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STUDIES OF THE ELECTRON TRANSPORT CHAIN OF EXTREMELY HALOPHILIC BACTERIA

V. MODE OF ACTION OF SALTS ON CYTOCHROME OXIDASE

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SUMMARY

1. Cytochrome oxidase from an extremely halophilic bacterium requires up to 5.0 M NaCl for maximal activity and stability. Differences in effectiveness among various salts in promoting enzyme activity were observed, but a minimal activity (about 30 %) could be supported by all salts tested including $MgCl_2$ and spermine at relatively low concentrations. The enzyme activity in the presence of low concentrations of $MgCl_2$ was shown to be more dependent on pH but less sensitive to hydrophobic bond-breaking agents, such as ethanol and especially *n*-propanol, than the enzyme activity in the presence of high concentrations of NaCl.

2. Spontaneous inactivation of the enzyme in the absence of salt demonstrated that the rate of inactivation of the $MgCl_2$-dependent activity has a greater temperature dependence than the rate of inactivation of the NaCl-dependent activity. The effect of temperature on the rate of inactivation of the NaCl-dependent activity was found to be greatest at neutral pH and became minimal at acid or alkaline pH. Inactivation of the enzyme in the presence of high concentrations of NaCl (by prolonged incubation) demonstrated that the enzyme was inactivated more rapidly at —10° than at 5° at pH 4.0, but not at pH 7.5.

3. These results suggest that hydrophobic forces predominate in promoting the major portion of the enzyme activity in the presence of high NaCl concentrations, while charge-shielding promotes partial enzyme activity in the presence of $MgCl_2$. Hydrophobic bonds also appear to be involved in the stability of the enzyme, particularly at acid and alkaline pH, where hydrogen bonding is expected to be less extensive.

INTRODUCTION

Previous studies from this laboratory have been concerned with the electron transport chain of an extreme halophile, *Halobacterium cutirubrum*. The cytochrome components of the chain have been identified[1] and the salt dependence of the NADH

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

oxidase system examined[2]. The mechanism of the effect of salt was studied in greater detail for the case of one of the components of the respiratory system, the NADH dehydrogenase (menadione reductase)[3,4], and it was found that the activity and stability of this enzyme reflects a requirement for extensive hydrophobic bonding between nonpolar side-chains in the protein[4]. It was proposed, therefore, that the reason for the high salt requirement is not merely the shielding of like charges, as suggested by others[5–7], but also the salting-out of solution of nonpolar side-chains, which are thus moved into the interior of the protein molecule and can form hydrophobic bonds.

Although both menadione reductase and the terminal component of the respiratory chain, cytochrome oxidase, are membrane-associated enzymes, the oxidase is much more tightly bound to the membrane than is the reductase[8]. Preliminary experiments in this laboratory revealed striking differences in the salt-dependence of binding to the membranes between menadione reductase and cytochrome oxidase. Thus, it is of special interest to compare the two enzymes with regard to the involvement of hydrophobic forces in their functional properties. Evidence is given in this report that hydrophobic bonding is primarily responsible for the activity, and to some extent the stability, of cytochrome oxidase.

MATERIALS AND METHODS

N,N,N',N'-Tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) and sodium azide were obtained from Eastman; Tris-HCl, spermine tetrahydrochloride and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) from Sigma; ascorbic acid (sodium salt) from Mann.

Preparation of enzyme

The growth and harvest of *H. cutirubrum* cells has been described before[1]. The crude, cell-free extract was prepared as described previously[1], except that in these studies the buffer used was 0.05 M HEPES, 3.4 M NaCl, pH 7.5. The crude extract was diluted 1:10 with the same buffer and centrifuged for 16 h at 150000 × *g* (av.) in the Spinco L2-65 ultracentrifuge. After centrifugation, three fractions were obtained: a reddish hard pellet; a clear, red, viscous liquid (soft pellet) at the bottom of the tube; and a colorless supernatant. The soft pellet was carefully withdrawn from the tubes, diluted about 25-fold with fresh HEPES buffer containing 3.4 M NaCl, and centrifuged as before. After the second centrifugation, both hard and soft pellets were collected and resuspended by homogenization with a Dounce-type tissue homogenizer (Kontes). This washed, particulate enzyme preparation was stored frozen at −78° and was used for all experiments except those involving release of the enzyme from the membrane. For the latter experiments the method of cell breakage and membrane preparation of STOECKENIUS AND ROWEN[9] was employed. For this preparation the HEPES buffer was used containing 3.4 M NaCl *plus* 2.0% (w/v) $MgCl_2$, 0.2% (w/v) KCl, and 0.02% (w/v) $CaCl_2 \cdot 2H_2O$.

