Studies of the Electron Transport Chain of Extremely Halophilic Bacteria

III. MECHANISM OF THE EFFECT OF SALT ON MENADIONE REDUCTASE

(Received for publication, February 10, 1969)

JANOS K. LANYI

From the Exobiology Division, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California 94035

SUMMARY

*Halobacterium cutirubrum* extracts exhibit no menadione reductase activity in the absence of salt; activity increases with salt concentration to a maximum at 2 M or higher concentrations of sodium chloride. The enzyme activity lost at low salt concentrations can be restored by adding 2 M NaCl. In addition to this reversible loss of activity, the enzyme also exhibits a time-dependent irreversible inactivation at salt concentrations below 2 M. The effects of salt on the initial enzyme activity and on the irreversible inactivation have a reciprocal relationship to one another. A mechanism for the salt effect is proposed, on the basis of kinetic data, whereby the active, stable form of the enzyme is in a salt-dependent equilibrium with an inactive, unstable form. The effect of NaCl, according to this model, is not to prevent the irreversible denaturation of the enzyme but rather to shift the equilibrium in favor of the active, stable form. The pH dependence of the inactivation rate constant and the reversibility of the pH effect indicate that there is an acid-base equilibrium, with pK = 9.3, between a less and a more stable intermediate form of the enzyme.

Low concentrations of MgCl₂, CaCl₂, and some polyamines also have activating and protecting effects on menadione reductase. The mode of action of these substances is shown to be unlike that of NaCl but to consist in the alteration of the intermediate form of the enzyme in such a way as to make it more stable and partially active.

Enzymes of extremely halophilic bacteria have been found to require high concentrations of salt for maximal activity and stability. Extensive reviews on the studies of these salt effects on halophilic enzymes have been written (1-3). More recently, DPNH oxidase and other electron transport components of *Halobacterium cutirubrum* and related species have been shown to be inactive without added salt and to require 2 to 3 M NaCl for full activity (4, 5). At low salt concentrations the enzyme was found to be irreversibly inactivated (5, 6). The salt requirement of DPNH oxidase could be satisfied by a large number of salts including those of divalent cations. One of the enzymes of the DPNH oxidase system, DPNH dehydrogenase, as assayed with indophenol, proved to be active and relatively stable when these salts were replaced with polyamines (6). Polyamines were effective at much lower concentrations than the salts in activating and stabilizing the enzyme.

In *H. cutirubrum* many of the salt-dependent characteristics of the DPNH oxidase system are due to the dehydrogenase component of the electron transport chain (5). In the study reported here we describe the effects of monovalent and divalent cations and of polyamines on DPNH dehydrogenase, as assayed with menadione as electron acceptor (henceforth referred to as menadione reductase), in order to explore the mechanism of the salt dependence of this enzyme.

METHODS

Sources of Chemicals—DPNH was obtained from Calbiochem; menadione, Tris-maleate, spermine tetrahydrochloride, and spermidine trihydrochloride, from Sigma; 1,4-diaminobutane, 1, 10-diaminodecane, diethyamine, and N₁,N₁-diethyl-1,5-pentanedi-amine, from Eastman.

Buffers—Tris-maleate and glycine-NaOH buffers were used in all experiments at 0.05 M concentration unless indicated otherwise.

Enzyme Assays—DPNH-menadione reductase activities were determined in the following assay mixture: enzyme, 30 to 40 μg of protein; menadione, 4 μmole; KCN, 2 μmoles; DPNH, 0.22 μmole per ml. All determinations and rate studies were carried out at room temperature. Enzyme activities are expressed in absorbance change at 340 nm per min, determined in a Beckman DK-2A spectrophotometer. Some of the rates measured were high, and care had to be taken to ensure that the method of starting and stopping the reactions was rapid enough. Additional to spectrophotometric cuvettes (5- or 10-μl solutions) were made on the tip of a plastic rod. Slopes of absorbance were measured within 5 sec of the additions. Low salt buffers to initiate inactivation and high salt buffers to stop the process were added by means of plunger delivery pipettes.

