Two K_m values for cytochrome *c* of aa_3-type two-subunit cytochrome *c* oxidase from *Nitrobacter agilis*

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Oxidation of ferrocytochrome *c* by aa_3-type two-subunit cytochrome *c* oxidase from *Nitrobacter agilis* was measured polarographically and the results obtained were analyzed by means of Eadie-Hofstee plots. Two apparent K_m values of $\sim 3.0 \times 10^{-8}$ and 2.0×10^{-6} M were obtained for horse cytochrome *c* in 25 mM phosphate buffer, pH 6.5. Also when *N. agilis* ferrocytochrome *c* was oxidized by the enzyme in 25 mM phosphate buffer (pH 6.5), two apparent K_m values of $\sim 2.0 \times 10^{-8}$ and 4.0×10^{-6} M were obtained although the break point in the Eadie-Hofstee plot was as clear as in the case of horse cytochrome *c*. The results show that two reactive sites for cytochrome *c* also occur in the bacterial cytochrome *c* oxidase composed of two subunits and will give further support to the idea that only the larger two subunits of eukaryotic cytochrome *c* oxidase are responsible for oxidation of ferrocytochrome *c*.

Bacterial cytochrome c *oxidase* *Cytochrome* aa_3 Nitrobacter agilis

1. INTRODUCTION

Cytochrome *c* oxidase of eukaryotes shows two K_m values for cytochrome *c* when oxygen reduction catalyzed by the oxidase is determined polarographically in a wide range of cytochrome *c* concentrations; the oxidase reacts with cytochrome *c* at two apparent K_m values of $\sim 10^{-8}$ and 10^{-6} M [1]. Although the eukaryotic oxidase is composed of 7 or more kinds of subunits (e.g., [2]), only the larger two subunits are thought to participate in oxidation of ferrocytochrome *c* [3]. Most bacterial cytochrome *c* oxidases so far purified are composed of 2 or 3 subunits, have heme *a* and Cu, and catalyze oxidation of some eukaryotic ferrocytochromes *c* [4–13]. These molecular and enzymatic features of the bacterial oxidases support strongly the idea mentioned above that only the larger two subunits of the eukaryotic enzyme are responsible for oxidation of ferrocytochrome *c*.

It was our interest to determine whether two K_m values for cytochrome *c* are also observed with the bacterial cytochrome *c* oxidase composed of two kinds of subunits. In this study, we found that two-subunit cytochrome *c* oxidase from *Nitrobacter agilis* also showed two apparent K_m values for cytochrome *c*.

2. MATERIALS AND METHODS

N. agilis cytochrome *c* oxidase and *N. agilis* cytochrome *c*-550 were purified as in [8,14]. Horse heart cytochrome *c* and cardiolipin were purchased from Sigma.

Cytochrome *c* oxidase activity was measured polarographically using an oxygen electrode (Beckman model 0260 oxygen analyzer) at 25°C. The standard reaction mixture contained 25 mM sodium phosphate buffer (pH 6.5), 4.5 mM sodium ascorbate, 0.16 mM tetramethylphenylenediamine (TMPD), cytochrome *c* at different concentrations and 0.14 μM *N. agilis* cytochrome *c* oxidase in a total volume of 1.1 ml. The reaction was started by addition of 15 μl enzyme (10 μM) dissolved in 10 mM Tris–HCl buffer (pH 8.0) containing 1% Tween 20. The concentration of Tween

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20 in the reaction mixture was ~0.014%. The nonenzymatic O_2 consumption by ascorbate was subtracted from the rate of enzymatic O_2 consumption. When the effect of cardiolipin was determined, 25 μl ethanol solution of the phospholipid (1.4 mg/ml) was added to 1.1 ml reaction mixture. The concentration of cardiolipin in the reaction mixture was ~0.0032%.

3. RESULTS

Fig.1 shows an Eadie-Hofstee plot for the reaction of *N. agilis* cytochrome *c* oxidase with horse cytochrome *c*. A biphasic feature is clearly observed in the plot and apparent K_m values were determined to be 3.0×10^{-8} and 2.0×10^{-6} M. V_{max} values (nmol O_2 consumed/min) were 0.5 and 2.8 for the high- and low-affinity sites, respectively.

