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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE

XI. REACTION WITH AZIDE

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SUMMARY

1. Addition of 100 μM azide to oxidized cytochrome *c* oxidase rapidly shifts the Soret peak from 424 to 421 nm, leaving the intensity of the band unchanged. Small changes are observed in the α and β region. The spectrum of the reduced enzyme is not affected by azide.

2. One molecule of azide is bound per molecule of cytochrome *c* oxidase (two hemes).

3. At pH 6.8 the value of the spectroscopic dissociation constant K_d ($2 \cdot 10^{-5}$ M) equals that of the inhibition constant K_i.

4. The "on" constant for azide binding, determined from the progressive inhibition of the cytochrome *c* oxidase activity by azide is $4.6 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ (pH 7.3, 20 °C), that is $1.6 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ when based on HN_3. The value for the "off" constant is 0.4 s^{-1}.

INTRODUCTION

In 1939, Keilin and Hartree[1] observed that in heart-muscle preparation, azide prevents the reduction of cytochrome a_3. Stannard and Horecker[2] ascribed the inhibition to HN_3 and this has been confirmed by others[3–5]. The type of inhibition was shown to be non-competitive[3,5,6], although an uncompetitive type of inhibition has also been reported[7]. The time course of the inhibition of cytochrome *c* oxidase activity after the addition of azide was studied by Yoshikawa and Orii[8]. They calculated an "on" constant for azide binding of $2.5 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ at pH 6.0 and room temperature, and furthermore concluded that the enzyme–azide complex is completely inactive.

Effects of high concentrations of azide on the spectrum of isolated oxidized cytochrome *c* oxidase were described by Muijsers *et al.*[9], but these slow spectral changes were not related to the inhibition reaction. In a more recent communication[5] we reported the much faster spectral changes at low azide concentrations (≤ 1 mM), and showed that the spectroscopic dissociation constant for these changes agree with

the inhibition constant. However, data such as the comparison of the rate of inhibition of the enzymic activity with the kinetics of spectral changes were not available. We have, therefore, reinvestigated the effects of azide on cytochrome *c* oxidase.

This paper will deal with the azide-induced spectral changes and the inhibition of the enzymic activity. The results agree well with those reported for cytochrome *c* oxidase *in situ*. The results have partly been reported in a preliminary form[10,11].

MATERIALS AND METHODS

Beef-heart cytochrome *c* oxidase was prepared as described before[12,13]. The preparations contained 8–10 μmoles heme *a* per g protein, and had an activity of 200–250 s^{-1} at infinite cytochrome *c* concentration and at 25 °C. Cytochrome *c* was isolated by a modification of the method of Margoliash and Walasek[14]. Ferrocytochrome *c* was prepared by Sephadex G-25 gel filtration as described by Yonetani and Ray[3]. Absorbance coefficients used were for cytochrome *c* (red–ox) 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm[15], and for cytochrome *c* oxidase (red–ox) 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm[16].

Chemicals were Analar grade, mainly obtained from British Drug Houses. Tween 80 was purchased from Koch–Light Laboratories. Asolectin (Associated Concentrates Inc., New York) sols were made according to the method of Wharton and Griffiths[17].

Spectrophotometric measurements were carried out on a Cary-14 or Cary-17 recording spectrophotometer or a Durrum–Gibson stopped-flow spectrophotometer with an optical pathlength of 2.0 cm. Low-temperature spectra were taken on a Perkin–Elmer 356 spectrophotometer with liquid-nitrogen attachment.

Inhibition of the enzyme activity

The enzymic activity of cytochrome *c* oxidase was measured spectrophotometrically. The enzyme was diluted to 6.4 μM in a medium containing 250 mM sucrose, 10 mM potassium phosphate (pH 7.0), 0.5% Tween 80 and 2 mg/ml Asolectin. The reaction was initiated by addition of 2.5 μl of this mixture to 2.5 ml of a solution containing 100 mM potassium phosphate ($pH < 8$) or Tris–sulphate ($pH > 8$), 1.0% Tween 80 and 10 μM ferrocytochrome *c*. The pH was measured with a Philips digital pH/mV meter. The inhibition constant was determined from the intercept on the abscissa of a Dixon plot (reciprocal value of the initial velocity against azide concentration). Since the inhibition is of the non-competitive type, the measurements were carried out at a single cytochrome *c* concentration.

