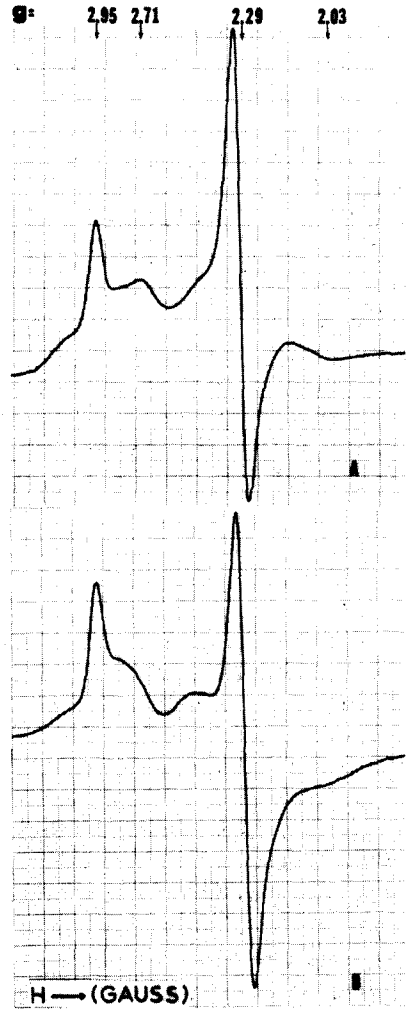


Fig. 3. Light absorption spectra at 77 K of ferrocytochrome c_3, reduced with sodium dithionite, at different pH values, but in 100 mM imidazole. The spectra were taken at constant protein concentration (19 μM). Each spectrum at a different pH was recorded with a different protein sample in a different low-temperature cell.

Fig. 4. EPR spectra at 8.5 K of ferricytochrome c_3 (0.77 mM in protein) in 100 mM imidazole (pH 7.5) before and after one reduction-oxidation cycle. The protein was reduced under anaerobic conditions as previously described [1,3,4] and then reoxidized with oxygen. Other EPR conditions were: modulation amplitude, 5.9 G; scanning rate of 1000 G/min; time constant, 0.3 s; frequency, 9.163 GHz and incident microwave power, 10 mW.

cytochrome. The position of heme III slightly shifted from $g = 2.75$ (cytochrome c_3 dissolved in 100 mM potassium phosphate buffer, pH 7.6) to 2.71 and reflects a slight interaction of imidazole with ferricytochrome c_3. The addition of sodium dithionite under anaerobic conditions to ferricytochrome c_3 results in the disappearance of all EPR signals due to reduction to the diamagnetic low-spin ferrous state. As previously discussed [1,4], reoxidation with oxygen leads to intermediate paramagnetic states due to reappearance of low-spin ferric heme signals from the individual hemes of cytochrome c_3. The individual ferric heme groups (I—IV) appear at different intensities indicating that the heme groups are not reoxidized to the same extent and may possess different redox potentials. As in the case of ferrocytochrome c_3 in 100 mM potassium phosphate buffer, pH 7.6 [1], heme III is preferentially reoxidized in this study. Likewise at an intermediate stage of reoxidation, a new resonance is found at $g = 1.62$ which disappears on reoxidation. On complete reoxidation the intensities of each heme group are approximately comparable to the initial low-spin ferric heme resonances (Fig. 4B). However, it is clear that the reoxidized EPR spectrum of ferricytochrome c_3 in Fig. 4B is not the same as in Fig. 4A. Hemes III and IV are altered in peak positions with heme III shifted 89 G to low-field with a g-value shift from 2.71 to 2.82 and heme IV sharpened and shifted 35 G to low-field with a g-value shift from 2.46 to 2.48. It appears that after a single redox cycle at least two of the hemes have apparently undergone reaction with imidazole resulting in the replacement of two histidines previously bound to heme iron, and leading to the subsequent lability of cytochrome c_3.

Discussion of results

Cytochrome c_3 is a remarkable hemoprotein containing four heme groups located on a single polypeptide chain. EPR and NMR studies [1—6] indicate the following: (i) the hemes are estimated to be about 10—15 Å apart; (ii) ferricytochrome c_3 exists in a low-spin ferric configuration with four non-equivalent ferric EPR signals suggesting that the four hemes are located in different environments within the hemoprotein; and (iii) each heme group is bound to two histidines and to two thioether links. EPR studies [1—4] indicate that at least two of the hemes are bound to histidine, based on known g-values for low-spin ferric heme-histidine liganded systems. However, two of the low-spin ferric signals show unusual g-values with g_z at 3.16 and 2.45, suggesting that the histidines bound to two heme groups are different. The recent studies of Brautigan et al. [12] suggest that a g_z value near 2.45 may be attributable to a low-spin ferric system bound to two ligands, such as imidazole or histidine with perhaps enhanced deprotonation plus contribution from a thioether type of bonding. Likewise a g_z value near 3.16 may be attributable to a low-spin ferric-ligand system in which a possible imidazole of histidine is in a protonated form, or perhaps in which both imidazoles of two histidines are protonated.

NMR spectroscopy [6] must be used to ascertain any unusual resonances which could explain the blue spectral shift observed since ferrocytochrome c_3 is EPR-inactive. Dobson et al. [6] found two remarkable resonances in

ferrocytochrome c_3 which disappeared on reoxidation. These two resonances were assigned to two histidines bound to heme iron located in a highly hydrophobic region of the protein. It is proposed that when cytochrome c_3 is reduced to the ferro state, the two iron-bound histidines located in the hydrophobic region, represented by hemes III and IV, are replaced by imidazole resulting in EPR shifts in their g-values to high-field on reoxidation, and spectrally in the 3.1 nm blue shift. The blue shift is also consistent with a change from a more polar to a less polar environment. Replacement of histidine with imidazole is then followed by eventual destruction of cytochrome c_3, since in reoxidized cytochrome c_3 the heme groups are unable to achieve correct alignment resulting in an unstable conformation. The spectral imidazole · cytochrome c_3 complex was not detected at 298 K but was reproducibly found at 77 K. This finding can be attributed to the enhanced sensitivity and resolution available at 77 K and is consistent with the low-temperature EPR results.

The pK values observed spectrally for ferrocytochrome c_3 in the presence of imidazole or histidine suggest that at least part of the observed spectral changes may be due to protonation/deprotonation phenomena. The actual pK values determined spectrally at 77 K are within the wide range reported in the literature at room temperatures, as, for example, for ionization of added imidazole in ferri-imidazole-hemoglobin (pK = 9.5) by Russell and Pauling [13] and for imidazole-heme undecapeptide (pK = 11) by Harbury and Loach [14].

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