We have shown in [15] that oxidation of horse and yeast ferrocytochromes *c* catalyzed by the bacterial oxidase is greatly stimulated by cardiolipin at the experimental concentrations of cytochrome *c* (~10 μM), when the reactions are determined spectrophotometrically; the apparent K_m value for cytochrome *c* decreases from 1×10^{-3} to 1.85×10^{-5} M, while the V_{max} value is hardly changed on addition of the phospholipid. In the polarographic determinations, addition of cardiolipin decreased the apparent K_m value at the high-affinity site from 3.0×10^{-8} to 0.8×10^{-8} M and increased the V_{max} value at the site from 0.5 to 2.0 nmol O_2 consumed/min. The apparent K_m value at the low-affinity site did not change on addition of the phospholipid, while the V_{max} value at the site increased from 2.8 to 25 nmol O_2 consumed/min (fig.2).

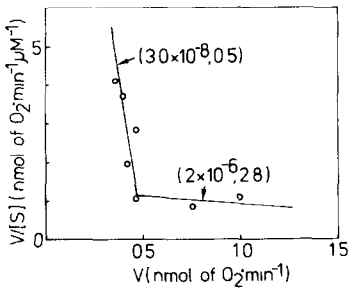

Fig.1. Eadie-Hofstee plot of oxidation of horse ferrocytochrome *c* by *N. agilis* cytochrome *c* oxidase in 25 mM phosphate buffer. The reaction mixture contained 25 mM phosphate buffer (pH 6.5), 0.14 μM *N. agilis* cytochrome *c* oxidase, 4.5 mM sodium ascorbate, 0.16 mM TMPD and various amounts of horse cytochrome *c* (0.02–1.6 μM). Figures in parentheses show apparent K_m and V_{max} values, respectively.

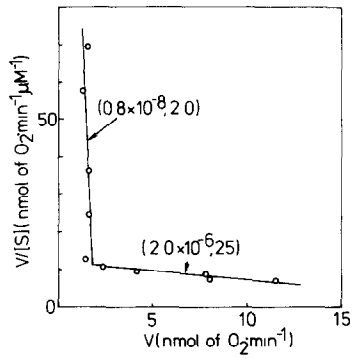

Fig.2. Eadie-Hofstee plot of oxidation of horse ferrocytochrome *c* in the presence of cardiolipin. The reaction conditions were the same as described in the legend for fig.1 except that 35 μg cardiolipin was added.

Fig.3 shows an Eadie-Hofstee plot for the reaction of *N. agilis* cytochrome *c* oxidase with *N. agilis* cytochrome *c*-550 in 25 mM phosphate buffer (pH 6.5). Although the biphasic feature was also clearly observed with the bacterial cytochrome *c* the kinetic pattern differed a little from that observed with horse cytochrome *c*; the break point in the Eadie-Hofstee plot was not sharp. Two apparent K_m values obtained were 2.0×10^{-8} and 4.0

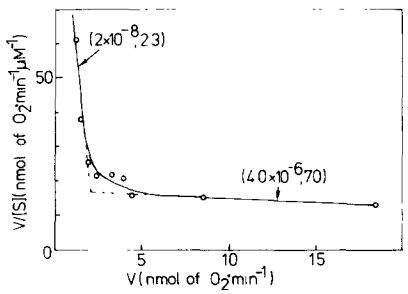

Fig.3. Eadie-Hofstee plot of oxidation of *N. agilis* ferrocytochrome *c* by *N. agilis* cytochrome *c* oxidase. The reaction conditions were the same as described in the legend for fig.1 except that various amounts of *N. agilis* cytochrome *c* (0.02–1.4 μM) were used in place of horse cytochrome *c*.

$\times 10^{-6}$ M at the high- and low-affinity sites, respectively.

4. DISCUSSION

N. agilis cytochrome *c* oxidase has two K_m values for horse ferrocytochrome *c* just as eukaryotic cytochrome *c* oxidase when the reactions are analyzed by means of an Eadie-Hofstee plot. This seems to be additional evidence which supports the idea that only the larger two subunits of the eukaryotic enzyme are responsible for oxidation of ferrocytochrome *c* [3]. Although when *N. agilis* cytochrome *c* is the electron donor for the bacterial oxidase two K_m values are observed, the break point in the Eadie-Hofstee plot is not so distinct; the data obtained appear to show that 3 K_m values might exist. This may show that the reaction mechanisms for the enzyme differ between *N. agilis* and horse cytochromes *c*. The difference should be elucidated in future study.

It has been reported [16] that although dimeric cow cytochrome *c* oxidase shows two K_m values, the monomeric oxidase shows only K_m value at the high-affinity site. This conflicts with the results in [17] that the monomeric camel oxidase shows two K_m values for cytochrome *c*. *N. agilis* cytochrome *c* oxidase exists as a monomer [18] and yet it shows two K_m values as shown by this study. The present study supports the idea that two K_m values are observable even with the monomer oxidase. Further, as the *N. agilis* oxidase does not contain appreciable amounts of phospholipid [15], the two K_m values are independent of phospholipid bound to the cytochrome *c* oxidase molecule. This is in good agreement with results obtained with the eukaryotic enzyme [19].

