Threonine Deaminase from Extremely Halophilic Bacteria. Cooperative Substrate Kinetics and Salt Dependence*

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ABSTRACT: The effect of salt on the activity, stability, and allosteric properties of catabolic threonine deaminase from *Halobacterium cutirubrum* was studied. The enzyme exhibits sigmoidal kinetics with the substrate, threonine; the Hill slope is 1.55 at pH 10 (near the pH optimum for enzyme activity). The enzyme is activated by ADP at low substrate concentrations and, in the presence of this effector, sigmoidal kinetics are no longer observed. At pH 10, in the absence of ADP, enzyme activity increases with increasing NaCl concentrations from 0 to 4 M while, in the presence of ADP, enzyme activity is greatly inhibited by increasing NaCl concentrations. In 4 M NaCl enzyme activity in the absence of ADP shows a sharp pH optimum at pH 9.75 but enzyme activity in the presence of ADP shows only slight pH dependence with the maximum shifted somewhat in the acid direction. Substrate cooperativity also shows a pH dependence, the Hill constant being lower at lower pH values (less than 1.1 at pH 8.5); the transition point is at pH 9.5. The enzyme is rapidly inactivated at low salt concentrations, with a half-life in 0.5 M NaCl at 0° of less than 60 sec. The half-life of the enzyme is a logarithmic function of the NaCl concentration from 0 to 5 M. The presence of substrate and ADP, however, is sufficient to stabilize the enzyme under some conditions in the absence of salt. At 5.0 M NaCl the enzyme showed cold sensitivity, being more rapidly inactivated at −18 and −10° than at +5°. The results are consistent with the existence of distinct conformational states of the enzyme in the presence or absence of ADP at pH 8.5 or 10. These conformational states differ in their cooperative or allosteric properties as well as their halophilic character.

Enzymes from extremely halophilic bacteria have been shown to require high concentrations of salt for both activity and stability (Larsen, 1962, 1967; Brown, 1964a). The mechanism of action of salts has been postulated to involve shielding of ionic charges on the enzyme by counterions (Baxter, 1959), as well as salting-out of solution of nonpolar side chains allowing them to form more stable hydrophobic bonds within the interior of the protein structure (Lanyi and Stevenson, 1970). The presence of salt thus allows these enzymes to attain an active and stable conformation. We thought, therefore, that it may be particularly interesting to study an allosteric enzyme from extreme halophiles, since these enzymes depend on subtle conformational changes to effect cooperative interactions between polypeptide subunits (Monod *et al.*, 1965; Koshland *et al.*, 1966). Allosteric enzymes that show substrate cooperativity have not been found previously in extreme halophiles. For example, one of the classic examples of allosteric enzymes from nonhalophilic systems, aspartate transcarbamylase, was found to be subject to feedback inhibition by CTP, but did not exhibit cooperative substrate kinetics with respect to either aspartate or carbamyl phosphate (Liebl *et al.*, 1969). Isocitrate dehydrogenase, an enzyme known to contain subunits in other systems, has been studied in extreme halophiles and did not show cooperativity (Hubbard and Miller, 1970; Aitken *et al.*, 1970).

In this investigation we examined the effect of salt on the activity, stability, and allosteric properties of threonine deaminase from *Halobacterium cutirubrum*. Among our findings is the demonstration that this enzyme exhibits sigmoidal substrate kinetics. The results are discussed in terms of the effect of salt on conformational changes in subunits of the enzyme and on the association between them.