Enzyme Preparation—Methods for growing and harvesting *H. cutirubrum* cells were described previously (7). For these studies a high speed particulate fraction (7), prepared in 3.4 M NaCl, was used. The specific menadione reductase activity of
this preparation was approximately 4 μmoles of DPNH oxidized per min per mg of protein; this value is nearly 10 times higher than that of crude extracts.

RESULTS

Dependence of Menadione Reductase Activity on NaCl Concentration—Menadione reductase was assayed at pH 9.4 at increasing concentrations of sodium chloride. The enzyme rates were determined after 0, 30, and 120 sec of exposure to the salt buffers. Rates for zero time were obtained by adding the enzyme to a complete assay mixture. The other determinations were carried out by adding DPNH to a solution of the enzyme, menadione, and KCN after 30 or 120 sec of incubation. The enzyme activities obtained under these conditions are shown in Fig. 1, plotted against NaCl concentration. In this graph the hyperbolic curve obtained at zero incubation time is seen to become progressively more sigmoid in shape as the enzyme is incubated prior to the assays, indicating that the enzyme was inactivated upon standing at the lower salt concentrations. This inactivation was greatest at low salt concentrations and diminished at higher salt concentrations up to approximately 2 M NaCl where inactivation was no longer observable.

Inactivation of Menadione Reductase at Various Salt Concentrations—The kinetics of inactivation of menadione reductase, upon exposure to low concentrations of NaCl, was determined by two methods. (a) The enzyme was incubated, together with menadione and KCN, at pH 9.4 in the presence of various concentrations of NaCl prior to the addition of DPNH. In these experiments the enzyme assays were conducted at the same salt concentrations as were the incubations. (b) The enzyme was incubated in pH 9.4 buffer at the appropriate salt concentration, and after a given time the salt concentration was brought to 2 M by adding a calculated volume of the same buffer containing higher concentrations of NaCl. Menadione and KCN were then added, and the enzyme was assayed.

The result obtained by Methods a and b are shown in Figs. 2A and 2B, respectively. The logarithm of the remaining enzyme activity plotted against the time of exposure to 0.5 M, 1.0 M, 1.5 M, and 2.0 M NaCl solutions yields straight lines, indicating that the inactivation can be described by first order kinetics. Consistent with the data in Fig. 1, no inactivation was observed in 2.0 M NaCl. From Fig. 2B it appears that, for the experiments in which salt was reintroduced, the inactivation curves extrapolate to full activity at zero time. Furthermore, the slopes of inactivation are the same by either method of determination. Thus, it appears that raising the salt concentration to 2.0 M stopped the inactivation process and restored some enzyme activity. Extrapolation to zero time of incubation (with a resolution of about 5 sec) indicates that adding salt fully reversed the initial loss of activity. The time-dependent inactivation, however, was not reversible.

First order rate constants for the inactivation of menadione reductase were calculated at various salt concentrations and are given, together with the initial enzyme activities, in Fig. 3A. This graph shows a direct relationship between the effects of NaCl on enzyme activity and on stability; the activity reached maximal value at 2 M NaCl, the same concentration at which the enzyme became completely stable. Plots of log (V/V₀ − 1) against log [NaCl], where V is rate and V₀ is maximal rate, have been made for other halophilic enzymes. For menadione reductase this plot gives slopes of 2.2 for enzyme activity and 2.4 for

---

Fig. 1. Salt dependence of menadione reductase activity. The enzyme was incubated in the assay mixture before the determinations as explained in the text. Incubation times: O, 0 sec; □, 30 sec; ∆, 120 sec.

