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Salt-Dependent Properties of Proteins from Extremely Halophilic Bacteria

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INTRODUCTION

The presence of large amounts of salt inhibits the growth of most familiar microorganisms. There are some bacteria, however, including the halobacteria and the halococci which have adapted successfully to high concentrations of NaCl. Indeed, as described in numerous reviews (21, 71, 81, 82), these organisms, collectively labeled "extreme halophiles," not only tolerate but require NaCl concentrations above 10 to 15% for survival, and optimal growth is often obtained above 20% NaCl. In the halobacteria the osmotic problems inherent at the high concentrations of salt are circumvented by the fact that the intracellular cation concentration is also very high and approximates that in the medium (34, 48, 79). The intracellular composition of salts is unlike that in the medium, however. While the growth medium contains Na^+ in several hundredfold excess over K^+, the cells concentrate K^+ to over 2 M concentration, and the intracellular [K^+] is several times the [Na^+] (34, 48, 79). Optimal growth also requires Mg^{2+} in unusually large amounts, up to 100 mM, in addition to the usual complement of trace metal ions (81, 82). The nutritional requirements of the halobacteria have been extensively reviewed before (49, 81, 82). Very little work has been done with the halococci.

The high intracellular salt concentration in these organisms raises questions about the possible mechanism of action for the various macromolecular components. The deleterious effects of monovalent salts at multimolar concentrations on biological macromolecules from various organisms have long been noted (65, 124) and seem to be caused to a large extent by dissociation of groups, subunits, etc. which are involved in ionic linkages. If such ionic bonds are lacking in halophilic cell constituents, the physical chemistry of these structures must be unusual.

The properties of specific enzymes and other components of extreme halophiles are discussed below in detail. In general, all structures investigated were indeed found to be stable only in the presence of at least 1 M salt. In addition, most systems required or were stimulated by salt at concentrations near or even above this value. Thus, rather than being destroyed at high salt concentration, the macromolecular structures responsible for biological activity in halophiles appear, in fact, to be dependent on the presence of salts. A dramatic example of this unique salt dependence is the behavior of the cell envelope of the halobacteria when the salt concentration is lowered. Under these conditions, cells (1, 81, 82) and isolated cell envelopes (20-23, 70, 73, 98, 108) disintegrate to give slowly sedimenting fragments, and several membrane-bound enzymes are inactivated (54, 75, 83). It is clear that upon lowering the salt concentration considerable changes take place in the structure of the cell envelope and its constituents.

A reasonable hypothesis to account for the salt dependence of halophilic enzymes and membranes had been developed by Baxter (5), Brown (20-23, 108), and others (70, 73, 98). These workers proposed that the protective effect of salt is due to the shielding of negative charges on these structures. It was presumed that in the absence of salt, charge repulsion caused unfolding and loss of activity. There is considerable evidence to support this view. The total bulk protein in halophilic cells (101) as well as membrane proteins (72, 92, 110, 113) and ribosomal proteins (9, 122) contain a large excess of acidic amino acids. In some cases low concentrations of $MgCl_2$ or $CaCl_2$ can substitute, at least partly, for high concentrations of monovalent salts (25, 54, 55, 76, 83, 96). Finally, Brown showed that succinylation of membranes of marine bacteria increased their halophilic character (22).

As pointed out recently (80), however, the

charge screening hypothesis seems to be deficient in several important respects. First, charge shielding by salt ought to be complete at a few tenths of molarity, as it was indeed found to be in the case of well-studied charged macromolecules, such as deoxyribonucleic acid (DNA) (64) and polyaminoacids (35, 125). Without additional assumptions, therefore, the requirement of *several molar* salt for the integrity of halophilic macromolecules is not predicted by the counter-ion hypothesis. Second, in many halophilic systems a specificity for various anions was observed (5, 54, 61, 80), which is unlikely to be a result of charge shielding alone. Third, in a highly charged macromolecule, such as polyglutamic acid at pH >5, the screening effect of the added cations is more than counteracted by the lowering of the pK of the acid groups at high salt concentrations, resulting in *destabilization* of the secondary structure (35). As these arguments suggest, effects other than charge screening must also be considered if the phenomenon of halophilic behavior is to be understood.

This review attempts to re-examine the literature of the enzymes of halophilic bacteria and their constituents in the light of what is known in general about the interaction of salts and macromolecules. It is the author's aim to arrive at a more unified view of halophilic systems, arising from the consideration of both the experimental observations available and the current theories of macromolecules.

GENERAL CONSIDERATIONS

Protein and assemblies of macromolecules exist in a dynamic state; their structure clusters about a most probable conformation. The existence of a particular geometry for the folding of the polypeptide chain, or an arrangement of subunits, is ensured by a free energy decrease obtained upon the interaction between various groups or sites. In some cases these interactions are brought about in the first place by the favorable or unfavorable influence of the solvent present. Thus, some residues are sequestered preferentially away from the aqueous phase, whereas others are exposed to it, due to a decrease of free energy on solvation. In other cases, competition by solvent molecules can change the stability of the residue interactions. In general, the "native" conformation is characterized by chain-chain interactions, whereas the disrupted conformation gives rise to increased chain-solvent effects (124). In this sense the nature of the solvent and its modification upon addition of salts and other agents may be expected to have a profound influence on the stability of macromolecular structures.

Although a large number of different kinds of groups are involved in the structure of proteins, the observed kinetics and thermodynamics of the denaturation process are often quite simple. Such simplicity arises from the cooperative nature of the residue interactions. In such cases one obtains a "two-state" system (17, 119), with no intermediate states having sufficient stability to exist as separate species and being accessible to experimentation. For irreversible processes like these, one may use the rates of denaturation to obtain activation energies for the unfolding. What such experimental data then reveal are the properties of the forces which act as barriers to the disruption of the structures.

The factors involved in the stability of macromolecular structures, defined in this manner, may be considered separately as: (i) ionic effects and (ii) hydrophobic effects. Among ionic effects Brandts (16, 17) distinguishes (i) long range electrostatic interactions and (ii) specific group or residue interactions, including hydrogen bonds. These factors are discussed in detail herein, insofar as they are relevant to the discussion on the proteins of halophiles.

