LOW-SPIN FORMS OF CYTOCHROME *c* OXIDASE

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1. Introduction

Studies of cytochrome *c* oxidase [1–5] have shown the presence of various signals in the EPR spectrum of the enzyme. The major signals have been carefully characterized, their intensity integrated [1–4] and assignments to paramagnetic components in the enzyme have been made. However, less is known about the additional signals observed in some preparations [2,3]. In this communication we report that in partially reduced cytochrome *c* oxidase resonances are present at *g* 3.27 and *g* 3.36 which are reactive towards ligands like CO and NO. These lines are detected in substantial amounts only when Tris–(hydroxymethyl)-aminomethane is used as a buffer and at pH values above of 7.5. It is suggested that the lines at *g* 3.27 and 3.36 represent the g_z lines of low-spin heme signals derived from a complex between Tris and the heme iron of cytochrome a_3.

2. Materials and methods

Beef-heart cytochrome *c* oxidase was prepared as described before [6,7]. The absorbance coefficient of cytochrome *c* oxidase was 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [8]. The preparation contained 10–11 μmol heme *a*/g protein. Chemicals were of analytical grade, mainly obtained from British Drug Houses. NADH, grade 2, was purchased from Boehringer. The concentration was calculated with an absorbance coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ at 340 nm [9]. Tris–(hydroxymethyl)-aminomethane (Tris) and phenazine methosulphate (PMS) were from Sigma. Anaerobic titrations were performed as described [10]. EPR experiments were carried out on a Varian E-9 or E-3 spectrometer equipped with either a helium transfer system (Air Products Inc. Model LTD-3-100) with automatic temperature controller or a low-temperature device as described [11,12]. Magnetic field and microwave-power were measured as reported [10].

3. Results

When cytochrome *c* oxidase is diluted in 100 mM potassium phosphate at neutral pH and partially reduced anaerobically with NADH and PMS, EPR spectra are observed which are similar to those reported earlier [1–5]. Upon increasing the pH to 8.0 (100 mM potassium phosphate) and partial reduction of the enzyme, the intensity of the *g* 6 signal decreases to about 30–50% of the intensity observed at pH 6.80 (not shown) and a minor low-spin signal (g_z 2.6, g_y 2.2 and g_x 1.86) appears with a clear splitting into two lines which may be assigned to hydroxyl species of cytochrome oxidase [3]. These observations are in line with the results [3,4]. In addition, a new broad resonance weak in intensity is observed at *g* 3.3.

The latter resonance (fig.1, trace A) showing clear peaks at *g* 3.27 and *g* 3.36, increases considerably in intensity when the enzyme is partly reduced in 100 mM Tris–SO_4, pH 8.1. Concomitantly a decrease is observed in intensity of the signals near *g* 6 to about 30% in comparison to those found in 100 mM potassium phosphate at pH 8.0. The new resonances near *g* 3.27 and *g* 3.36 appear only on partial reduction of the enzyme, disappear on complete reduction of the enzyme and parallel closely the redox behav-

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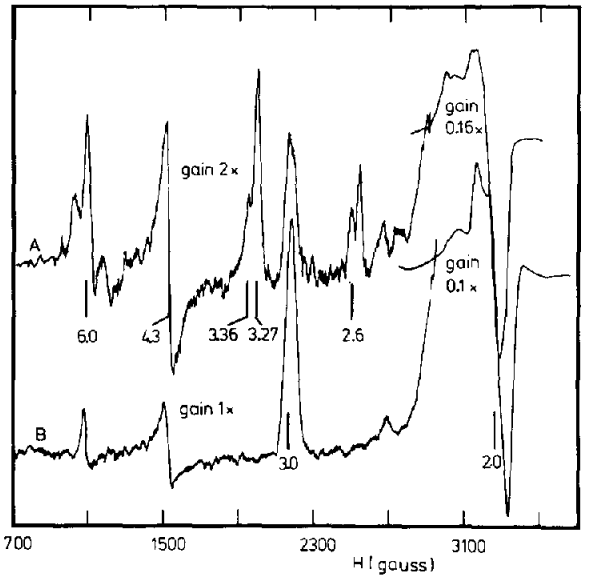

