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A comparison of three preparations of cytochrome c oxidase. 
Optical absorbance spectra, EPR spectra and reaction towards ligands

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Abstract

Three preparations of cytochrome c oxidase, the preparation as traditionally prepared in our laboratory as described by Van Buuren (1992; PhD Thesis, University of Amsterdam), a preparation according to Volpe and Caughey (Biochem. Biophys. Res. Commun. 61 (1974) 502-509) and a preparation of ‘fast’ cytochrome c oxidase (Brandt, U., Schiigger, H. and Von Jagow, G. (1989) Eur. J. Biochem. 182, 705-711), are compared in their reaction with cyanide and carbon monoxide. The reaction with cyanide is nearly as fast for the Van Buuren preparation as for the ‘fast’ preparation, but much slower for the Volpe-Caughey preparation. Mixed-valence cytochrome c oxidase (cytochrome a3 and CuB reduced with carbon monoxide bound and cytochrome a and CuA oxidized) is prepared by anaerobic incubation with carbon monoxide. With the Van Buuren preparation complete formation of the species takes 4 h, whereas with the Volpe-Caughey preparation it takes 20 h. Longer incubation under CO results in partial reduction of cytochrome a and CuA. With the ‘fast’ preparation mixed-valence cytochrome c oxidase is formed after more than one day of incubation with CO, but it is stable for at least 3 days. The presence of oxidized cytochrome c did enhance the reactivity towards cyanide and towards carbon monoxide in cytochrome c oxidase of all three preparations. Furthermore, optical and EPR spectra of the preparations of cytochrome c oxidase are compared. The Volpe-Caughey preparation has an intense g’= 12 EPR-signal, the Van Buuren preparation has hardly any g’= 12 signal and the ‘fast’ preparation has no g’= 12 signal. In the ‘fast’ preparation the low-spin heme signal is shifted (from g = 3.00 to g = 2.97). The absorbance spectra of the three preparations in the Soret region are similar with a maximum at 424 nm. Only the ‘fast’ preparation as isolated was completely oxidized, whereas the other preparations were partially reduced. It was concluded that differences in the reaction of cytochrome c oxidase with ligands are determined by the internal or external ligand bound to the cytochrome a3-CuB couple.

Key words: Cytochrome c oxidase; Ligand; Preparation method; Cyanide; Optical absorbance spectroscopy; EPR

1. Introduction

Cytochrome c oxidase, the last enzyme in the respiratory chain, catalyses the oxidation of cytochrome c by reducing oxygen to water. Cytochrome c oxidase contains at least four metal centres; two cytochromes, cytochrome a and cytochrome a3, and two copper atoms CuA and CuB. Cytochrome a and/or CuA accept electrons from cytochrome c and the electrons are subsequently transferred to the haem a3-CuB couple. This couple binds oxygen and reduces oxygen by twice transferring two electrons.

The redox-couple cytochrome a3-CuB binds various ligands in the reduced state as well as in the oxidized state. Depending on the nature of the ligand, a certain redox state is stabilized. For example, carbon monoxide stabilizes the reduced state, whereas ligands such as azide and formate stabilize the oxidized state. Cyanide binds to oxidized as well as to reduced cytochrome a3-CuB. Carbon monoxide can be used in stabilizing fully reduced (4 e−) or mixed-valence (2 e−) cytochrome c oxidase.

Internal electron transfer was found to be slow in some preparations, the so-called resting or ‘slow’ cytochrome c oxidase [1,2,5–7]. Resting cytochrome c
oxidase is heterogeneous and the properties of ligand binding or spectral properties vary slightly among those 'slow' preparations [8–10]. During turnover or after reduction and subsequent reoxidation cytochrome c oxidase is turned into the 'pulsed' or 'fast' form [11], which has fast internal electron transfer and reacts rapidly with cyanide and carbon monoxide [4,8,11]. The Soret maximum of 'slow' cytochrome c oxidase is blue-shifted compared to that of the 'fast' enzyme, which has its maximum at 424 nm. 'Slow' preparations are characterized by a $g' = 12$ EPR signal, a low rate of cyanide binding and slow internal electron transfer [4,10]. The $g' = 12$ EPR signal is accompanied by a broad signal at $g' = 2.95$ [12,13].