LONG-RANGE ELECTROSTATIC INTERACTIONS

Long-range electrostatic effects arise from the difference in electrostatic-free energies between the folded and unfolded states of a protein that carries charges. Tanford considered the free energy of a solvent-impermeable sphere, of a size typical of many proteins, with charges on its surface (as calculated from the Debye-Hückel theory) and concluded that a substantial fraction of the electrostatic-free energy remains even at high concentrations of counterions (117). On the other hand, it could be shown that counter-ion shielding eliminates nearly all of the electrostatic-free energy of a similar macromolecule if it is permeable to the solvent and ions. It is apparent that the distribution and the sign of the charges uniquely determines the electrostatic contribution to the thermodynamic properties of actual proteins. Nevertheless, as implied by these calculations (117), it is probably a general principle that in proteins the decrease in free energy, obtained on the expansion of the molecule, is due not only to the separation of the charges, but also to the increased possibility for charge screening. It follows from Tanford's discussion that polypeptide chains can reduce their electrostatic-free energy even by limited unfolding, particularly in the presence of adequate concentrations of

counter-ions. Because the electrostatic effect is expected to influence the folding of the proteins, hence their volume and extent of hydration, it can be followed by studying hydrodynamic properties. The pH (and therefore charge)-dependent changes of volumes are indeed readily observed in hemoglobin (13, 116). The persistence of residue interactions, which contribute to the stability of the structure, ordinarily prevents gross unfolding by such means, however. In extreme cases, residue interactions and disulfide linkages can completely overcome the electrostatic effects. Ribonuclease and beta-lactoglobulin, for example, do not show preferential unfolding even at pH values where they are highly charged (18, 121).

The need for considering such electrostatic effects in the macromolecular components of extreme halophiles becomes evident when the amino acid compositions of various proteins or protein fractions are examined. It long has been recognized that halophilic proteins are, in general, acidic (21, 81, 82). Table 1 contains a summary of the published data. The cytoplasmic (soluble) fraction of whole cells of various halophiles, of the *Halobacterium* and *Halococcus* type, are seen, from Reistad's results (101), to exhibit an excess of acidic groups as

TABLE 1. *Acidic and basic amino acids in cells and cell constituents and hydrophobicity parameters*

Material	Source	Acidic	Mole % basic	Excess acidic	$H\Phi_{ave}$[a]	p[b]	Ref
Cytoplasmic protein	*Halobacterium salinarium*	26.8	9.7	17.1	0.958	1.089	101
Cytoplasmic protein	*Halococcus* no. 24	27.8	9.9	17.9	0.936	1.107	101
Cytoplasmic protein	*Halococcus* no. 46	26.8	10.3	16.5	0.956	1.071	101
Cytoplasmic protein	*Pseudomonas fluorescens*	20.9	13.8	7.1	1.054	0.953	101
Cytoplasmic protein	*Sarcina lutea*	21.2	12.6	8.6	1.025	0.917	101
Cytoplasmic protein	*Escherichia coli*	18.4	18.2	0.2	1.114	1.003	109
Ribosomes, 70*s*	*Halobacterium cutirubrum*	28.2	13.7	14.5	0.943	1.445	9
Ribosomes, 70*s*	*H. cutirubrum*	26.6	13.1	13.5	0.845	1.321	122
Ribosomes, 30*s*	*H. cutirubrum*	24.8	13.0	11.8	0.849	1.330	122
Ribosomes, 50*s*	*H. cutirubrum*	26.5	14.1	12.4	0.834	1.373	122
Ribosomes, 70*s*	*E. coli*	21.6	12.5	9.1	1.086	0.965	109
Ribosomes, "RNA-rich fraction"[c]	*H. cutirubrum*	25.4	19.2	6.2	0.986	1.531	9
Ribosomes, 50*s*[d]							
Fraction S-1	*H. cutirubrum*	29.0	10.9	18.9	0.857	1.308	122
Fraction S-2	*H. cutirubrum*	24.1	15.2	8.9	0.841	1.400	122
Fraction S-3	*H. cutirubrum*	21.1	16.6	4.5	0.787	1.350	122
Cell envelope, total	*H. cutirubrum*	27.2	7.0	20.2	0.946	1.075	72
Cell envelope, total	*H. salinarium*	25.6	5.9	19.7	0.886	1.149	110
Cell envelope, total	*H. cutirubrum*	27.6	6.9	20.7	0.905	1.204	
Cell envelope, red pellet[e]	*H. cutirubrum*	21.5	6.5	15.0	1.019	0.758	
Cell envelope, outer layer	*Halobacterium halobium*	28.0	7.3	20.7	0.790	1.423	92
Cell envelope, membrane fraction	*H. halobium*	25.7	8.2	17.5	0.934	1.124	92
Cell envelope, red pellet[f]	*H. halobium*	24.0	7.2	16.8	0.949	1.022	113
Cell envelope, supernatant	*H. halobium*	29.2	7.1	22.1	0.855	1.371	113
Purple membrane	*H. halobium*	12.2	11.5	0.7	1.241	0.723	113
Purple membrane	*H. halobium*	13.6	7.4	6.2	1.261	0.610	97
Gas vacuoles	*H. halobium*	20.2	12.7	6.5	0.928	1.189	113
Gas vacuoles	*H. halobium*	22.2	9.0	13.2	1.076	0.804	69

[a] Hydrophobicity index (in kcal/residue) calculated as described by Bigelow (14), who used the free energies of transfer for amino acids from water to ethanol (118).

[b] Ratio of volume for polar and nonpolar residues, according to Fisher (41).

[c] Dialysis of ribosomes against buffer containing Mg^{2+}, and a low concentration of monovalent cations, followed by sedimenting the RNA-rich particles.

[d] Dialysis of ribosomes against 0.3 mM Mg^{2+}, centrifugation; supernatant is S-1. Incubation in 3.5 M LiCl-ethylenediaminetetraacetic acid (EDTA), centrifugation. Supernatant is S-2, followed by incubation in 3.5 M LiCl-urea, which yielded supernatant S-3.

[e] Cell envelopes were dialyzed against 0.01 M EDTA. Centrifugation at 105,000 × *g* yielded red pellet (J. K. Lanyi and E. L. Bugna, unpublished data).

