Biochimica et Biophysica Acta, 593 (1980) 17—23

BBA 47941

THE EFFECT OF pH AND IONIC STRENGTH ON THE STEADY-STATE ACTIVITY OF ISOLATED CYTOCHROME *c* OXIDASE

J. WILMS, J.L.M.L. VAN RIJN and B.F. VAN GELDER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam (The Netherlands)

(Received March 17th, 1980)

Key words: Cytochrome c oxidase; pH effect; Ionic strength effect; Turnover number

Summary

1. The turnover number and apparent K_m of isolated beef-heart cytochrome *c* oxidase were found to increase continuously when the pH was lowered from 8.6 to 4.6 (turnover number 32—630 s^{-1}). In this pH range neither irreversible denaturation of the enzyme nor an optimum for the turnover number was observed.

2. The turnover number of cytochrome *c* oxidase was found to be independent of ionic strength. It was concluded that the dependence of the activity of cytochrome *c* oxidase on ionic strength is caused by a change in the value of K_m for cytochrome *c*.

3. The pH dependence of the turnover number of cytochrome *c* oxidase can be described by a simple model in which at least three sites on the complex of cytochrome *c* oxidase with cytochrome *c* (pK_a 8.0, 6.5 and 4.8) can take up a proton.

Introduction

The effect of pH on the activity of cytochrome *c* oxidase has frequently been investigated, but the results seemed often contradictory [1—10]. This may be due to differences in reaction conditions, such as the concentrations of cytochrome *c* and buffer at which the activity was measured. In most cases an optimal pH for the enzymic activity of cytochrome *c* oxidase was found, but the position of the optimum varied considerably in the reports.

The effect of pH on the activity of cytochrome *c* oxidase is particularly important because protons are used as substrate in the steady-state oxidation of ferrocytochrome *c* by dioxygen, as shown by the reaction equation:

$$4 \text{ ferrocytochrome } c + 4\,H^+ + O_2 \xrightarrow{\text{cytochrome } c \text{ oxidase}} 4 \text{ ferricytochrome } c + 2\,H_2O$$

Furthermore, cytochrome *c* oxidase may be involved in proton transport across the mitochondrial inner membrane [11,12] and may act as a proton pump.

Therefore, we investigated in detail the effect of pH and ionic strength on the activity of cytochrome *c* oxidase at various concentrations of cytochrome *c*. The activity at infinite cytochrome *c* concentration was found to increase continuously with the proton concentration (pH range 4.6—8.6), but it was unaffected by ionic strength. The effects causing an apparent pH optimum in the activity of cytochrome *c* oxidase will be discussed.

Materials and Methods

Preparation and determination of the concentration of cytochrome *c* and cytochrome *c* oxidase have been described previously [13]. Cytochrome *c* was reduced at pH 8.0 by incubation with excess potassium ascorbate, after which the ascorbate was removed by gel filtration (Sephadex G-25, medium). Ionic strength (I) of potassium phosphate, varying in concentration from 25 to 125 mM in the pH range 4.6—8.6, was calculated from:

$$I = \frac{K_w}{[H^+]} + \frac{\frac{K_{a1}}{[H^+]}\left[1 + \frac{3K_{a2}}{[H^+]}\left(1 + \frac{2K_{a3}}{[H^+]}\right)\right] P_i}{1 + \frac{K_{a1}}{[H^+]}\left[1 + \frac{K_{a2}}{[H^+]}\left(1 + \frac{K_{a3}}{[H^+]}\right)\right]}$$

where P_i is the total phosphate concentration. The dissociation constants used were: $pK_{a1} = 2.12$, $pK_{a2} = 7.21$, $pK_{a3} = 12.67$ and $pK_w = 14.0$.

At low buffer capacity the pH was checked with a micro pH electrode before and after the reaction. The pH change never exceeded 0.1 unit.