Enzyme assay

Cytochrome oxidase was assayed by oxidation of TMPD and measurement of the accompanying increase in absorption at 610 nm. This method of assaying cyto-

chrome oxidase was made possible by the fact that Wurster's Blue (extinction coefficient $1.2 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$), the semiquinone free radical product of the one-electron oxidation of TMPD[10], is not oxidized further in the *H. cutirubrum* system, and is very stable in aqueous solution[10,11]. TMPD solutions (0.25 M), containing a small amount of ascorbic acid (about 0.05 % (w/v)) to prevent autoxidation, were prepared fresh before use and were kept on ice throughout the day. In each assay the autoxidation of 5 μl of the substrate solution in 3.0 ml of buffer was followed for 2–3 min in a Cary 14 recording spectrophotometer. This autoxidation rate was less than 2 % of the enzyme-dependent oxidation rate at high NaCl concentrations, 10–20 % of the enzyme rate at low salt concentrations or in $MgCl_2$, and up to 50 % of the enzyme rate at high concentrations of $NaClO_4$. The autoxidation rate was substracted from the rate obtained after addition of enzyme to obtain the enzyme-dependent rate. The net enzyme activity obtained was a linear function of enzyme concentration within the range of concentrations used. The final concentration of TMPD in the assay (0.417 mM) produced about 80–90 % of the maximal velocity obtainable and was from 4–10 times the K_m value, depending upon the conditions of assay. (The K_m value was increased in buffers with low salt concentrations or salts other than NaCl, as noted below.) The cytochrome oxidase activity by this assay method could be inhibited by 75 % with 2 mM sodium azide and by 90 % with 10 mM azide.

For calculation of specific enzymatic activities protein determinations were carried out by the biuret method[12] using a biuret reagent containing 1 % (w/v) deoxycholate. Specific activities are given in terms of nmoles of O_2 reduced per min per mg of protein. Protein concentrations in the assays were 0.5–0.7 mg/ml. The molar quantities of O_2 were calculated using an equivalence of 4 electrons (4 molecules of TMPD oxidized) for each O_2 reduced. The specific activities obtained by the new assay method calculated in this manner are about 2.5 times higher than the specific activities obtained by the conventional oxygen uptake assay method[13] under identical conditions. Experimental results, such as dependence of enzyme activity on salt concentration, however, were very similar with both assay systems.

Since CHEAH[14] has found multiple cytochrome oxidases in *H. cutirubrum* by spectrophotometric identification of their chromophores, it is probable that this procedure assays more than one oxidase.

Carbon monoxide difference spectra

Difference spectra were determined in a Phoenix Dual Beam Spectrophotometer. Suspensions of particulate preparations (containing 3–4 mg/ml protein) were reduced by either dithionite or TMPD (0.1 mM, containing a small amount of ascorbate) and were divided into two 10-mm pathlength cuvettes. One of the cuvettes was flushed with carbon monoxide in the dark for about one minute and the difference spectrum was recorded. When KCN was added (2 mM) the spectra were recorded after 10 min of incubation.

RESULTS

Dependence of cytochrome oxidase activity on salt

Cytochrome oxidase activity was determined in the presence of increasing concentrations of 3 different salts: NaCl, $NaNO_3$, and $NaClO_4$. The salt-dependence of

enzyme activity in these experiments is shown in Fig. 1. It is apparent that maximal activity, about 33.4 nmoles O_2 reduced per min per mg protein, is obtained only with 5 M NaCl. $NaClO_4$ could support only about 30 % of the maximal activity, at 1–1.5 M, with no further stimulation or slight inhibition seen at higher concentrations. As shown in Fig. 1, $NaNO_3$ promotes an intermediate level of activity. The salt dependence curves show complex behavior at lower concentrations. Thus, there is a perceptible plateau in the $NaNO_3$ curve at 0.6 M to 1.0 M, but only a slight inflection in the NaCl curve at about 1 M, while the $NaClO_4$ curve begins to level off in this region. This plateau region will be discussed below.

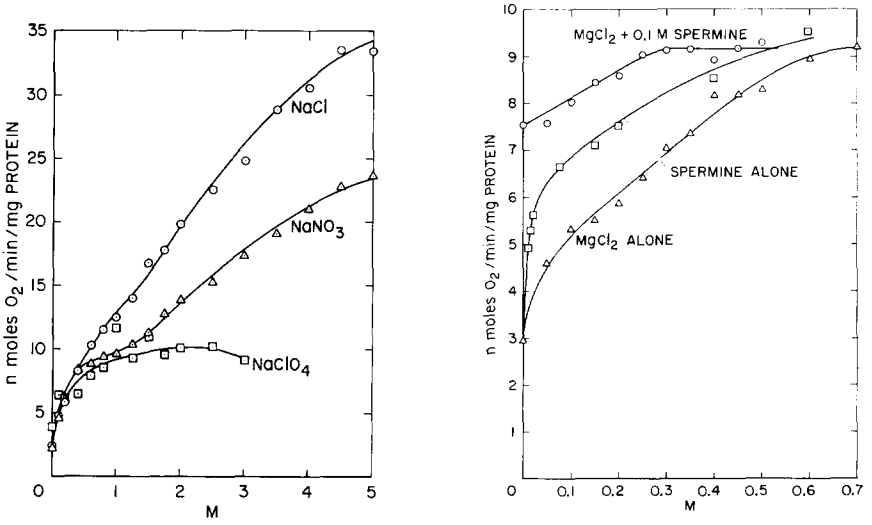

Fig. 1. Salt dependence and specificity of cytochrome oxidase activity. The buffer used was 0.05 M Tris–HCl, pH 7.5.