[f] Exhaustive dialysis of cell membranes against distilled water, sedimentation at 105,000 × *g* for 20 to 30 h.

high as 17 to 18 mole percent, unlike those of representative nonhalophilic cells, included in Table 1. The cell envelopes of *Halobacterium* are apparently even more highly charged, the excess of acidic groups amounting to 19 to 20 mole percent. Some cellular structures of extreme halophiles have been separated into fractions by lowering the salt concentration. *Halobacterium cutirubrum* ribosomal proteins, somewhat more acidic than those of *Escherichia coli* on the average, were fractionated by Bayley (9, 11) and Visentin et al. (33, 122) by exposure to buffers which lacked the high KCl concentrations found in the cells (34), but which contained 0.05 M $MgCl_2$. A considerable fraction of the proteins was removed by this treatment from the ribosomes; the detached proteins (fraction S-1) were found to be more acidic than the original ribosomes (122), whereas the residual bound proteins were less acidic (9). Removal of Mg^{2+} produced a second solubilized fraction (S-2), less acidic than the first fraction, leaving behind proteins which were the least charged and which were solubilized only in the presence of urea (122). This kind of salt-dependent fractionation was performed by Stoeckenius and Kunau (113) with *Halobacterium halobium* cell envelopes and by J. K. Lanyi and E. L. Bugna with *H. cutirubrum* cell envelopes (unpublished data), and it was found that exhaustive dialysis against distilled water resulted in the solubilization of some of the membrane proteins, including the proteins of the outer envelope. As shown in Table 1, in both organisms these components were more acidic than the rest of the membrane proteins, which were retained in lipid-containing membrane fragments after the treatment. It appears from these results that the proteins of extreme halophiles which are most responsive to the removal of salt, at least by the criterion of solubilization, also show the greatest excess of acidic groups. There are components of halophiles, on the other hand, which seem to be little affected by low-salt treatment such as the purple membrane of *H. halobium* (97, 113), which exhibits a considerable degree of crystalline structure after removal of salt (15), and the gas vacuoles, found in some strains of *H. halobium* (69, 113), which are morphologically unchanged in distilled water. As expected, the protein components of these structures are much less acidic than the bulk protein of the organism (Table 1). Unfortunately, reliable estimates of the amide content of the above proteins are not available, for amide content either is not given or is estimated from NH_3 in the hydrolysate. For *H. cutirubrum* cell envelopes, at least, it was possible to show (J. K. Lanyi and R. E. Mack, unpublished data) that approximately 80% of the acidic groups were free to react with glycine in the presence of dicyclohexylcarbodiimide (24). More work of this type is clearly needed to establish this point. The only purified halophilic enzymes for which amino acid analyses are given are the DNA-dependent and ribonucleic acid (RNA)-dependent RNA polymerases of *H. cutirubrum* studies by Louis and Fitt (89, 90). These enzymes are not unusually acidic and will be discussed in a later section.

Baxter first suggested (5) that the screening of negative charges accounts for the various observations associated with halophilic enzymes. The salt-dependent properties of these systems may be separated, for the purposes of this discussion, into: (i) enzyme activity, (ii) stability, and (iii) subunit association and allosteric effects. The salt response of the enzyme activity is highly variable among these proteins. Earlier reports seemed to show that most enzymes from extreme halophiles exhibited no activity in the absence of salt (5, 7, 57, 81, 82, 95). However, in many cases the possibility of time-dependent inactivation at the lower salt concentrations, before or during the enzyme assays, was not taken into account. More recently, it has been established that some of those enzymes, indeed, show little or no activity at NaCl concentrations below 0.1 M. These enzymes are aspartate transcarbamylase (85), nicotinamide adenine dinucleotide (NADH)-menadione reductase (75, 76), cytochrome oxidase (32, 75, 83), polynucleotide phosphorylase (99), an amino acid incorporating system (10, 51), citrate synthase (25) and malic enzyme (26) from *H. cutirubrum*, NADH oxidase from an extreme halophile, AR-1 (54, 55), and glycerol dehydrogenase from *Pseudomonas salinaria* (6). These enzymes exhibited maximal activity between 2 to 4 M NaCl or KCl, and several were not inhibited at the highest salt concentration tested. Another class of enzymes, including malic dehydrogenase (59), ornithine transcarbamylase (37, 38), arginine desimidase (38) and isocitrate dehydrogenase (3) of *Halobacterium salinarium*, isocitrate dehydrogenase (62, 63) and threonine deaminase (84) of *H. cutirubrum*, and NADH dehydrogenase of AR-1 (56), showed maximal activity at intermediate salt concentrations, between 0.5 and 1.5 M NaCl, many being inhibited at higher concentrations. Finally, some enzymes from halophilic bacteria do not require salt or are inhibited by low concentrations, such as the fatty acid synthetase (100) and RNA-dependent RNA polymerase (87) of *H. cutirubrum* and amylase (50) of *H.*

halobium. Typical graphs showing the effects of salt on enzyme activity in halophiles are given in Fig. 1.

Although the salt requirement of enzyme activity is variable in halophilic systems, all enzymes investigated have shown a time-dependent inactivation at lower salt concentrations and especially in the absence of salt. In many cases the inactivation followed first-order kinetics (3, 5, 60, 75, 76, 84, 96). Lanyi found (76) that apparent first-order inactivation rate constants could be calculated at various salt concentrations for menadione reductase, an NADH dehydrogenase linked to the membrane-bound respiratory chain (28, 29, 74, 75) of *H. cutirubrum*. When the rate constant was plotted against NaCl concentration, the curve obtained was a mirror image of the salt dependence of enzyme activity, both reaching limiting values at 2 M NaCl (Fig. 2). These findings were consistent with the kinetics expected for a rapid, salt-dependent equilibrium of the native, active form of the enzyme with an inactive, unstable form. The salt dependence of the apparent rate of inactivation, as well as that of enzyme activity, was assumed to reflect a shift in the above equilibrium toward the unstable, presumably partially unfolded, state, as the salt concentration was lowered. The half-life of this species was 40 s at neutral pH and room temperature; the inactivation was faster at pH values above 9, reaching a half-life of 5 to 6 s above pH 10. The midpoint of transition was at pH 9.3 and seemed to reflect the ionization of a group (amino or tyrosyl-hydroxyl) critically involved in the stability of the structure. Lanyi had shown later (78) that the salt-dependent properties of this enzyme are little influenced by the membrane matrix, where it is found, because detachment from the membrane occurs at higher salt concentrations and is much faster than inactivation.

