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EFFECT OF CARDIOLIPIN ON THE ENZYMATIC ACTIVITY OF *NITROBACTER AGILIS* CYTOCHROME *c* OXIDASE

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Effects of cardiolipin on the reaction rates of *Nitrobacter agilis* cytochrome *c* oxidase with cytochrome *c* were studied at various concentrations of phosphate buffer. Cardiolipin stimulated greatly the oxidation by the enzyme of horse and yeast ferrocytochromes *c*, especially at higher ionic strengths. However, the oxidation by the enzyme of *N. agilis* ferrocytochrome *c*-550, the physiological electron donor for the oxidase, was not accelerated by addition of cardiolipin. Analysis of the lipid compositions showed that neither the cell membranes of *N. agilis* nor the enzyme preparation contained cardiolipin. These results suggest that cardiolipin is not necessary for the reaction of *N. agilis* cytochrome *c* oxidase with *N. agilis* cytochrome *c*-550. On the basis of these results, the difference in the reactivity with cytochrome *c* of cytochrome *c* oxidase between the bacterial and mitochondrial enzymes is discussed.

Introduction

Many aerobic prokaryotes as well as eukaryotes possess aa_3-type cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) [1]. In previous studies, we have purified the aa_3-type cytochrome *c* oxidases from the chemoautotrophs, *Thiobacillus novellus* and *Nitrobacter agilis*, and determined some of their properties [2–4]. Although the spectral properties of these enzymes are similar to those of the mitochondrial enzymes, their subunit structures are found to be very different from those of the latter enzymes [2–4]; the bacterial enzymes are composed of two different subunits, while the mitochondrial enzymes are composed of five to seven different subunits [5,6]. The larger subunit (subunit I) of the *N. agilis* enzyme is similar to subunit I of the mitochondrial enzyme in amino acid composition, molecular weight and 'abnormality' in polyacrylamide gel electrophoresis in the presence of SDS [4,7]. Ludwig [6] has shown that subunit II of *Paracoccus denitrificans* cytochrome *c* oxidase cross-reacts immunologically with subunit II of the yeast enzyme. The findings suggest that the bacterial enzyme may be functionally equivalent to an assembly of subunits I and II of the mitochondrial enzyme. Indeed, Winter et al. [8] have shown that it may be subunits I and II of the mitochondrial enzyme that catalyze oxidation of ferrocytochrome *c*, while other subunits may be related to ion transport.

Although *N. agilis* cytochrome *c* oxidase reacts with eukaryotic cytochrome *c* derived from horse, *Candida krusei* and *Saccharomyces oviformis*, its reactivity varies considerably among the different cytochromes [7]. Further, the reaction rates are very sensitive to ionic strength; they decrease greatly with an increase in concentration of phos-

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phate in the reaction mixture [3,4], while the reaction rates of the mitochondrial enzyme with cytochromes *c* show maximal values at certain concentrations of phosphate [9–13]. Recent studies show that mitochondrial cytochrome *c* oxidase requires cardiolipin for its reaction with cytochrome *c* [14].

In the present investigation, we studied effects of phospholipids, especially cardiolipin, on the reactivity of *N. agilis* cytochrome *c* oxidase with cytochromes *c*, and lipid compositions of the cells and oxidase preparation of the bacterium. Cardiolipin affected greatly the reactions of the oxidase with eukaryotic cytochromes *c*, while its effect was not observed in the reaction of the enzyme with *N. agilis* cytochrome *c*.

Materials and Methods

N. agilis cytochrome *c* oxidase was purified according to the method reported previously [4], and the oxidase prepared was dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 1% Tween 20 and stored in liquid nitrogen until use.

