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EPR STUDY OF THE EFFECT OF FORMATE ON CYTOCHROME c OXIDASE

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SUMMARY

The addition of formate to oxidized cytochrome c oxidase (ferrocytochrome c:oxygen oxidoreductase, EC 1.9.3.1) causes the appearance of a high spin heme signal at g = 6 and a splitting of g = 3 signal to g = 2.98 and 3.07. When formate-cytochrome c oxidase is reduced, the g = 2.98 signal decreases significantly. The spectrophotometric studies showed that formate is a specific ligand to cytochrome a_3. Data suggest that binding of formate to oxidized cytochrome c oxidase produces a ligand-a_3 interaction leading to the splitting of g = 3 signal hitherto considered as due to cytochrome a. Thus both cytochrome a and a_3 contribute to the resonance of g = 3 signal of cytochrome c oxidase.

INTRODUCTION

Cytochrome c oxidase catalyzes the four electrons reduction of oxygen to water with concomittant synthesis of ATP at site III of the mitochondrial respiratory chain. The catalytic site of the protein contains two hemes in the form of heme a and two atoms of copper (1,2). Electron paramagnetic resonance (EPR) studies of cytochrome c oxidase have showed the presence of various signals in the spectrum of the enzyme (3-8). The most accepted view at the present time is that a low spin signal at g = 3 is due to cytochrome a while a high spin signal at g = 6 is due to a_3. Minor signals between g = 2.6 and g = 2.2 due to heme a have been also described. A broad signal at g = 2 is assigned to both free radical and visible copper. In the oxidized enzyme, cytochrome a_3 and Cu-a_3 appear to be antiferromagnetically coupled and EPR silent (9). Formate has

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been showed to bind only to the oxidized form of cytochrome c oxidase to give a formate-a_3 complex (10). Upon reduction of the oxidase, with ascorbate-TMPD, the formate-a_3 complex remains oxidized while cytochrome a becomes reduced. However, an EPR study of cytochrome c oxidase with formate as ligand has not been reported previously; such a study is reported in this paper.

MATERIALS AND METHODS

Chemicals : The ammonium sulfate used in the purification of cytochrome c oxidase was of enzyme grade and was obtained from Schwarz/Mann, Orangeburg, New York. Sodium cholate, Tween 80, dithiothreitol (DTT), TMPD (N,N,N',N'tetramethyl-p-phenylene diamine) and L-ascorbic acid, were purchased from Sigma Chemicals Co., St Louis, Mo. Formate was made with formic acid from Fisher, Springfield, New Jersey.

Cytochrome c oxidase purification : Beef heart cytochrome c oxidase was isolated according to the Yonetani (11) procedure. Using as extinction coefficient 16 mM^{-1} at 605 nm (12), preparations contained between 9 and 12 nmoles heme a/mg protein and were free of contamination from other hemoproteins. Sodium dodecyl sulfate polyacrylamide gel electrophoresis performed according to reference 13, showed 6-7 bands as reported for beef heart cytochrome c oxidase by others (14,15).

Spectroscopy : The determination of optical spectra of cytochrome c oxidase was carried out with a dual wavelength scanning spectrophotometer built in the Johnson Foundation (16).

EPR spectroscopy : About 250 μM cytochrome c oxidase in 0.1 M phosphate buffer, pH 7.4 was divided in 2 ml test tubes. 20 mM sodium formate was added to each tube, followed by increasing concentrations of dithiothreitol as reductant. The tubes were incubated aerobically at 22°C. At various intervals the reaction mixtures were transferred to calibrated 3 mm i.d. quartz EPR sample tubes and quickly frozen by immersion in a liquid mixture of isopentane and methylcyclohexane (5:1) at approximately 80°K. After freezing, the samples were maintained at liquid nitrogen temperature, until the EPR spectra were measured. The EPR measurements were carried out in a Varian E-109 or Varian E-4 spectrometer with sample temperature maintained at 10°K by a flow of liquid helium from an external dewar through an Air Products cryo-tip control.