Fig. 2. Cytochrome oxidase activity in the presence of various concentrations of $MgCl_2$ alone and with 0.1 M spermine. The buffer used was 0.05 M Tris–HCl, pH 7.5.

It was found that $MgCl_2$ or spermine, a polyamine, could also promote enzyme activity (Fig. 2). The activity obtainable with either $MgCl_2$ (at 0.5–0.6 M) or spermine (at 0.1 M) is 20–30 % of the maximal activity obtainable with NaCl. Spermine causes a steep increase in activity up to 0.1 M; this activity (about 20 % of maximal) can be further increased to 30 % with spermine concentrations up to 1.0 M. In order to determine if $MgCl_2$ and spermine were in fact acting in the same manner on the enzyme, we examined their effects for additivity. If the polyvalent cations show nonspecific interactions with the enzyme, then the effects of $MgCl_2$ and spermine should be additive at low concentrations, but not at higher concentrations. In this experiment enzyme activity was measured (a) as a function of $MgCl_2$ concentration alone, and (b) as a function of $MgCl_2$ concentration in the presence of 0.1 M spermine. As shown in Fig. 2, the effects of $MgCl_2$ and spermine are additive up to 0.3 M $MgCl_2$, since the $MgCl_2$ plus 0.1 M spermine curve continues to increase up to 0.3 M $MgCl_2$. However, at this point the maximal level of activity obtainable with $MgCl_2$ alone is reached, and the $MgCl_2$ and spermine dependent activities are no longer found to be additive.

Comparison of NaCl- and $MgCl_2$-dependent activities

The pH dependence of the enzyme activity in the presence of NaCl was compared with that in the presence of $MgCl_2$. The results are given in Fig. 3 and show that the NaCl-dependent activity has a broad pH curve with a maximum at about pH 7.5. The $MgCl_2$-dependent activity has a much steeper pH curve with a maximum at pH 6.5. The ratio of activity in the presence of $MgCl_2$ to that in the presence of NaCl continuously decreases with increasing pH. At pH 5.5 the $MgCl_2$-dependent activity was 43 % of the NaCl-dependent activity, at pH 7.0 it is 32 %, while at pH 8.5 it is only 8 % of the NaCl-dependent activity.

Kinetic parameters for the enzyme were examined under various conditions and it was found that the K_m for TMPD was lowest when the enzyme was assayed in 5.0 M NaCl (about 50 μM TMPD) and was increased somewhat at low NaCl concentrations or when assayed in other salts including polyvalent cations. However, the increases were not great, with maximal differences less than two-fold. The inhibition constant K_i for sodium azide (60 μM in 5.0 M NaCl) showed differences in the same range, being about 2.5 times higher in 0.5 M $MgCl_2$ than in 5.0 M NaCl.

The sensitivity of the enzyme activity to inhibition by protein denaturants was investigated under various conditions. Ethanol and *n*-propanol were tested as inhibitors, in the presence of either 3.5 M NaCl or 0.5 M $MgCl_2$. In 16 % ethanol, the highest concentration used, 11.0 % of the $MgCl_2$-dependent activity but only 3.1 % of the NaCl-dependent activity remained. At 6 % *n*-propanol, the highest concentration used, 17.6 % of the $MgCl_2$-dependent activity but only 2.8 % of the NaCl-

