EFFECTS OF HEXACYANOFERRATE ON CYTOCHROME *c* OXIDASE

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1. Introduction

Tiesjema et al. [1,2] have reported distinctly different redox behaviours of cytochrome *c* oxidase in the absence and presence of cytochrome *c*. In studying this effect we obtained divergent results in redox titrations using ferrocytochrome *c* or hexacyanoferrate(II), respectively, as the reducing agent. In view of the frequent use of hexacyanoferrate in studies of cytochrome oxidase (see, for example, [1–5]), we deemed it important to investigate this effect further. While our investigation was in progress, Yu et al. [6] reported that hexacyanoferrate(II) inhibits cytochrome oxidase and also affects its optical spectrum.

Our anaerobic redox titrations show that the heme optical properties in cytochrome oxidase depends upon the reducing agent, different results being obtained with ferrocytochrome *c* and with hexacyanoferrate(II). Furthermore, the reduction with hexacyanoferrate(II) follows a complicated time course with slow changes in the optical spectrum, accompanied by a decrease in the high-spin heme EPR-signals. In addition, the EPR-signal from hexacyanoferrate(III) formed by anaerobic reduction of the oxidase differs in line-width from that obtained on mixing hexacyanoferrate(III) with the resting oxidase. These results suggest that hexacyanoferrate(III) interacts with cytochrome oxidase, thereby changing its redox properties. Thus, many results in the literature may not reflect the intrinsic properties of the oxidase but rather those of its complex with one of the mediators.

2. Materials and methods

The preparations of cytochrome oxidase and cytochrome *c* are described in [7], as well as the methods used for making anaerobic EPR-samples and integration of EPR-signals. For the millimolar extinction coefficients, reduced minus oxidized, the following values have been used: for cytochrome oxidase at 605 nm and 550 nm, 24.0 and −2.6, respectively, and for cytochrome *c* at 605 nm and 550 nm, −1.2 and 21.1, respectively.

Optical spectra were recorded with a Gilford 240 scanning spectrophotometer provided with a special cuvette holder for anaerobic experiments and coupled to an *XY*-recorder, and EPR-spectra with a Varian E-9 spectrometer.

The hexacyanoferrate(II)/(III) titrations were performed in an anaerobic cuvette with side bulbs from which enzyme additions were made. The system was freed from oxygen ($[O_2]<5\cdot10^{-7}$ M) by repeated degassing and flushing with oxygen-free nitrogen. In some experiments catalytic amounts of fungal laccase, which is readily reduced by hexacyanoferrate(II) was added in an attempt to lower the oxygen concentration further [8]. Before the degassing of the system was started, the potential of the buffer was set potentiometrically with a Calomel-Pt electrode containing 1 M KCl. When anaerobiosis had been achieved, the potential was checked from the hexacyanoferrate(III) absorption at 420 nm using a redox potential of 425 mV [9].

Titrations with ferrocytochrome *c* as reductant were either performed with the same system as used

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in the hexacyanoferrate titrations or by anaerobic additions of reductant from a syringe to a sealed cuvette containing cytochrome oxidase. In this case the cell compartment was flushed with nitrogen during the experiment. All titrations were performed at 25°C.

3. Results

A complex series of optical changes occurs when cytochrome oxidase is added anaerobically to a hexacyanoferrate(II)/(III) buffer (fig.1). There is an initial rapid increase in the absorbance at 605 nm and 445 nm, ascribable to the reduction of a heme component. The α-band reaches its maximum within five minutes and then decreases slowly for about 50 min. During the latter phase there is a simultaneous increase in the absorbance between 445 nm and 421 nm and a decrease between 421 nm and 390 nm. At low potentials the Soret band is split at a time when the α-band has its maximal strength, but at equilibrium there is a single peak at 430 nm. The spectrum obtained at equilibrium bears resemblance to that reported by Yu et al. [6]. The possibility that the decrease in the α-band should be due to a slow oxidation by oxygen was excluded by experiments in which preincubations of the hexacyanoferrate buffer with catalytic amounts of fungal laccase were made.