Fig. 2. Time-dependent irreversible inactivation of menadione reductase at various NaCl concentrations. As explained in the text, enzyme assays were carried out at the indicated NaCl concentrations (A) and after raising NaCl concentrations to 2.0 M (B).
Electron Transport Chain of Halophilic Bacteria. III

Vol. 244, No. 15

Fig. 3. Relationship of initial enzyme activity and first order inactivation rate constant at various salt concentrations. A, enzyme activity and stability in sodium chloride solutions; B, enzyme activity in magnesium chloride solutions and stability in the presence of MgCl₂ as determined after adding NaCl to 2.0 M concentration to stop inactivation. O, initial enzyme activity; □, inactivation rate constant determined before raising the NaCl concentration; Δ, inactivation rate constant determined after adding NaCl to 2.0 M.

Fig. 4. Dependence of the first order inactivation rate constant on pH. O and ●, the inactivation rate constants were determined by incubating aliquots of the enzyme in 0.025 M buffer at various pH values for increasing lengths of time, followed by adding an equal volume of 0.1 M Tris-maleate buffer, pH 7.0, containing 4.8 M NaCl. □ and ■, the enzyme was incubated in 0.025 M Tris-maleate buffer, pH 7.0, followed by adding an equal volume of 0.1 M buffer, giving varying pH after mixing, containing 4.8 M NaCl. ○ and ●, the enzyme was incubated in 0.025 M glycine-NaOH buffer, pH 10.0, followed by adding an equal volume of 0.1 M buffer, giving varying pH after mixing, containing 4.8 M NaCl. Open symbols, Tris-maleate buffer; closed symbols, glycine-NaOH buffer.

Fig. 5. Reversibility of the pH-dependent change in the irreversible inactivation of menadione reductase. O, the enzyme was incubated in 0.025 M Tris-maleate, pH 7.0, in the absence of added salt, and NaOH was added to shift the pH to 10.0. ●, incubation in 0.025 M glycine-NaOH, pH 10.0, was followed by adding HCl to shift the pH to 7.0. Enzyme assays of aliquots were carried out after adding equal volumes of 0.1 M Tris-maleate, pH 7.0, containing 4.8 M NaCl.

Inactivation curves for menadione reductase in the presence of MgCl₂ at various concentrations were determined in the manner described above for sodium chloride. In these experiments the incubation of the enzyme was terminated by adding an equal volume of the same buffer containing 4.0 M NaCl. The first order inactivation rate constants obtained and the initial enzyme activities, determined separately in the presence of MgCl₂, are plotted against MgCl₂ concentration in Fig. 3B. The reciprocal relationship between enzyme activity and rate of inactivation observed with NaCl was not obtained in the case of MgCl₂. Instead, as shown in Fig. 3B, the inactivation rate constant decreased rapidly with increasing MgCl₂ concentration. It reached a minimum value at concentrations as low as 2 mM MgCl₂ and remained constant up to 0.36 M MgCl₂. Enzyme activity, on the other hand, was negligible at 2 mM MgCl₂ and reached maximal value at about 0.2 M MgCl₂. Similar lack of correspondence between enzyme activity and stability was found with spermine and spermidine. These compounds were effective at concentrations about 10-fold lower than those for MgCl₂ for activating and partially stabilizing the enzyme.
culated from the time dependence of inactivation, are plotted in 
Fig. 4 as a function of pH during the inactivation process. The 
data show that at higher pH values the rate of inactivation is 
about 7-fold greater than it is at lower pH and that the midpoint 
of the curve is at pH 9.3. The effect of pH on the reactivation of 
the enzyme on the addition of salt was determined after inactiva-
tion at both pH 7.0 and pH 10.0. The incubation of the enzyme 
at these two pH values without added salt was terminated at vari-
ous times by adding an equal volume of 0.1 M buffer of varying 
pH, containing 4.8 M NaCl, menadione, and KCN. The pH 
after mixing was determined and the enzyme was assayed. The 
data in Fig. 4 show that under these conditions the first order 
rate constant of inactivation is only slightly affected by pH. The 
observed rate of inactivation is dependent on both the rate of 
inactivation and the extent of reactivation. If reactivation, 
which takes place instantaneously, were dependent on pH, this 
dependency would appear as an effect on the apparent rate of in-
activation. Since varying the pH during reactivation had little 
fluence on the observed rate of inactivation, it appears that the 
extent of reactivation is not affected by pH.

