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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE

XII. KINETICS OF AZIDE BINDING

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SUMMARY

1. The reaction of oxidized cytochrome *c* oxidase with low concentrations of azide (1 mM) is biphasic. A fast bimolecular binding reaction measured at 558 nm, strongly pH-dependent, is followed by a slow, nearly pH-independent first-order reaction measured at 432 nm.

2. The pH dependence suggests binding of hydrazoic acid. The rate constant ($k = 2.5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$) and the low energy of activation of 1 kcal $\cdot$ mole^{-1} point to a diffusion-controlled but sterically hindered reaction.

3. The second reaction, with a rate constant ($k = 0.03$ s^{-1}) independent of the azide concentration and an energy of activation of 14 kcal $\cdot$ mole^{-1} is interpreted as an azide-induced conformational change in the protein.

4. Study of the dissociation of the enzyme–azide complex by the pH-jump technique indicates that the azide anion is released. In order to explain the kinetics a cyclic mechanism is proposed.

INTRODUCTION

In the preceding paper[1] it was shown that purified oxidized cytochrome *c* oxidase (EC 1.9.3.1) reacts with azide and that only one azide molecule is responsible for the observed spectral changes and the inhibition of its activity. From the kinetics of the progressive inhibition observed when the enzyme was mixed with ferrocytochrome *c* and azide in a stopped-flow spectrophotometer an 'on' constant for azide binding of $4.6 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ at pH 7.3 has been calculated. The strong pH dependence of both K_i and K_d suggests that hydrazoic acid is the reactive species (but see the preceding paper[1]); in this case the 'on' constant increases to a value of $1.6 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. In rat-liver mitochondria Wilson[2] demonstrated that on addition of excess azide in the aerobic steady state a pseudo first-order reduction of cytochrome *a* occurs. Based on hydrazoic acid he calculated a rate constant for binding of $2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ which is in excellent agreement with our value obtained from progressive-inhibition kinetics[1].

This paper will deal with the kinetics of azide binding to isolated oxidized

cytochrome *c* oxidase, which are found to be similar to those observed with particulate enzyme. Some of the results are published in a preliminary form[3–5].

MATERIALS AND METHODS

Enzyme preparations

For a description of enzyme preparations, chemicals and instruments, see the preceding paper[1].

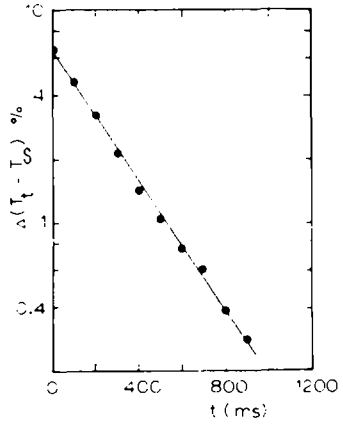
RESULTS

Kinetics of formation of cytochrome c oxidase–azide complex

As was demonstrated in the preceding paper (*cf.* Fig. 1 of ref. 1) azide affects the spectrum of the oxidized enzyme. The formation of the peaks and troughs in the Soret region with 1 mM azide at pH 7.3 and 20 °C was complete in about 3 min, whereas the peaks in the α and β regions in the difference spectrum (enzyme + azide *minus* enzyme) were formed at least two orders of magnitude more rapidly.

The reaction visible at 558 nm is first order with respect to enzyme and monophasic as jugded from the semilogarithmic plot of Fig. 1. The first-order rate constant measured at 558 nm is proportional to the azide concentration (Fig. 2), indicating that the reaction is first order with respect to azide. Thus the reaction of oxidized cytochrome *c* oxidase with azide is bimolecular. For an equilibrium reaction the measured rate constant (k'_1) is given by Eqn 1

$$k'_1 = k_1 I^\circ + k_{-1} \tag{1}$$



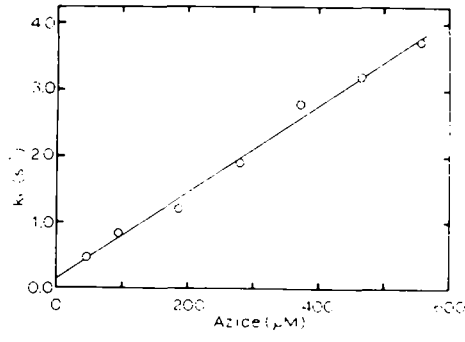


Fig. 1. Semilogarithmic plot of the transmission changes observed after mixing 930 μM azide with an equal volume of 56 μM cytochrome *c* oxidase. The reaction was followed at 558 nm using a stopped-flow spectrophotometer; 100 mM potassium phosphate buffer (pH 7.3) and 1% Tween 80; temperature 20 °C.