In an attempt to find the nature of the slow spectral changes in the anaerobic mixtures of hexacyanoferrate(II)/(III) and cytochrome oxidase we repeated the experiment with four-times higher concentrations of the reactants and followed the reaction in an EPR-spectrometer. Hexacyanoferrate buffer

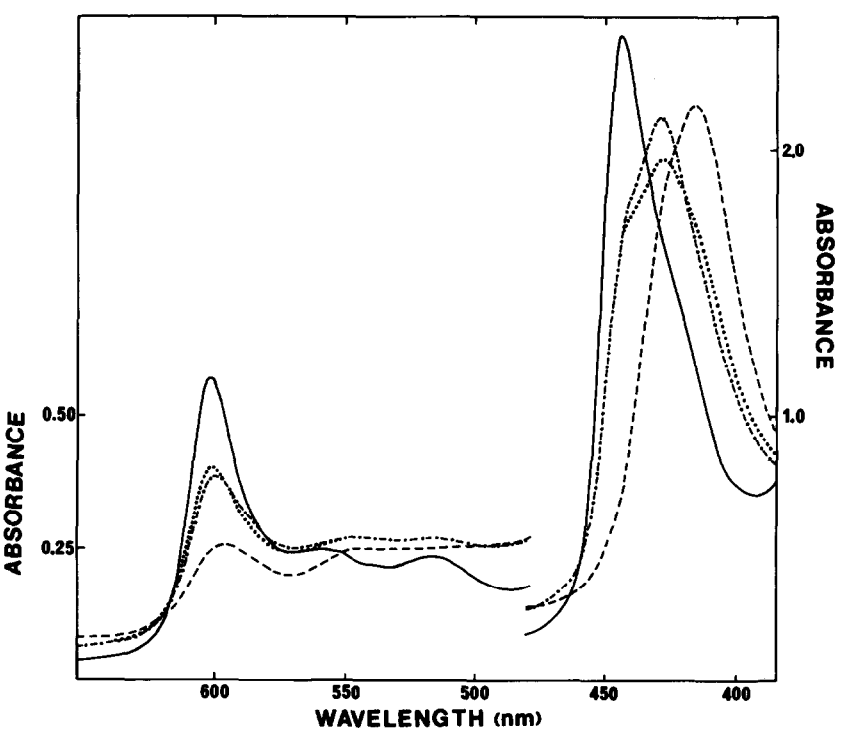

Fig.1. Optical spectrum of cytochrome oxidase after different incubation times with anaerobic hexacyanoferrate at approximately 330 mV. The potential, as well as the pH, was buffered with a solution containing 4.3 mM potassium hexacyanoferrate(II)/(III) and 0.1 M potassium phosphate, pH 6.4, 0.5% Emasol 4130. Fungal laccase was added to the buffer 15 min before cytochrome oxidase. The concentration of cytochrome oxidase and laccase were 26 μM and 0.5 μM, respectively. The optical path length was 0.5 cm. Incubation times: (· · · ·) 10 min and (·—·—·—·) 50 min. Spectra for the oxidized (- - -) and the dithionite reduced (——) oxidase are also given.

and cytochrome oxidase were mixed anaerobically and frozen in liquid nitrogen after varying incubation times, ranging from 3 min to 1 h. The only signals that show significant changes over this period are the high-spin heme signals (g 6). These signals decrease in concentration by at least 50% from 3–60 min, without any noticable change in structure. For example at 380 mV the g 6 signals decrease in concentration from 0.1–0.05 heme/functional unit.

Figure 2 shows a Nernst plot of the results of optical hexacyanoferrate(II)/(III) titrations of cytochrome oxidase, in the presence and absence of cytochrome c, followed at 605 nm. The pH, 6.4, was chosen to obtain a high activity of laccase, but similar results were obtained at pH 7.4, the condition used in the titrations with ferrocytochrome c (see below). In experiments with starting potentials lower than about 340 mV the buffer capacity is not sufficient, so that there is a potential change due to the reduction of the oxidase. In these cases, however, one is sufficiently close to the redox potential of cytochrome c, 255 mV [10], to use this protein as a potential probe. As can be seen at the higher potentials, the redox behaviour of cytochrome oxidase is the same whether cytochrome c is present or not. A simulation of the titration, on the assumption that a high potential chromophore contributes 50% of the spectral change, is included into fig.2.