Recently, isolation methods for cytochrome c oxidase that result in 'fast' preparations have been described [3,4,10]. Those preparations turn into the 'slow' conformation upon incubation at low pH or after addition of formate [3,4,14,15]. Even the binding of cyanide to the 'fast' form of cytochrome c oxidase is not as

![Fig. 1. Spectra of the Soret-band of oxidized cytochrome c oxidase. (A) Absolute spectra; (B) Second derivative spectra. (-----), Van Buuren preparation; (-----), Volpe-Caughey preparation; (-----), 'fast' preparation. The experimental conditions are described in detail in the Materials and methods section.](image-url)
rapid as inhibition of the enzyme by this ligand during turnover. The transition of 'slow' to 'fast' cytochrome c oxidase was attributed to reduction of both cytochrome a and Cuₐ [16,17] or of Cu₉ [18], which would induce a conformational change.

Cytochrome c was mentioned to trigger the transition of 'slow' into 'fast' cytochrome c oxidase [19] and to accelerate binding of cyanide to cytochrome c oxidase [20,21]. The effect of cytochrome c would be to induce a conformational change when bound to cytochrome c oxidase [22]. A 'fast' and a 'slow' preparation were studied by Moody et al. [4]. It was found [23] that a certain fraction, that was one-electron reduced, reacted just as fast with cyanide as cyanide inhibits the enzyme during turnover. The presence of cytochrome c stimulated this extremely fast binding of cyanide, since this cytochrome mediate electron transfer between cyanide-bound one-electron reduced and oxidized molecules [23].

The preparation, mostly used in our laboratory, was isolated according to Van Buuren [1,24] and has properties of 'slow' cytochrome c oxidase. The Volpe-

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**Fig. 2.** Spectra of the alpha-band of cytochrome c oxidase (left panels) and difference spectra of oxidized minus reduced cytochrome c oxidase between 620 and 820 nm (right panels). (---) oxidized cytochrome c oxidase as isolated; (-----) and (------) ferricyanide-oxidized cytochrome c oxidase. (A) Van Buuren preparation; (B) Volpe-Caughey preparation; (C) 'fast' preparation. The experimental conditions are described in detail in the Materials and methods section.
Caughter preparation is also a ‘slow’ preparation, but it has a higher respiratory control ratio when reconstituted in vesicles [25]. For both ‘slow’ preparations [1,2] a mitochondrial membrane precipitate is used and the protein is solubilized by detergents and purified by ammonium sulphate precipitation steps. For purification of cytochrome c oxidase according to the method of Brandt et al. [3] mitochondria are used as starting material and cytochrome c oxidase is purified using hydroxy apatite. During the isolation procedure [3] the pH never drops below 7.2, which results in a ‘fast’ cytochrome c oxidase preparation. From those three preparations, optical and EPR spectra were compared as well as the reaction with cyanide and carbon monoxide. The effect of the presence of cytochrome c on the reaction towards ligands was also studied for the three preparations.

### 2. Materials and methods

Cytochrome c oxidase was isolated from bovine heart according to three procedures [1–3]. Cytochrome c was isolated from horse heart according to [26] or was purchased from Sigma, USA (horse heart type VI). The concentrations were determined spectrophotometrically using an ε_{550nm} (red-ox) of 21.1 mM \(^{-1}\) cm \(^{-1}\) for cytochrome c [27] and an ε_{605nm} (red-ox) of 24 mM \(^{-1}\) cm \(^{-1}\) for cytochrome c oxidase [28]. Chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or BDH (UK). CO was obtained from Matheson (USA).

