THE INVOLVEMENT OF THE FULLY OXIDIZED STATE IN CYTOCHROME OXIDASE REACTION WITH OXYGEN STUDIED WITH THE 655 nm BAND AS A PROBE

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1. Introduction

The use of a low temperature technique as developed by Chance et al. [1] led to the discovery of intermediates in the reaction of cytochrome oxidase with oxygen [2–5]. The reaction is initiated by photolysis of CO bound mitochondria in presence of oxygen. From both the fully reduced state and the mixed valence state the reaction yields first the formation of Compound A [4] which is accounted for by O_2 binding. The following reaction gives rise to different spectral changes depending on the starting redox level: the mixed valence state leading to Compound C formation [4] and the fully-reduced state to Compound B [4]. We describe elsewhere the spectral properties of these intermediates (manuscript in preparation). One questionable point has been the redox level of hemes *a* and a_3 in the stage of Compound C and Compound B formation. Several identifications have been proposed [4–9] which did or did not involve the fully oxidized state. On the basis of their heme-heme interaction model, Wikstrom et al. [8] suggested that the mechanism of oxygen reduction by cytochrome oxidase does not involve the fully oxidized form. Recently Beinert et al. [10] characterized specifically the a_3^{3+} form with the 655 nm band that they pointed out previously by reflectance spectroscopy [11]. In this study this 655 nm band is used as a probe of the a_3^{3+} state in Compound B and Compound C formation.

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The lack of the 655 nm band has been pointed out already [12] in Compound C formation. Here we present the evidence for the formation of this band after Compound A formation from the fully reduced state.

2. Materials and methods

The technique, a modification [12] of the previous low temperature trapping [1,4] will be described in detail elsewhere (manuscript in preparation). However, in this particular study the scanning investigation has been done at fixed temperature.

The beef heart mitochondria were kindly provided by Dr C. P. Lee. The succinate reduced mitochondria are bound to CO and suspended in 30% ethylene glycol. A 30% ethylene glycol oxygenated solution (2 mM O_2) is mixed with mitochondria at −20°C in a few seconds and the samples are promptly frozen at about −80°C. The experiments are done in the frozen state at different temperatures. Our purpose here being the study of the last stage of the reaction rather than the beginning involving Compound A, we used the wavelength scanning technique at temperatures as high as −40°C. All chemicals used were reagent grade.

3. Results

In ferricyanide supplemented mitochondria the formation of Compound C in the frozen state does

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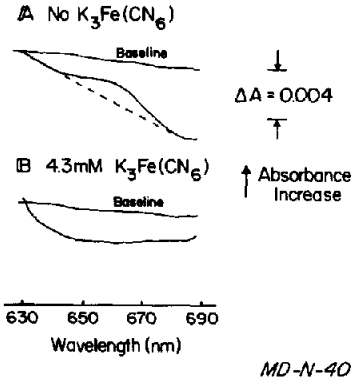

Fig.1. Difference spectra after CO dissociation from cytochrome oxidase in the fully-reduced state (A) and mixed-valence state (B) at −40°C. Photolysis is operated with a 200 J xenon flash lamp. The reference spectrum is the state before photolysis. Final concentrations in the samples: 10 mg/ml beef heart mitochondria, 25 mM inorganic phosphate, 2.5 mM succinate, 0.6 mM carbon monoxide and 1 mM oxygen. In the case of fig.1B potassium ferricyanide is added at −20°C to the reduced CO bound mitochondria 30 s before the oxygenated solution.

not give rise to the 655 nm band as shown on fig.1B. In the absence of ferricyanide, the result of the reaction at the same temperature, −40°C, shows a well-defined band superimposed on a distorted background level compared with the baseline. A dotted line has been drawn as an empirical correction [13] in order to give a better representation of this band. Although the A_{max} appears to be closer to 660 nm than 655 nm we keep the previous 655 nm band label [10] to avoid confusion.

The investigation has been carried out mainly in the range −40°C to −80°C. At lower temperature the spectral changes at 655 nm becomes less significant as the decrease at 606 nm following the formation of Compound A becomes also barely detectable.

