Changes induced by hydrazine in optical spectra of cytochrome oxidase

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Under aerobic conditions hydrazine reduces haem *a* of cardiac cytochrome oxidase and brings about the formation in optical spectra of a new band at 845 nm, whereas under anaerobic conditions hydrazine reduces both haems, *a* and a_3, as well as EPR-detectable copper, Cu_A, and results in the band at 845 nm. The formation of this band was sensitive to inhibitors of oxygen binding. It is suggested that the band at 845 nm reflects changes induced by hydrazine in Cu_B which in the resting enzyme is EPR-undetectable.

Cytochrome oxidase *Hydrazine, effect of*

1. INTRODUCTION

Cytochrome oxidase (EC 1.9.3.1) catalyzing the 4-electron reduction of oxygen contains cytochrome *a* and a_3 as well as 2 copper atoms, Cu_A and Cu_B. One of the copper atoms, Cu_A, possesses the EPR signal at $g = 2.0$ [1–3] and has in the optical spectrum of the enzyme the band located at 830 nm [4–7]. The visualization of the other copper atom, Cu_B, is problematic, although EPR and optical spectra of this copper are a subject of many recent studies [7–15]. Cu_B may be visualized during its interaction with some inhibitors [16–18].

Hydrazine is known to be an inhibitor of cytochrome oxidase [19]. However, the mechanism of its interaction with the enzyme has not been established. We have observed the formation of a new band, centered at 845 nm, in the optical spectrum of cytochrome oxidase treated by hydrazine [20]. Here, we describe the results of the more detailed study of the hydrazine effect on optical spectra of cytochrome oxidase.

2. MATERIALS AND METHODS

Cytochrome oxidase was isolated from bovine heart mitochondria as in [21]. The last sedimentation by ammonium sulphate was carried out in the presence of 10^{-4} M EDTA. The enzyme was dissolved in 0.05 M Na-phosphate buffer (pH 7.4) containing 1% cholate or 0.5–3% Tween 20. The haem content in the preparations obtained was 12 nmol/mg protein. The spectral purity index of the enzyme, $A_{280}/A_{420(ox.)}$, was 2.2. The turnover number at pH 7.0 in 0.05 N Na-phosphate buffer–0.5% Tween 20 was 100–120 s^{-1}. The concentration of cytochrome oxidase was determined from millimolar extinction $\Delta E_{(605red.-630ox.)}$ of 16.5.

Optical spectra were recorded on a Beckman M-26 spectrophotometer in 10 mm cells at 22°C. EPR spectra were obtained on a Varian E-4 instrument operating at: microwave frequency, 9.08 GHz; microwave power, 10 mW; modulation amplitude, 10 G; temp., 77 K.

Anaerobic conditions were reached by bubbling of argon in the reaction cells for 20 min. To follow the effect of oxygen, the stoppers of the cells were opened, closed again and thoroughly shaken.

Hydrazine hydrochloride or sulphate (Sigma) was added to cytochrome oxidase as aliquots, the pH of which was previously adjusted to desirable values.

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3. RESULTS

Fig.1 shows changes in the optical spectrum of cytochrome oxidase under anaerobic conditions after addition of hydrazine. These data show that hydrazine results in the reduction of EPR-detectable copper as followed by the signal at g = 2.0 and the absorbance at 830 nm. Moreover, hydrazine reduces both haems practically completely and brings about the formation of a new band at 845 nm. The intensity of this band is 2.5-times higher as compared with the band at 830 nm.

Fig.2 illustrates changes induced by hydrazine in the cytochrome oxidase spectrum under aerobic conditions. It is clear that under aerobic conditions, hydrazine reduces only a half of haem