Optical absorbance measurements were carried out with a Hewlett-Packard diode array spectrophotometer (8452A). With this instrument spectra can be taken between 190 and 820 nm. For this reason it was not possible to measure at 830 nm the absorbance maximum of the Cu A band. EPR measurements were carried out on a Bruker ECS106 spectrometer, equipped with a cryostat of Oxford Instruments.

For measuring optical spectra, the concentration of cytochrome c oxidase was 6–20 µM. Ferricyanide-oxidized spectra were recorded after addition of a few grams of potassium ferricyanide and dithionite-reduced cytochrome c oxidase was prepared by adding a few grams of sodium dithionite. When no further absorbance changes occurred the spectrum was recorded.

The method of preparing mixed-valence carboxy-cytochrome c oxidase by incubating the ‘resting’ enzyme under carbon monoxide has been described [29]. A 6 µM cytochrome c oxidase solution, in 100 mM potassium phosphate (pH 7.4) and 0.5% Tween-80 was made anaerobic by flushing with He, and was subsequently incubated at a CO-pressure of 50 cmHg for 6–20 h (at 20°C). In some experiments cytochrome c was present in a 1:1 ratio with cytochrome c oxidase.

In the reaction with cyanide a 2 µM solution of cytochrome c oxidase in phosphate buffer (100 mM, pH 7.4) was used either in the absence or in the presence of 2 µM cytochrome c. From a neutralized stock-solution (1 M potassium cyanide) 25 mM of KCN were added to the cuvette. The reaction with cyanide was measured at 434 minus 412 nm for 2 min [3].

### 3. Results and discussion

Fig. 1 shows spectra of oxidized cytochrome c oxidase in the region between 320 and 520 nm. The absolute spectra are shown in panel A and the second derivative spectra in panel B. No large differences are observed in the absolute spectrum of the Soret-bands of the three preparations of cytochrome c oxidase. The maximum is at 424 nm for all preparations, but the shape of the band is not the same. The second-derivative spectra show that the largest differences are found in the region between 410 and 430 nm. The three preparations have a negative band at 429 nm, as also was found by Sherman et al. [30]. In addition, the Van Buuren preparation has a shoulder at 414 nm and the Volpe-Caughey preparation has a shoulder at 412 nm. This shoulder is missing from the spectrum of the ‘fast’ preparation, which indicates a difference at the cytochrome a\(_3\) site between the three preparations [30].

Fig. 2 shows absolute spectra in the region of 520 to 820 nm of oxidized (as isolated) and ferricyanide-oxidized cytochrome c oxidase. The effect of ferricyanide on the spectrum shows clearly that the ‘fast’ preparation (panel C) is completely oxidized and that the Van Buuren preparation (panel A) and the Volpe-Caughey preparation (panel B) are partially reduced. Upon addition of ferricyanide to the Van Buuren and Volpe-Caughey preparations the absorbance at 600 nm decreases and the absorbance at 655 nm increases. The maximum of the a-band in both the Van Buuren preparation after oxidation by ferricyanide and the ‘fast’ preparation was found to be at 598 nm. In the Volpe-Caughey preparation, however, the absorbance maximum remains at 600 nm after oxidation by ferricyanide and the intensity of the band is high when compared to the absorbance at 598 nm in the other two preparations.

