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BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME *c* OXIDASE

XV. REACTION WITH FLUORIDE

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SUMMARY

1. Fluoride is a mixed-type inhibitor of the cytochrome *c* oxidase activity with a K_i for the free enzyme of 10 mM and a K_i for the cytochrome *c*-complexed enzyme of 35 mM.

2. Fluoride shifts the γ-band of the enzyme from 423 to 421 nm and the α-band from 597 to 598 nm. The difference spectrum (oxidized enzyme in the presence of fluoride minus oxidized enzyme) has peaks at 400, 453, 482, 605 and 638 nm and troughs at 430, 520, 552 and 674 nm. The changes in absorbance are small (about 3% at absorbance maxima) with respect to those of other hemoproteins.

3. On addition of fluoride to isolated cytochrome *c* oxidase 3 reactions can be distinguished: (I) a bimolecular binding reaction ($k_{on} = 4\ M^{-1} \cdot s^{-1}$ and $k_{off} = 2.9 \cdot 10^{-2} s^{-1}$ at 25 °C, pH 7.4) contributing at 638 nm and 430 nm; (II) a first-order reaction ($k = 2.4 \cdot 10^{-2}\ s^{-1}$ at 22 °C, pH 7.2) visible mainly at 430 nm and (III) a very slow reaction with a half-time in the order of 10 min.

4. The spectroscopic dissociation constants for the fluoride binding, determined from Hill plots using the absorbance changes at 638 and 430 nm, are similar (7 and 10 mM, respectively, at 22 °C, pH 7.2).

5. A mechanism for the reaction is discussed in which the bimolecular binding reaction is followed by a conformational change of the enzyme–fluoride complex.

INTRODUCTION

In 1939 Keilin and Hartree [1] reported inhibition of oxygen uptake when fluoride was added to succinate-oxidizing heart-muscle preparation. This inhibition was accompanied by small spectral shifts in the cytochrome oxidase region. In their paper, where the cytochromes *a* and a_3 were distinguished for the first time, it was stated that the ferric form of cytochrome a_3 combines with cyanide, sulphide, azide, hydroxylamine and possibly also with fluoride. The inhibitory effects of fluoride on the components of the respiratory chain were further investigated in 1945 by Borei [2] who observed a competitive type of inhibition.

More recently [3], we reported on spectral effects observed after addition of

fluoride to isolated cytochrome *c* oxidase but the slow absorbance changes, requiring several days for completion, were due to ageing of cytochrome *c* oxidase rather than to effects of fluoride. (For detailed information on spectral properties of cytochrome *c* oxidase, see Muijsers et al. [4]).

Since the observation that fluoride interferes with the anaerobic reduction of cytochrome *c* oxidase by NADH plus phenazine methosulphate [4, 5] could not always be reproduced with the improved preparations now available (cf. ref. 6), it is necessary to reinvestigate the effects of fluoride on isolated cytochrome *c* oxidase.

Part of this work has been published in Muijsers' thesis [7].

MATERIALS AND METHODS

Cytochrome *c* oxidase was isolated as described by Van Buuren [8]. The procedure follows the principles of the Fowler method [9] and uses the cholate–$(NH_4)_2SO_4$ fractionation reported by MacLennan and Tzagoloff [10]. The concentration was determined [11] using a $\Delta A_{605\ nm}$ (red minus ox) of 24 $mM^{-1} \cdot cm^{-1}$. The activity was measured spectrophotometrically as described before [12]. For spectroscopic measurements a Cary-17 recording spectrophotometer or a Durrum–Gibson stopped-flow apparatus was used.

Tween 80 (Sigma) was used from a 10% (v/v) stock solution, stored at 0–4 °C. Cholic acid (British Drug Houses) was recrystallized in ethanol after treatment with active charcoal [13] and 20% (w/v) stock solutions of the potassium salt were stored in dark bottles. All other chemicals were of reagent grade, mainly from Baker.