Materials and Methods

H. cutirubrum cells were grown in a medium containing (per liter of distilled water): 5 g of Hy-Case SF protein hydrolysate (Sheffield Chemical Co.), 5 g of yeast extract (Difco), 200 g of NaCl, 20 g of $MgCl_2 \cdot 6H_2O$, 2 g of KCl, and 0.2 g of $CaCl_2 \cdot 2H_2O$ (Hochstein and Dalton, 1968). The pH was adjusted to 7.0 using a Corning triple-purpose glass electrode (with low Na error). The cells were grown by inoculating 10 ml of a stationary-phase culture into 1-l. portions of medium in 2-l. flasks and incubating at 37° on a rotating shaker for 4 days (early stationary phase). The cells (from 20 l.) were harvested by centrifugation and resuspended in a total volume of 150 ml with 0.05 M *N*-2-hydroxypiperazine-*N'*-2-ethanesulfonic acid–3.4 M NaCl (pH 7.5). The cell-free extract was prepared by freezing the cell suspension overnight at −78° and thawing. The resultant extremely viscous extract was incubated with deoxyribonuclease (approximately 10–20 mg) for 1 hr at 37° with occasional stirring. Another 10–20 mg of deoxyribonuclease was then added and incubation was continued another hour. The extract was centrifuged at 65,000*g* (av) for 4 hr, and the supernatant was diluted 4-fold with 0.05 M K_2HPO_4–5.0 M NaCl (pH 7.5) and centrifuged at 250,000*g* (av) in a Spinco Model L2-65 ultracentrifuge with a type 60 Ti rotor for 20 hr. The resulting supernatant was used as crude enzyme and stored frozen at −78° in separate tubes that were thawed only once before use. These extracts were occasionally dialyzed but no differences were observed between dialyzed and undialyzed enzyme.

L-Threonine, ADP, pyridoxal phosphate, 2,4-dinitrophenylhydrazine, α-ketobutyric acid, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid were purchased from Sigma Chemical Co.

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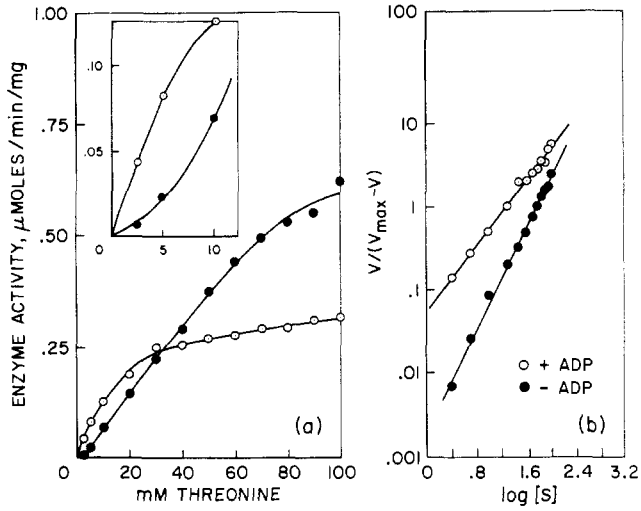

FIGURE 1: Substrate kinetics. (●) Without ADP, (○) with ADP. (a) Enzyme rate *vs.* substrate concentration. Assay conditions: 0.10 M glycine, 4.0 M NaCl, and 10 μg of pyridoxal phosphate/ml, pH 10.0. ADP, when used, was at 1.0 mM. Enzyme protein was 116 μg in 1.0-ml reaction volume. (b) Hill plot of substrate kinetics. The slope, *n*, is 1.55 in the absence of ADP, and 0.99 in the presence of ADP.

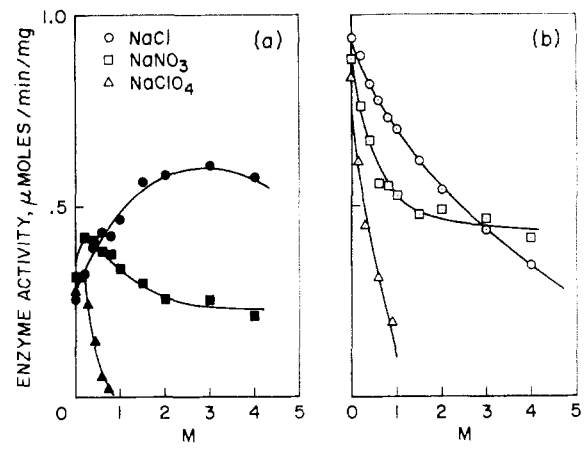

FIGURE 2: Salt response of enzyme activity. Circle, NaCl; squares, $NaNO_3$; triangles, $NaClO_4$. Assay conditions: 0.10 M glycine, 10 μg of pyridoxal phosphate/ml, and 100 mM threonine, pH 10.0. Enzyme protein was 116 μg in 1.0-ml reaction volume. (a) Without ADP, (b) with ADP (1.0 mM). In cases where the enzyme reaction was not linear with time, such as in the absence of ADP at low NaCl concentrations or in $NaNO_3$ or $NaClO_4$ (due to inactivation of the enzyme during assay), the initial enzyme velocity was determined as follows: enzyme activity was measured after various lengths of incubation and the activity per unit time was plotted (logarithmically) *vs.* the time of incubation. This plot yielded a straight line and the initial velocity was determined by extrapolating to zero time.