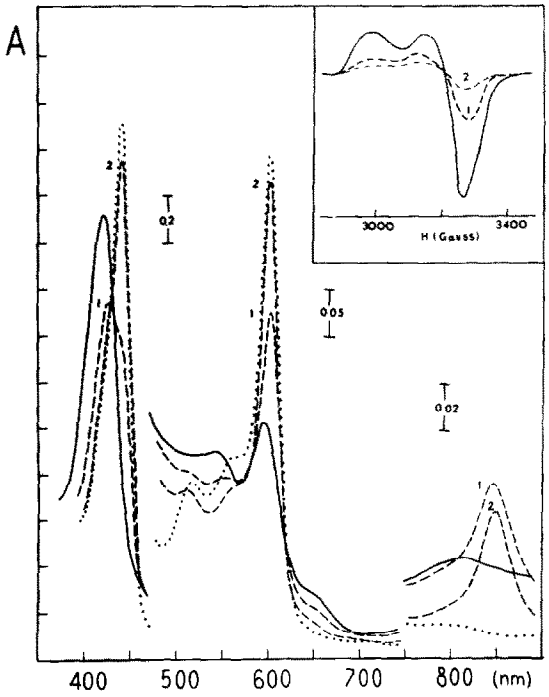

Fig.1. The effect of hydrazine on optical and EPR (inset) spectra of cytochrome oxidase under anaerobic conditions: (——) oxidized enzyme in 1% cholate at pH 7.4; (···) enzyme reduced by dithionite; (– – –) oxidized enzyme incubated with hydrazine for 5 min (1) and 1 h (2). Concentrations of cytchrome oxidase and hydrazine for optical measurements were 2.3×10^{-5} M and 3.0×10^{-2} M, respectively, and for EPR measurements 3.8×10^{-4} M and 3.0×10^{-1} M, respectively.

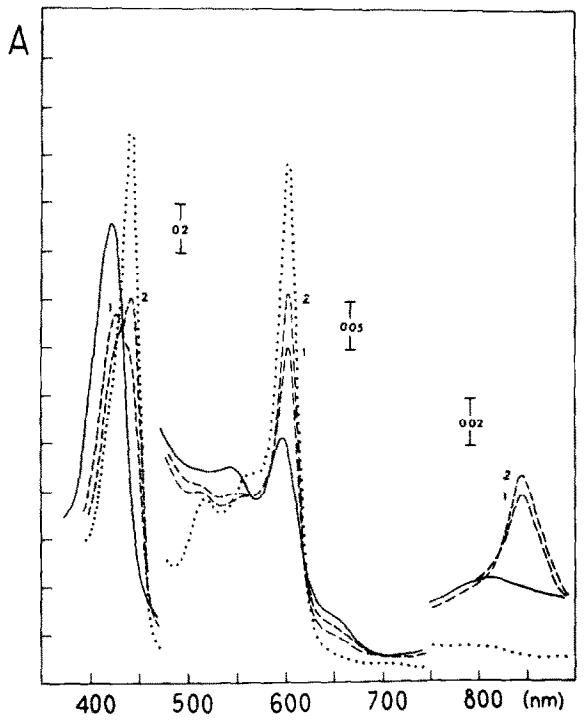

Fig.2. The effect of hydrazine on the optical spectrum of cytochrome oxidase under aerobic conditions: (——) oxidized enzyme (2.3×10^{-5} M) in 1% cholate at pH 7.4; (···) enzyme reduced by dithionite; (– – –) oxidized enzyme incubated with 3.0×10^{-2} M hydrazine for 5 min (1) and 1 h (2).

groups. It was found that the intensity of the band at 845 nm increases with the increasing of the hydrazine concentration, reaching the maximal intensity at 100-fold molar excess of hydrazine over cytochrome oxidase. However, at 10-fold excess the band at 845 nm is also observed but its intensity was lower and the band disappears rapidly, while at 1000-fold excess the rate of the 845 nm band formation was significantly accelerated as compared with 100-fold excess.

The effect of hydrazine on the oxygenated form of cytochrome oxidase is presented in fig.3. It is of importance to note that in this case the band at 845 nm arises more rapidly than with the oxidized enzyme.

The difference spectrum shown in fig.4 illustrates that for the formation of the band at 845 nm, besides of hydrazine, the presence of oxygen is necessary.

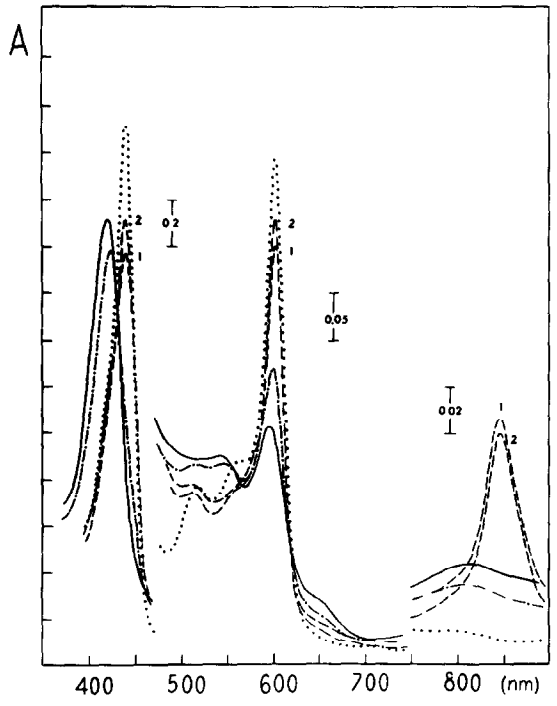

Fig.3. The effect of hydrazine on the optical spectrum of oxygenated cytochrome oxidase: (——) oxidized enzyme (2.3×10^{-5} M) in 1% cholate at pH 7.4; (···) enzyme reduced by dithionite; (– – –) oxygenated enzyme formed after the exposition of the reduced enzyme to aerobic conditions; (– – –) oxygenated cytochrome oxidase treated by 3.0×10^{-2} M hydrazine for 2 min (1) and 1 h (2).