Membrane particles of *N. agilis* were prepared by the following procedure: The cells suspended in deionized water were treated with a sonic oscillator at 20 kHz for 20 min and then treated twice with a French pressure cell at 400 kg/cm^2. The suspension thus treated was centrifuged at $3000 \times g$ for 10 min, the debris obtained was discarded, and the resulting supernatant was further centrifuged at $90000 \times g$ for 60 min. The debris thus obtained was suspended in 10 mM Tris-HCl buffer, pH 8.0, and used as membrane particles. The concentrations of cytochromes in the membrane particles were determined spectrophotometrically using $\Delta\epsilon_{550-580}$ (reduced minus oxidized) = 22 mM^{-1} for cytochrome *c* [16], $\Delta\epsilon_{589}$ (reduced minus oxidized) = 13 mM^{-1} for cytochrome a_1 [17] and $\Delta\epsilon_{605-630}$ (reduced minus oxidized) = 11.5 mM^{-1} for cytochrome aa_3 [4]. *N. agilis* cytochrome *c*-550 was prepared according to the method of Yamanaka et al. [16].

C. krusei and *S. oviformis* cytochromes *c* were kindly supplied by Sankyo Co., Ltd. (Tokyo). Horse cytochrome *c* (type VI), cardiolipin, phosphatidylserine, phosphatidylcholine, phosphatidylglycerol and phosphatidylethanolamine were purchased from Sigma Chemical Co. (U.S.A.).

Extraction of lipids from *N. agilis* cells was performed by the following procedure: The cells (5 g wet weight) suspended in 10 ml deionized water were mixed with 13 ml chloroform and 25 ml methanol, and the resulting mixture was shaken vigorously for 5 min. After being allowed to stand for 10 min at room temperature, a further 13 ml of chloroform and deionized water were added to the mixture, and the suspension thus obtained was centrifuged at $700 \times g$ for 5 min. The separated chloroform layer was carefully collected, evaporated without heating, and the solid materials thus obtained were redissolved in 0.5 ml chloroform. The lipids included in the *N. agilis* cytochrome *c* oxidase preparation were extracted according to the method of Awasthi et al. [18]. Extracted lipids were stored at −20°C until required.

Lipids were analyzed by thin-layer chromatography on 0.25 mm thick silica gel 60 (Merck, F.R.G.) [19]. The solvent system used was chloroform/methanol/water (65:25:4, v/v). The spot of each lipid which appeared on the plate was detected by fluorescence after 0.03% rhodamine 6G dissolved in ethanol was sprayed on the plate [20]. Phospholipids were stained with Zinzadze reagent [21]. Identification of the separated components was performed by cochromatography of each authentic compound.

Enzymatic activity of cytochrome *c* oxidase was determined spectrophotometrically with a Hitachi spectrophotometer, model 220A, using a 1 cm light path cuvette. The reaction was started by addition of the enzyme to ferrocytochrome *c* solution, and the decrease in absorbance at 550 nm was followed spectrophotometrically over time. The reaction proceeded according to first-order kinetics under all experimental conditions tested. Ferrocytochrome *c* was prepared by dialysis of $Na_2S_2O_4$-reduced cytochrome *c* against 10 mM Tris-HCl buffer, pH 8.0.

Results

Effect of cardiolipin on the enzymatic activity of N. agilis cytochrome c oxidase

The enzymatic activity of *N. agilis* cytochrome *c* oxidase was greatly dependent on the ionic strength