RESULTS

Spectra

Fig. 1 shows the absorption spectra of oxidized cytochrome *c* oxidase and its azide complex at 20 °C. Azide in low concentration shifts the Soret peak from 424 to 421 nm, without affecting its intensity. In the α and β region the changes are small. At liquid-nitrogen temperatures essentially the same spectral shifts were observed as at room temperature. At 77 °K the Soret maximum at 423 nm shifted to 421 nm in the presence of azide, while no significant changes were seen in the α and β region.

Small changes in absorbance can best be studied by means of difference spec-

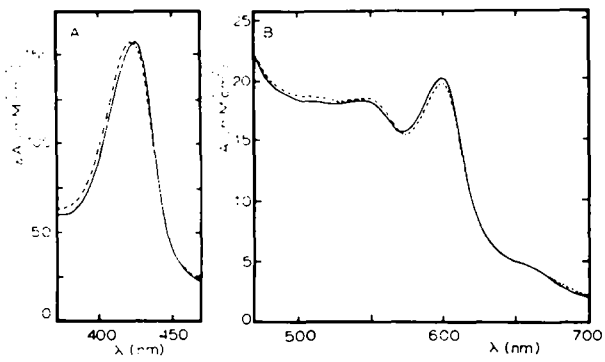

Fig. 1. Absorption spectra of oxidized cytochrome *c* oxidase and its azide complex. 100 mM potassium phosphate buffer (pH 7.2); 1.0% Tween 80; temperature 20 °C. A, Soret region, 3.9 μM cytochrome *c* oxidase; B, visible region, 27.5 μM cytochrome *c* oxidase; ———, no addition; -------, with 100 μM azide.

troscopy. This is illustrated in Fig. 2 where the difference spectrum cytochrome *c* oxidase + azide *minus* cytochrome *c* oxidase is shown. In the visible region more details can be observed. Peaks are present at 395, 415, 470, 520, 558 and 678 nm and troughs at 590 and 432 nm. No effect could be observed in the near infrared region (up to 1000 nm). Addition of azide to $Na_2S_2O_4$-reduced enzyme does not affect the absolute spectrum from 400 to 700 nm, indicating that azide does not perturb the ligand field of the heme iron. Therefore, if azide interacts with the reduced enzyme it is not bound close to the heme.

Spectroscopic dissociation constant

It is possible to determine the value of the dissociation constant of the enzyme–ligand complex from titrations of ligand-induced spectral changes. Information on the number of ligand molecules bound to the enzyme becomes available from these data when the value of the dissociation constant is comparable in magnitude to the

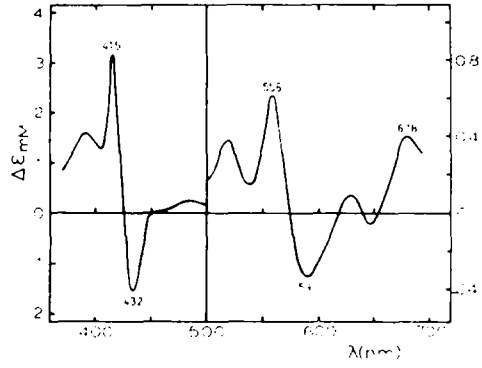

Fig. 2. Difference spectrum of oxidized cytochrome *c* oxidase + 1 mM azide *minus* cytochrome *c* oxidase; 100 mM potassium phosphate buffer (pH 7.3) and 0.7% cholate. Spectrum was taken 3 min after azide addition. Absorbance scale normalized to mM heme *a*.

enzyme concentration, since in this case a correction in the total ligand concentration must be applied for enzyme-bound ligand. For the equilibrium reaction:

$$E + nI \rightleftharpoons EI_n$$

the following expression can be obtained[18]:

$$\log(1-\alpha)/\alpha = \log K_d - n\log([I_t] - n\alpha[E_t])$$

Here $[I_t]$ and $[E_t]$ are the total ligand and enzyme concentration, respectively; α is the degree of saturation *i.e.* the ratio of the spectral effect at a definite ligand concentration to that at infinite concentration. In the correction term ($n\alpha[E_t]$), n stands for the number of ligand molecules bound to the enzyme molecule. For the azide-induced peak in the difference spectrum at 558 nm, plots (Fig. 3) of log $(1-\alpha)/\alpha$ against log $([I_t]-n\alpha[E_t])$ are constructed on basis that either one azide molecule is bound per two heme *a* molecules (●—●) or one azide molecule is bound per one heme *a* molecule (○—○). A straight line is obtained only in the former case, indicating that one molecule of azide is bound per molecule (two hemes) of cytochrome *c* oxidase.