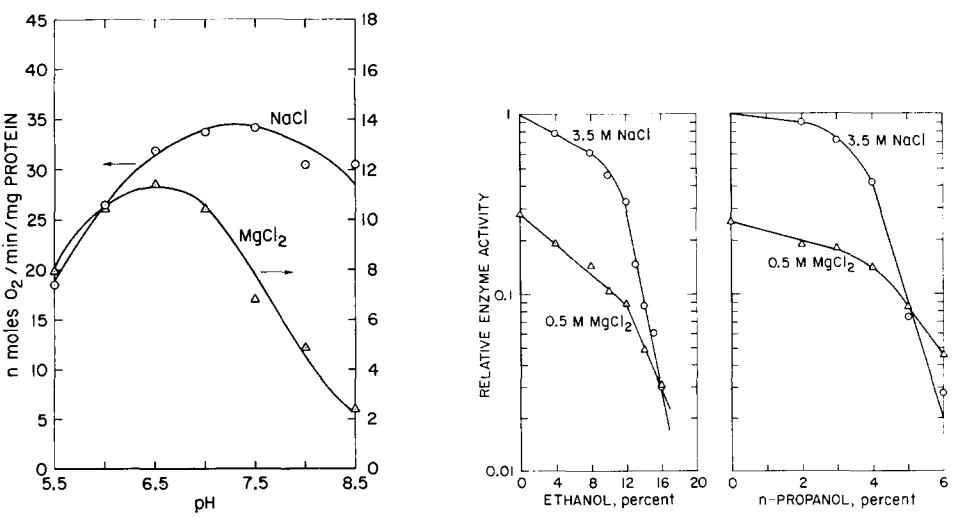

Fig. 3. pH dependence of enzyme activity in the presence of 5.0 M NaCl or $MgCl_2$ at the concentration which gave maximal activity at that particular pH. This concentration of $MgCl_2$ was 0.8–1.0 M for all pH values tested except pH 8.5 where 0.3 M $MgCl_2$ gave maximal activity. The buffers used were 0.05 M Tris–maleate from pH 5.5 to pH 7.0 and 0.05 M Tris–HCl from pH 7.5 to pH 8.5.

Fig. 4. Effect of ethanol and *n*-propanol on enzyme activity in the presence of 3.5 M NaCl or 0.5 M $MgCl_2$. Relative enzyme activities, based on the activity at 3.5 M NaCl in the absence of alcohol as 1.0, are plotted logarithmically against the concentration of the alcohol. The buffer used was 0.05 M Tris–HCl, pH 7.5.

dependent activity remained. Thus the activity at 3.5 M NaCl was found to be more sensitive to ethanol and especially to *n*-propanol than the activity at 0.5 M $MgCl_2$. In Fig. 4 the logarithm of relative enzyme activity in the presence of these agents is plotted against the concentration of the denaturant. The NaCl and $MgCl_2$ curves intersect at about 16 % ethanol or 5 % *n*-propanol; hence at these denaturant concentrations the salting-out effect of NaCl is apparently cancelled and high concentrations of NaCl no longer stimulate enzyme activity over the level of activity obtainable with low concentrations of $MgCl_2$.

Carbon monoxide difference spectra

Dithionite-reduced preparations exhibited a CO difference spectrum (Fig. 5a) similar in amplitude to that reported by CHEAH[14], for a given concentration of membranes, although the positions of the main peak and trough were somewhat different, 424 and 443 nm, respectively, for preparations in this study, as compared to the reported values of 419 and 442 nm.

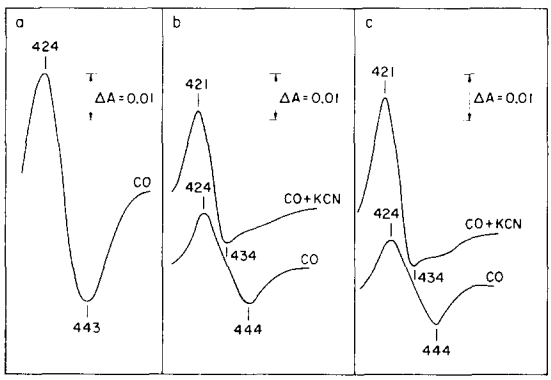

Fig. 5. Carbon monoxide difference spectra at room temperature. (a) dithionite + CO *minus* dithionite in 5.0 M NaCl, (b) TMPD + CO *minus* TMPD, after recording the difference spectra, KCN was added to both cuvettes and the CO + KCN difference spectra were recorded in 5.0 NaCl, (c) experimental conditions identical to (b) but performed in 0.2 M $MgCl_2$. The buffer used was 0.05 M Tris–HCl, pH 7.5.

The CO difference spectra of TMPD-reduced preparations were very similar when determined either in 5.0 M NaCl (Fig. 5b) or in 0.2 M $MgCl_2$ (Fig. 5c). Comparison with the dithionite-reduced CO-difference spectrum (Fig. 5a) indicated that (a) TMPD reduces only about a third of the CO-reactive pigments and (b) no significant differences in the positions of the peak and trough were observed in either solvent used, or between the TMPD-reduced and dithionite-reduced preparations.

Adding KCN to both cuvettes after the CO-difference spectra were recorded resulted in a shift of the peak from 424 to 421 nm and of the trough from 444 to 434 nm, leaving a shoulder at 444 nm. Such changes were reported in *Halobacterium halobium* by CHEAH[15] who interpreted them as revealing of the cytochrome o component of the oxidase in addition to the *a*-type cytochrome. No significant differences were observed between the CO–KCN spectra obtained from 5.0 M NaCl and 0.2 M $MgCl_2$.