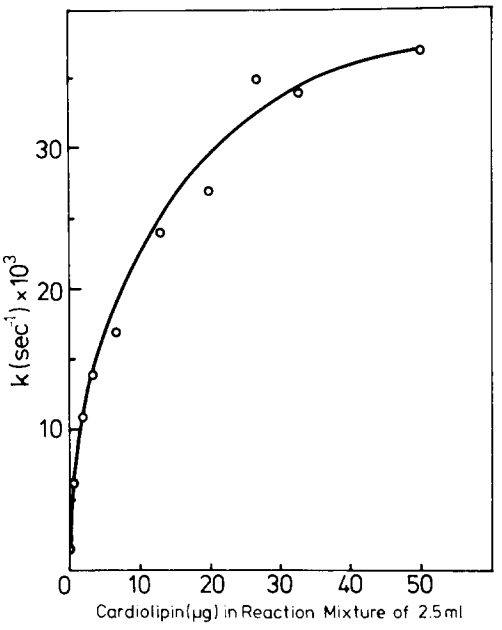

Fig. 1. Effect of cardiolipin on the reaction rate of horse cytochrome *c* with *N. agilis* cytochrome *c* oxidase. The reaction mixture contained 40 mM phosphate buffer, pH 6.5, 8.5 μM horse ferrocytochrome *c*, 6 nM oxidase and cardiolipin in various amounts in a total volume of 2.5 ml. The enzyme which had been dissolved in 10 mM Tris-HCl buffer, pH 8.0, containing 1% Tween 20 at a concentration of 117 μM and stored in liquid nitrogen was diluted to 1 μM by 10 mM Tris-HCl buffer, pH 8.0 containing 1% Triton X-100 before use; the enzyme solution (volume 15 μl) was then added to the reaction mixture. Cardiolipin in ethanol was added to the reaction mixture prior to addition of the enzyme, and the reaction was started by addition of the enzyme.

of the reaction mixture; horse ferrocytochrome *c* was rapidly oxidized by the enzyme in 10 mM phosphate buffer, pH 6.5, while the reaction rates were very low when the concentrations of phosphate were higher than 40 mM. Depression of the reaction rates occurred with NaCl, KCl and $NaNO_3$ in a similar way to the case with phosphate. Fig. 1 shows the effect of cardiolipin on the oxidation rate of horse ferrocytochrome *c* catalyzed by *N. agilis* cytochrome *c* oxidase using 40 mM phosphate buffer.

Cardiolipin greatly stimulated the reaction rate under the experimental conditions. The reaction rate reached the maximal value when 30 μg cardiolipin per reaction mixture was added. The molar ratio of cardiolipin added to the oxidase was about $3 \cdot 10^3$. Nonionic detergents such as Triton X-100 or Tween 20 did not accelerate the reaction rate at concentrations from 0.02 to 0.16%.

Fig. 2A show the dependency on ionic strength of the oxidation of horse ferrocytochrome *c* catalyzed by the oxidase in the presence and absence of cardiolipin. Cardiolipin accelerated greatly the oxidation rate at concentrations of phosphate from 20 to 60 mM, while the acceleration was not so marked at lower concentrations of the salt (5–10 mM). The optimum concentration of phosphate for the reaction was displaced towards higher values in the presence of cardiolipin. It seemed interesting that the dependency on ionic strength of the