Fig. 2. Rate constant observed at 558 nm, for the reaction of cytochrome *c* oxidase with azide as a function of the azide concentration; 100 mM potassium phosphate buffer (pH 7.3) and 1% Tween 80; 28 μM cytochrome *c* oxidase; temperature 20 °C.

where I^0 is the initial azide concentration and k_1 and k_{-1} are the rate constants for the forward and backward reaction, respectively. The slope in Fig. 2 gives a value of $6.5 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ for the rate constant k_1 at pH 7.3. Based on free hydrazoic acid ($pK_a = 4.76$) a value of $2.5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ is obtained. The rate constant k_{-1} of the back reaction calculated from the intercept on the ordinate is 0.15 s^{-1}.

Fig. 3 shows that the slow reaction as measured at 395, 415, 432, 590 and 678 nm is first order with respect to enzyme with a value for the rate constant of 0.03 s^{-1}. Furthermore, the rate constant is independent of the azide concentration (not shown). In this context it should be mentioned that at 678 nm there is also a contribution of the fast reaction to the difference spectrum (see later). It is likely that the slow reaction represents a conformational change of the protein induced by the binding of the ligand. These findings can be summarized by the following scheme:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI^{558} \overset{k_2}{\rightleftharpoons} EI^{432,558} \tag{2}$$

where EI^{558} represents the rapidly formed enzyme-azide complex and $EI^{558,432}$ results from the subsequent conformational change. The following expression for the observed rate constant (k'_2) can be obtained[6] from Eqn 2

$$k'_2 = k_{-2} + \frac{k_2}{1 + \dfrac{k_{-1}}{k_1 I^0}} \tag{3}$$

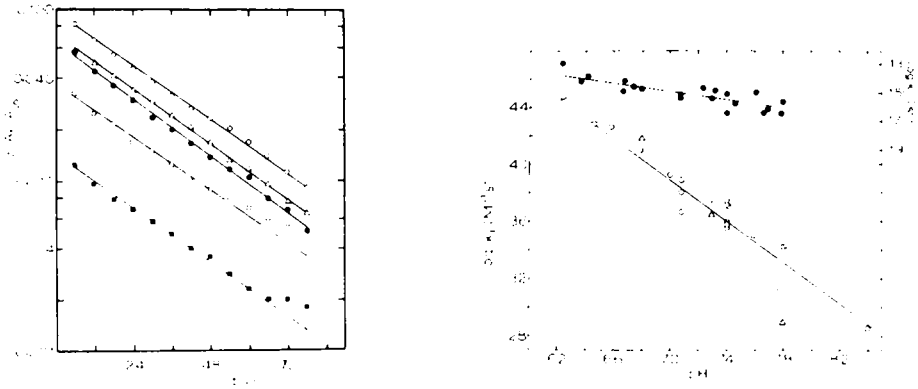


Fig. 3. Semilogarithmic plot of the absorbance difference at five different wavelengths as a function of time. The reaction was started by the addition of 400 μM azide to cytochrome *c* oxidase; 1.0% Tween 80, 100 mM potassium phosphate buffer (pH 7.1); temperature 20 °C. Enzyme concentration for measurements in Soret region, 24 μM, other wavelengths, 145 μM. ○—○, $-\Delta A_{432}$; ●—●, ΔA_{415}; △—△, ΔA_{395}; □—□, $-\Delta A_{590}$; ■—■, ΔA_{678} nm.

Fig. 4. Logarithm of the rate constant of the reaction of cytochrome *c* oxidase with azide, measured at 558 and 432 nm, as a function of pH; temperature 20 °C; 1% Tween 80; 100 mM potassium phosphate buffer or, above pH 7.9, 100 mM Tris–sulphate buffer. ○—○, at 558 nm, enzyme 25–50 μM, azide 0.05–1 mM; ●—●, at 432 nm, enzyme 10–20 μM, azide 1 mM; △—△, values obtained from Table I of ref. 2.