RESULTS

Fig. 1 illustrates the Soret region spectra of four states of cytochrome c oxidase. Spectrum 1 is that of the oxidized enzyme with a peak at 424 nm. The spectrum 2 is taken after 5 minutes incubation in the presence of 20 mM formate, showing a shift of 4 nm to the blue. Spectrum 3 is the half reduced state ($a^{2+}a_3^{3+}$)

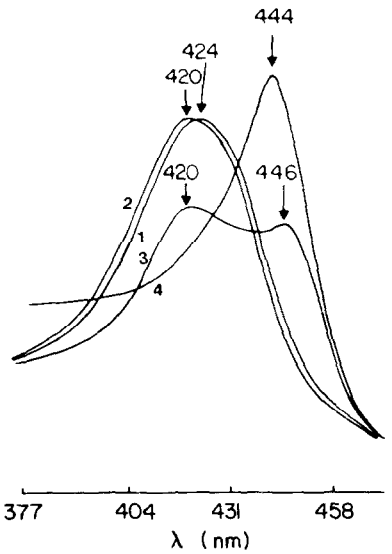

Fig. 1 - Effect of formate on the absolute spectra of purified cytochrome c oxidase. 20 μM cytochrome c oxidase in 20 mM phosphate buffer plus 0.1% Tween 80, pH 7.4, at room temperature. (1) oxidized enzyme; (2) oxidized + 20 mM formate; (3) formate-cytochrome c oxidase reduced by DTT ($a^{2+}a_3{}^{3+}$) and (4) fully reduced by dithionite.

induced by the addition of dithiothreitol. The Soret peak of the a_3^{3+} component remains at 420 nm, but the peak of reduced cytochrome a shifts to 446 nm. Upon addition of dithionite (spectrum 4), reduction of cytochrome a_3 occurs, as seen by the peak at 444 nm. The α band region around 600 nm did not show any significant change upon addition of formate to the oxidized form of the enzyme, but reduction of cytochrome a induced by ascorbate-TMPD or dithiothreitol showed the expected α peak at 606 nm (spectra not showed).

The EPR spectrum of oxidized cytochrome c oxidase and formate-cytochrome c oxidase is showed in Fig. 2. There were no significant changes in the intensity or position of the copper signal upon addition of formate to oxidized cytochrome c oxidase. The low spin heme signal at g = 3 was split to two new signals with resonance at g = 2.98 and 3.07. The band at g = 2.98 has amplitude similar to the g = 3 signal, while the g = 3.07 signal has approximately

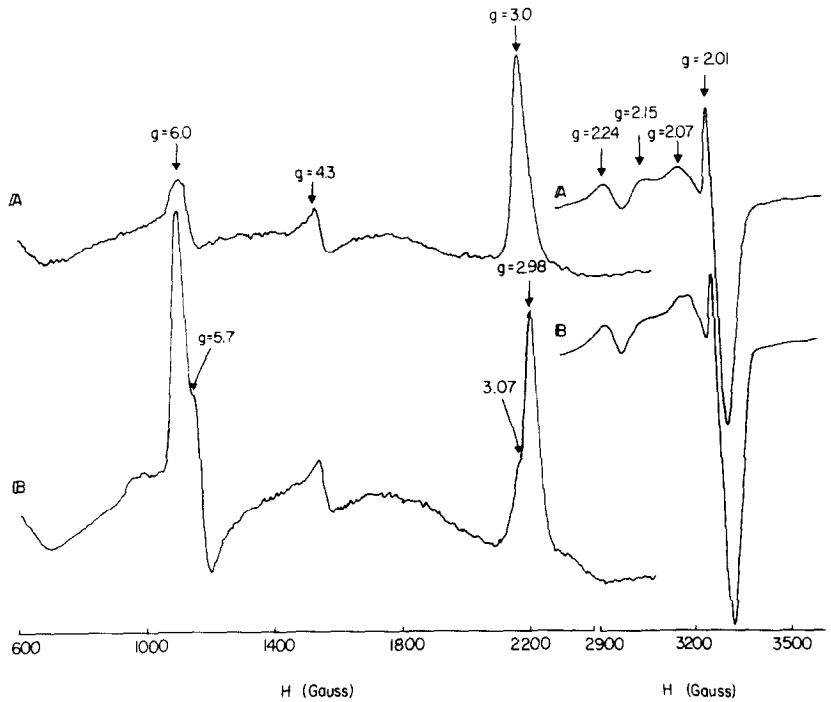

Fig. 2 - EPR spectra of cytochrome c oxidase. 250 μM cytochrome c oxidase in phosphate buffer 0.1 M, Tween 80 0.1%, pH 7.4; (A) oxidized enzyme and (B) after addition of 20 mM formate. Addition of formate produces an enhancement of g = 6 signal and a splitting of g = 3 signal to g = 2.98 and g = 3.07. Conditions : temperature, 10°K; microwave power, 10 mW; modulation amplitude, 20 G; microwave frequency, 9.26 GHz.