Inactivation of the enzyme

Cytochrome oxidase undergoes a time-dependent irreversible inactivation in the absence of salt. The rate of inactivation is logarithmic until about 50 % inactivation has occurred, beyond which point inactivation becomes slower. The rates of inactivation given below are calculated from the initial (logarithmic) part of the time-dependent inactivation curves. Since cytochrome oxidase is more stable at lower salt concentrations than menadione reductase[4], the inactivation experiments with cytochrome oxidase were carried out over longer periods of time than was previously done for the other enzyme[4]. In order to study the inactivation of the NaCl-dependent activity separately from the inactivation of the $MgCl_2$-dependent activity, the enzyme was incubated in the absence of salt to inactivate it, then assayed at either high concentrations (2.5 M) of NaCl, or at low concentrations (0.5 M) of $MgCl_2$. The inactivation buffer was 0.05 M Tris–HCl, pH 7.5, and inactivation was stopped by adding an equal volume of the same buffer containing either 5.0 M NaCl or 1.0 M $MgCl_2$. The inactivation rates obtained in this experiment, carried out at different temperatures, are shown in Table I, where it is seen that at 0° the inactivation rates of both activities are similar, but at 30° the rate of inactivation of the $MgCl_2$-dependent activity is much greater than the rate of inactivation of the NaCl-dependent activity. Thus, the inactivation rate of the NaCl-dependent activity shows a lesser temperature dependence than the inactivation rate of the $MgCl_2$-dependent activity.

TABLE I

TEMPERATURE DEPENDENCE OF INACTIVATION OF NaCl AND $MgCl_2$-DEPENDENT ACTIVITIES

Inactivation was carried out without added NaCl at pH 7.5, for increasing periods of time at the given temperature, and terminated by addition of either NaCl or $MgCl_2$ (final concentrations 2.5 M and 0.5 M, respectively). Enzyme activities were determined at room temperature. Inactivation rates were calculated from logarithmic plots as described in the text.

Temperature (°)	*Rate of inactivation* (h^{-1})	
	Assayed in NaCl	*Assayed in $MgCl_2$*
0	0.68	0.74
23	2.8	4.2
30	5.7	31.2

The inactivation of the NaCl-dependent activity was investigated further as a function of pH at 5° and 23°. The enzyme was inactivated in the absence of salt at varying pH values at 5° or 23°, and then assayed in the presence of high NaCl concentrations at room temperature. At both 5° and 23° the rate of inactivation is less at neutral pH than at acid or alkaline pH values, but the effect of pH on inactivation is much greater at 5° than at 23°. In Fig. 6 the ratio of the rate of inactivation at 5° to the rate of inactivation at 23° is plotted as a function of pH. The curve is seen to have a minimum between pH 5.5 and 6.5 with a ratio of about 0.1. The ratio approaches 1.0 at both acid and alkaline pH values, leveling off at about 0.95 at pH 10.0 and reaching 0.7 at pH 4.0.

The enzyme can be inactivated even in the presence of high concentrations of salt when incubated over long periods of time. The effect of temperature on the inac-

tivation of the enzyme was tested in the presence of 3.4 M NaCl at pH 7.5 and pH 4.0. The results, given in Fig. 7, show that in 3.4 M NaCl at pH 7.5 the enzyme is protected from inactivation by lowering the temperature. However, at pH 4.0, in 3.4 M NaCl, the enzyme is inactivated more rapidly at —10° than at 5°. Thus, at pH 4.0, but not at pH 7.5, the enzyme shows cold-sensitivity.