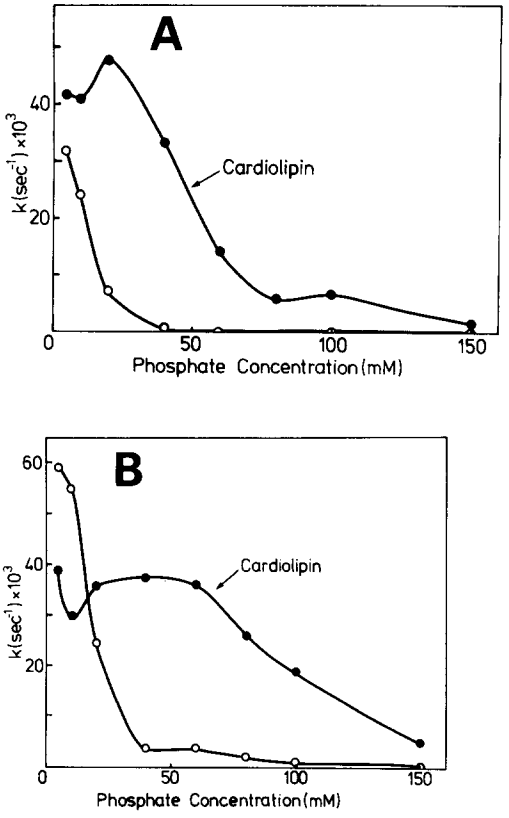

Fig. 2. Effect of cardiolipin on the oxidation rates of horse and *C. krusei* ferrocytochromes *c* with *N. agilis* cytochrome *c* oxidase at various concentrations of phosphate buffer in the reaction mixture. The reaction mixture contained 33 μg cardiolipin, cytochrome *c* and phosphate buffer, pH 6.5, in a total volume of 2.5 ml. Other reaction conditions were as described in the legend to Fig. 1, except that phosphate buffers of various concentrations were used. ○———○, without addition of cardiolipin; ●———●, with added cardiolipin. (A) Horse cytochrome *c* (9 μM), (B) *C. krusei* cytochrome *c* (7.7 μM).

enzymatic activity of the oxidase in the presence of added cardiolipin was similar to that of the mitochondrial cytochrome *c* oxidase to which the phospholipid was bound [17,18]. The addition of cardiolipin affected greatly K_m for horse cytochrome *c* of the enzyme; the values were 1 mM and 18.5 μM (at 40 mM phosphate) in the absence and presence of the phospholipid, respectively. The latter values is very close to that obtained with *N. agilis* cytochrome *c*-500. The oxidation of *C. krusei* ferrocytochrome *c* by the *N. agilis* oxidase was also activated by cardiolipin (Fig. 2B). Cardiolipin stimulated the oxidation rate at concentrations of phosphate from 20 to 100 mM in a similar way to the case of the oxidation of horse ferrocytochrome *c*, although the reaction was inhibited by the phospholipid at concentrations of the salt lower than 20 mM. The oxidation of *S. oviformis* ferrocytochrome *c* was also activated by cardiolipin. Although the reactivity of this cytochrome was lower, as a whole, than that of the *C. krusei* cytochrome, its activation profile by cardiolipin resembled that of the *C. krusei* cytochrome (data not shown).

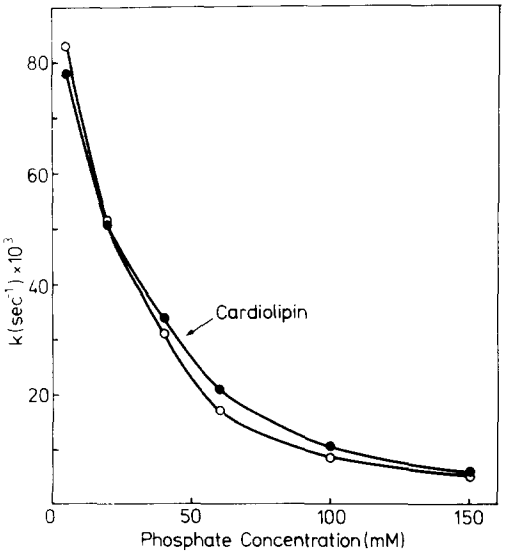

Fig. 3. Effect of cardiolipin on the oxidation rate of *N. agilis* cytochrome *c*-550 with *N. agilis* cytochrome *c* oxidase at various concentrations of phosphate buffer in the reaction mixture. The reaction conditions were the same as those described in legend to Fig. 2, except that *N. agilis* cytochrome *c*-550 (6.1 μM) was used in place of horse cytochrome *c*. ○——○, without addition of cardiolipin; ●——●, with added cardiolipin.