The dissociation constant calculated from the point of intersection on the abscissa in Fig. 3 is 10 μM at 22 °C and pH 6.7. The same value was found when the dissociation constant was determined at a different wavelength (432 nm), although the changes at this wavelength are caused by a consecutive reaction (see the following paper).

When the negative logarithm of the dissociation constant is plotted against the pH (Fig. 4), a linear relationship ($pK_d = 12.3 - 1.1$ pH) is found both at 432 (○—○) and 558 nm (●—●).

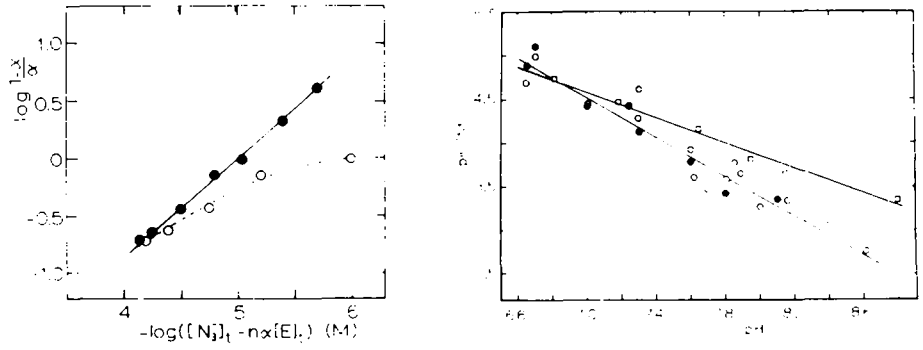

Fig. 3. Determination of the dissociation constant for the oxidase–azide complex at 558 nm; 22.5 μM cytochrome *c* oxidase; 100 mM potassium phosphate buffer (pH 6.7); temperature 22 °C. ●—●, Hill plot obtained on the assumption that one azide molecule is bound per two heme *a* molecules; ○—○, Hill plot obtained on the assumption that one azide molecule is bound per one heme *a* molecule.

Fig. 4. The pH dependence of the spectroscopic dissociation and inhibition constants. The spectroscopic dissociation constant was calculated from Hill plots of the azide-induced spectral changes; 1.0% Tween 80 or 0.75% cholate; temperature 20 °C; ●—●, 558 nm; ○—○, 432 nm. The inhibition constant (□—□) was obtained as described in Materials and Methods. 1.0% Tween 80; 100 mM potassium phosphate buffer; above pH 8.0, 100 mM Tris–sulphate buffer; 10 μM ferrocytochrome *c*; 6.4 nM cytochrome *c* oxidase; temperature 20 °C.

The inhibition of cytochrome c oxidase activity

Azide inhibits cytochrome *c* oxidase activity non-competitively (refs. 3, 5, 6). The inhibition constant was shown to be dependent on the pH[3,5]. Fig. 4 also gives K_i as a function of pH. As can be seen K_i and K_d are equal at pH 6.8, but at high pH values the pK_i becomes increasingly larger than the pK_d. In agreement with our previous publication[5] we find $pK_i = 9.67 - 0.73$ pH.