Fig. 2 also shows difference spectra of oxidized (as isolated) and ferricyanide-oxidized minus reduced cytochrome c oxidase in the region of 620 to 820 nm in order to compare the Cu A band, with its maximum at 830 nm, and the 655 nm band, that is attributed to the oxidized cytochrome a\(_3\)-Cu B couple [31]. The left flank of the 830 nm band is not affected by ferricyanide and is the same for all three preparations. This indicates that the conformation of Cu A is not affected by the isolation procedure and moreover that Cu A was not
reduced in the partially reduced preparations. The absorbance band with its maximum at 655 nm differs considerably among the three preparations and is affected strongly by oxidation with ferricyanide. Before reoxidation by ferricyanide the 655 nm band of the Van Buuren preparation (panel A) corresponds to that of the 'fast' preparation (panel C). After addition of ferricyanide, however, the absorbance at 655 nm of the Van Buuren preparation increases, whereas the absorbance of the 'fast' preparation is unaffected. The absorbance at 655 nm relative to the absorbance at 710 nm is higher for the Volpe-Caughey preparation (panel B) than for the other two preparations and it increases upon addition of ferricyanide. Since Schoonover and Palmer [15] reported that upon addition of formate to a 'fast' preparation of cytochrome c oxidase [10] the 655 nm band shifts to 665 nm, this high absorbance at 655 nm indicates that in the Volpe-Caughey preparation a formate-like ligand, such as a carboxyl group [4], could be bound to the cytochrome a3-CuB couple. The fact that upon oxidation of the partially reduced preparations by ferricyanide the absorbance at 655 nm is increased indicates that the cytochrome a3-CuB couple is partially reduced. The absorbance decrease at 605 nm found upon addition of ferricyanide to oxidized cytochrome c oxidase, as isolated, can also be attributed to oxidation of cytochrome a3 or to oxidation of CuB that could affect the absorbance of cytochrome a3 in this region. The results suggest that in the partially reduced preparations cytochrome a as well as CuA are completely oxidized.

EPR spectra of the three preparations between almost zero field and high field are shown in Fig. 3. The Van Buuren preparation (spectrum A) has a very small g' = 12 signal and the Volpe-Caughey preparation (spectrum B) has a large g' = 12 signal, whereas in the 'fast' preparation (spectrum C) no g' = 12 signal is present.

In the EPR spectrum of the Volpe-Caughey preparation (Fig. 3, spectrum B) a radical signal at g = 2 is observed as well as some additional signals at g = 1.7 and g = 1.8. These signals were found previously for an almost completely oxidized intermediate in the reaction of reduced carbon monoxide cytochrome c oxidase by oxygen [32]. Addition of formate to oxidized cytochrome c oxidase induces the development of broad EPR signals at g' = 12 and at g' = 2.9 [13]. Those EPR signals are related to the activation state of cytochrome c oxidase [12,15] and to the ligand bound to cytochrome a3-CuB [4]. The g' = 2.95 is only clearly discerned in preparations with a very intense g' = 12 [4]. However, in the EPR spectrum of the Volpe-Caughey preparation the broad signal at g' = 2.9 was not observed. Schoonover and Palmer [15] observed that shortly after addition of formate the g' = 12 EPR signal arises, whereas the shift of the Soret band to shorter wavelength is much slower. This intermediate form in formate binding might be related to the ligand state of the cytochrome a3-CuB couple in the Volpe-Caughey preparation.

The cytochrome a EPR signal of the 'fast' preparation (Fig. 3, spectrum C) has a slightly smaller rhombicity than that of the other preparations, g = 2.97, 2.22, 1.47 versus g = 2.99, 2.20, 1.44, and the signals are sharper. Also the high-spin haem signal of the 'fast' preparation at g = 6 has a higher intensity than that of both other preparations. Both an intense g = 6 EPR signal and a smaller rhombicity are described for 'slow' cytochrome c oxidase as induced by incubation at low pH or after addition of formate [15] and for a 'fast' as well as for a 'slow' preparation in the presence of chloride [4 and 33, respectively]. Since no g' = 12 EPR signal is noticeable and since, during the isolation procedure chloride is present and the pH was kept high, we conclude that the preparation as prepared according to Brandt et al. [3] is a 'fast' preparation with a chloride bound to the cytochrome a3-CuB couple.

Fig. 4 shows the difference spectra recorded at various times after addition of cyanide to oxidized cytochrome c oxidase. Upon addition of cyanide to the Van Buuren preparation (panel A) the absorbance at
412 nm decreases and at 434 nm it increases. About 80% of these changes occur in the first 2 min and they reach their maximum within 30 min. Between 0.5 and 2 h after the addition of cyanide some absorbance increase is observed at 444 nm.