Standard assay conditions for threonine deaminase were as follows. Enzyme preparation (50–500 μg) was incubated with 100 μmoles of L-threonine, 10 μg of pyridoxal phosphate, 4.0 mM NaCl, and 100 μmoles of glycine (pH 10.0) in a total volume of 1.0 ml. ADP, when used, was at a final concentration of 1.0 mM. Other modifications used are given in the text when appropriate. Incubation was carried out at 37° for varying lengths of time (the reaction was linear for over 60 min under the usual assay conditions). The reaction was stopped by the addition of 1.0 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl. After 5 min (room temperature), 2 ml of absolute ethanol was added, followed by 5 ml of 2.5 N NaOH with immediate and rapid mixing (Greenburg, 1962). The absorbance values were read after 10 min at 435 mμ on a Gilford Model 300-N spectrophotometer. Blanks containing equal amounts of pyridoxal phosphate were always included and subtracted from the experimental samples because of a small constant reading from the pyridoxal phosphate in the assay mixture (about 0.3 OD_{435}/10 μg). An OD_{435} of 1.50 corresponded to 1.02 μmoles of product (α-ketobutyrate) per ml of assay mixture and was a typical result (the assay was linear to over 3.0 OD). Duplicate assays usually showed less than 10% variance; enzyme activities of less than 0.005 μmole/min per mg could easily be detected.

Protein determinations were carried out by the biuret method (Gornall *et al.*, 1949). Specific activities are given in terms of micromoles of α-ketobutyrate formed per minute per milligram of protein.

Kinetic parameters for sigmoidal kinetics were determined with the aid of a computer program obtained from Professor H. J. Wieker (1970), modified for use with a time-sharing system and an on-line terminal.

Results

Substrate Kinetics. Enzyme activity was determined at increasing substrate concentrations in the presence and absence of ADP at 4 M NaCl. The results shown in Figure 1a, plotting enzyme activity *vs.* threonine concentration, demonstrated that the kinetics are sigmoid in the absence of ADP, but hyperbolic in the presence of ADP. ADP activates the enzyme by as much at 6- to 7-fold at low concentrations of substrate such at 2.5 mM, while at high concentrations of threonine, above 50 mM, ADP appears to inhibit enzyme activity. In Figure 1b these results are plotted according to the Hill equation (Hill, 1913), using a maximum velocity determined from a computer program, based on reiterative linear transformations with a least-squares fit (Wieker *et al.*, 1970). The Hill plots were found to be linear over most of the range of substrate concentrations and yield a slope or Hill constant (*n*) of 1.55 in the absence of ADP and 1.0 in the presence of ADP. The K_m for threonine is about 65 mM in the absence of ADP and about 20 mM in the presence of ADP. The same values for *n* and K_m were obtained when determined in 1.0 M NaCl as in 4.0 M NaCl.

Salt Response. Enzyme activity was assayed at 100 mM threonine as a function of salt concentration using three different salts: NaCl, $NaNO_3$, and $NaClO_4$. The results (Figure 2a) show that the enzyme increases in activity with increasing NaCl concentrations in the absence of ADP and is 2–2.5 times more active in 3–4 M NaCl than in 0.05 M NaCl. Low concentrations of $NaNO_3$ increased activity somewhat, while higher concentrations were slightly inhibitory. Low concentrations of $NaClO_4$ also increased activity very slightly, but became greatly inhibitory at higher concentrations. The same experiments were repeated in the presence of ADP (Figure 2b) and showed that increases in the concentrations of all three salts resulted in greatly inhibited enzyme activities. The order of specificity is retained, however, $NaClO_4$ being most inhibitory and NaCl the least. The effect of ADP on the salt response of enzyme activity at two different pH values is presented in Figure 3. At pH 10.0 (Figure 3a) the presence of ADP reverses the effect of NaCl on enzyme activity (data from Figure 2a,b). At pH 8.5 (Figure 3b) NaCl has only a slightly stimulatory effect on enzyme activity in the absence of ADP, whereas low concentrations of NaCl increase enzyme activity but higher NaCl