The inactivation of other halophilic enzymes at lowered salt concentrations does not follow such simple kinetics. In most systems the salt dependence of enzyme activity and of stability are clearly separable. Thus, the enzyme activi-

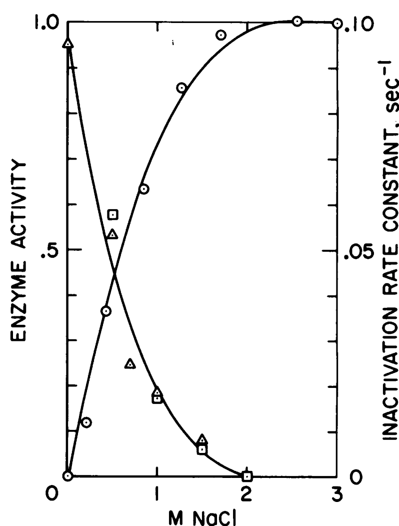

FIG. 2. *Dependence of enzyme activity and apparent first-order inactivation rate constant on NaCl concentration for menadione reductase of H. cutirubrum. Enzyme activity, O; rate constants calculated from inactivation kinetics and determined at decreasing salt concentrations, □; and after restoring NaCl concentration to 2 M, Δ. Reprinted with permission from J. K. Lanyi (1969), J. Biol. Chem.* **244**:*4168 (ref. 76).*

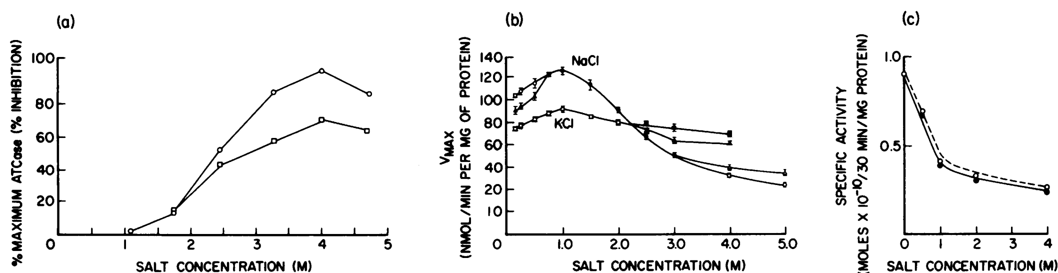

FIG. 1. *Salt dependence of enzyme activity in typical halophilic systems. (a) Aspartate transcarbamylase from H. cutirubrum in 1.1 M NaCl solutions with added KCl to give the stated total salt concentrations. Enzyme activity, O; inhibition by CTP, □. Similar results were obtained with NaCl alone. Reprinted with permission from V. Liebl et al. (1969), Can. J. Biochem.* **47**: *1095 (ref. 85). (b) Isocitrate dehydrogenase from H. salinarium in NaCl or KCl solutions. Apparent V_{max} in NaCl; isocitrate concentration varying, O; apparent V_{max} in NaCl; NADPH concentrations varying, Δ; apparent V_{max} in KCl, NADPH varying, ■; apparent V_{max} in KCl, isocitrate varying, ▲. Reprinted with permission from D. M. Aitken et al. (1970), Biochem. J.* **116**: *125 (ref. 3). (c) Fatty acid synthetase from H. cutirubrum in NaCl or KCl. Solutions of NaCl, O; solutions of KCl, ●. Reprinted with permission from E. L. Pugh et al. (1971), Can. J. Biochem.* **49**: *953 (ref. 100).*

ties of isocitrate dehydrogenase (62, 63) and threonine deaminase (84) of *H. cutirubrum* and of malic dehydrogenase of *H. salinarium* (58–60) are optimal at an intermediate NaCl concentration, but the stabilities are enhanced by higher salt concentrations, up to saturation. As Hubbard and Miller have shown (61), the most stable form of isocitrate dehydrogenase, that obtained in 4 M salt, has little activity. It may be assumed that the polypeptide chain in these enzymes is capable of a reversible, tighter folding at the higher salt concentrations, which increases the activation energy of unfolding. Unlike the case of menadione reductase, lowering the NaCl concentration did not result in inactive states for these enzymes, but under these conditions the structures were unstable, and both isocitrate dehydrogenase (62) and malic dehydrogenase (58, 60) were inactivated in a time-dependent manner. Both of these enzymes, as well as several others (58), could be reactivated by dialysis against high concentrations of NaCl, although not by directly adding salt. The inactive form of these enzymes, obtained after incubation at low-salt concentrations, is probably extensively unfolded. Holmes and Halvorson found (60) that upon inactivation the sedimentation constant of malic dehydrogenase decreased from $s_{20,w}$ = 9.5 to 2.4, whereas the corresponding values of isocitrate dehydrogenase were $s_{20,w}$ = 5.3 and 2.0 (63). Such decreases in sedimentation velocity are consistent with the results obtained by Tanford and co-workers (119, 120) on exposing various proteins to guanidinium hydrochloride, a treatment generally thought to result in extensive unfolding of the polypeptide chains. Because of these results, as well as the finding that elution of inactivated isocitrate dehydrogenase from Sephadex columns indicated higher Stokes radii than for the native species (62), Hubbard and Miller concluded that the inactivation process caused an expansion of the molecule to a less folded state. Circular dichroism measurements indicated (126) that the helix content of the enzyme decreased from about 35% to near zero under these conditions and that it returned partially upon reactivation. Reactivation was apparently affected by the fate of sulfhydryl groups exposed during unfolding. Holmes and Halvorson found (60) that after reaction with *N*-ethyl maleimide in the denaturated state no reactivation could be obtained. The reactivation of isocitrate dehydrogenase was complete only when the inactivated form was protected with dithiothreitol (63). The recovery of full enzyme activity, and apparently the native form of the enzyme, from such unfolded conformations is a surprising result and may provide a useful system for studying the mechanism and thermodynamics of the folding of polypeptide chains (4).