When the oxidation rate of horse ferrocytochrome *c* catalyzed by the *N. agilis* oxidase is determined spectrophotometrically, the K_m value for cytochrome *c* of the enzyme decreases from 1 mM to 18.5 μM on addition of cardiolipin [15], while the value at the low-affinity site remains unchanged on addition of the phospholipid when the reaction is determined polarographically. Even in the latter determination, V_{max} is increased 9-fold. It has been reported that the K_m value for cytochrome *c* of bovine cytochrome *c* oxidase determined spectrophotometrically differs from that determined polarographically as dissociation of ferricytochrome *c* from the oxidase is included in the spectrophotometric determinations [20]. The results that the K_m value determined spectrophotometrically decreases greatly in the presence of cardiolipin may be attributable to prevention by cardiolipin of an inhibitory interaction of ferricytochrome *c* with the oxidase. Thus, the K_m value at the low-affinity site remains unchanged in the polarographic determinations where most of the cytochrome *c* molecules may exist in the reduced state. However, there seems to be no simple reason why the V_{max} value at the low-affinity site in the polarographic determinations does increase on addition of cardiolipin. The phospholipid does not seem to act as an effector for the enzyme [15], since the activity of the *N. agilis* enzyme is not affected by cardiolipin when *N. agilis* cytochrome *c* is used as the electron donor.

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REFERENCES

[1] Ferguson-Miller, S., Brautigan, D.L. and Margoliash, E. (1978) J. Biol. Chem. 253, 149–159.
[2] Kadenbach, B. (1983) Angew. Chem. Int. Ed. Engl. 22, 275–283.
[3] Winter, D.B., Bruyninckx, W.J., Foulke, F.G., Grinich, N.P. and Mason, H.S. (1980) J. Biol. Chem. 255, 11408–11414.
[4] Yamanaka, T., Fujii, K. and Kamita, Y. (1979) J. Biochem. 89, 265–273.
[5] Yamanaka, T. and Fujii, K. (1980) Biochim. Biophys. Acta 591, 53–62.
[6] Ludwig, B. and Schatz, G. (1980) Proc. Natl. Acad. Sci. USA 77, 196–200.
[7] Fee, J.A., Choc, M.G., Findling, K.L., Lorence, R. and Yoshida, T. (1980) Proc. Natl. Acad. Sci. USA 77, 147–151.
[8] Yamanaka, T., Kamita, Y. and Fukumori, Y. (1981) J. Biochem. 89, 265–273.
[9] Sone, N. and Yanagita, Y. (1982) Biochim. Biophys. Acta 682, 821–824.

[10] Sone, N., Yanagita, Y., Hon-nami, K., Fukumori, Y. and Yamanaka, T. (1983) FEBS Lett. 155, 150–154.
[11] Gennis, R.B., Casey, R.P., Azzi, A. and Ludwig, B. (1982) Eur. J. Biochem. 125, 189–195.
[12] De Vrij, W., Azzi, A. and Konigs, W.N. (1983) Eur. J. Biochem. 131, 97–103.
[13] Yamanaka, T. and Fukumori, Y. (1981) Plant Cell Physiol. 22, 1223–1230.
[14] Yamanaka, T., Tanaka, Y. and Fukumori, Y. (1982) Plant Cell Physiol. 23, 441–449.
[15] Fukumori, Y. and Yamanaka, T. (1982) Biochim. Biophys. Acta 681, 305–310.
[16] Nalęcz, K.A., Bolli, R. and Azzi, A. (1983) Biochem. Biophys. Res. Commun. 114, 822–826.
[17] Darley-Usmar, V.M., Alizai, N., Al-ayash, A.I., Jones, G.D., Sharpe, A. and Wilson, M.T. (1981) Comp. Biochem. Physiol. 68B, 445–456.
[18] Sato, M., Tanaka, N., Kakiuchi, K., Fukumori, Y., Yamanaka, T., Kasai, N. and Kakudo, M. (1983) Biochem. Int. 7, 345–352.
[19] Al-Tai, W.F., Jones, M.G., Rashid, K. and Wilson, M.T. (1983) Biochem. J. 209, 901–903.
[20] Smith, L., Davies, H.C. and Nava, M.E. (1979) in: Cytochrome Oxidase (King, T.E. et al. eds) pp.293–304, Elsevier, Amsterdam, New York.