The enzymic activity (v/e) of cytochrome *c* oxidase was determined spectrophotometrically at 550 nm and 25°C, using a Zeiss PMQ II spectrophotometer equipped with a logarithmic converter and a recorder [14]. The reaction was started by adding cytochrome *c* oxidase, that was previously diluted to a concentration of about 2 μM in a mixture of 0.25 M sucrose, 2 mg/ml Asolectine, 0.5% (v/v) Tween 80 and 10 mM potassium phosphate (pH = 7.0). The turnover number of cytochrome *c* oxidase was extrapolated from the rate of ferrocytochrome *c* oxidation (6.5—37.3 μM) and was expressed as μM ferrocytochrome *c* oxidized per second per μM cytochrome *c* oxidase (2 heme *a*) at infinite cytochrome *c* concentration.

Results

Fig. 1A shows the oxidation of cytochrome *c* catalyzed by cytochrome *c* oxidase at extreme low pH (4.6). It is evident that also at this low pH the oxidation of ferrocytochrome *c* follows the same type of first-order kinetics as found at physiological pH [14,15]. Fig. 1B shows the Lineweaver-Burk type plot of the enzymic activity of cytochrome *c* oxidase versus the cytochrome *c* concentration at several potassium phosphate concentrations (pH 4.6). Obviously, the enzymic activity at infinite cytochrome *c* concentration is unaffected by ionic

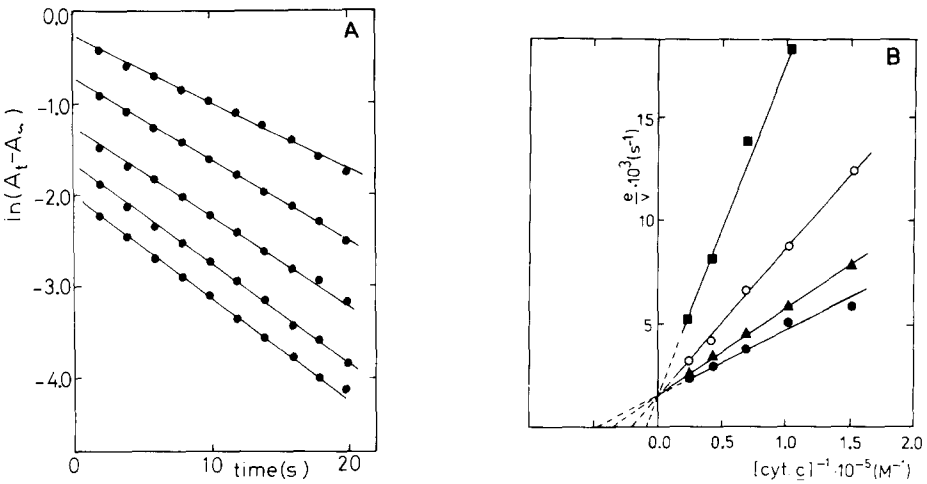


Fig. 1. A. Effect of phosphate on the oxidation of ferrocytochrome *c*, catalyzed by cytochrome *c* oxidase at pH 4.6. The absorbance was followed at 550 nm; 100 mM potassium phosphate, 0.5% (v/v) Tween 80; 25°C. Cytochrome *c* oxidase, 20 nM; cytochrome *c*, 37.3, 23.3, 14.0, 9.3 and 6.5 μM (from top to bottom). B. Effect of ionic strength on the enzymic activity of cytochrome *c* oxidase at pH 4.6. Potassium phosphate: ●———●, 50 mM; ▲———▲, 75 mM; ○———○, 100 mM; ■———■, 125 mM.

strength; thus the ionic strength dependence of the cytochrome *c* oxidase activity at finite substrate concentrations is due to a change in the apparent K_m value. These phenomena were observed over the entire pH range investigated (4.6—8.6). Control experiments were carried out in which part of the potassium phosphate was substituted by potassium chloride without changing the ionic strength of the medium. This substitution did not affect the enzymic activity, indicating that an ionic strength effect was observed [16] and not a specific phosphate effect (cf. Refs. 17—20).