Muijsers *et al.*[5] observed almost instantaneous inhibition of cytochrome *c* oxidase activity after addition of the inhibitor. Therefore, a stopped-flow spectrophotometer was used for a study of the transient state of the inhibition. After mixing ferrocytochrome *c plus* azide with cytochrome *c* oxidase the oxidation of cytochrome *c* was monitored at 550 nm as a function of time. Two phases in the reaction can be distinguished, an initial rapid oxidation of ferrocytochrome *c* which gradually slows down due to the formation of the inhibitory enzyme–azide complex, and a final slow oxidation of ferrocytochrome *c*. The rate of this slow oxidation depends on the concentration of free cytochrome *c* oxidase. The rate constant for the reaction of azide with catalytically active cytochrome *c* oxidase can be calculated from the biphasic region by extrapolating the slow reaction, approximately zero order in cytochrome *c*, to zero time. The small differences between extrapolated and measured transmissions at different times were plotted semi-logarithmically against time. Straight lines were obtained for each azide concentration used and from the slope of the lines the rate constants, corresponding to the formation of the inhibitory complex were obtained. The reaction for the formation of this complex is first order in azide as shown in Fig. 5, where the observed rate constants are given as a function of the azide concentration. From the slope of the straight line in Fig. 5 the "on" constant (k_i) for azide binding can be calculated to be $4.6 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ at pH 7.3 and 20 °C. The value of the "off" constant (k_{-i}) of the reaction, obtained from the intercept of the line on the ordinate, equals 0.4 s^{-1}. The inhibition constant K_i given by the ratio k_{-i}/k_i is 87 μM, in fair agreement with the value of 43 μM for the inhibition constant of cytochrome *c* oxidase activity at the same pH calculated from Fig. 4. The discrepancy may be due to an inaccuracy in the determination of k_{-i}.

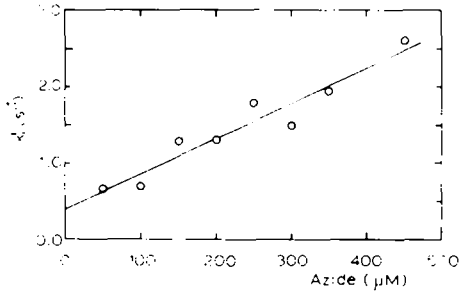

Fig. 5. Rate constant for the progressive inhibition of cytochrome *c* oxidase activity as a function of the total azide concentration. 3.6 μM ferrocytochrome *c* containing variable amounts of azide was mixed with 0.26 μM cytochrome *c* oxidase; 100 mM potassium phosphate buffer (pH 7.3); 1.0% Tween 80; temperature 20 °C.

DISCUSSION

The extinction coefficients of the azide-induced effects in the difference spectrum cytochrome *c* oxidase + azide *minus* cytochrome *c* oxidase are small in comparison to

those of other ferric hemoprotein–azide complexes[19]. This suggests that the azide molecule is not a ligand of the iron atom, and that its binding to the ferric enzyme only perturbates slightly the environment of the heme group.

The pH dependence of both the dissociation and inhibition constants suggests that hydrazoic acid is bound to the enzyme. However, the pH dependency can also be explained as an effect of a dissociating group in the enzyme on azide binding. To decide between the two possibilities measurements at pH values close to the pK_a of HN_3 (4.8) are required, but this cannot be carried out because of the instability of the enzyme at low pH. Since the inhibition constant is about equal to the dissociation constant, and only one molecule of azide is bound per molecule of cytochrome *c* oxidase, it may be concluded that this molecule is responsible for both the inhibition and the induced spectral changes. The discrepancy between K_i and K_d at higher pH values may indicate the existence of inactive spectroscopically undetectable enzyme–azide complexes.

The K_i for the isolated enzyme (*cf.* Fig. 4) is close to the dissociation constant of 43 μM at pH 7.2 reported by Wilson[14] for the changes observed at 445–455 nm after addition of azide to anaerobic mitochondria, but differs from the value (100–200 μM) reported in uncoupled mitochondria[20]. No explanation can be offered for this phenomenon.

The rate constant for the formation of inhibited enzyme–azide complex is $4.6 \cdot 10^3\ M^{-1} \cdot s^{-1}$ at pH 7.3 corresponding to a value of $1.6 \cdot 10^6\ M^{-1} \cdot s^{-1}$ when based on hydrazoic acid. This equals the value ($2 \cdot 10^6\ M^{-1} \cdot s^{-1}$) found for the binding of hydrazoic acid to membrane-bound cytochrome *c* oxidase[20]. This indicates that our isolation procedure[13] of cytochrome *c* oxidase does not modify the azide binding site.

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