RESULTS

Inhibition

The inhibition by fluoride of the catalytic activity of cytochrome *c* oxidase was measured spectrophotometrically. The reaction was initiated by addition of small aliquots of enzyme, preincubated with fluoride, to the reaction vessel containing various concentrations of ferrocytochrome *c* and the same fluoride concentration as during the preincubation. The reaction was terminated after 50–100 s by addition of $K_3Fe(CN)_6$.

Both in the absence and presence of fluoride straight lines are obtained when the ferrocytochrome *c* concentration was plotted semilogarithmically against time. The inversed initial rates calculated from the slope of these lines are plotted against the inversed substrate concentration in Fig. 1A. The straight lines for different fluoride concentrations in this Lineweaver–Burk plot intersect in the second quadrant. From this it is concluded that fluoride is a mixed-type inhibitior. In this type of inhibition [13] the ligand has affinity for the free enzyme (E) as well as for the cytochrome *c*-complexed enzyme (ES and EP). The K_i values for the free enzyme (K_i^E) and the complexed enzyme ($K_i^{ES, EP}$) determined from the Dixon plot (Fig. 1B) are 10 and 35 mM, respectively.

The relatively weak inhibition of the enzymic activity by fluoride might be explained as an aspecific salt effect. This is ruled out by the observation that an increase of the phosphate concentration from 65 to 80 mM had no appreciable effect on the catalytic parameters of our purified cytochrome *c* oxidase preparations.

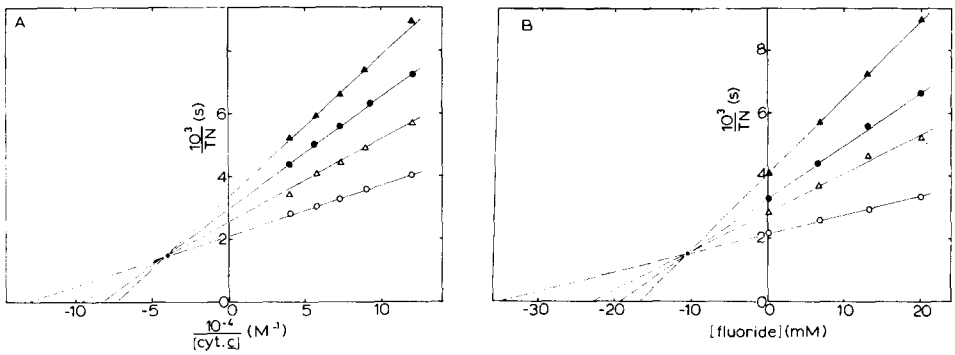

Fig. 1. Inhibition of the cytochrome *c* oxidase activity by fluoride. The enzyme was preincubated with 6.6–20 mM fluoride for 1–2 h in 0.2 % Asolectin, 10 mM phosphate buffer (pH 7.0), 0.25 M sucrose and 0.5 % Tween 80. The assay medium contained 65 mM phosphate buffer (pH 6.0), 0.5 % Tween 80, 1 mM EDTA, 20 μg Asolectin per ml, 8.3–25 μM ferrocytochrome *c* and the same fluoride concentration as in the incubation medium. The reaction was initiated by the addition of cytochrome *c* oxidase to a final concentration of 1 nM. The turnover number (TN) is expressed as moles cytochrome *c* oxidized per mole cytochrome *c* oxidase per s. Temp., 22 °C. A. Lineweaver–Burk plot. Fluoride: ○–○, none; △–△, 6.6 mM; ●–●, 13.3 mM and ▲–▲, 20 mM. B. Dixon plot. The points are obtained from Fig. 1A. Cytochrome *c*: ○–○, infinite concentration (determined by extrapolation of the lines in Fig. 1A); △–△, 25 μM; ●–●, 15 μM and ▲–▲, 8.3 μM.