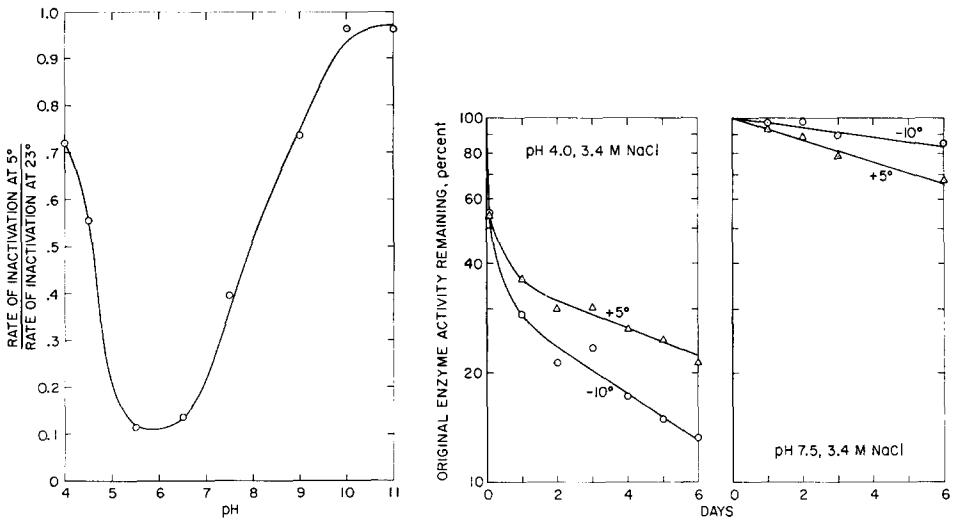

Fig. 6. Effect of pH on the temperature-dependence of inactivation of the enzyme in the absence of salt. The ratio of the initial rate of inactivation at 5° to the initial rate of inactivation at 23° is plotted against pH. The enzyme was inactivated by incubating in the following buffers (not containing added NaCl): 0.05 M acetate at pH 4.0 and 4.5, 0.05 M Tris–maleate at pH 5.5, 0.10 M NaH_2PO_4–K_2HPO_4 at pH 6.5 and 7.5, and 0.05 M glycine at pH 9.0, 10.0, and 11.0. The inactivation was terminated after appropriate incubation times by adding an equal volume of 0.10 M NaH_2PO_4–K_2HPO_4 containing 5.0 M NaCl and the pH was adjusted to neutrality with a pre-determined amount of either 0.5 M NaOH or 1.0 M HCl. Initial rates of inactivation were calculated as described in the text.

Fig. 7. Inactivation of the enzyme at high salt concentrations. The percent remaining activity is plotted logarithmically against time. The buffers used for inactivation were 0.05 M acetate containing 3.4 M NaCl, at pH 4.0, and 0.05 M HEPES containing 3.4 M NaCl, at pH 7.5. The samples were handled for assay as described in the legend to Fig. 6.

Release of the enzyme from the membrane vesicles

During the course of studies in this laboratory concerning the dissolution of membranes from *H. cutirubrum* at low salt concentrations, it became apparent that exposure to low salt concentrations could be a way of solubilizing the enzyme. Membrane vesicles prepared by the method of STOECKENIUS AND ROWEN[9] were exposed to various concentrations of NaCl, from 0.1 M to 0.8 M, for 60 min at 0°, and then centrifuged at 34000 × *g* (max.) for 15 min in the Sorvall centrifuge (which effectively sediments the membrane vesicles at high salt concentrations). An equal volume of 0.05 M Tris–HCl, pH 7.5, containing 5.0 M NaCl was added to the supernatants to stop inactivation and the same buffer was used for resuspension of the pellets. The enzyme activity of the pellet and supernatant fractions of each sample were assayed. The results are shown in Fig. 8, where the percent of the activity released from the membranes into the supernatant as well as the total activity recovered,

are plotted as a function of NaCl concentration. It is evident that enzyme activity is readily released from the membranes at concentrations of NaCl below 0.4–0.5 M. When the enzyme released from the membranes was tested for inactivation in the absence of salt, the released activity was found to be inactivated at the same rate as the enzyme activity still associated with the membrane vesicles. Thus the inactivation of the enzyme at low salt concentrations is not associated with the observed detachment from the membrane vesicles, but appears to be an independent process.

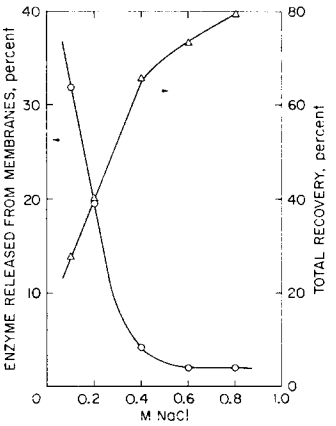

Fig. 8. Release of cytochrome oxidase from membrane vesicles. The percent enzyme activity released from the membranes and the percent total recovery after one hour incubation at 0° is plotted against concentration of salt. Experimental details are given in the text.

The sedimentation properties of released enzyme were studied because STOECKENIUS AND ROWEN[9] showed that the product of the low salt treatment of membranes still contained membrane fragments. A sample of the cytochrome oxidase preparation released from the membrane vesicles at 0.2 M NaCl that remained in the supernatant after centrifuging as described above, was adjusted to 2.6 M NaCl and centrifuged at 50000 × *g* (av.) for 2 h. All of the enzyme activity was found to sediment as a pellet, and thus the enzyme detached from membrane vesicles at low salt concentrations was either still associated with membrane fragments, or participates in membranous aggregates on addition of salt.