At high pH values the enzyme is shown to become more labile. 
The reversibility of the effect of pH on the enzyme was tested by 
sequentially exposing the enzyme to low and high pH buffers at a 
low salt concentration. Thus, the enzyme was first incubated in 
0.025 M Tris-maleate buffer, pH 7.0, and after a period of time 
the pH was shifted to 10.0 by adding a predetermined amount of 
NaOH. In another experiment the enzyme was incubated in 
0.025 M glycine-NaOH buffer, pH 10.0, and the pH was shifted 
with HCl to 7.0. The remaining enzyme activity was tested pe-
riodically during this treatment. Enzyme assays were carried 
out after salt reactivation with an equal volume of 0.1 M Tris-
maleate buffer, pH 7.0, containing 4.8 M NaCl. The results in 
Fig. 5, shown as remaining enzyme activity with respect to time 
of exposure to the low salt buffer, indicate that on shifting the pH 
from 7.0 to 10.0 and from 10.0 to 7.0 the characteristic rates of in-
activation found previously at these pH values are re-established.

**Effect of Polyamines on Menadione Reductase Activity**—The en-
zyme was assayed in 0.05 M Tris-maleate buffer, pH 8.0, without 
added salt, in the presence of various amines. Fig. 6 shows 
initial enzyme activity as a function of the concentration of sper-
mine, spermidine, 1,4-diaminobutane (putrescine), N₁,N₁-
diethyl-1,5-diaminopentane, diethylamine, and 1,10-diamo-
decane. The order of effectiveness in activating the enzyme was 
spermine > spermidine > putrescine; the other amines had vir-
tually no activating effect.

**Determination of Michaelis-Menten Constant for Menadione** 
**in Presence of Various Cations**—Initial rates of menadione reductase 
activity at increasing menadione concentrations were determined 
separately in the presence of 1.0 M NaCl, 3.4 M NaCl, 0.3 M MgCl₂, 
0.3 M CaCl₂, 20 mM spermine, and 20 mM spermidine. The 
double reciprocal plots calculated from these data are given in 
Fig. 7. The points for spermidine virtually coincide with those 
obtained for CaCl₂ and are not included in the graph. As shown 
in Fig. 7, the value of $K_m$ is different in sodium chloride solutions 
from those obtained in the presence of the other substances. The 
$K_m$ for menadione with NaCl is calculated to be 0.044 mM; with 
multivalent cations it is 0.10 mM.
DISCUSSION

Previous studies (5) indicated that at pH 9.4 DPNH oxidase and DPNH dehydrogenase activities exhibit sigmoid dependence on NaCl concentration. It has now been shown that such dependence is observed only when the enzyme is incubated in the assay mixture before the determinations are carried out. Thus, the shape of the salt-dependence curves is influenced by the effect of NaCl on both enzyme activity and stability, and the sigmoid shape is caused by a time-dependent inactivation of the enzyme at lower concentrations of salt.

Other halophilic enzymes have been shown (8, 9) to exhibit some differences in the salt requirement for activity and for stability. For isotropic dehydrogenase of *H. cutirubrum* the requirements for enzyme activity and for stability appear to differ greatly since the optimum salt concentration for activity is about one-fourth the salt concentration that provides maximum stability. Nevertheless, as Baxter suggested (8), these two phenomena probably do have a common basis, and the effects of salts on halophilic enzymes can be attributed to charge interaction between the polypeptide chain and the ions.

The salt dependence of menadione reductase in *H. cutirubrum* shows that even though the *K*ₐ for menadione is unchanged, enzyme activity is much reduced at low concentrations of NaCl. The inactivation rate does not reach zero at lower pH but approaches a constant value below pH 8, and above pH 10 the rate does not become more accelerated but appears to level off. Therefore, the pH-dependent transition, which is shown to be reversible, probably reflects an acid-base equilibrium between two alternate forms of the enzyme, differing in stability. The midpoint of the transition is at pH 9.3 and may thus be due to the ionization of one or more amino groups of the inactive intermediate in the scheme described above. The reactivation of the inactive form appears to be much less affected by pH changes. Presumably, the active form of the enzyme also undergoes an acid-base transition. However, such dissociation is not apparent from the dependence of menadione reductase activity on pH, which is similar to the broad pH optimum of *H. cutirubrum* DPNH oxidase (7).