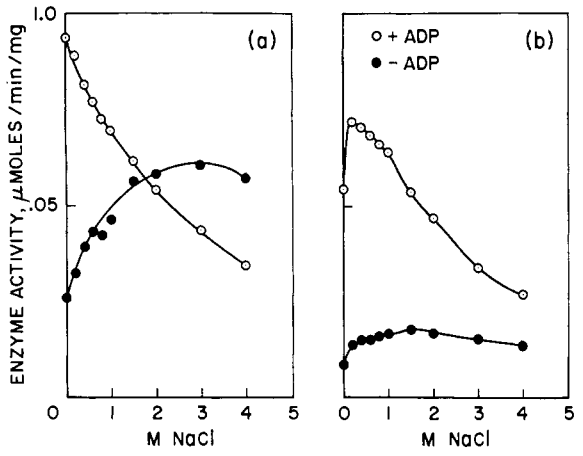

FIGURE 3: Effect of ADP on salt response of the enzyme. (●) Without ADP, (○) with ADP (1.0 mM). (a) pH 10.0 Conditions as described in the legend to Figure 2. (b) pH 8.5. Conditions same as in (a) except 0.05 M Tris·HCl used as buffer in place of 0.10 M glycine.

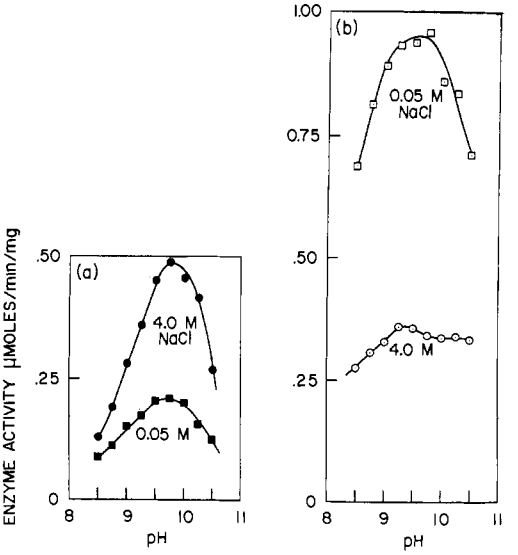

FIGURE 4: pH dependence of enzyme activity. Conditions as described in the legend to Figure 2 (except for pH changes). (a) Without ADP (■) 0.05 M NaCl, (●) 4.0 M NaCl. (b) With ADP (□) 0.05 M NaCl, (○) 4.0 M NaCl.

concentrations markedly inhibit enzyme activity in the presence of ADP.

pH Dependence of Enzyme Activity. Enzyme activity was determined as a function of pH at both 0.05 and 4.0 M NaCl. In the absence of ADP (Figure 4a) enzyme activity shows a sharp pH optimum at pH 9.75 at both high and low salt concentrations. In the presence of ADP (Figure 4b) in 0.05 M NaCl, the pH dependence of enzyme activity is similar, but in 4.0 M NaCl, enzyme activity shows very little pH dependence with the maximum shifting about 0.25–0.5 pH unit in the acid direction. Substrate cooperativity was also measured as a function of pH. These results (Figure 5) demonstrate that the Hill constant, n, decreases at lower pH values, and is less than 1.1 at pH 8.5. The curve resembles an acid-base transition with a pK of 9.5. The K_m for threonine at pH 8.5 in the absence of ADP is somewhat lower than at pH 10.0 (about 55 mM compared to 65 mM) reflecting a shift from a sigmoid substrate saturation curve at higher pH to a hyperbolic curve at lower pH. However, the K_m for threonine in the presence of ADP is still much lower, about 20 mM at both pH 8.5 and 10.0. Thus ADP still activates the enzyme at pH 8.5 at low substrate concentrations, even though at this pH in the absence of ADP substrate kinetics are not sigmoid.