Cooperative or allosteric effects have been observed in a number of halophilic systems (3, 37, 84, 85, 96), but the influence of salt on the regulatory properties of these enzymes has not been extensively investigated. Liebl et al. (85) found that the inhibition of aspartate transcarbamylase from *H. cutirubrum* by cytidine 5′-triphosphate reached maximal value only at 4 M salt, a concentration similar to that required for enzyme activity (Fig. 1a). Although the low-salt inactivation of threonine deaminase from *H. cutirubrum* showed some dependence on protein concentration (84), implying dissociation of this tetrameric protein, upon lowering the salt concentration neither this enzyme nor aspartate transcarbamylase yielded enzymically active subunits. Such active subunits of aspartate transcarbamylase could be obtained after polyethylene glycol treatment however (96). A salt-dependent effect on subunit interaction was observed by Louis and Fitt (88) in DNA-dependent RNA polymerase from *H. cutirubrum*, where the β subunit was seen to be preferentially inactivated in the absence of salt and was then unable to form an active complex with the more stable alpha subunit.

The suggestion that the salt-dependent properties of halophilic enzymes are the consequence of the need to screen negative charges (5) received support from results of Hochstein and Dalton (54, 55), Lanyi (76), Lieberman and Lanyi (83), Cazzulo (25), and Norberg et al. (96), who found that the high concentrations of monovalent cations, required for enzyme activity and stability in various systems, could be replaced with lower concentrations of divalent cations, such as Mg^{2+} or Ca^{2+} or polyvalent cations, such as spermine. Because these agents possess much greater charge densities than do monovalent cations, they may be expected to participate more readily in ionic interactions required to reduce the electrostatic-free energy of charged proteins. The above results are consistent with this kind of stabilization in halophilic proteins. However, for cytochrome oxidase (83) and menadione reductase (76) of *H. cutirubrum* polyvalent cations were only partially effective, the latter enzyme showing increased Michaelis constant (K_m) for menadione in the presence of Mg^{2+}, Ca^{2+}, or spermine, without added NaCl.

As discussed in the beginning of this section, the possibility of electrostatic screening in a macromolecule is dependent on its permeability to solvent, that is, the closeness of folding. Naturally, the degree of folding is a variable

property among proteins. Charges on macromolecules which are freely permeable to the solvent are effectively shielded at a monovalent ion concentration near 0.2 M. Such a situation is obtained for DNA (64) and polyglutamic acid (125). Solvent-impermeable structures, on the other hand, are not completely shielded even at high counter-ion concentrations. In fact, it might be expected (as discussed in a subsequent section) that at very high salt concentrations the volume of proteins decreases and the polypeptide chains collapse into a tighter conformation, allowing for *decreased* charge screening. The stability of *H. cutirubrum* aspartate transcarbamylase was indeed found to be further enhanced by 0.1 M $MgSO_4$ in the presence of 4 M NaCl (96). The requirement for *very high* salt concentration in halophilic systems, thus, probably does not originate from the shielding of charges. Additional difficulties with the charge-screening hypothesis are the specificities with respect to anions, found with several halophilic enzymes (5, 54, 61, 79), and the finding that the alpha-helical structure of polyglutamic acid, a molecule obviously dependent on charge effects, is unstabilized at high concentrations of NaCl or KCl (35). The analogy with polyglutamic acid is thus unworkable, and it appears that halophilic proteins cannot be represented simply as highly charged polypeptides.

For these reasons it is clear that even though long-range electrostatic forces must be important components of the interaction of salts and halophilic enzymes, the unusually high salt requirement for preserving the integrity of these structures is not fully explained by such effects.

RESIDUE INTERACTIONS

Specific residue interactions in macromolecules include what might be termed anomalous titration effects due to the influence of the overall structure of the molecule on the behavior of individual residues (16, 17). In these cases side groups of polypeptide chains, for example, may show different electrochemical free energy (pK, dipole moment, ion binding, etc.) or chemical reactivity in the folded and unfolded states. An increase in the free energy of such groups upon unfolding contributes to the stability of the macromolecule. Hydrogen bonds are thought to belong in this category, but binding of ions may be also involved in such specific residue interactions. The tendency of the alkali earth metal ions to participate in coordinate complexes allows them to stabilize structures where potential ligands are brought into close proximity (4). Such complexes may be formed with monovalent ions as well, however. Saroff (104) suggested that Na^+ binding of beta-lactoglobulin and myosin takes place in a pocket involving a basic group, a carboxylate and two carboxyl groups, forming a 4-coordinate complex. The stoichiometry of Na^+ binding by pepsin and pepsinogen (36) supports this hypothesis. Another example of complex formation by monovalent ions is the behavior of polyvinylbutylpyridinium bromide at high ionic strengths. Fuoss and co-workers proposed (44, 46) that the decrease in the size of molecule observed was due to the formation of $-N^+-Br^--N^+$ bridges. Insofar as hydrogen bonds arise from charge interaction, their stability is reduced at high salt concentrations. In contrast, if the possibility of direct participation by ions in residue interactions exists, such interactions are favored at increased concentrations of the salt.

Specific residue interactions are easiest to separate from gross structural phenomena when they occur near the active site of the enzyme. The effect of salt on the binding constant of substrates has been investigated by Aitken et al. (3) for *H. salinarium* isocitrate dehydrogenase. In contrast to menadione reductase, whose K_m for the substrate was independent of NaCl concentration (76), Aitken et al. found that the binding of isocitrate in the *H. salinarium* dehydrogenase showed a maximum at 0.75 M NaCl or at 1.5 M KCl, whereas nicotinamide adenine dinucleotide phosphate (NADPH) binding decreased continually with salt concentration. Similarly, according to Baxter (5), the K_m for the substrate increased with salt concentration for the lactic dehydrogenase of *H. salinarium*, even though the maximal rate (V_{max}) increased under these conditions. In these systems the binding of substrates is affected by the salt concentration. Conversely, the salt response of other halophilic enzymes was found to be influenced by the binding of substrates. The spontaneous inactivation of isocitrate dehydrogenase (62, 63), aspartate transcarbamylase (96), and threonine deaminase (84) of *H. cutirubrum*, NADH dehydrogenase from AR-1 (55), and malic dehydrogenase of *H. salinarium* (60) at low salt concentrations appeared to be greatly reduced when one of the substrates was present. This kind of protective effect is probably confined to the immediate vicinity of the active site, because the reactivation of isocitrate dehydrogenase (62, 63) and malic dehydrogenase (60) could not be brought about by substrates, but required high concentrations of NaCl once extensive unfolding upon prolonged exposure to low salt concentrations