For the Volpe-Caughey preparation and the 'fast' preparation difference spectra are also shown at a short time after addition of cyanide (Fig. 4, panel B and panel C). A few seconds after addition of cyanide to those preparations an absorbance decrease is found at 426 nm and an absorbance increase at 450 nm. About ten seconds after addition of cyanide a trough starts to develop at 412 nm and a further increase is observed at 450 nm. From then on continuous absorbance changes at 412 and at 444 nm are noticeable for the Volpe-Caughey preparation (panel B). For the 'fast' preparation the absorbance at 412 nm also decreases further, but the maximum of the absorbance increase shifts to 434 nm. The absorbance at 434 nm is maximal after 3 min. Upon longer incubation of the 'fast' preparation, 0.5 or 2 h, there is still a slight decrease of the absorbance at 412 nm and an absorbance increase at 450 nm, which causes a shift of the maximum to 440 nm.

In the binding of cyanide two processes seem to be involved. One causes an absorbance increase at 434 nm and another causes an absorbance increase at 450 nm. Concomitantly with both changes the absorbance at 412 nm decreases. The most rapid reaction at 434 nm is found for the 'fast' preparation, whereas the reaction at 450 nm is fastest in the Volpe-Caughey preparation. The 434 nm reaction in the Van Buuren preparation is at an intermediate rate, whereas the 450 nm reaction is slowest in this preparation.

The time courses of the binding of cyanide to cytochrome c oxidase, as measured at 434 minus 412 nm, in the absence and in the presence of cytochrome c, are shown in Fig. 5. Cytochrome c itself can also react with cyanide and therefore the absorbance changes in the presence of cytochrome c were corrected for this reaction. The fastest binding reaction is observed for the 'fast' preparation (panel C). Although the rate is nearly twice as high in the presence of cytochrome c it is by far not as high as that of cyanide inhibition during turnover. This might be related to the fact that this preparation is completely oxidized, since Mitchell et al. [23] reported that the one-electron reduced enzyme is the state that binds cyanide fastest. On the other hand, the presence of chloride may play a role in the rate of cyanide binding and in the effect of cytochrome c on this rate. The Van Buuren preparation also reacts relatively fast with cyanide and the rate is enhanced about 3 times in the presence of cytochrome c (panel A). The Volpe-Caughey preparation reacts slowly and the rate is only slightly enhanced in the presence of cytochrome c (panel B). It is interesting to note that the reduction of the Volpe-Caughey preparation, that occurs slowly during cyanide binding in the absence of cytochrome c, is largely enhanced in the presence of cytochrome c (results not shown). The fact that the Volpe-Caughey preparation and the Van Buuren preparation are partially reduced, apparently does not result in strong stimulation of cyanide binding by cy-

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![Difference spectra of cytochrome c oxidase at various time intervals after addition of 25 mM cyanide to the oxidized enzyme.](image)
cytochrome c [cf. 23]. Furthermore, cytochrome c does not induce the transition of a 'slow' preparation into a 'fast' preparation.

A further differentiation between the preparations is the formation of mixed-valence cytochrome c oxidase that is formed upon incubation of the oxidized enzyme with carbon monoxide under anaerobic conditions (results not shown). For the Van Buuren preparation the conversion to the mixed-valence state takes 7 h in the absence of cytochrome c, whereas the formation of the mixed-valence species in the Volpe-Caughey preparation in the absence of cytochrome c takes 20 h. In the presence of cytochrome c the conversion was much faster and was completed within 7 h. The mixed-valence state of 'fast' cytochrome c oxidase is formed only very slowly; it takes over 27 h. However, in the presence of cytochrome c the reaction is enhanced and the conversion to the mixed-valence state takes about 20 h. The formation of mixed-valence cytochrome c oxidase is very slow for the 'fast' preparation. Even in the presence of cytochrome c that enhances this reaction, mixed-valence cytochrome c oxidase is formed only after one day of incubation with carbon monoxide. This might be caused by the presence of chloride at the cytochrome $a_3$-$Cu_B$ couple or by the fact that the preparation is completely oxidized.