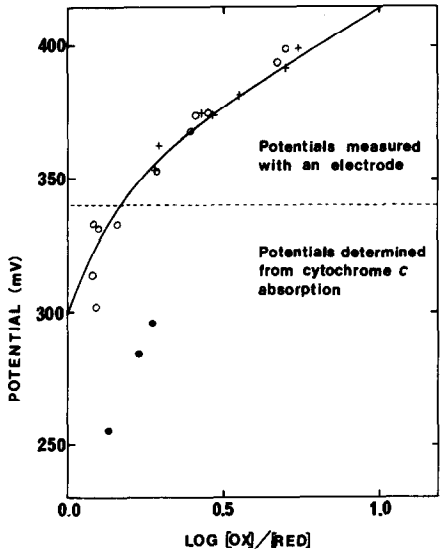

Fig.2. Nernst plot of titrations of the 605 nm band with hexacyanoferrate(II)/(III) buffer (in some of which stoichiometric amounts of cytochrome c were present) and titrations with different amounts of ferrocytochrome c. Hexacyanoferrate(II)/(III) titrations were performed with the same buffer and enzyme concentrations as in fig.1. The ratio [ox]/[red] was estimated from the fractional change in absorbance at 605 nm. Above 340 mV the potentials of the solutions were determined with an electrode and below this value from the ratio of oxidized to reduced cytochrome c. (+) and (○) represent experiments performed in the absence and presence of cytochrome c, respectively. The simulation (——) was made for two electron acceptors at 375 mV and 225 mV with equal absorbance contributions (see ref. [11]). Titrations with ferrocytochrome c as reductant (•) in 0.1 M potassium phosphate, pH 7.4, 0.5% Emasol 4130. The concentration of cytochrome oxidase was 10 μM or 20 μM, and the potentials were obtained from the degree of reduction of cytochrome c.

Anaerobic titrations of cytochrome oxidase with ferrocytochrome c leads to equilibrium after at most 20 min, i.e., no further changes in optical spectra or EPR-signals occur after this time. Three points from this kind of titration are included in fig.2. The potentials have been calculated from the ratio of reduced to oxidized cytochrome c. These results are consistent with the assumption that the species with the highest potential contributes 35–40% of the change at the α-band on dithionite reduction.

When cytochrome oxidase preincubated with hexacyanoferrate(II) or (III) (5–10 mM) is reduced with dithionite aerobically, the Soret band does not attain the same strength as in the absence of hexacyanoferrate. However, if dithionite reduced cytochrome oxidase is mixed anaerobically with hexacyanoferrate(II) no effect on the optical spectrum is observed. If oxygen is allowed into this mixture the Soret band decreases even in the presence of large excess of dithionite.

We have also performed anaerobic EPR titrations of cytochrome oxidase with hexacyanoferrate(II)/(III) at several potentials between 420 and 300 mV, freezing the sample after 20 or 30 min. The course of the disappearance of the copper (g 2) and the low-spin heme (g 3) signals does not differ from other kinds of titrations, i.e., the g 3 signal disappears before the g 2 signal. Of course, the heights of the g 6 signals at these long incubation times are very small.

The hexacyanoferrate(III) signal with a peak at 250 mT formed in the reduction of cytochrome