A proposed mechanism of the salt effect on menadione reductase that incorporates the above mentioned features is shown in Fig. 8. In this figure the two active, stable forms of the enzyme are designated $E$ and $EH^+$, the two inactive, unstable intermediates are $[E]$ and $[EH^+]$. The salt-dependent equilibria, proposed between $E$ and $[E]$ and between $EH^+$ and $[EH^+]$, and the pH-dependent equilibria, proposed between $E$ and $EH^+$ and between $[E]$ and $[EH^+]$, are drawn. As discussed above, the rate of irreversible inactivation of $[E]$ is much greater than that of $[EH^+]$. No explanation can be offered yet as to how the dissociation of one or more groups can cause a drastic change in the stability of the intermediate form of the enzyme.

In addition to monovalent salts, other substances such as MgCl₂, CaCl₂, spermine, spermidine, and polyamines have been shown to activate halophilic respiratory enzymes (4, 6). The requirement of Mg²⁺ or spermine for nonhalophilic bacterial oxidative phosphorylation (11) and the stabilizing effect of these substances on protoplaists, ribosomes, and various enzymes in a large number of systems have been described (12). In *H. cutirubrum* these multivalent ions are effective at much lower concentrations than is NaCl in activating and stabilizing enzymes. However, we find that the effect of these substances on menadione reductase is not equivalent to that of sodium chloride. This conclusion is supported by the following evidence. (a) The maximal enzyme activity attained with multivalent cations is considerably less than the activity in the presence of NaCl. (b) The *K*ₐ for menadione is higher in the presence of MgCl₂, CaCl₂, and the polyamines than it is with NaCl (Fig. 7). It is remarkable, however, that this altered affinity constant is the same for the various polyvalent ions. (c) In contrast to the findings with NaCl, lowering of inactivation rate by polyvalent ions occurs at low concentrations at which enzyme activity is not yet observed. On the other hand, even at concentrations where maximum enzyme activity is obtained, the rate of inactivation does not decrease below a low but measurable level. Thus, at low concentrations of MgCl₂ or spermine there may exist a form of menadione reductase that is inactive but considerably more stable than it would be in the absence of any protective substance. Multivalent cations, therefore, could not act by shifting the

---

1. J. S. Hubbard and A. L. Miller, presented at the 68th Meeting of the American Society for Microbiology (10).
equilibrium toward the stable, active form of the enzyme (Fig. 8) but appear instead to interact with the unstable, inactive intermediate to produce a more stable species that becomes partially active at higher concentrations of the cations.

A comparison of the stimulation of activity by different polyamines shows that in the spermine-spermidine-putrescine-diethylenamine series the molecule with the greatest number of amino groups has the greatest effect on the enzyme. For effectiveness in enzyme activation it is also necessary to have (a) amino groups reasonably close to one another, as suggested by the lack of activity with 1,10-decanediamine, and (b) sterically unhindered interaction between the amino groups of the activator, as shown by the low activity in the presence of N1,N2-diethyl-1,5-pentanediamine. The physiological role of spermine and of divalent cations in *H. cutirubrum* is uncertain. The instability, the decrease in maximal rate, and the increased *Km* for the substrate indicate that in the presence of these substances menadione reductase is not in its most effective conformation.

REFERENCES

Studies of the Electron Transport Chain of Extremely Halophilic Bacteria: III. MECHANISM OF THE EFFECT OF SALT ON MENADIONE REDUCTASE
Janos K. Lanyi

J. Biol. Chem. 1969, 244:4168-4173.

Access the most updated version of this article at http://www.jbc.org/content/244/15/4168

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/244/15/4168.full.html#ref-list-1