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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE

XX. REACTION WITH SULPHIDE

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SUMMARY

1. Upon addition of sulphide to oxidized cytochrome *c* oxidase, a low-spin heme sulphide compound is formed with an EPR signal at $g_x = 2.54$, $g_y = 2.23$ and $g_z = 1.87$. Concomitantly with the formation of this signal the EPR-detectable low-spin heme signal at $g = 3$ and the copper signal near $g = 2$ decrease in intensity, pointing to a partial reduction of the enzyme by sulphide.

2. The addition of sulphide to cytochrome *c* oxidase, previously reduced in the presence of azide or cyanide, brings about a disappearance of the azido-cytochrome *c* oxidase signal at $g_x = 2.9$, $g_y = 2.2$ and $g_z = 1.67$ and a decrease of the signal at $g = 3.6$ of cyano-cytochrome *c* oxidase. Concomitantly the sulphide-induced EPR signal is formed.

3. These observations demonstrate that azide, cyanide and sulphide are competitive for an oxidized binding site on cytochrome *c* oxidase. Moreover, it is shown that the affinity of cyanide and sulphide for this site is greater than that of azide.

INTRODUCTION

In 1939, Keilin and Hartree [1] discovered that sulphide prevents the reduction of cytochrome a_3 by succinate in heart-muscle preparation. Gilmour et al. [2] confirmed this and reported that sulphide acts rather like cyanide. The only other study of the effect of sulphide on cytochrome *c* oxidase (cf. ref. 3) is that of Wilson and Leigh [4], who showed a small increase of the half-reduction potential of cytochrome *a* upon binding of sulphide to oxidized cytochrome a_3.

In this paper we report on the effect of sulphide on the EPR spectrum of cytochrome *c* oxidase, and in particular describe a new low-spin heme signal of sulphido-cytochrome *c* oxidase.

MATERIALS AND METHODS

Cytochrome *c* was isolated from horse-heart according to the method of Margoliash and Walasek [5]. Cytochrome *c* oxidase was isolated from heart-muscle preparation according to the methods of Fowler et al. [6] and MacLennan and Tzagoloff [7] as modified by Van Buuren [8]. Absorbance coefficients used were for cytochrome *c* (reduced minus oxidized) 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm [9], for cytochrome *c* oxidase (reduced minus oxidized) 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm and for metmyoglobin 9.5 $mM^{-1} \cdot cm^{-1}$ at 505 nm [11].

Chemicals were analytical grade mainly obtained from British Drug Houses. Sodium sulphide crystals (May and Baker) were rinsed in distilled water in order to remove possible oxidation products. Solutions (0 °C) of these crystals, neutralized with KH_2PO_4, were prepared just before use.

EPR experiments were carried out on a Varian E-9 or a Varian-4501A spectrometer both equipped with a helium transfer system (Air Products Inc. model LTD-3-100) with automatic temperature controller. Temperature, magnetic field and microwave frequency were measured as previously described [12].

RESULTS AND DISCUSSION

The EPR spectrum at 15 °K of cytochrome *c* oxidase (Fig. 1A) shows a line at $g = 3$ and intense resonance near $g = 2$ originating from low-spin ferric heme *a* and copper [13], respectively. Upon incubation (2 min) of the enzyme with sulphide both the low-spin heme signal at $g = 3$ and the signal near $g = 2$ decrease in intensity, whereas an intense low-spin heme signal appears at $g = 2.54$, 2.23 and 1.87 (Fig. 1B). After 12 min incubation (Fig. 1C) the signals at $g = 3$ and near $g = 2$ disappear concomitantly with an intensification of the new low-spin heme signal. This spectrum also shows the appearance of a minor sulphide-induced signal with lines at the low-field side of the g_x and g_y and the high-field side of g_z of the major signal. Since the *g* values of these new signals correspond to those reported for sulphide complexes of other iron(III) hemoproteins [14,15], we assign them to two species of sulphido-cytochrome *c* oxidase.

In the presence of higher concentrations (100 instead of 10 mM) of sulphide the reactions are more rapid (not shown), and the only EPR signals observed after mixing the ligand with the enzyme are the sulphide-induced low-spin heme signals. Essentially the same EPR spectrum is obtained when cytochrome *c* oxidase is incubated with low concentrations (1–5 mM) of the ligand and subsequently reduced with ascorbate and a catalytic amount of cytochrome *c* under aerobic conditions. Thus, sulphide, known to be an electron donor, partly reduces cytochrome *c* oxidase before it induces the low-spin heme signals. It is interesting to note that the cyanide and azide-induced signals are also observed only in the partially reduced enzyme [13, 16, 17].

The intensity of the sulphide-induced signals in cytochrome *c* oxidase (0.20 mM) of Fig. 1C corresponds to 0.19 mM heme iron, as determined from double integration and comparison with the low-spin heme signal of azido-metmyoglobin. Thus, sulphide reduces one of the hemes of the functional unit (2 Fe, 2 Cu) of cytochrome *c* oxidase and prevents the reduction of the second heme.