Fig. 2A shows that the activity of cytochrome *c* oxidase at infinite cyto-

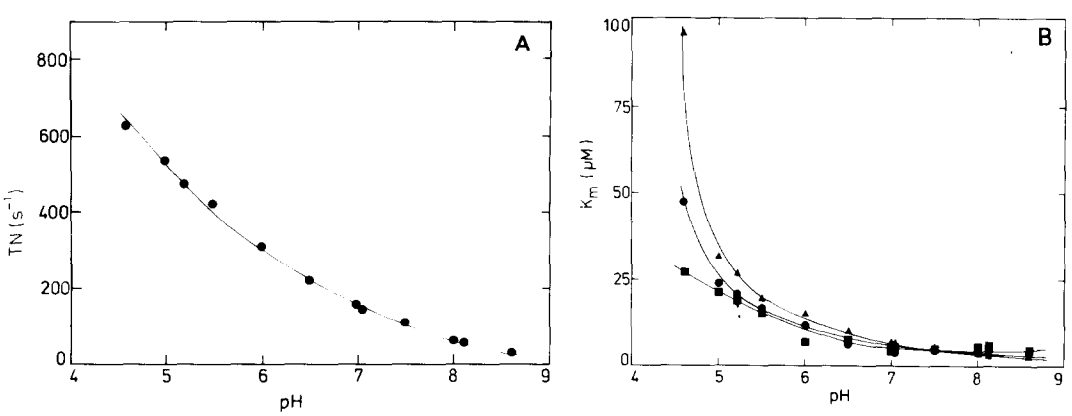
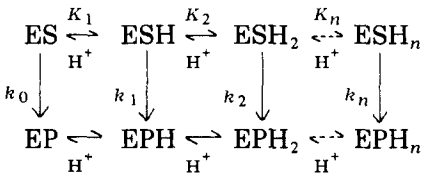


Fig. 2. A. Effect of pH on the turnover number (TN) of cytochrome *c* oxidase. ●, experimental data. The solid line is a simulation of Eqn. 2 using the constants presented in Table I. B. The apparent K_m for cytochrome *c* as function of pH. The apparent K_m was determined from plots as shown in Fig. 1B and interpolated to the following ionic strength values: ▲———▲, 125 mM; ●———●, 100 mM; ■———■, 75 mM.

chrome *c* concentration increases when the pH is lowered to 4.6, whereas above pH 8.0 cytochrome *c* oxidase is almost inactive. At these extreme pH values, no denaturation phenomena were observed: the enzyme, incubated for 30 min at either pH 5.0 or pH 8.0, showed the same activity determined at pH 7.0 as the preparation that was not subjected to pH treatment. Fig. 2B illustrates that the apparent K_m of cytochrome *c* oxidase for cytochrome *c* also increases upon lowering of the pH and is clearly affected by ionic strength.

The increase in turnover number upon lowering of the pH can be interpreted with a model in which protonation of sites on the cytochrome *c*-cytochrome *c* oxidase complex is essential for activation:



The turnover number (TN) of an enzyme reacting according to this model will be a function of rate constants (k_0, k_1, k_2, ..., k_l, ..., k_n) and the dissociation constants (K_1, K_2, ..., K_l, ..., K_n) of the various enzyme-substrate complexes. This function can be expressed as (cf. Ref. 21):

$$TN = \left\{k_0 + \sum_{l=1}^{n}\left(k_l[H^+]^l \Big/ \prod_{j=1}^{l} K_j\right)\right\} \Big/ \left\{1 + \sum_{l=1}^{n}\left([H^+]^l \Big/ \prod_{j=1}^{l} K_j\right)\right\} \quad (1)$$

In a range where one of the sites becomes protonated, this expression can be simplified if the values of the rate constants are of the same order of magnitude, and the dissociation constants differ at least one order of magnitude, i.e.

$K_1, ..., K_{l-1} < [H^+] < K_{l+1}, ..., K_n$ and

$K_1, ..., K_{l-1} < K_l < K_{l+1}, ..., K_n$.