had taken place. The specificity of the substrate protection is demonstrated by the finding of Holmes and Halvorson (60), that the half-life of malic dehydrogenase in 0.07 M NaCl increased from a fraction of a minute to 48 h in the presence of 0.9 mM NADH, whereas malate, oxaloacetate, nicotinamide adenine dinucleotide, NADPH, or nicotinamide were virtually completely ineffective, even at higher concentrations. Similarly, Hubbard and Miller found (62) that *H. cutirubrum* isocitrate dehydrogenase was protected only by a combination of isocitrate and $MnCl_2$.

The importance of *local* interactions in the overall salt response of the enzyme was emphasized by Lieberman and Lanyi (84). These workers found that the binding of the allosteric effector, adenosine 5'-diphosphate (ADP), which caused a shift from sigmoid to hyperbolic substrate kinetics in *H. cutirubrum* threonine deaminase, changed the salt dependence of enzyme activity (at saturating threonine concentrations) from mild enhancement at 2 to 3 M NaCl to extensive inhibition even at low NaCl concentrations. Because it appears that subtle conformational changes, involving the interactions responsible for cooperative substrate binding, are sufficient to alter the overall effect of salt on this enzyme, it is unlikely that more than a few residues are responsible for the salt-dependent behavior.

Louis and Fitt found (86, 88, 89, 91) that the salt response of *H. cutirubrum* DNA-dependent RNA polymerase was influenced by the source of the template DNA supplied. Although the enzyme was active in the presence of all templates tested except T_4 phage DNA, bacterial and phage DNA (from *H. cutirubrum*, *Bacillus subtilis*, *E. coli*, and T_7 coliphage) were effective only above 2 M NaCl or KCl, calf thymus and salmon sperm DNA were effective only at very low salt concentrations, and the activity with poly [d(A-T)] was unchanged by salt (89). Some of these results are reproduced in Fig. 3. The *H. cutirubrum* enzyme is a dimer, containing a single alpha and beta subunit (88, 89, 91). Louis and Fitt had shown that the beta subunit is responsible for chain initiation and that in a primed reaction, catalyzed by the alpha subunit, the template-dependent salt effects are no longer observed (91). The template specificity of this enzyme probably resides in a site which recognizes short nucleotide sequences on the DNA, preferred for chain initiation. If this is the case, a possible explanation for the above-described effects is that the beta subunit carries two or more initiation sites with different salt-dependent properties. However, the *H. cutirubrum* enzyme, unlike that from *E. coli*, is very small; the molecular weight of the subunits is approximately 18,000 (86). It is probable, therefore, that there is only one site, and the effect of salt is to cause a conformational change in the protein, resulting in altered reading of the nucleotide sequences.

Chazan and Bayley (27) reported a DNA-dependent RNA polymerase in *H. cutirubrum* with entirely different properties from that studied by Louis and Fitt (86, 88, 89, 91), having higher molecular weight (300,000 to 400,000), being insensitive to rifampin and lacking the salt-dependent template specificity. The activity of this enzyme reached maximal value near 0.2 M KCl or NH_4Cl and, particularly in the purified state, declined considerably above this concentration. The relationship of the two enzymes, apparently from the same organism, is not known.

On thermodynamic grounds, charged residues are expected to be on the surface of the proteins, in contact with the solvent. The active sites of most enzymes are influenced by these charges (65), as well as any charges on the substrates, and the pH optima for K_m and V_{max} reflect the ionization constants of the groups involved. In the presence of high concentrations of salt fully exposed charges are well screened. Indeed, although the enzyme activities of malic dehydrogenase (60) and isocitrate dehydrogenase (3) from *H. salinarium*, of amylase from *H. halobium* (50), as well as of cytochrome oxidase of *H. cutirubrum* (83), exhibit sharp pH dependence at low salt concentrations, high concentrations (2 to 4 M) of NaCl or KCl cause the

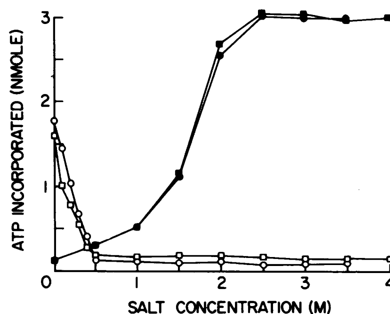

FIG. 3. *Salt dependence of DNA-dependent RNA polymerase from H. cutirubrum assayed with template DNA from different sources. Template, calf thymus DNA, ○ and □; template, H. cutirubrum DNA, ● and ■. Solutions of KCl, ○ and ●; solutions of NaCl, □ and ■. Reprinted with permission from B. G. Louis and P. S. Fitt (1972), Biochem. J.* **127**:*69 (ref. 89).*

pH curves to flatten. Other halophilic enzymes, however, show narrow pH optima even at high salt concentrations. Among these are the aspartate transcarbamylase (85), polynucleotide phosphorylase (99) and citrate synthase (25) of *H. cutirubrum*, ornithine carbamoyltransferase of *H. salinarium* (37), and glycerol dehydrogenase of *Ps. salinaria* (6). It is clear that in these cases charge-dependent effects prevail despite the presence of overwhelming concentrations of counter-ions. Interestingly, Lieberman and Lanyi found (84) that in the case of threonine deaminase of *H. cutirubrum* the effect of salt on the shape of the pH curves was reversed when the allosteric effector, ADP, was added. As shown in Fig. 4, in the absence of ADP, increasing the NaCl concentration from 0.05 to 4 M caused a narrowing of the pH optimum of enzyme activity. In the presence of ADP, however, when the cooperative substrate kinetics were no longer observed, the pH response of the enzyme was broadened in the presence of the salt. It was suggested by these authors that the conformational shift in threonine deaminase, caused by the binding of ADP, involves the exposure of charged groups near the catalytic site, previously shielded from the solvent. Binding of ADP thus reverses the effect of 4 M salt which, in the absence of this effector, causes the protein to be more folded and enhances the charge effects on the active site.