As the rate constant of the slow reaction is independent of the azide concentration, at least from 0.1 to 1 mM (not shown), it is required that: $I^0 \gg k_{-1}/k_1$. Substitution of the observed k_1 and k_{-1} values shows that this condition is satisfied with the azide concentrations used.

pH and temperature dependence of the reactions of azide with cytochrome c oxidase

Because the spectroscopic dissociation constant and the inhibition constant in the catalytic activity measurements are pH dependent, the fast and the slow reaction were studied in more detail at different pH values. The rate constant of the fast reaction is extremely pH dependent. When the logarithm of the observed rate constant is plotted as a function of pH a linear relationship is obtained (Fig. 4), which obeys the expression:

$$\log k'_1 = 9.0 - 0.7 \text{ pH} \tag{4}$$

In Fig. 4 the points (△—△) found by Wilson[2] for rat-liver mitochondria are also given, showing agreement between isolated and particulate enzyme in the reaction with azide. The pH dependence can be explained by a preferential binding of the hydrazoic acid to the cytochrome. The deviation of the slope of the line (0.7) from unity may be due to a relatively increasing contribution of k_{-1} to the total measured rate constant at high pH values. In contrast to the fast reaction, the rate constant of the slow reaction (○—○) in Fig. 4 is nearly independent of the pH in the range studied (pH 6.2–7.8).

The fast bimolecular reaction is only slightly dependent on temperature ($Q_{10} = 1.05$; energy of activation 1.0 kcal·mole^{-1}). The slow reaction, however, has a Q_{10} of 2.5, corresponding to an energy of activation of 14 kcal·mole^{-1}.

Dissociation reactions

In order to test the reaction scheme given in Eqn 2, the dissociation was investigated by means of the pH-jump technique as described in detail in the legend of Fig. 5. Because the K_D and K_i are strongly pH dependent and the azide reactions are reversible it is possible to study the kinetics of the dissociation reaction by suddenly raising the pH of a solution containing the enzyme–azide complex.

The dissociation reaction as measured at 558 nm by the pH-jump technique (pH 6.8→8.0) is ten times faster than that measured at 432 nm. Both reactions are first order and monophasic (not shown).

Fig. 5 shows that the inverse values of the apparent dissociation rate constant as measured at 558 nm and the sum of the free enzyme and N_3^- concentration are linearly dependent. This result suggests a dissociation of the azide anion instead of hydrazoic acid. To account for the observed relaxation kinetics (see refs 6 and 7), an additional intermediate, undetectable at 558 nm, but having the azide bound, must be incorporated into the reaction scheme

$$EI^{558,432} \rightleftharpoons EI^{432} \rightleftharpoons E^{432} + N_3^- \tag{5}$$

In order to determine the kinetic parameters of the reaction at 432 nm the following experiment was carried out: The enzyme–azide complex was first formed at pH

6.7, after which it was dissociated by adding alkaline Tris–sulphate buffer (pH 10.0) to a final pH of 9.0. The reversibility of the reaction was checked by lowering the pH again to its original value (6.6) by the addition of acidic phosphate. In both cases the rate constants of the monophasic 'on' and 'off' reactions are equal (0.03 s^{-1}) and independent of the azide concentration.

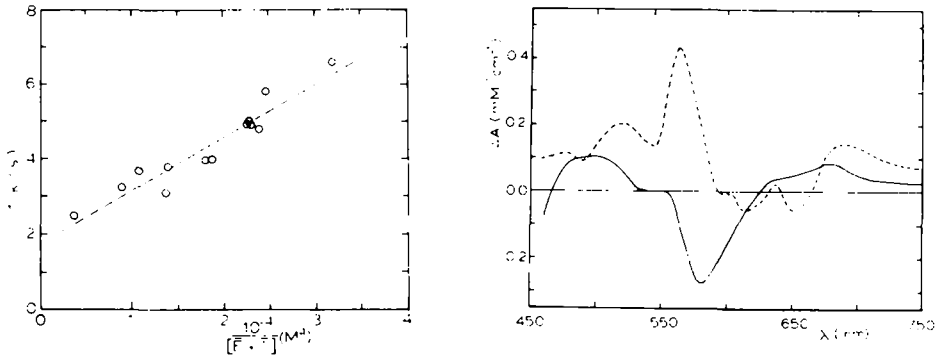


Fig. 5. Reciprocal value ($1/k$) of the dissociation rate constant measured at 558 nm, as a function of the reciprocal sum of the equilibrium values of the initial free enzyme ($\bar{E}$) and N_3^- (I) concentration. Dissociation of the enzyme–azide complex was brought about by mixing in a stopped-flow spectrophotometer a solution of 20 mM potassium phosphate buffer (pH 6.8), containing variable amounts of the enzyme–azide comples (30–126 μM in enzyme) with equal volumes of 200 mM Tris–sulphate buffer (pH 8.0), having the same concentration of azide (16–300 μM) as in the other syringe. All solutions contained 1% Tween 80. Final pH 7.8, temperature 20 °C. Free enzyme and azide concentration at pH 6.8 were calculated from the dissociation constant at pH 6.8.