The following expression is obtained under these restrictions:

$$TN - k_{l-1} \simeq \{k_l - k_{l-1}\}/\{1 + K_l/[H^+]\} \quad (2)$$

and a plot of $(TN - k_{l-1})^{-1}$ versus $[H^+]^{-1}$ will result in a straight line with intersections that yield k_l and K_l.

At high pH all enzyme will be in unprotonated ES-form and hence $TN = k_0$. The data shown in Fig. 2A indicate that for pH > 9 the enzymic activity becomes negligible small ($k_0 \simeq 0$). Thus in the pH range where the first activity stimulating site becomes portonated, i.e. $l = 1$, the turnover number will be dependent on concentration and reactivity of ESH. For $l = 1$ and $k_0 = 0$ Eqn. 2 can be written as:

$$TN = \{k_1[H^+]/K_1\}/\{1 + [H^+]/K_1\} \quad (3)$$

As shown in Fig. 3A, the plot of TN^{-1} versus $[H^+]^{-1}$ yields a straight line and values of $k_1 = 105\ s^{-1}$ and $K_1 = 10^{-8}$ M are found. With these values the experimental data for $l = 2$ and $l = 3$ can be plotted according to Eqn. 2 (Figs. 3B and 3C, respectively).

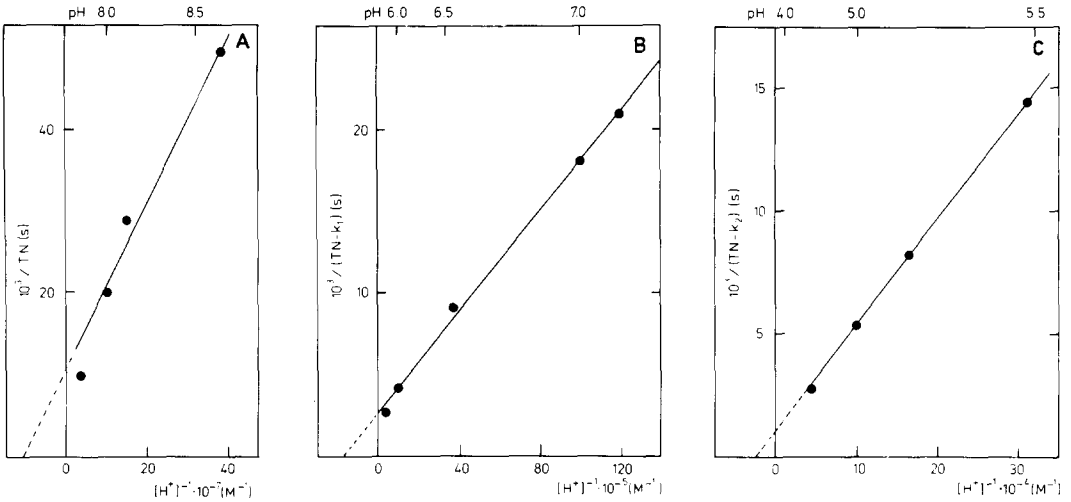


Fig. 3. Determination of dissociation and rate constants of various activity-regulating acid-base sites. Double reciprocal plot of $(TN - k_{l-1})$ versus $[H^+]$ according to Eqn. 2 are shown. A. $l = 1$, $TN = k_1/(1 + K_1/[H^+])$; from Eqn. 1 with $k_0 = 0$ and $[H^+]$, $K_1 < K_2, \ldots, K_n$. B. $l = 2$, $(TN - k_1) = (k_2 - k_1)/(1 + K_2/[H^+])$; from Eqn. 1 with $K_1 < [H^+]$, $K_2 < K_3, \ldots, K_n$. C. $l = 3$, $(TN - k_2) = (k_3 - k_2)/(1 + K_3/[H^+])$; from Eqn. 1 with $K_1, K_2 < [H^+]$, $K_3 < K_4, \ldots, K_n$.