Spectra

Addition of 100 mM fluoride to oxidized cytochrome *c* oxidase shifts the α-band from 597 to 598 nm with an increase of absorbance of about 3% (Fig. 2). At 638 nm, where the absorbance increases by 15%, a shoulder is formed. The γ-band shifts from 423 to 421 nm and decreases 3% in intensity. The difference spectrum (cytochrome *c* oxidase+fluoride minus cytochrome *c* oxidase), shown in Fig. 3, has

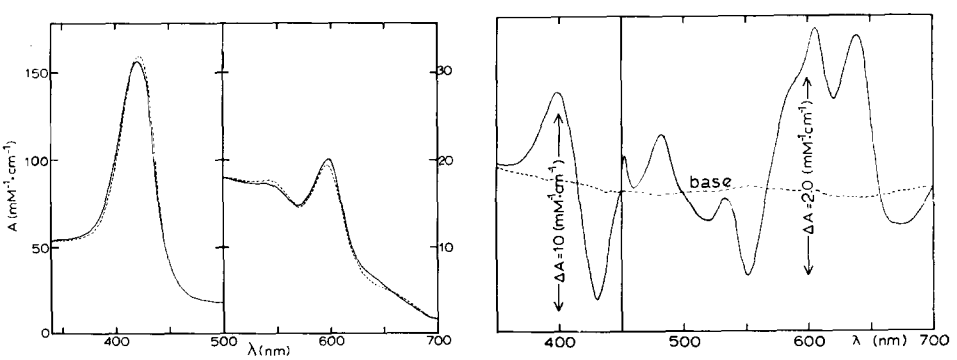

Fig. 2. Absorbance spectrum of cytochrome *c* oxidase in the absence (- - -) and presence (———) of 100 mM KF. The enzyme was diluted in 0.1 M phosphate buffer (pH 7.2) and 0.5 % cholate. The spectroscopic measurements were carried out at 22 °C. Absorbance scale based on 2 hemes per cytochrome *c* oxidase molecule.

Fig. 3. Difference spectrum of cytochrome *c* oxidase in the absence and presence of fluoride (enzyme +fluoride minus enzyme). Conditions as described in Fig. 2.

peaks at 400, 453, 482, 605 and 638 nm, a shoulder at 585 nm, and throughs at 430, 520, 552, and 674 nm. Isosbestic points are found at 415, 450, 497, 566, 657 and 700 nm.

Kinetics

The rate of reaction of fluoride with cytochrome *c* oxidase was studied at 638 and 430 nm. At 638 nm the main part of the absorbance change is associated with a relatively rapid reaction (I) while the remaining 10–20% of the absorbance change stems from a very slow reaction (III) with a half-time of 800 s, which was not further investigated.

In Fig. 4A where the changes in absorbance are plotted semilogarithmically against time, straight lines are observed with slopes depending on the fluoride concentration. This indicates, that the reaction is first-order with respect to enzyme.