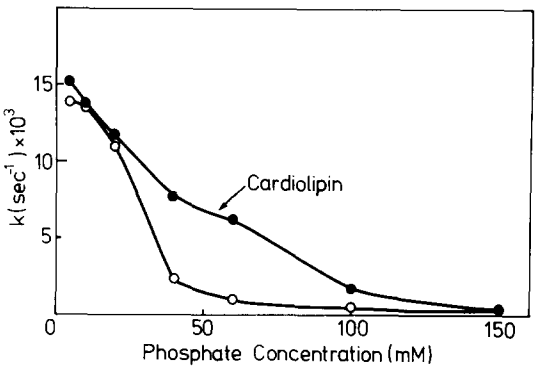

Fig. 4. Effect of cardiolipin on the oxidation rate of horse ferrocytochrome *c* with *N. agilis* membrane particles at various concentrations of phosphate buffer in the reaction mixture. The membrane particles contained 0.79 μM cytochrome *c* oxidase, 1.2 μM cytochrome a_1 and 0.81 μM cytochrome *c*. The reaction conditions were the same as those described in Fig. 2A except that membrane particles were used in place of the purified enzyme and the concentration of horse ferrocytochrome *c* was 7.7 μM. ○——○, without addition of cardiolipin; ●——●, with added cardiolipin.

The effect of cardiolipin on the oxidation of *N. agilis* ferrocytochrome *c*-550 by the *N. agilis* oxidase is shown in Fig. 3. In this case, cardiolipin scarcely accelerated the oxidation rate at concentrations of phosphate from 5 to 150 mM; the oxidation rate decreased markedly with increase in phosphate concentration, even in the presence of cardiolipin. Thus, the profile of the dependency on ionic strength of the reaction rate in the case of *N. agilis* cytochrome *c*-550 differed greatly from those in the case of the eukaryotic cytochromes *c* mentioned above.

The dependency on ionic strength of the oxidation rate of horse ferrocytochrome *c* with membrane particles is shown in Fig. 4. The membrane particles oxidized rapidly horse ferrocytochrome *c* at lower ionic strengths as well as the solubilized oxidase. The oxidation rate with the membrane particles was also accelerated by addition of cardiolipin, although the degree of activation was lower with the particles than that with the solubilized enzyme.

Phospholipids in N. agilis cells

The phospholipid composition of the *N. agilis* membrane was analyzed by thin-layer chromatog-

raphy. Major phospholipids found were phosphatidylethanolamine and phosphatidylcholine. The lipid composition of the bacterium was similar to that of the mitochondrial inner membrane [22]. Although the lipid which had the same R_f value as cardiolipin was detected on the plate when it was stained with rhodamine 6G, the spot was not stained with Zinzadze reagent. As Zinzadze reagent stains phospholipids, particularly cardiolipin, the result suggests that the amount of cardiolipin in the membrane of *N. agilis* is too small to be detected even though it is present. The lipids extracted from the *N. agilis* cytochrome *c* oxidase preparation were also analyzed by thin-layer chromatography. Phosphatidylcholine and phosphatidylethanolamine were not detected. Two spots were detected with rhodamine 6G staining on the plate. One spot with higher mobility was due to Triton X-100, while the other had a mobility very similar to that of cardiolipin. However, this spot was not stained with Zinzadze reagent. At present, we have not succeeded in characterizing this lipid.

Effect of various kinds of phospholipids on N. agilis cytochrome c oxidase activity

Effects of several kinds of phospholipids on the enzymatic activity of the oxidase were determined. Phosphatidylglycerol and phosphatidylserine in addition to cardiolipin stimulated the oxidation of horse ferrocytochrome *c* by *N. agilis* cytochrome *c* oxidase, while phosphatidylcholine and phosphatidylethanolamine had no effect on the reaction (data not shown). The lipids extracted from *N. agilis* cells hardly activated the oxidation of horse ferrocytochrome *c* catalyzed by the enzyme. None of the phospholipids tested stimulated the oxidation of *N. agilis* ferrocytochrome *c*-550 by the oxidase.

Discussion

It is generally accepted that biological membranes are constructed of lipid bilayers and that phospholipids play important roles in the structural and functional behavior of intrinsic membrane enzymes [19]. In the present investigation, we have studied the effects of phospholipids, especially cardiolipin, on the oxidation of ferrocytochrome *c* catalyzed by *N. agilis* cytochrome *c* oxidase.