Inactivation of the Enzyme at Low Salt Concentrations. The enzyme was inactivated by incubation at low salt concentrations at pH 8.5 for varying lengths of time at 0°. The inactivation was stopped by the addition of buffer containing high concentrations of salt and then assayed at pH 8.5 to determine the level of enzyme activity remaining. The results, presented in Figure 6a in percentages of remaining activity as a logarithmic function of the inactivation time, show that the enzyme is inactivated more rapidly as the salt concentration decreases.[1] In 0.5 M NaCl at 0° at pH 8.5 the enzyme has a half-life of less than 60 sec. The rate of inactivation was not significantly affected by the pH at which inactivation was carried out. The half-life of the enzyme is a logarithmic function of the NaCl concentration from 0 to 5 M NaCl (Figure 6b). The half-life values range from 10 to 15 sec in 0.05 M NaCl (at 0°) up to about 4×10^5 sec (about 5 days) in 5.0 M NaCl.

[1] Essentially identical results were obtained with a 15-fold purified enzyme preparation.

However, the presence of substrate (10 mM threonine), but not ADP, during inactivation stabilized the enzyme at 0° even in the absence of salt. In contrast, at 37° in the absence of NaCl, or in the presence of $NaNO_3$ or $NaClO_4$, substrate was not sufficient to stabilize the enzyme, but the addition of ADP resulted in complete stabilization. The latter results are given in Table I and show that in the absence of ADP the enzyme is inactivated at concentrations of NaCl below 1.0 M whereas in the presence of ADP no inactivation could be detected (for up to 30-min incubation). Furthermore, in the absence of ADP the enzyme is increasingly rapidly inactivated by increasing concentrations of $NaNO_3$ or $NaClO_4$, and ADP afforded protection against this type of inactivation as well.

Cold Sensitivity of the Enzyme. The enzyme was inactivated by incubation in 5.0 M NaCl over long periods of time at +5, +10, and −18°. After 4-days incubation there was about 70% enzyme activity remaining at +5°, about 40% at −10°, but only about 10% at −18°. The kinetics of inactivation (Figure 7) demonstrate that the enzyme is inactivated more rapidly at lower temperatures (below +5°).

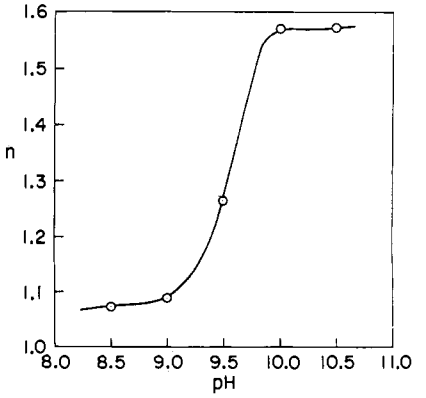

FIGURE 5: pH dependence of substrate cooperativity. The Hill constant, n, is plotted against pH. Conditions as described in the legend to Figure 1a.

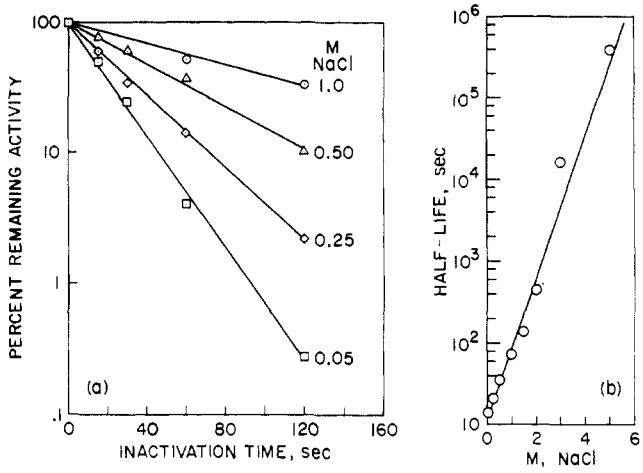

FIGURE 6: Inactivation of the enzyme in low salt concentrations. (a) Inactivation was carried out by diluting the enzyme 40-fold (protein concentration during inactivation was 0.29 mg/ml) into buffer containing 0.05 M Tris·HCl, 10 μg of pyridoxal phosphate/ml, and varying concentrations of NaCl, pH 8.5 (total volume = 0.8 ml). After inactivation by incubation at 0° for various lengths of time, 0.2 ml of cold buffer containing 0.05 M Tris·HCl, 1.0–5.0 M NaCl, and 50 mM threonine (pH 8.5) were added to stop inactivation and start the assay. The inactivated enzyme was assayed at a final concentration of 10 mM threonine in 1.0 M NaCl at pH 8.5. (b) Stability of the enzyme as a function of salt concentration. Inactivations and assays performed as described in the legend to Figure 6a, except that in some cases assays were at 3 or 4 M NaCl. Half-lives were determined from plots such as shown in Figure 6a.