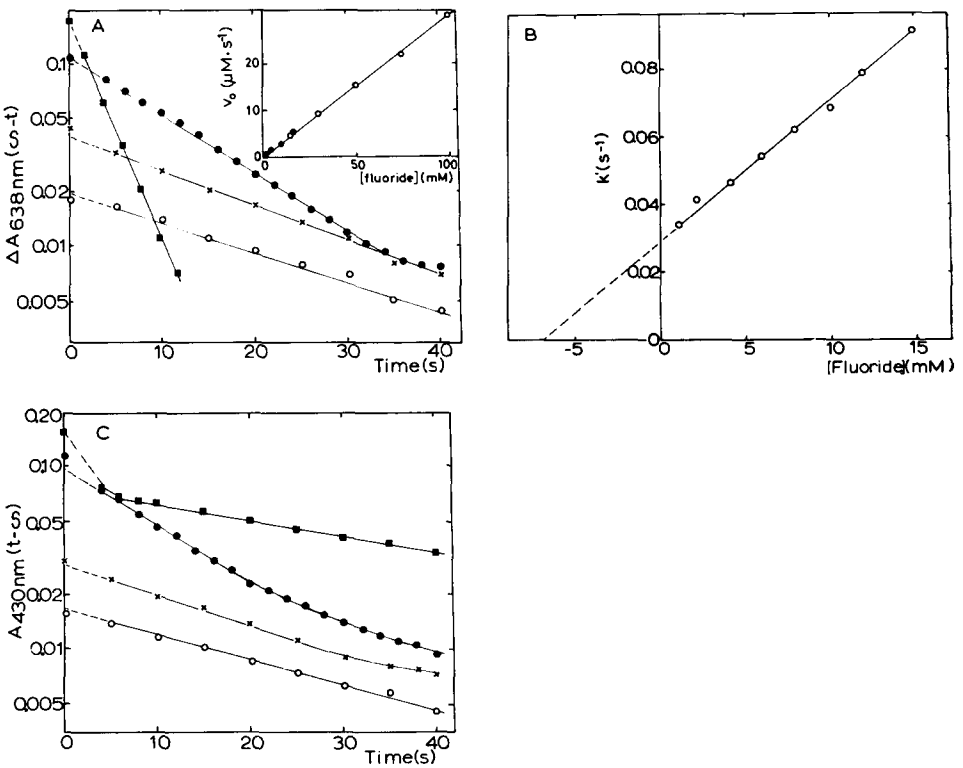

Fig. 4. Time course of the reaction of fluoride with cytochrome *c* oxidase. Enzyme was dissolved in 100 mM phosphate buffer (pH 7.4) and 1 % (v/v) Tween 80 at 25 °C. Fluoride was added at zero time. A. Semilogarithmic plot for the change in 638-nm absorbance with time. Cytochrome *c* oxidase, 80 μM KF^-: ○-○, 1 mM; ×-×, 2 mM; ●-●, 6 mM and ■-■, 50 mM. Inset: dependence of the initial rate on the fluoride concentration. The closed circles are obtained from the experiments similar to those shown in Fig. 4A, open circles are from a stopped-flow experiment under the same conditions. B. The observed first-order rate constant for the reaction of fluoride with cytochrome *c* oxidase at 638 nm as a function of the fluoride concentration. The points are obtained from experiments similar to those described in Fig. 4A. C. Semilogarithmic plot for the change in absorbance at 430 nm. Fluoride was added at zero time. 25 μM cytochrome *c* oxidase; other conditions and symbols as in Fig. 4A.

Since the initial rate of fluoride binding is proportional to the fluoride concentration (inset Fig. 4A), the reaction is also first-order in fluoride and hence the binding of fluoride to cytochrome *c* oxidase is a bimolecular process.

$$E + F \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} EF \qquad (1)$$

The second-order rate constant for the binding of fluoride, calculated from the slope of the line in the inset of Fig. 4A is $3.9\ M^{-1} \cdot s^{-1}$, at 25 °C. When fluoride is present in excess with respect to cytochrome *c* oxidase the following equation can be derived:

$$k' = k_{on}[\text{fluoride}] + k_{off} \qquad (2)$$

where k' represents the first-order rate constant for the approach to equilibrium between fluoride and cytochrome *c* oxidase. Eqn 2 predicts a straight-line relationship between k' and the fluoride concentration. This is shown in Fig. 4B. From the slope of the line and the intercept on the ordinate the rate constants for the association ($4.1\ M^{-1} \cdot s^{-1}$) and dissociation ($0.029\ s^{-1}$) of fluoride were determined. The dissociation constant K calculated from these rate constants is 7 mM, in good agreement with the spectrophotometrically determined dissociation constant for the cytochrome *c* oxidase–fluoride complex (see later Fig. 5).

When studied at 430 nm the kinetic pattern for the reaction of fluoride with cytochrome *c* oxidase is more complex (Fig. 4C). At low fluoride concentrations the reaction corresponds well with the bimolecular Reaction I. At higher fluoride concentrations, however, the time-course of the reaction is clearly biphasic. The initial rate of reaction increases linearly with the fluoride concentration (not shown) but the rate of the final reaction (II) is independent of the fluoride concentration. At a fluoride concentration above 100 mM the first reaction is rapid enough to permit separate study of the kinetics of Reaction II. As can be seen from Table I this reaction is independent of the fluoride concentration and has a first-order rate constant of $0.024\ s^{-1}$ at 22 °C.