Fig. 6. Calculated contribution of the enzyme–azide complexes EI^{558} and $EI^{558,432}$ to the absorbance changes in the difference spectrum enzyme + azide *minus* enzyme; azide 100 μM; 100 mM potassium phosphate buffer (pH 7.2); 10% Tween 80; temperature 20 °C. Dotted line, spectral changes due to formation of the first complex (EI^{558}). Solid line, spectral change caused by the formation of the second complex ($EI^{558,432}$). Spectra were calculated as described in Results.

We propose the following cyclic mechanism, based on the experimental results for the association (Eqn 2) and dissociation (Eqn 5) reactions:

$$\begin{array}{ccc} E + HN_3 & \rightleftharpoons & EI^{558} \\ \rightleftharpoons & & \rightleftharpoons \\ E^{432} + N_3^- & \rightleftharpoons & EI^{432} \rightleftharpoons EI^{558,432} \end{array} \qquad (6)$$

It is assumed that E is the main enzyme constituent in the absence of azide. Starting with E the main pathway of the association reaction goes clockwise *via* EI^{558} to $EI^{558,432}$ and the dissociation reaction mainly *via* the component EI^{432} and E^{432} back to E.

Difference spectra of the two azide complexes

The large difference in rate between the binding reaction and the conformational change permitted us to calculate the contributions of both azide complexes to

the final difference spectrum. After addition of azide, spectra were taken at regular intervals with known scanning speed. Using the first-order rate constant (0.03 s^{-1}) and extrapolation to zero time, the spectral changes due to the slow reaction were calculated. Subtracting this from the total spectral change yielded the contribution of the rapid reaction. Fig. 6 shows that the bands in the difference spectrum contain elements of both reactions with a notable exception between 542 and 558 nm, where only the rapid, and at 590 nm where only the slow reaction contributes.

The bands at 432, 415 and 395 nm are formed slowly (not shown). No contribution of the fast reaction could be detected in this region. If this reaction is present its contribution to the absorbance must be less than 5% of the final spectral effect.

DISCUSSION

The binding reaction and the conformational change

Wilson[2] calculated from the rate of reduction of cytochrome *a*, observed after mixing azide with rat-liver mitochondria, an 'on' constant for the binding of hydrazoic acid of $2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$. This value corresponds well with that found from azide-induced spectral changes. Because the 'on' constant for the formation of the inhibitory oxidase–azide complex as determined by us[1] from transient-inhibition kinetics, is equal to the 'on' constant determined from the first visible spectral change at 558 nm, the primary inhibitory complex is the EI^{558} component. The conformation induced by azide ($EI^{558,432}$) is as effective in the inhibition as the EI^{558} component, since no biphasic inhibition kinetics are observed.

The rate constant of $2.5 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ for the diffusion-controlled azide binding is rather low compared to the rate constant of a reaction between two small molecules ($k = 10^9$–10^{10} $M^{-1} \cdot s^{-1}$, see ref. 8). However, Schmitz and Schurr[9] pointed out that moderate angular constraints imposed on an enzyme and a reactant drastically decrease the diffusion-controlled rate constant. In cytochrome *c* oxidase an angular constraint of this nature might be caused by a sterically hindered entry of the HN_3 molecule into a cavity of the enzyme. A low energy of activation for the enzyme–ligand reaction similar to that observed for the azide binding reaction (1 kcal $\cdot$ $mole^{-1}$) has been reported recently by Erecińska and Chance[10] for the reaction between reduced cytochrome a_3 and O_2 (4 kcal $\cdot$ $mole^{-1}$) or CO (3 kcal $\cdot$ $mole^{-1}$) in intact pigeon-heart mitochondria. A sterically hindered entry of azide has also been observed in methemoglobin, where the azide ion lies deeply buried in the haem pocket. Perutz and Mathews[11] conclude from their X-ray model that before the azide ion is bound, one of the side chains blocking the entrance of the ligand site must swing out in order to admit it.

Our proposal that azide induces a conformational change is supported by the observation of Van Buuren *et al.*[12] and Wilson *et al.*[13] that the enzyme–azide complex reacts more rapidly with cyanide than the free oxidized cytochrome *c* oxidase.