Fig.1. Effect of Tris and NO on the EPR spectrum of partially reduced cytochrome *c* oxidase. (A) 0.4 mM cytochrome *c* oxidase in 100 mM Tris–SO_4 (pH 8.1). (B) after addition of NO (0.5 atm). Conditions of EPR spectroscopy were: frequency, 9.11 GHz; microwave power, 0.63 mW; modulation amplitude, 10 G; scanning rate, 500 G · min^{-1}; time constant, 1.0 s; temp., 15°K.

iour of the high-spin heme signals during reductive titrations. At higher pH values the line at g 3.36 intensifies whereas the intensity of the signals near g 6 decreases further (not shown).

An approach to identify these resonances is to add ligands to the enzyme like CO and NO that are specific for reduced cytochrome a_3. Fig.1, trace B illustrates the effect of NO on partially reduced enzyme at pH 8.1 (100 mM Tris–SO_4). As shown this ligand causes diminuation in intensity of the signals near g 6, while the low-spin heme signals with g_z 2.6, g_y 2.2 and g_x 1.86 and the resonances at g 3.27 and g 3.36 disappear completely. Concomitantly with formation of the distinctive EPR signal at g 2 of the NO compound of reduced cytochrome a_3 [13] the major low-spin heme signals (g_z 3.0, g_y 2.2 and g_x 1.5) increases in intensity. Similar changes in intensity of the various signals are observed on addition of CO to the partially reduced enzyme (not shown) which is in line with previous results [14].

4. Discussion

Our experiments demonstrate the presence of two new resonances which seem to represent some form of cytochrome *c* oxidase in equilibrium with its high-spin species as judged from the redox behaviour of these signals. It was found that they are only present in partially reduced enzyme and have maximal intensity at halfreduction (2 electrons/4 metal ions). This concept is also supported by the effects on the EPR spectrum induced by CO and NO. These ligands cause the simultaneous disappearence of both the resonances at g 3.27 and g 3.36, the low-spin heme signal with g_z 2.6, g_y 2.2, and g_x 1.86 as well as the high-spin heme signals. As proposed [10,14–16], these ligand-induced changes are likely to be due to an electron redistribution brought about by changes in the redox potentials of the heme and copper components in the enzyme upon binding of ligands to cytochrome a_3.

It is well known that the azide [1,15], sulphide [17] and cyanide complexes [18] of cytochrome a_3 are detectable only in partially reduced enzyme while the intensity of the high-spin signals is decreased. This suggests that the resonances near g 3.3 observed in this study are due to a complex between cytochrome a_3 and a ligand. Since these signals are only observed in substantial amount in the presence of Tris, the ligand may be the amine group of this compound. This suggestion is supported by the observations [19] which showed that heme iron liganded to both ϵ amino and imidazole in ferrihemochromes is low-spin having g_z values ranging from 3.3–3.4. Thus, the resonances near g 3.3 observed in cytochrome *c* oxidase may represent the g_z lines of low-spin heme complexes of cytochrome a_3 with the primary amine group of Tris. In this context it is interesting to note that Tris has been reported to act as an exogenous ligand with cytochrome c_3 [20].

The g_y and g_x are not resolved in our spectra. It is likely that g_y is obscured by the copper signal at g 2 and that the g_x is too broad to be detected since it is located at very high magnetic field [19]. Therefore determination of the amount of heme *a* involved [21] is not possible under our conditions.

The observations that the signals near g 3.3 appear only at slightly alkaline pH might suggest that they represent some OH^- derivative. However, this is

unlikely since hydroxyl hemoprotein derivatives have much more isotropic g values [22]. A simple explanation for the observed pH-dependency might be that only the deprotonated amine group of Tris (pK_a 8.3) acts as a ligand for heme iron. The weak signal observed on dilution of the enzyme in phosphate buffer (cf. Results) is probably due to the use of Tris as buffer during isolation of cytochrome c oxidase from beef heart [6,7]. Our results indicate that Tris could better not be used as a buffer for cytochrome c oxidase.

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