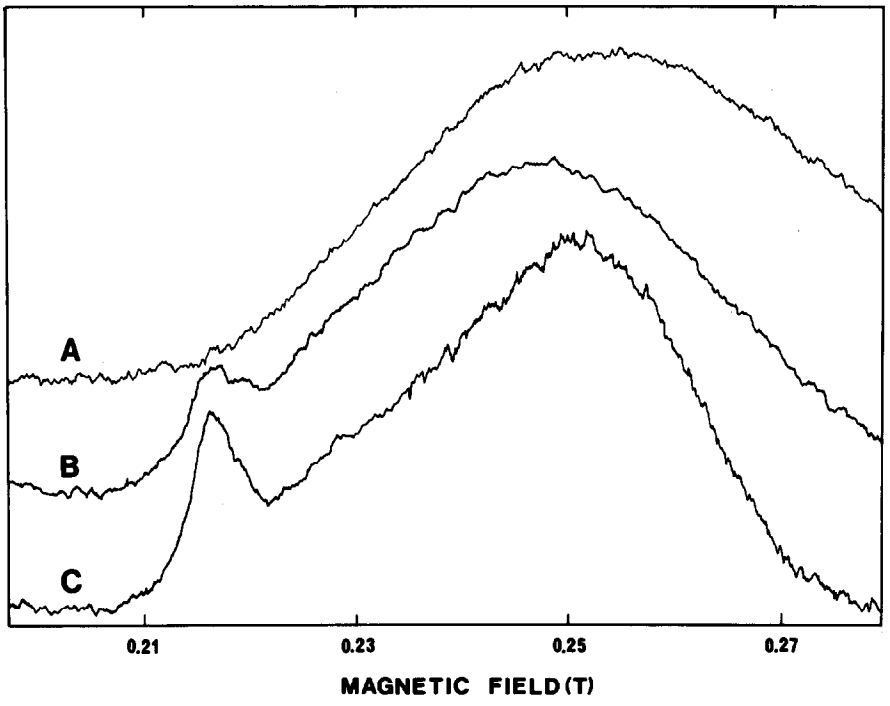

Fig.3. EPR-Spectra of the hexacyanoferrate(III) signal in the presence and absence of cytochrome oxidase. The buffer solution contained 20 mM potassium hexacyanoferrate(II)/(III) and 0.1 M potassium phosphate, pH 7.4, with 0.5% Tween-80. The concentration of cytochrome oxidase was 100 μM. (A) Buffer at 330 mV; (B) cytochrome oxidase in buffer at approximately 330 mV; (C) cytochrome oxidase in buffer at approximately 310 mV. In (B) and (C), buffer and cytochrome oxidase were mixed anaerobically and frozen in liquid nitrogen after 20 min. In order to simplify the comparison of the line shapes, the height of the hexacyanoferrate(III) signals were normalized by adjustment of the gain. To minimize the contribution of the low-spin heme signal at 250 mT, a high (200 mW) microwave power was chosen. The spectra were recorded at the following conditions: microwave frequency, 9.13 Hz; modulation amplitude, 2 mT; temperature, 15°K.

oxidase (fig.3) is much more narrow and slightly shifted compared to the hexacyanoferrate(III) signal in pure hexacyanoferrate(II)/(III) buffer or the signal obtained on mixing oxidized cytochrome oxidase and hexacyanoferrate(III) aerobically (not shown).

4. Discussion

Several redox titrations of cytochrome oxidase have been carried out in recent years. Some of them (for example, [1,3]) have indicated the presence of one high-potential heme, the reduction of which contributes about 50% of the total spectral change of the α-band on full reduction. This picture is in agreement with our hexacyanoferrate(II)/(III) titrations (fig.2). The presence or absence of cytochrome *c* does not affect the results (fig.2, cf. [1,2]), but if the titration is carried out with ferrocytochrome *c* as the reductant without hexacyanoferrate, then a distinctly different redox behaviour is observed. The difference could be the result of the binding of hexacyanoferrate affecting the heme absorption bands, though this is not a unique interpretation.

The EPR-results in fig.3 strengthen the suggestion that the effect observed is caused by the binding of hexacyanoferrate(III) to the partially reduced oxidase. Of course, from this kind of experiment one cannot exclude the possibility that hexacyanoferrate(II) interacts with the enzyme, as suggested by Yu et al. [6]. It may be noted that changes in the hexacyanoferrate(III) EPR-signal has also been observed in redox

titrations of tree laccase (B. Reinhammar, unpublished results) and that hexacyanoferrate affects the redox potentials of this enzyme [12].

A striking difference in the results with the hexacyanoferrate system as redox buffer, compared to the titrations with ferrocytochrome *c* alone, are the slow changes in the optical spectrum (fig.1), which continue for about one hour. They are parallelled by the disappearance of the *g* 6 signals. Possible corresponding changes in the other EPR-signals cannot be detected.

Yu et al. [6] observed spectral changes with the dithionite-reduced oxidase on the addition of hexacyanoferrate(II). We found that such changes take place only if oxygen is present, indicating either that it is hexacyanoferrate(III) which becomes bound or that binding occurs exclusively to the partially reduced oxidase.

In view of the complications in the redox behaviour of cytochrome oxidase introduced by the hexacyanoferrate system, we consider it important to study the redox properties of the enzyme with the natural substrate, cytochrome *c*, as the sole reductant. Further investigations of this kind are in progress in our laboratory.

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