TABLE I

PARAMETERS USED FOR THE SIMULATION OF THE TURNOVER NUMBER VERSUS pH CURVE (FIG. 2A, SOLID LINE) USING EQN. 1 WITH $k_0 = 0$ AND THREE ACTIVITY-REGULATING ACID-BASE SITES

l	1	2	3
k_l (s^{-1})	120	340	850
pK_l	8.0	6.5	4.8

The set of reaction and dissociation constants obtained from Fig. 3 can be used for the calculation of a turnover number versus pH profile (cf. Eqn. 1) which fits the experimental data well. However, as shown by the solid line in Fig. 2A, a very close fit to the experimental data is obtained with the set of slightly corrected constants, presented in Table I.

This result indicates that at least three acid-base sites are required with pK values of 8.0, 6.5 and 4.8 to explain the pH-dependence of the turnover number. When protonated, these sites promote the enzymic activity.

Discussion

Our results show that in the pH region of 4.6—8.6 the enzymic activity of isolated cytochrome *c* oxidase at infinite cytochrome *c* concentration is independent of ionic strength, in contrast to the Michaelis constant with respect to cytochrome *c*. This is in line with observations in literature where it was shown that the enzymic rate is dependent upon ionic strength when measured at a single cytochrome *c* concentration [1—6,8].

The independence of the turnover number and the dependence of K_m upon ionic strength point to an electrostatically governed binding reaction between cytochrome *c* and cytochrome *c* oxidase, as was also observed for the reduction of cytochrome *c* by cytochrome b_5 [22,23]. It has frequently been reported that in the association reaction of cytochrome *c* oxidase and cytochrome *c* charges of opposite sign are important [7,20,24,26].

The pH-dependence of cytochrome *c* oxidase activity has often been investigated at a single cytochrome *c* and at a single buffer concentration [1—6,8,27]. Under these conditions three effects of pH can be discerned: a direct effect of pH on the turnover number and K_m, as well as an indirect effect of pH on ionic strength and thus on K_m. In such an experiment the appearance of a pH optimum (also found by us) is caused by the opposite effect of pH on the turnover number and on K_m. It is evident that the position of the pH optimum of the activity is not constant, but depends on concentration, pK_a and electrical charge of the applied buffer. When using the polarographic assay method additional pH effects, due to reactions in which ascorbate and/or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine are involved, must be considered [25].

Several authors [1—3,5,6,10,27] have reported a pH optimum in the activity of cytochrome *c* oxidase at low pH; however, in the turnover number of our enzyme preparation no such optimum was found. It is conceivable that our preparation is more stable at low pH.

The dependence of the turnover number of pH has been explained in a model in which three proton-binding sites affect the reaction rate. For enzymic activity the protonation of the site with a pK_a of 8.0 is required. This site might be the same as that which affects the redox potential of the high-potential heme [28]. From our kinetic data we cannot discern whether a proton acts as an activator of the enzymic activity, or that a hydroxyl ion forms an inhibitory complex with the oxidase. Lanne et al. [29] have suggested that a hydroxyl ion can bind to cytochrome a_3 as the sixth ligand to the heme iron. If the binding of a hydroxyl ion to the site with pK_a 8.0 causes cytochrome *c* oxidase to become inactive, a K_i of 1 μM can be calculated from our results. The nature of the two other sites (pK_a 6.5 and 4.8, respectively) is not clear yet. Maybe the rate of proton uptake by oxygen intermediates increases upon protonation of these sites.

An alternative explanation of the pH-dependence of the turnover number is that the rate-determining step in the steady-state mechanism is a conformational change in the cytochrome *c* oxidase molecule, induced by binding of cytochrome *c* [30,31]. Protonation of the three sites may cause formation or breaking of salt bridges. This will govern the rate of the conformational change of cytochrome *c* oxidase, thus facilitating the electron transfer by the enzyme.