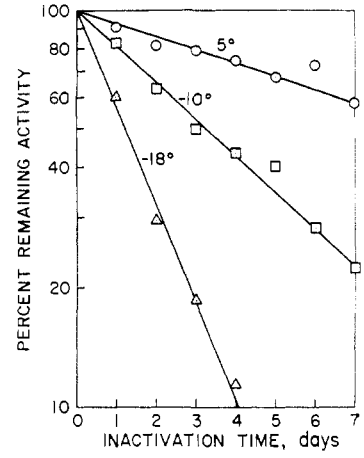

FIGURE 7: Cold sensitivity of the enzyme. Inactivation was carried out by diluting the enzyme 40-fold (protein concentration during inactivation was 0.29 mg/ml) into buffer containing 0.10 M glycine, 5.0 M NaCl, and 10 μg of pyridoxal phosphate/ml, pH 10.0. Incubation was at either +5, −10, or −18°. Assays were carried out with 100 mM threonine, in 4.0 M NaCl, pH 10.0.

Discussion

Allosteric enzymes, such as threonine deaminase, are characterized by their ability to undergo subtle but significant changes in physical conformation (Monod *et al.*, 1965; Koshland *et al.*, 1966). These changes typically result in differences in kinetic behavior, which are considered important from the standpoint of metabolic regulation *in vivo*, through control of enzyme function. It has been well documented that the physical conformation of proteins (von Hippel and Wong, 1962, 1964), as well as subunit associations (Wolff, 1962; Nagy and Jencks, 1965) are greatly affected by high concentrations of salts. Since it has been established that extremely halophilic bacteria maintain a high internal concentration of salt (3–4 M) (Gibbons and Baxter, 1953; Christian and Ingram, 1959; Christian and Waltho, 1962), it is of special interest to determine if allosteric halophilic enzymes can undergo subtle conformational changes and maintain cooperative interactions between subunits of the enzyme while under the extreme environmental influence of high salt concentrations.

Threonine deaminase has been studied in nonhalophilic systems and two such enzymes found: a biosynthetic enzyme subject to feedback inhibition by isoleucine and valine (Sharma and Mazumder, 1970; Harding *et al.*, 1970) and a catabolic enzyme activated by ADP (Nakazawa and Hayaishi, 1967). The latter enzyme (which is of the type reported here) was studied in *Clostridium tetanomorphum* and found to consist of several subunits (at least eight molecular species were detected) (Whitely, 1966), and exhibited cooperative substrate kinetics (Whitely and Tahara, 1966; Nakazawa and Hayaishi, 1967).

Because of differences observed in kinetic behavior and salt response by the threonine deaminase from *H. cutirubrum* in the presence or absence of ADP and at pH 8.5 or pH 10, the results obtained here can be analyzed by postulating four distinct states of the enzyme. (1) At pH 10 in the absence of ADP the enzyme exhibits typical allosteric behavior (Figure 1) consistent with cooperative interactions between subunits of the enzyme-containing substrate binding sites, as well as interactions between effector binding sites and substrate binding sites (Monod *et al.*, 1965; Koshland *et al.*, 1966). In this conformation the enzyme is greatly halophilic in character, increasing in activity at high salt concentrations (Figures 2a and 3a). (2) At pH 8.5 in the absence of ADP the enzyme no longer exhibits cooperative substrate kinetics (Figure 5) (but is still activated by ADP), and is less halophilic in character than the enzyme in conformation 1 (Figure 3b). (3) At pH 10 in the presence of ADP the enzyme is noncooperative (Figure

TABLE I: Effect of ADP on Enzyme Stability at 37° in the Presence of Substrate.[a]

Salt	Concn (M)	Inactivation Rate (hr^{-1}) −ADP	Inactivation Rate (hr^{-1}) +ADP
NaCl	0.05	0.60	0
	0.2	0.40	0
	0.5	0.32	0
	1.0	0.08	0
$NaNO_3$	0.2	0.62	0
	0.4	0.74	0
	0.6	0.82	0
$NaClO_4$	0.15	0.88	0
	0.3	1.44	0
	0.6	1.70	0.28

[a] The enzyme was assayed for various lengths of time, as described in the legend to Figure 2, in the presence of the concentrations of NaCl, $NaNO_3$, or $NaClO_4$ indicated in the table. The enzyme activities were plotted also as described in the legend to Figure 2. The slope of the line obtained from this plot was used to calculate the rate of inactivation during the assay.