40% of the height of g = 2.98. Fig. 3 shows that, when formate-cytochrome c oxidase is reduced, the low spin resonance at g = 2.98 decreases significantly. Table I shows the change in the amplitude of g = 2.98 and 3.07 after addition of reductant. Approximately 70% of the signal at g = 2.98 disappeared upon consecutive additions of dithiothreitol, while the magnitude of the signal at g = 3.07 remained unchanged. The high spin heme signal at g = 6 was also intensified upon addition of formate (Fig. 2), although the absolute intensity of this signal varied from preparation to preparation.

DISCUSSION

In this work we have studied the interaction of formate with oxidized cytochrome c oxidase. Formate binds to oxidized a_3 and

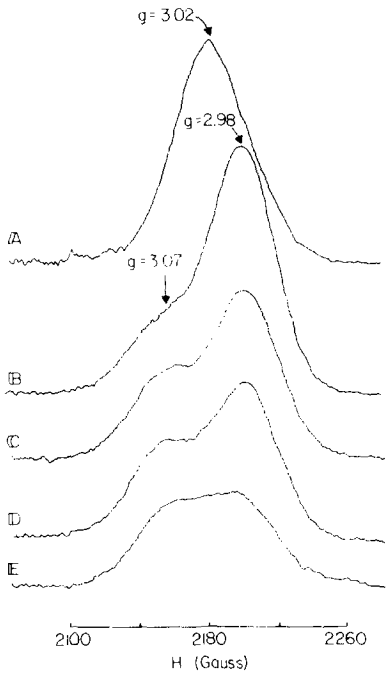

Fig. 3 - EPR spectra of g = 3 region of formate-cytochrome c oxidase. 250 μM cytochrome c oxidase in phosphate buffer 0.1 M plus 0.1% Tween 80; (A) oxidized enzyme; (B) oxidized enzyme + 20 mM formate; (C), (D), (E) formate-cytochrome c oxidase after addition of 3.2 mM, 6.5 mM and 13 mM dithiothreitol as reductant. Conditions : temperature, 10°K; microwave power, 10 mW; modulation amplitude, 20 G; microwave frequency, 9.26 GHz.

induces a splitting of g = 3 signal of the unliganded enzyme into two resonances at g = 2.98 and g = 3.07. Spectrophotometric studies showed that addition of a reductant such as DTT or ascorbate-TMPD

TABLE I
Effect of reductant on the g = 2.98 and g = 3.07 resonance of formate-cytochrome c oxidase (a)

	g = 2.98	g = 3.07
F-cytochrome c oxidase[b]	72	25
F-cytochrome c oxidase		
+ 3.2 mM DTT	47	27
+ 6.5 mM DTT	44	27
+ 13 mM DTT	26	24

(a) The values are expressed in arbitrary units.

(b) Formate-cytochrome c oxidase.

to formate-cytochrome c oxidase produces a reduction of cytochrome a, while cytochrome a_3 remains oxidized under these conditions. EPR studies indicate a significant decrease in the resonance at g = 2.98 on adding those reductants, suggesting that this signal is due to cytochrome a. The magnitude of the signal at g = 3.07 remains unchanged under these conditions, implying that it is due to formate-liganded cytochrome a_3. Our results suggest that the low spin heme signal in the unliganded oxidized enzyme at g = 3 is heterogenous, and is composed by two superimposed signals due to cytochrome a and a_3 respectively. These signals become resolvable due to shift in position on addition of formate. These results would explain a number of anomalies of the g = 3 signal as reported by Hartzell and Beinert (17) and by Wilson and associates (18). In addition, binding of formate intensifies the signal seen at g = 6 in the unlinganded oxidase. Because formate binds specifically to cytochrome a_3, the intensification of the high spin signal further supports the view that this signal is attributable to cytochrome a_3, in accord with the report of Stevens et al. (19) on nitric oxide binding to the oxidase. But because of heme-heme interaction in cytochrome c oxidase (5, 6), it is not yet possible to conclude positively that the high spin signal intensification by formate or nitric oxide binding can be attributed entirely to cytochrome a_3.

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