Cardiolipin accelerates greatly the oxidation of horse and *C. krusei* ferrocytochromes *c* catalyzed by the *N. agilis* oxidase at higher ionic strengths where the activity of the enzyme was very low without addition of the phospholipid, while Triton X-100 and Tween 20 have no effects on the reaction. Even the oxidase in the membrane fragments of the organism oxidizes horse ferrocytochrome *c* more rapidly in the presence of cardiolipin than in its absence. These results suggest that the activation by cardiolipin of the oxidase is not caused by a change of the aggregation state or conformational state of the enzyme.

On the other hand, the oxidation of *N. agilis* cytochrome *c*-550 by the *N. agilis* oxidase is not affected by addition of cardiolipin. Further, the reaction rate of *N. agilis* cytochrome *c*-550 with the enzyme is much more rapid than the rates for horse cytochrome *c* or *C. krusei* cytochrome *c* (Figs. 2 and 3). The K_m value for horse cytochrome *c* of the enzyme is decreased to that for *N. agilis* cytochrome *c*-550 in the presence of the phospholipid. *N. agilis* cytochrome *c*-550 is the physiological electron donor for the *N. agilis* oxidase [16,24]. Therefore, the acceleration effect on the *N. agilis* oxidase activity observed with the nonphysiological cytochromes *c* as electron donors does not seem to be physiological. Thus, cardiolipin has not been detected in the membrane particles of *N. agilis*. Further, the lipids extracted from the organism have not shown any acceleration effect on the oxidation of nonphysiological cytochromes *c* by the oxidase.

Although *N. agilis* cytochrome *c*-550 is homologous with eukaryotic cytochrome *c*, its isoelectric point is lower than that of horse cytochrome *c*, the value being 7.5 (20°C) [16,24]. Therefore, *N. agilis* cytochrome *c*-550 is not so positively charged in the reaction mixture as is horse cytochrome *c*. Phosphatidylglycerol and phosphatidylserine as well as cardiolipin activate the oxidation of horse ferrocytochrome *c* by the oxidase, while phosphatidylethanolamine or phosphatidylcholine does not. The phospholipids which stimulate the oxidase activity are negatively charged in the reaction mixture. Therefore, the results obtained in the present studies suggest that the activation effect on the oxidase of cardiolipin and other phospholipids may be attributable to controlling positive charges

around the domain of the cytochrome *c* molecule which reacts with the oxidase. However, the activation of the oxidase with cardiolipin may not be caused only by the electrostatic interaction between cytochrome *c* and phospholipids. Thus, the stimulation of the oxidase activity by the phospholipid is dependent on the ionic strength and differs between horse and *C. krusei* cytochromes *c* (Fig. 2A,B).

Recently, Vik et al. [15] have reported that cardiolipin tightly bound to mitochondrial cytochrome *c* oxidase participates in binding cytochrome *c* at the low-affinity site. At present, it has not been determined whether the *N. agilis* enzyme molecule has two (high and low) affinity sites. Kinetic experiments are now under investigation with the *N. agilis* enzyme in our laboratory and the results will be published elsewhere. Although we have not succeeded in clarifying the activation mechanism of cardiolipin on the *N. agilis* oxidase, the present studies have revealed that cardiolipin is not essential for the physiological function of the oxidase; the phospholipid does not accelerate the oxidation of cytochrome *c*-550 by the enzyme. The idea that the activation by lipids of the *N. agilis* oxidase does not occur in vivo is also supported by the results that the phospholipids extracted from *N. agilis* membrane or the oxidase preparation do not show the activation effects on the reactions with the oxidase of eukaryotic cytochromes *c* as well as of *N. agilis* cytochrome *c*-550.

In the previous study [7], we have shown that the specificity for cytochrome *c* of the *N. agilis* oxidase differs considerably from that of the bovine enzyme, and discussed the evolutional significance of this difference. Although the *N. agilis* oxidase has been used without addition of cardiolipin in the study, its reported specificity for cytochrome *c* seems significant, as the effect of cardiolipin is not of physiological significance for the enzyme.

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