1) and nonhalophilic, being significantly inhibited by high salt concentrations (Figures 2b and 3a). (4) At pH 8.5 in the presence of ADP the enzyme is noncooperative, but its salt response falls between those of states 2 and 3 (Figure 3b). There is also a large difference in K_m for threonine between state 2 and state 4 (55 and 20 mM, respectively), a consequence of ADP binding. The K_m values in states 1 and 3 (at pH 10, in the absence and presence of ADP), 65 and 20 mM, respectively, are very similar to those reported for purified threonine deaminase from *Clostridium tetanomorphum* (Vanquickenborne *et al.*, 1969) of 75 and 20 mM.

These hypothetical conformational states are used to facilitate the discussion of the effect of salt on the activity, stability, and allosteric properties of threonine deaminase. The action of salts on other halophilic enzymes has been studied and two basic mechanisms have been proposed. First, the salt can shield ionic charges on the enzyme by providing counter ions, thereby reducing electrostatic repulsion from like charges and allowing hydrogen bonds to form (Baxter, 1959; Brown, 1965; Holmes and Halvorson, 1965; Kushner and Bayley, 1963; Kushner and Onishi, 1966). This hypothesis has been supported by the finding that halophilic proteins have an abundance of acidic acids (Kushner and Onishi, 1966; Bayley, 1966; Brown, 1963), and that succinylation of a nonhalophilic enzyme gives the enzyme some halophilic characteristics (Brown, 1964b). However, charge shielding should be complete by 0.1–0.2 M salt (Inman and Jordan, 1960; Edsall *et al.*, 1950); hence, this hypothesis would not account for the high concentrations of NaCl (2–4 M) required for maximal activity of some halophilic enzymes (Lanyi, 1969a,b; Lanyi and Stevenson, 1970; Lieberman and Lanyi, 1971), nor would it account for the salt specificity, especially among the anion components of the salt, demonstrated for these enzymes (Hochstein and Dalton, 1968; Lanyi and Stevenson, 1970). The second mechanism proposed for the effect of salt on halophilic enzymes is based on the salting-out power of certain salts such as NaCl (Lanyi and Stevenson, 1970). In the presence of high concentrations of NaCl, nonpolar side chains on the enzyme are salted-out of solution and are thus moved into the interior of the structure where they can form stable hydrophobic bonds (Lanyi and Stevenson, 1970). Other salts, such as $NaNO_3$ and especially $NaClO_4$ which follow NaCl in the lyotropic series (Hatefi and Hanstein, 1969), do not salt-out as well or even salt-in and thus could not support maximal enzyme activity. These two mechanisms for the action of salts are not mutually exclusive, but probably complement one another. In the case of threonine deaminase, both mechanisms are suggested by the salt response of the enzyme at pH 10 (Figure 2a). Low concentrations (0.2–0.4 M) of NaCl, $NaNO_3$, or $NaClO_4$ increase enzyme activity somewhat, suggesting a nonspecific charge shielding effect. However, maximal enzyme activity is obtained only in the presence of high concentrations (3–4 M) of NaCl. Because of difficulty in obtaining accurate results at very low substrate concentrations in NaCl concentrations below 1.0 M, the Hill constant, n, could not be determined at very low NaCl concentrations. But when determined at 1.0 M NaCl, the Hill constant was the same as at 4.0 M NaCl. High concentrations of $NaNO_3$ inhibit the enzyme to some extent and high concentrations of $NaClO_4$ inhibit greatly. Thus the enzyme under these conditions appears to be typically halophilic in character.

At pH 8.5 in the absence of ADP the enzyme exhibits the charge-shielding effects of NaCl at 0.2–0.4 M, but not the salting-out effects at concentrations up to 4 M (Figure 3b). At pH 10 in the presence of ADP, the enzyme exhibits neither the charge-shielding nor the salting-out effects of NaCl (Figures 2b, 3a) and increasing the NaCl concentrations from 0 to 4 M results in increasing inhibition of enzyme activity. However, the enzyme still shows salt specificity in that $NaClO_4$ inhibits to a greater extent than NaCl. Thus, it appears that although the enzyme is sensitive to salting-in effects, under these conditions it becomes nonhalophilic in character. At pH 8.5 in the presence of ADP enzyme activity is increased by low concentrations of NaCl (0.2–0.4 M), but becomes significantly inhibited by high concentrations of NaCl up to 4 M (Figure 3b).