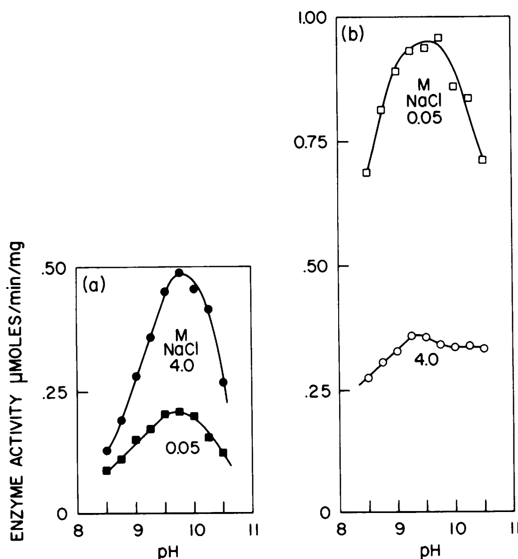

FIG. 4. *pH dependence of H. cutirubrum threonine deaminase. The enzyme was assayed at NaCl concentrations of the indicated molarity in the absence of ADP (a) or in the presence of 1 mM ADP (b). Reprinted with permission from M. M. Lieberman and J. K. Lanyi (1972), Biochemistry* **11**:*211 (ref. 84); copyright by the American Chemical Society.*

It is evident from the studies described above that in some halophilic systems the effect of salt may be restricted to a small region on the protein molecule. The possibility of such local effects, perhaps involving a few residues near the active site, needs to be further explored in more rigorous studies on purified halophilic enzymes.

CATION SPECIFICITY

Binding of cations by macromolecules implies the existence of binding sites and some degree of selectivity. Until recently it had been thought that the specificity of binding observed in many biological systems was necessarily due to the presence of several ligands which, in a cooperative manner, define a cavity of unique geometry, and only such steric effects could lead to a preference toward a cation of a certain radius (40, 115). The solvation of alkali metal ions by single non-cooperative ligands, such as exemplified by alkyl-substituted formamides as model compounds, is indeed characterized by a lack of specificity, showing a featureless increase in binding energy with decreasing ionic radius (106, 107). However, Eisenman and Krasne (39) have pointed out the need to consider the presence of water, because the affinity of a cation to its binding site depends on the *net* difference in free energy obtained upon removing the ion from its hydration shell and placing it near the site of binding. For many simple ligands capable of binding cations by electrostatic forces, such as the carbonyl groups of formamide in an aqueous environment, this *net* free energy difference is not a monotonous function of the ionic radii, but shows a peak near the value for K^+. Such deviations from linear behavior result in a preference for K^+ (or for cations of different radii in other systems), which can amount to 2 kcal/mol of enthalpy. Calculated values of net free energy of binding, based on simple coulombic considerations, agree well with the experimental results (39).

Thus, if the analogy between the formamide and peptide groups is made, it appears that the binding of alkali metal ions by simple sites, such as the polypeptide backbone carbonyls, can account, under certain conditions, for a several-fold preference toward a given ion. The selectivity observed with cation exchange resins of the carboxyl and phosphate type for various alkali metal ions (19) substantiates this view.

If the function of an enzyme is affected by the binding of ions, as may be the case for halo-

philic systems, the kinetics of enzyme activity should reflect any preference exhibited by the binding site. It is important to recognize that the selectivity of the enzyme for a given ion can increase over that shown by a single binding site, if there are several sites which influence enzyme activity.

Evans and Sorger (40) and Suelter (115) listed a large number of enzymes for which salt effects and specificities for various monovalent cations have been observed. For most of these systems the salt requirement was relatively slight (<0.1 M), consistent with purely ionic effects and distinct binding sites. Evans and Sorger (40) treated the data in terms of cation-binding sites, analogous to substrate-binding sites, and suggest that the specificities are largely due to differences in the size of the ions. Thus, for many enzymes the effects of K^+, NH_4^+, and Rb^+, which have similar ionic radii, were similar, as distinct from the effects of Na^+ or Li^+.

An assessment of the ionic specificities of halophilic systems is hindered by the fact that many of the reports do not separate stability from enzyme activity at lower salt concentrations. The salt-dependence curves of these enzymes is not complicated by such considerations at the higher salt concentrations, however, and the data cited here refer to salt concentrations above 1 M. Most of the information available concerns the relative effect of KCl over NaCl on enzyme activity, because the intracellular concentration of K^+ is 2 to 3 times that of Na^+ (34, 48, 79) and the salt responses of halophilic enzymes have been expected to reflect this fact. As shown in Table 2, halophilic enzymes exhibit various degrees of specificity, ranging from little or no preference for K^+ over Na^+ to fairly high selectivities. Of other cations, Li^+ was found to be relatively ineffective for some halophilic enzymes (6, 54), but not for others (79). Ammonium ion supported partial enzyme activity in some systems (27, 51, 54).

If the effect of salts on the activity of extremely halophilic enzymes is interpreted in terms of binding sites, as it has been done by several authors (3, 5, 60), the kinetics yield dissociation constants in the neighborhood of molar concentrations of salt and up to 4 to 5 binding sites. Dissociation constants of ion pairs, such as K^+Cl^-, formed by random encounter of a monovalent ion with another, are also in the order of 1 M (45, 68). It is difficult to understand how sites with such low-cation affinities can provide the geometrical requirements for selectivity. It is more likely that, in fact, there are no specific cation-binding sites on these proteins, which are analogous to substrate-binding sites, but rather loci of higher electron densities, which show cation selectivity through the energetics of the coulombic interaction, as described at the beginning of this section.

The envelopes of whole cells of halobacteria are stabilized much better by NaCl or LiCl than by KCl or NH_4Cl (1, 108), whereas isolated envelopes show little selectivity (108). To explain this difference, Kushner and Onishi (73, 98) suggested a differential distribution of binding sites for Na^+ and K^+ on the inside and outside surfaces of the cell envelopes. Soo-Hoo and Brown (108) disputed this view and attributed the cation selectivity of whole cells to differential permeability and osmotic effects. Halobacteria cells appear to be passively permeable to K^+ (47, 48), but a conclusive study of the independent permeabilities of various ions in isolated envelope vesicles has yet to be carried out.