TABLE I

FIRST-ORDER RATE CONSTANT OF REACTION II OF CYTOCHROME *c* OXIDASE WITH FLUORIDE

Medium, 100 mM potassium phosphate buffer (pH 7.2) and 0.8 % cholate; temperature 22 °C; λ, 430 nm.

Cytochrome *c* oxidase (μM)	[Fluoride] (mM)	$10^2 \cdot k$ (s^{-1})
8.4	600	2.7
8.4	200	2.6
8.4	120	2.2
5.0	200	2.4
3.3	200	2.2

Equilibrium studies

The reversibility of the reactions with fluoride was tested by passing the cytochrome *c* oxidase–fluoride complex through a Sephadex G-25 column or by dialysis against a saturated $CaSO_4$ solution, by which fluoride was removed as a precipitate. In both cases the γ-peak shifted back to its original position while the enzymic activity was restored for 80–100%. Furthermore, addition of azide to the cytochrome *c* oxidase-fluoride complex results in a spectrum also found with azide alone (not shown).

The equilibrium of Reaction I was studied at 638 nm. In the determination of the saturation degree α the contribution of the slow Reaction III was not included. The Hill plot (Fig. 5A) shows a straight line with slope $h = 0.8$ and from the intercept with the ordinate a dissociation constant (K_D) of 7 mM can be calculated. The Hill plot (Fig. 5B) from the absorbance changes at 430 nm, which include both Reactions I and II, shows a straight line with $h = 0.9$ and a K_D of 10 mM.

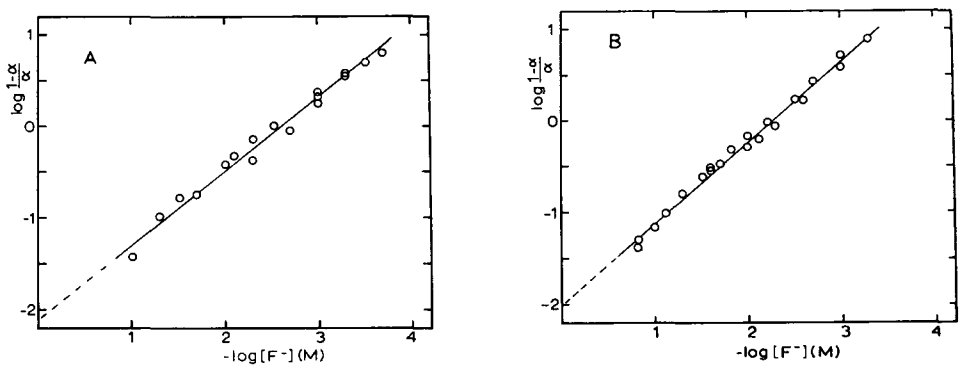

Fig. 5. Hill plot for the equilibrium between cytochrome *c* oxidase and its fluoride complex. Conditions as described in Fig. 2. The saturation degree α is defined as the ratio of the spectral effect of a certain fluoride concentration to that of an infinite fluoride concentration. The samples with 0.2–0.5 M fluoride were taken as saturated ($\alpha = 1$). A. 53 μM cytochrome *c* oxidase, spectral changes measured at 638 nm. B. 10 μM cytochrome *c* oxidase, spectral changes measured at 430 nm.

DISCUSSION

Studies of the effect of pH on the reaction of hemoproteins with fluoride revealed large variations in reaction pattern. Three cases have been reported: (i) reaction with and binding of HF; (ii) reaction with HF and binding of F^-, and (iii) reaction with F^-, with a rate strongly dependent on the charge of a heme-linked acidic group.

The effect of the pH on the reaction of fluoride with cytochrome *c* oxidase is still under investigation. It would be, therefore, premature to make a detailed comparison with kinetic constants of other hemoproteins. In general the kinetic parameters for cytochrome *c* oxidase fall in the same range as those given in the hemoprotein literature, but there is no close similarity with one in particular [14–17].