Figure 2 displays the result that the absorbance increase of the 655 nm band follows the decrease at 606 nm. The trace 7 has been drawn with a different origin on the chart recorder to enhance the resulting band, the progression of which remaining quite clear at −75°C. We must point out that the decrease at 606 nm is accompanied also by a significant absorbance decrease at 549 nm due to oxidation of cytochrome *c*. We shall demonstrate elsewhere (manuscript

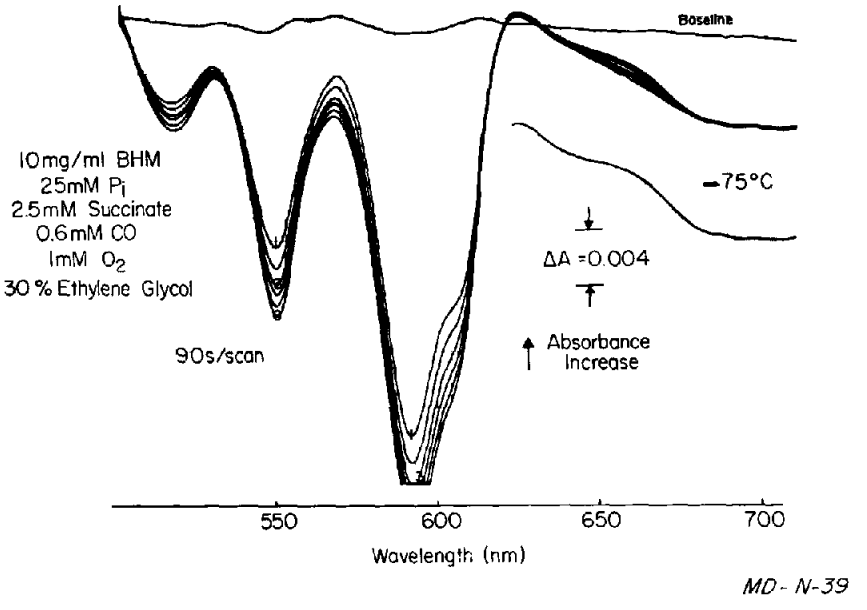

Fig.2. Difference spectra after photolysis of CO bound mitochondria in presence of oxygen. The reference spectrum is the state before photolysis. The experimental conditions are the same as in fig.1A except temperature which is −75°C.

in preparation) that this absorbance decrease at 549 nm is not the effect of any intermediate but rather the result of cytochrome *c* oxidation.

4. Discussion

On the basis of the 655 nm band being characteristic of the a_3^{3+} form [10] we have demonstrated on the hand that the reaction with oxygen of mitochondrial cytochrome oxidase in the mixed valence state does not lead to the formation of the fully oxidized state. This result is consistent with the prominent absorbance increase observed at 606 nm [4,5,12] when Compound C forms which strongly supports a reduced state of the iron. These considerations have suggested the formulation of Compound C as $a_3^{2+} - 0_2^- - Cu^{2+}$ [9]. On the other hand we have shown that a_3^{3+} forms before the complete disappearance of Compound A expressed by the absorbance decrease at 590 nm on fig.2.

The results in fig.2 provide evidence that cytochrome *c* oxidation may occur at temperatures lower than −50°C previously suggested as a limit [5]. The oxidation of cytochrome *c* implies the one of cytochrome *a* to a certain extent. Therefore the oxidation of hemes *a* and a_3 as demonstrated above, is sufficient to account for the absorbance decrease at 606 nm as observed under the conditions of fig.2. This would reduce to an earlier stage of the reaction the possibility to assign some spectral change at 606 nm to any form of Compound B as proposed earlier by Chance et al. [4]. Although the above data do not allow to conclude that all a_3 reacting with 0_2 goes to the fully oxidized form, on the basis of the 655 nm band, at least a significant amount of a_3^{2+} reaches the a_2^{3+} state. Furthermore, considering the oxidation of cytochrome *a* implied by the one of cytochrome *c*, the above data support strongly a progression of cytochrome oxidase to the fully oxidized state which was not expected from heme–heme interaction considerations [8].

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