DISCUSSION

Cytochrome oxidase from *H. cutirubrum* has been shown to require high concentrations of sodium chloride for maximal activity[2,16]. Differences in effectiveness among various salts have been previously demonstrated for other membrane-bound enzymes, such as the complete NADH oxidase system of *Halobacterium salinarium*[17], and menadione reductase (NADH dehydrogenase) from *H. cutirubrum*[4]. In these systems, the enzymes are more selective among the anions than the cations. In the case of menadione reductase[4] the anion specificity was demonstrated to follow the lyotropic series Cl^-, $PO_4^{3-} > Br^- > NO_3^- > ClO_4^-$, SCN^-, the order of effectiveness in promoting enzyme activity being the same as the order of salting-out power. This observation, in addition to other evidence, led to the suggestion that the role of salt in activating and stabilizing menadione reductase is primarily due to the stabiliza-

tion of hydrophobic interactions. In the case of cytochrome oxidase, reported here, the anion specificity is also consistent with the lyotropic series. The salt dependence curves (Fig. 1) show that at concentrations above 1 M the order of effectiveness of the salts tested was $NaCl > NaNO_3 > NaClO_4$. Below 1 M concentrations, however, little difference in effectiveness is found among these salts. The data suggest, therefore, that at lower salt concentrations the salts act in a nonspecific manner, perhaps by charge shielding, thereby supporting a relatively small amount of activity. At 1 M salt, charge shielding may be expected to be complete and at this concentration about 30 % of the maximal enzyme activity is obtained. The high concentrations of salt required for the major portion of the enzyme activity and the observed specificity suggest that the effect of salt on the enzyme consists mainly of promoting the formation of new hydrophobic bonds by the salting-out effect at high concentrations of salt. Thus the plateau in the $NaNO_3$ curve (Fig. 1) might reflect the region where charge shielding is complete, but salting-out is not yet effective. This plateau is not as pronounced with NaCl (Fig. 1), consistent with the fact that this salt is a better salting-out agent than $NaNO_3$. In this system $NaClO_4$, which generally salts in, may participate in charge shielding but not in salting-out. If this hypothesis concerning the action of salts is valid, agents such as $MgCl_2$, or spermine, which do not salt-out at low concentrations but effectively shield charges, should be able to support about the same level of activity as 0.5–1.0 M concentrations of NaCl, $NaNO_3$, or $NaClO_4$, or about 30 % of the maximal activity. This expectation is borne out by the data obtained (Fig. 2). The results suggest that, as in the case of menadione reductase[4], charge shielding by low concentrations of salt or by $MgCl_2$ causes only partial cytochrome oxidase activity, with the greater portion of enzyme activity dependent on hydrophobic bonds, stabilized by the presence of high concentrations of a salting-out type salt.

The complexity of halophilic cytochrome oxidases has been documented by spectrophotometric studies[14,15]. The existence of various cytochrome oxidase species were best revealed in a combination of CO and KCN difference spectra[15]. Since the possibility existed that the two plateaus in Fig. 1 were due to the participation of different portions of the cytochrome oxidase complex, the TMPD-reduced CO difference spectra, with and without KCN, were recorded in 0.2 M $MgCl_2$ and in 5 M NaCl. Since no detectable differences were seen in the spectra obtained in these solvents, it appears that TMPD reduces the same components of the oxidase complex even when the salt concentration is varied or when divalent salts are substituted. The differences in enzyme properties observed when the salt concentrations were changed must then have been due to the conformation of the oxidase or to the membrane environment surrounding the complex. At this time we are unable to distinguish between these effects. For brevity the two portions of the cytochrome oxidase activity, distinguished on the basis of the experiments discussed above, have been named NaCl-dependent and $MgCl_2$-dependent enzyme activity.

While the NaCl-dependent portion of the enzyme activity appears to involve hydrophobic bonding, all that can be said about the $MgCl_2$-dependent portion of activity at this stage is that negative charges must be shielded to obtain activity. However, we have sought to find differences in the behavior of the two parts of enzyme activity which might indicate the relative importance of hydrogen and hydrophobic bonds.

In contrast to hydrogen bonding[18], hydrophobic interactions should be little

affected by the state of ionization of charged groups. EDELHOCH[19], in a study of the effects of ethanol and pH on the inactivation of pepsin, found that at lower ethanol concentrations the rate of inactivation was more dependent on pH than at higher ethanol concentrations. This was interpreted as indicative of the breaking of hydrogen bonds at lower ethanol concentrations and hydrophobic bonds as well at higher ethanol concentrations. Correspondingly, one might expect that in our system the $MgCl_2$-dependent enzyme activity would show more variation with pH than the NaCl-dependent activity. The results show, indeed, that the activity in the presence of $MgCl_2$ declines more rapidly with increasing pH above 6.5 than the activity in the presence of NaCl. The preferential loss of $MgCl_2$-dependent activity at higher pH values may reflect the loss of a proton from positively charged groups with a pK of 7.0–7.5, involved in the structure of the enzyme. In contrast, the NaCl-dependent activity does not show much pH dependence in this region.