The dramatic difference in salt response in the presence and absence of ADP of *H. cutirubrum* threonine deaminase is an unexpected result. The salt dependence of halophilic enzymes has been previously thought to be the consequence of major departures in their amino acid composition (Kushner and Onishi, 1966; Bayley, 1966; Brown, 1963) from that of the equivalent nonhalophilic proteins. The conversion of a typically halophilic enzyme to a salt-inhibited form through the binding of the allosteric effector suggests, however, that the salt effects may not involve the entire protein molecule. The coincidence of the transition from cooperative to noncooperative kinetics with the transition from NaCl-requiring to NaCl-inhibited enzyme activity may, in fact, reflect the response of a relatively small region in the vicinity of the active site.

In the absence of ADP, the addition of salt at high concentrations (4 M) has little effect on the pH dependence of enzyme activity (Figure 4a), but in the presence of ADP, pH dependence is greatly affected by salt (Figure 4b). Since high concentrations of salt decrease the pH dependence of enzyme activity in the presence of ADP, it seems likely that the salt is shielding ionizable functional groups involved in catalytic activity. Such effects of salt on the pH dependence have been observed with other halophilic enzymes (Aitken *et al.*, 1970; Holmes and Halvorson, 1965). The anomalous persistence of the sharp pH optimum for threonine deaminase at high salt concentrations in the absence of ADP raises the possibility that under these conditions the functional groups might not be available for shielding by counter ions. The basis for this inaccessibility is not clear since ionic groups are generally not stable in a hydrophobic environment (Tanford, 1961).

Studies of the inactivation of the enzyme at low salt concentrations indicate that unlike one halophilic enzyme reported (Lanyi and Stevenson, 1970), the stability of threonine deaminase is not directly related to enzyme activity in regard to salt effects. Since the logarithm of the half-life of the enzyme is directly proportional to the salt concentration over the entire range tested from 0 to 5 M (Figure 6b), the effect of salt on stability of the enzyme cannot be readily divided into charge-shielding and salting-out effects. Also, since ADP does not prevent inactivation at low salt concentrations, the enzyme is halophilic in character (requires salt) with respect to stability both in the presence or absence of ADP. Changes in pH during inactivation do not affect the halophilic character of the enzyme with respect to stability, nor does purification of the enzyme change the above properties of the crude system.

Hydrophobic interactions are known to be cold sensitive (Brandts, 1964; Havir *et al.*, 1965; Rosenberg and Lumry, 1964; Sheraga *et al.*, 1962). Several halophilic enzymes have been shown to be cold sensitive, a fact argued to be a consequence of the involvement of hydrophobic bonds in their structure (Lanyi and Stevenson, 1970; Lieberman and Lanyi, 1971). The finding that threonine deaminase is cold sensitive at high salt concentrations (Figure 7) is another piece of evidence suggesting the involvement of hydrophobic bonds in

the stability of the enzyme. In the case of menadione reductase (Lanyi and Stevenson, 1970) the increase in the free-energy barrier of inactivation at 0° in the presence of 3.0 M NaCl over that in the absence of salt was calculated to be 3.5 kcal. This figure was suggested to represent the sum of contributions from electrostatic shielding and hydrophobic bonding. The same calculation for threonine deaminase, using the formula $\Delta F^{\ddagger} = RT \ln (k_1/k_2)$, where k_1 (inactivation rate in the absence of salt, calculated from Figure 6b) = 2.73 min^{-1}, and k_2 (inactivation rate in 3.0 M NaCl, calculated from Figure 6b) = 0.00476 min^{-1}, yields $\Delta F^{\ddagger}$ = 3.4 kcal. The remarkable agreement in the calculated free energies of inactivation of the two enzymes as well as the demonstrated cold sensitivity of these enzymes, suggest that the requirement for salt for the stabilization of widely different halophilic enzymes may have some common structural origin.

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