As the spectral changes observed upon addition of fluoride to cytochrome *c* oxidase are small [18–24] (cf. the $\Delta A_{638\,nm}$ of 1.7 $mM^{-1} \cdot cm^{-1}$ with the value [25] of 4–6 $mM^{-1} \cdot cm^{-1}$) we suggest that fluoride does not bind directly to the heme iron but that its reaction with the enzyme perturbates slightly the ligand field of the heme iron. In accord with this suggestion is the observation that the EPR spectrum of the ferric enzyme is not affected by fluoride [6].

The experiments shown in Fig. 5 do not provide an answer to the question whether one mole of fluoride is bound per mole of cytochrome *c* oxidase or per mole heme *a*. The first possibility is more likely in view of our recent investigations on ligand binding to cytochrome *c* oxidase where it was found that only one mole of cyanide [8, 12, 26, 27] or azide [7, 27–30] per mole of cytochrome *c* oxidase completely inhibited the enzymic activity. If this is the case, the slope of the line in a Hill plot should equal 1.0 (ref. 30). The observation that the slope of the lines is less than one suggests multiple binding sites with different K_D values. Whether these multiple binding sites are caused by different binding sites on the enzyme (having no or a negative-cooperative interaction) or by an inhomogeneity of the preparation [31] can not be decided.

The kinetic pattern of the reactions of fluoride with cytochrome *c* oxidase resembles that of azide [27–29, 32] The azide reaction was interpreted in terms of a bimolecular binding reaction followed by a ligand-induced conformational change of the protein. This model is schematically presented in Eqn 3 for the case of fluoride

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI^{638} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^{638,430} \tag{3}$$

where E is the free enzyme, EI^{638} the rapidly formed enzyme–fluoride complex, and $EI^{638,430}$ the final enzyme–inhibitor complex. Our observations fit this mechanism when in the difference spectrum EI^{638} and $EI^{638,430}$ are spectroscopically distinct at 430 nm but not at 638 nm.

As was shown by Wever et al. [32], the observed rate constant for the formation of $E^{638,430}$ will resemble that of a monomolecular reaction provided $k_{-1}/k_1 \ll (I)$. This was the case for the conditions used in Table I. In this context it is interesting to note that Van Buuren et al. [33], who preferred a similar mechanism for the reaction of cytochrome *c* oxidase with cyanide, and Wever et al. [32], who studied the reaction with azide, both reported similar values for the first-order rate constant (0.018 s^{-1} and 0.03 s^{-1}, respectively) for the ligand-induced conformational transition as was found for fluoride (0.024 s^{-1}).

Yoshikawa and Orii [34], who studied inhibitory effects of various ligands, found similar values for the first-order rate constant (0.015–0.029 s^{-1}) which were also independent of the inhibitor concentration. For the explanation of their results, Yoshikawa and Orii preferred a model in which two conformations of cytochrome *c* oxidase are in equilibrium, of which only one is capable to react with ligands such as fluoride. A scheme of their mechanism is given in Eqn 4 in which we have included our spectroscopic observations.

$$E^{638} \rightleftharpoons E \underset{I}{\overset{I}{\rightleftharpoons}} EI^{638,430} \tag{4}$$

where E^{638} and E are the respective enzyme conformations and $EI^{638,430}$ is the enzyme–fluoride complex. The time courses of Figs 4A and 4C can be explained by this mechanism when E^{638} and $EI^{638,430}$ are spectroscopically distinguishable at 430 nm but not at 638 nm.

According to Yoshikawa and Orii [34] their model also accounts for the mode of action of cyanide, hydroxylamine, azide and salicylaldoxim. Our data on the kinetics of the cyanide-induced [33] spectral changes of cytochrome *c* oxidase could only be explained with a mechanism similar to that of Eqn 4 if the two conformations of the unliganded enzyme are spectroscopically indistinguishable entities. The latter assumption is untenable in the case of azide and fluoride. Since we prefer a common scheme for the reaction of ligands with cytochrome *c* oxidase as a minimum hypothesis, the model given by Eqn 3 is proposed.

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