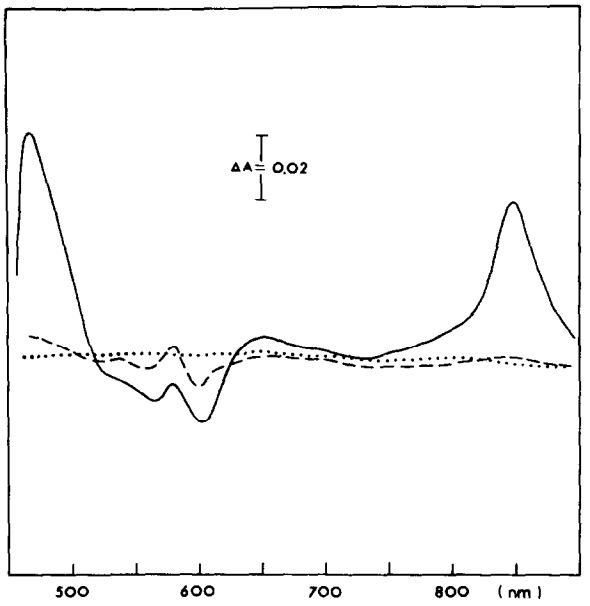

Fig.4. Changes in the difference spectrum of cytochrome oxidase–dithionite–hydrazine minus cytochrome oxidase–dithionite after the exposition to aerobic conditions; 2.0×10^{-5} M cytochrome oxidase in 1% Tween 20 (pH 7.4) was incubated with 10^{-2} M dithionite (as a reference) or dithionite (10^{-2} M)–hydrazine (10^{-2} M) (as a sample) for 30 min; (···) cytochrome oxidase reduced by dithionite was in reference and sample cells; (– – –) the enzyme–dithionite–hydrazine minus the enzyme–hydrazine. When both cells were aerated for 30 s the positive band at 845 nm was observed. This band reaches the maximal value after 30 min (——).

We found that after the treatment of the reduced cytochrome oxidase by CO and after the addition of cyanide (10^{-2} M), azide (10^{-2} M) or nitrite (3×10^{-2} M) to the oxidized enzyme in the presence of hydrazine the band at 845 nm is not observed. It was noted that the reduction of the enzyme by hydrazine in 1% Tween 20 is more slow than in 1% cholate. Moreover, in 3% Tween 20 hydrazine does not reduce cytochrome oxidase, and the band at 845 nm has not been observed. However, when the preparation in 3% Tween 20 was first reduced by dithionite and then hydrazine was added the band at 845 nm was again observed. When cytochrome oxidase was preincubated with Na-dodecylsulphate (0.5%) or urea (6 M) the band at 845 nm was not observed.

4. DISCUSSION

Under aerobic conditions at pH 7.0–7.4 only one haem, haem *a*, is reduced by hydrazine whereas under anaerobic conditions both haem groups, *a* and a_3, are reduced. Thus, first of all hydrazine acts as a reductant of haems. Further, under anaerobic conditions hydrazine acts also as a reductant for EPR-detectable copper, Cu_A as indicated by the drop of intensities both the EPR-signal at $g = 2.0$ and the band at 830 nm. However, under aerobic conditions hydrazine does not reduce EPR-detectable copper. Further, the band at 845 nm is observed independently of the reduced or oxidized state of haem a_3. Therefore, the 845 nm band, observed under both aerobic and

anaerobic conditions, should reflect changes induced in Cu_B, which in the resting enzyme is EPR-undetectable, rather than in Cu_A or in haems.

Cu_B is known to be the component of the oxygen binding centre of cytochrome oxidase, haem a_3–Cu_B [22,23]. During the interaction of hydrazine with oxygenated cytochrome oxidase the intensity and the rate of the formation of the band at 845 nm were higher than with the resting form of the enzyme. This is a piece of evidence that suggests that the band reflects the oxygenated state of Cu_B, a conclusion which is supported by the results obtained with inhibitors of the oxygen binding. The data obtained has led us to consider that perhaps there is oxygen in the surrounding of Cu_B even under the conditions which are generally considered as strictly anaerobic.

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