A shoulder is observed at 450 nm in the difference spectrum of mixed-valence minus oxidized cytochrome c oxidase in the absence of cytochrome c, but not in its presence. Absorbance at 450 nm was also found in the reaction of cytochrome c oxidase with cyanide in the absence of cytochrome c. Reduction of cytochrome $a$ can be excluded, because no absorbance increase at 605 nm was observed (results not shown). The absorbance at 450 nm can be caused by a conformational change at cytochrome $a$ as described to occur during turnover [34]. The absorbance band was also described to be present in cyanide-bound cytochrome c oxidase with cytochrome $a$ reduced, but not in reduced or reduced CO-bound cytochrome c oxidase [35,36]. The band disappears upon addition of cytochrome c [35] or at low pH [36]. It is likely that this band is related to a certain conformation of cytochrome c oxidase induced by binding of a ligand at the cytochrome $a_3$-$Cu_B$ site that is released when cytochrome c is present [36]. Since in our experiments cytochrome $a$ is oxidized we suggest that the 450 nm band also represents a certain conformation of oxidized cytochrome $a$.

An overview of the properties of the three preparations of cytochrome c oxidase studied in this paper is presented in Table 1. The Van Buuren preparation has its Soret-band maximum at 424 nm and a $g' = 12$ EPR signal with very low intensity. Although those spectral properties are an indication for 'fast' cytochrome c oxidase, the reaction towards cyanide is slow. A bridging ligand could be present at the cytochrome $a_3$-$Cu_B$

<table>
<thead>
<tr>
<th>Feature</th>
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<tr>
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<tr>
<td>Soret-band maximum</td>
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<tr>
<td>EPR signal at $g' = 12$</td>
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<tr>
<td>$g$ values of low-spin heme signal</td>
<td>2.99, 2.20</td>
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<tr>
<td>Additional signals</td>
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<td>Cyanide binding rate</td>
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<td>Cyt. c-enhancement</td>
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<td>Mixed-valence formation</td>
<td>4 h</td>
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<td>Idem + cytochrome c</td>
<td>n.d.</td>
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Table 1
An overview of the properties of three preparations of cytochrome c oxidase

The Van Buuren preparation, the Volpe-Caughey preparation and the 'fast' preparation were prepared according to [1], [2] and [3], respectively.
couple in this preparation [37,38]. The Volpe-Caughey preparation has an intense \( g' = 12 \) EPR signal, which is indicative of a 'slow' preparation, but the Soret-maximum is at the same position as that of 'fast' cytochrome \( c \) oxidase [10]. The reactivity towards ligands is slow. A carboxyl-group might be present as ligand at the cytochrome \( a_3 \)-Cu-B couple.

The 'fast' preparation, as studied by us, is completely oxidized and shows no \( g' = 12 \) EPR signal, but the cytochrome \( a_3 \)-Cu-B couple. The 'fast' preparation reacts fast with cyanide but its reactivity towards carbon monoxide is extremely slow. The difference in reactivity of the 'fast' preparation towards cyanide and carbon monoxide can be explained by the fact that cyanide only has to bind to the cytochrome \( a_3 \)-Cu-B couple, whereas carbon monoxide has to reduce this site first. Since the cytochrome \( a_3 \)-Cu-B couple is not the natural entrance site for electrons, we conclude that the 'fast' preparation is most close to native cytochrome \( c \) oxidase as present in mitochondrial membranes.

We conclude that the isolation procedure that is used might change the ligand present at the cytochrome \( a_3 \)-Cu-B site, which determines the reactivity of cytochrome \( c \) oxidase towards ligands. It is obvious that in order to define whether a preparation of cytochrome \( c \) oxidase is 'fast' or 'slow', it is important to study more than one of the characteristics of 'slow' cytochrome \( c \) oxidase, such as the \( g' = 12 \) EPR signal, the maximum of the Soret band or the rate of cyanide binding.

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