Acknowledgements

The authors wish to thank Mr. G.J.A. Schilder for stimulating discussion and Dr. R. Wever for his interest. This work has had the support of the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

References

1 Wainio, W.W., Person, P., Eichel, B. and Cooperstein, S.J. (1951) J. Biol. Chem. 192, 349—360
2 Wainio, W.W., Eichel, B. and Gould, A. (1960) J. Biol. Chem. 235, 1521—1525
3 Yonetani, T. (1961) J. Biol. Chem. 236, 1680—1688
4 Davies, H.C., Smith, L. and Wasserman, A.R. (1964) Biochim. Biophys. Acta 85, 238—246
5 Orii, Y. and Okunuki, K. (1965) J. Biochem. 58, 561—568
6 Yonetani, T. and Ray, G.S. (1965) J. Biol. Chem. 240, 3392—3398
7 Ferguson-Miller, S., Brautigan, D.L. and Margoliash, E. (1976) J. Biol. Chem. 251, 1104—1115
8 Bomhoff, G.H. and Spencer, M. (1977) Can. J. Biochem. 55, 1114—1117
9 Wrigglesworth, J.M. (1977) in Proc. 11th FEBS Meeting, Vol. 45, pp. 95—103, North-Holland, Amsterdam
10 Maurel, P., Douzou, P., Waldmann, J. and Yonetani, T. (1978) Biochim. Biophys. Acta 525, 314—324
11 Wikström, M. and Krab, K. (1978) FEBS Lett. 91, 8—14
12 Moyle, J. and Mitchell, P. (1978) FEBS Lett. 88, 268—272
13 Van Buuren, K.J.H. (1972) Ph.D. Thesis, University of Amsterdam, Gerja, Waarland
14 Smith, L. and Conrad, H. (1956) Arch. Biochem. Biophys. 63, 403—413
15 Minnaert, K. (1961) Biochim. Biophys. Acta 50, 23—34
16 Goldkorn, T. and Schejter, A. (1979) J. Biol. Chem. 254, 12562—12566
17 Barlow, G.H. and Margoliash, E. (1966) J. Biol. Chem. 241, 1473—1477
18 Nicholls, P. (1974) Biochim. Biophys. Acta 346, 261—310
19 Taborsky, G. (1972) Biochemistry 11, 729—734
20 Osherhoff, N., Koppenol, W.H. and Margoliash, E. (1978) in Frontiers of Biological Energetics (Dutton, P.L., Leigh, J.S. and Scarpa, A., eds.), Vol. 1, pp. 439—449, Academic Press, New York
21 Dixon, M. and Webb, E.C. (1964) Enzymes, pp. 116—145, Longmans, London
22 Ng, S., Smith, M.B., Smith, H.T. and Millett, F. (1977) Biochemistry 16, 4975—4978
23 Stonehuerner, J., Williams, J.B. and Millett, F. (1979) Biochemistry 18, 5422—5427
24 Van Gelder, B.F., van Buuren, K.J.H., Wilms, J. and Verboom, C.N. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E.C. and Siliprandi, N., eds.), pp. 63—68, North-Holland, Amsterdam
25 Smith, L., Davies, H.C. and Nava, M.E. (1979) Biochemistry 18, 3140—3146
26 Veerman, E.C.I., Wilms, J., Casteleijn, G. and van Gelder, B.F. (1980) Biochim. Biophys. Acta 590, 117—127
27 Douzou, P. and Maurel, P. (1977) Trends Biochem. Sci. 2, 14—17
28 Van Gelder, B.F., van Rijn, J.L.M.L., Schilder, G.J.A. and Wilms, J. (1977) in Structure and Function of Energy-Transducing Membranes, Vol. 14, BBA Library (van Dam, K. and van Gelder, B.F., eds.), pp. 61, 68, Elsevier/North Holland Biomedical Press, Amsterdam
29 Lanne, B., Malmström, B.G. and Vänngård, T. (1979) Biochim. Biophys. Acta 545, 205—214
30 Wainio, W.W. (1974) in Molecular Oxygen in Biology (Hayaishi, O., ed.), pp. 275—314, North-Holland, Amsterdam
31 Kornblatt, J.A. (1977) Can. J. Biochem. 55, 458—464