It has been demonstrated that cyanide, azide and CO compete for cytochrome *c* oxidase [16]. In order to determine any competition of sulphide with the other inhi-

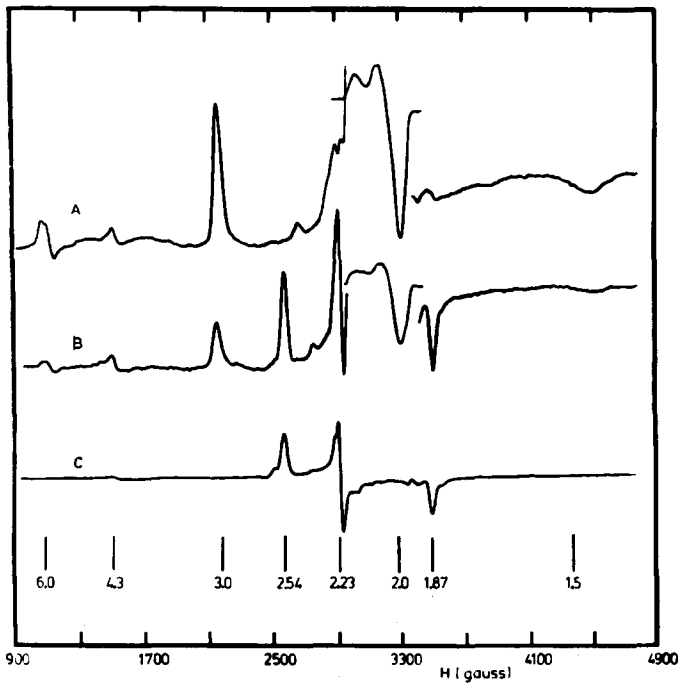

Fig. 1. The effect of sulphide on cytochrome *c* oxidase. (A) 0.20 mM cytochrome *c* oxidase in 100 mM potassium phosphate (pH 7.0), 1.0 % (v/v) Tween-80. (B) After addition of sulphide (10 mM) and incubation at room temperature for 2 min. (C) After incubation of B at room temperature for 10 min. Conditions of EPR spectroscopy: frequency, 9.092 GHz; microwave power, 2 mW; modulation amplitude, 10 G; scanning rate, 500 G · min^{-1}; time constant, 1.0 s; temperature, 15 °K. The copper signal in A and B was recorded at a 10-fold lower receiver gain. The gain was 5 times greater in A and B than in C.

bitors of cytochrome *c* oxidase, the following experiments were designed. Cytochrome *c* oxidase was incubated with azide (10 mM) and reduced with ascorbate and cytochrome *c*. The EPR spectrum (Fig. 2A) after this treatment shows the typical [13] azide-induced low-spin heme signal with $g_x = 2.9$, $g_y = 2.2$ and $g_z = 1.67$ and the copper signal near $g = 2$, with an intensity 15 % of that of the oxidized enzyme. Upon addition of 10 mM sulphide to this sample the azide-induced signal and the copper signal around $g = 2$ disappear and EPR resonances (Fig. 2B) are observed which are typical for the sulphide compounds of cytochrome *c* oxidase. This observation demonstrates that sulphide replaces azide at cytochrome *c* oxidase and, moreover, shows that the affinity of sulphide towards partially reduced cytochrome *c* oxidase is greater than that of azide.

Experiments similar to those shown in Fig. 2 were carried out with cyanide instead of azide. The EPR spectrum (not shown) of cytochrome *c* oxidase incubated with cyanide and subsequently reduced with ascorbate and cytochrome *c* shows the typical low-spin heme resonance at $g = 3.6$ [17] derived from cyano-cytochrome *c* oxidase. Upon incubation of the cyano-cytochrome *c* oxidase with sulphide (10 mM), the sulphide-induced signals appear very slowly with a concomitant decrease in intensity (about 60 % after 2 h) of the cyanide-induced signal at $g = 3.6$. These observations

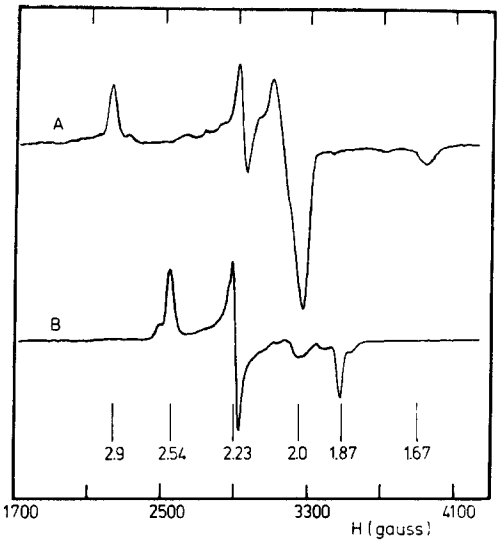

Fig. 2. Competition between azide and sulphide for cytochrome *c* oxidase. (A) Cytochrome *c* oxidase (0.20 mM), in 1.0 % (v/v) Tween-80, 100 mM potassium phosphate (pH 7.0), 10 mM azide was reduced by incubating with cytochrome *c* (25 μM) and ascorbate (19 mM) for 6 min at room temperature. (B) After addition of sulphide (10 mM) to A and incubation for 45 s at room temperature. Conditions of EPR spectroscopy as in Fig. 1. The gain in A was twice that in B.

indicate that cyanide and sulphide compete for cytochrome *c* oxidase. The affinity of the two ligands is of the same order of magnitude, since incubation of sulphido-cytochrome *c* oxidase with cyanide (10 mM) shows after prolonged incubation (2 h) a signal at $g = 3.6$, which is, however, relatively weak (about 20 %) in intensity in comparison with that found in the absence of sulphide.

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