The mechanism of the reactions and the site of action

Although the proposed cyclic mechanism is rather tentative it gives a satisfactory explanation for all observations.

Support for a cyclic mechanism is provided by the observation that on disso-

ciation the reaction at 558 nm is monophasic. If the reaction pathway for association and dissociation were the same (compare Eqn 2), two reactions would be observed at 558 nm on dissociation of the complex. However, the possibility can not be ruled out that the absorbance at 432 nm of the $E^{558,432}$ component is large and the amount present is very small compared to the amount of EI^{558}, thus masking the detection of a biphasic dissociation reaction.

It has been demonstrated[1] that one HN_3 molecule is responsible for the induced spectral changes. Because the rate of binding of azide to isolated oxidized cytochrome *c* oxidase equals the rate of formation of the inhibitory complex either for the enzyme *in situ*[2] or the isolated enzyme[1], we conclude that in all cases a binding occurs to the oxidized form of the enzyme.

A recent detailed investigation by Wilson and coworkers[14] of the effect of azide on the redox potentials of the two heme groups showed that the oxidized low-potential heme (cytochrome *a*) binds azide. A discrepancy still exists between the value of dissociation constant determined from potentiometric studies[14] (250 μM at pH 7.2), and those reported[1,2,15-17] for the inhibition and dissociation constants (20–60 μM at pH 7.2).

Unfortunately there is no direct evidence available concerning the actual binding site of azide. It can only be said that azide binds to partially reduced[18] and oxidized cytochrome *c* oxidase and that it affects the ligand field of the heme.

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REFERENCES

1 Wever, R., Muijsers, A. O., Van Gelder, B. F., Bakker, E. P. and Van Buuren, K. J. H. (1973) *Biochim. Biophys. Acta* 325, 1d7
2 Wilson, D. F. (1971) in *Probes of Structure and Function of Macromolecules and Membranes* (Chance, B., Yonetani, R. and Mildvan, A. S., eds), Vol. II, pp. 593–600, Academic Press, New York and London
3 Van Gelder, B. F., Wever, R. and Muijsers, A. O. (1973) in *Mechanisms in Bioenergetics* Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E. and Siliprandi, N., eds), pp. 571–577, Academic Press, New York
4 Van Gelder, B. F., Wever, R. and Muijsers, A. O. (1972) *Fed. Proc.* 31, Abstr. 1114
5 Van Gelder, B. F., Tiesjema, R. H., Muijsers, A. O. ,Van Buuren, K. J. H. and Wever, R. (1973) *Fed. Proc.*, in the press
6 Czerlinski, G. H. (1966) *Chemical Relaxation*, Marcel Dekker Inc., New York
7 G. G. Hammes (1968) in *Adv. Proc. Chem.* (Anfinsen, Jr, C. B., Anson, M. L., Edsall, J. T. and Richards, F. M., eds), 23, 1–57
8 Eigen, M. and Hammes, G. G. (1963) in *Adv. Enzymol.* (Nord, F. F., ed.), Vol. 25, pp. 1-38 Interscience Publishers, New York

9 Schmitz, K. S. and Schurr, J. M. (1972) *J. Phys. Chem.* 76, 534–545
10 Erecińska, M. and Chance, B. (1972) *Arch. Biochem. Biophys.* 151, 304–315
11 Perutz, M. F. and Mathews, F. S. (1966) *J. Mol. Biol.* 21, 199–202
12 Van Buuren, K. J. H., Nicholls, P. and Van Gelder, B. F. (1972) *Biochim. Biophys. Acta* 256, 258–276
13 Wilson, D. F., Erecińska, M. and Brocklehurst, E. S. (1972) *Arch. Biochem. Biophys.* 151 180–187
14 Wilson, D. F., Lindsay, J. G. and Brocklehurst, E. S. (1972) *Biochim. Biophys. Acta* 256, 277–286
15 Wilson, D. F. (1967) *Biochim. Biophys. Acta* 131, 431–440
16 Yonetani, T. and Ray, G. S. (1965) *J. Biol. Chem.* 240, 3392–3398
17 Muijsers, A. O., Slater, E. C. and Van Buuren, K. J. H. (1968) in *Structure and Function of Cytochromes* (Okunuki, K., Kamen, M. D. and Sekuzu, I., eds), pp. 129–137, Univ. of Tokyo Press, Tokyo
18 Van Gelder, B. F. and Beinert, H. (1969) *Biochim. Biophys. Acta* 189, 1–24