Another approach in separating the two types of bonds presumed to be involved in cytochrome oxidase activity was to subject the enzyme preparation to agents which have less effect on hydrogen bonds than on hydrophobic interactions, such as ethanol and *n*-propanol[20–23]. The results showed that the contribution to enzyme activity by the presence of high NaCl concentrations was decreased relative to the $MgCl_2$-dependent activity in the presence of alcohols, indicating that the former portion of enzyme activity is more dependent on hydrophobic bonding. This suggestion is supported by the fact that the differences in sensitivity between the NaCl- and $MgCl_2$-dependent activities is greater for *n*-propanol than for ethanol, since the effect of *n*-propanol on hydrophobic bonds relative to its effect on hydrogen bonds is greater than the effect of ethanol. It is interesting to note that by manipulating the conditions of assay as discussed above, one can greatly reduce either the charge-shielding or the hydrophobic bonding-dependent portions of the enzyme activity. Thus, at pH 8.5 the $MgCl_2$-dependent activity is only 8 % of the NaCl-dependent activity, whereas at lower pH values $MgCl_2$ contributes a much larger proportion of the NaCl-dependent activity, up to 43 % at pH 5.5 (Fig. 3). On the other hand, by assaying the enzyme in the presence of 16 % ethanol or 5 % *n*-propanol, one can effectively inhibit any stimulation of the enzyme activity by high concentrations of NaCl over the level of activity obtainable with low concentrations of $MgCl_2$. Thus, the enzyme assayed at pH 8.5 appears to be virtually completely dependent on hydrophobic bonding for activity, whereas the enzyme assayed in the presence of 16 % ethanol or 5 % *n*-propanol behaves as if it were dependent mostly on charge shielding for activity.

Hydrophobic interactions are reportedly cold-sensitive[24–27]. In the previous paper of this series[4] it was demonstrated that menadione reductase shows decreasing temperature dependence of inactivation under conditions of increasing hydrophobic bonding, and becomes cold-sensitive in the presence of high NaCl concentrations. Cytochrome oxidase was examined for cold-lability also. This investigation was carried out in three parts: (a) separate inactivation of the NaCl-dependent and the $MgCl_2$-dependent activities at low salt concentrations, (b) pH dependence of the inactivation at low salt concentrations, and (c) inactivation of the enzyme at high concentrations of salt. In the first case the results demonstrated that the spontaneous inactivation of the $MgCl_2$-dependent activity at low salt concentrations has a much greater temperature dependence than the inactivation of the NaCl-dependent activity (Table I). As found with menadione reductase[4], the temperature coefficient of enzyme

inactivation may be resolved into a hydrophobic bond component which has a negative sign and a hydrogen bond component with a positive sign. SHERAGA *et al.*[27] have shown that even though the stability of a protein may depend to a large extent on hydrophobic bonding, the temperature coefficient of inactivation may still be positive due to the influence of the positive enthalpy of breaking hydrogen bonds that are present. The lower temperature coefficient of the inactivation of the NaCl-dependent cytochrome oxidase activity, compared to the $MgCl_2$-dependent activity, may thus be interpreted as the consequence of the greater involvement of hydrophobic bonding. The pH dependence of the ratio of inactivation rates of the NaCl-dependent activity at 5° and 23° (Fig. 6) demonstrates that at acid or alkaline pH values, but not at neutral pH, change of temperature (in this range) has little effect on the inactivation of the enzyme. At extreme pH values the relative importance of hydrophobic bonds in the stability of the enzyme thus appears to be increased. The inactivation curves of the enzyme in the presence of high concentrations of NaCl (Fig. 7) show cold-lability at pH 4.0, but not at pH 7.5, indicating that at high salt concentrations and at acid pH, inactivation involves primarily breaking hydrophobic bonds.

The results of the studies on the inactivation of the enzyme suggest that at neutral pH hydrogen bonding is predominant in providing stability to the enzyme. At acid or alkaline pH and especially at high salt concentrations, however, the component of hydrophobic bonding is revealed by the temperature dependence of the inactivation process.

It has been assumed throughout this paper that the inactivation of cytochrome oxidase at low salt concentration is due to the unfolding of the enzyme, the effect thus being analogous to the generally observed instability of halophilic enzymes in the absence of salt[5–7]. In other systems, however, membrane lipids have been shown to oxidize on solubilization[28] and the resulting peroxides might be destructive to enzyme activity. In extreme halophiles, however, few or no unsaturated lipids were found[29], excluding lipid oxidation as a factor in the stability of cytochrome oxidase.

The experiments which show release of the enzyme from membrane vesicles (Fig. 8) are of interest in two respects. First, they demonstrate that unlike enzyme activity which is affected by salt concentrations of 0–5 M, the release of the enzyme takes place in a narrow concentration range of 0–0.5 M. Since the association of the enzyme with the membrane vesicles is complete at 0.5 M NaCl, it is suggested that salting-out has little effect on the attachment. Secondly, since the released enzyme activity was shown to be inactivated at the same rate as the membrane vesicle-associated enzyme activity, the inactivation observed at low salt concentrations in the first part of this paper cannot be ascribed to increased lability due to the detachment of the enzyme from the membrane vesicles.

It was not possible at this time, however, to study any effect of the immediate membrane environment on the low-salt stability of this halophilic enzyme since we had not been able to obtain it in a fully solubilized form.

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