HYDROPHOBIC EFFECTS

When the effective concentration of salt is small, the binding of ions by a macromolecule may be thought to be specific and it may be possible to identify a binding site. However, when larger concentrations of salts are required to affect the proteins, they act in a less specific manner and exert their effects also through changing the structure of the solvent. To consider such solvent-dependent phenomena it is necessary to introduce the concept of the hydrophobic bond (67).

The entropy decrease observed on dissolving nonpolar molecules in water has long been recognized to be due to the ordering of water structure (42, 43). The transfer of nonpolar groups, such as alkyl or phenyl residues, from the interior of a protein to its exterior, with partial exposure to water, may be expected to be analogous to the dissolution of these residues in water and to contribute an unfavorable free-energy term to the overall process of unfolding. It has been, indeed, possible to account for the denaturing or stabilizing effect of various salts on a large number of proteins and other macromolecules on the basis of the "salting-out" or "salting-in" effect of the salts on organic molecules, reflected in the Hofmeister series (65, 124). Such effects are, for the most part, properties of anions only, because these are often large, their change density is low and, consequently, they do not always attract water molecules. The presence of the larger anions,

TABLE 2. K^+/Na^+ *preference of various halophilic proteins*

K^+/Na^+ selectivity[a]	Enzyme	Source	Ref
<2 (low)	Malic dehydrogenase	*Pseudomonas salinaria*	7
	Lactic dehydrogenase	*P. salinaria*	7
	Cytochrome oxidase	*P. salinaria*	7
	Isocitrate dehydrogenase	*Halobacterium salinarium*	2,3
	Fumarate hydratase	*H. salinarium*	2
	Malate dehydrogenase	*H. salinarium*	2
	Aconitate hydratase	*H. salinarium*	2
	Glutamate dehydrogenase	*H. salinarium*	2
	Glucose-6-PO_4 dehydrogenase	*H. salinarium*	2
	Lactic dehydrogenase	*H. salinarium*	5
	Ornithine carbamoyltransferase	*H. salinarium*	37
	Polynucleotide phosphorylase	*Halobacterium cutirubrum*	99
	Cytochrome oxidase	*H. cutirubrum*	32
	Fatty acid synthetase	*H. cutirubrum*	100
	DNA-dependent RNA polymerase	*H. cutirubrum*	89
	Menadione reductase	*H. cutirubrum*	79
	NADH oxidase	AR-1	54
~2 (medium)	Glycerol dehydrogenase	*P. salinarium*	6
	Succinic dehydrogenase	*P. salinarium*	7
	α-Ketoglutarate dehydrogenase	*H. salinarium*	2
	Citrate synthetase	*H. salinarium*	2
	Succinyl-CoA synthetase	*H. salinarium*	2
	Isocitrate lyase	*H. salinarium*	2
≥5 (high)	Isocitrate dehydrogenase	*P. salinaria*	7
	Cysteine desulphydrase	*P. salinaria*	8
	Succinic dehydrogenase	*H. salinarium*	2
	Glutamate uptake system[b]	*H. salinarium*	112
	Aminoacyl transfer RNA synthetase	*H. cutirubrum*	51
	Ribosomal proteins	*H. cutirubrum*	11

[a] Ratio of enzyme activities or stability in the presence of KCl and NaCl.
[b] Selectivity in favor of Na^+.

such as ClO_4^- and SCN^-, and to a lesser degree NO_3^-, interrupts the formation, dissociation, and reformation of water aggregates (43, 94), resulting in lowered mobility for water molecules, and thus increased cluster size. In the presence of these anions, introducing a nonpolar molecule requires less energy expenditure. The dissolution of nonpolar molecules in water is also encouraged by lowering the temperature, because thermal motion is reduced and the size of the water clusters is again increased. Hydrophobic bonds, which arise because of the low solubility of nonionic residues, in water, are thus destabilized under these conditions. Conversely, the presence of salting-out-type salts, such as those of Cl^-, $HPO_4^=$, $SO_4^=$, and increasing temperatures (up to 50 to 60 C) encourage the formation of hydrophobic interactions.

Jencks and co-workers (93, 102, 103, 111) and others (12, 105) have disputed the view that salts and other agents act on proteins only through their effect on the structure of water. Recently, in a series of publications (52, 123), von Hippel and collaborators presented evidence for another mechanism of action, in which salts are assumed to bind directly to the components of proteins, e.g., to the polypeptide backbone, while exerting their effects on hydrophobic interactions through the ordering of water molecules in the vicinity of the binding site.

Many enzymes (65, 124), including those of extremely halophilic bacteria (80, 83, 84), are denatured by high concentrations of salting-in-type salts, suggesting the involvement of hydrophobic forces in their structure. In view of the presence of nonpolar residues in all proteins, this result is not surprising. However, the dena-

turing effect of such salts *combined* with the stabilizing effect of salting-out-type salts on some halophilic enzymes led Lanyi and coworkers (80, 83, 84) to suspect that in these enzymes hydrophobic forces may have a more dominant role than is usually the case. This possibility was examined in detail for menadione reductase of *H. cutirubrum*. As discussed in a previous section, this enzyme shows an absolute requirement for NaCl or KCl, both enzyme activity and stability reaching maximal values above 2 M (76). Lanyi and Stevenson (80) described the effect of various protein denaturants, such as urea, formamide, and their alkyl derivatives, on the activity of the enzyme. This information was compared with solubility data, obtained by Robinson and Jencks (102, 103) in the presence of these agents, of the model compounds toluene and acetyltetraglycine ethyl ester (ATGEE). It was found that greater inhibition of menadione reductase enzyme activity was obtained with those agents which caused an increase in the solubility of toluene, in contrast with other enzymes (93, 102, 103) which showed such correlation with the solubility of ATGEE, a model for the polypeptide backbone. These authors concluded that the halophilic enzyme is dependent, to a considerable degree, on the interaction of nonpolar residues (resembling toluene) for enzyme activity. Similar conclusions were reached from studies of the thermodynamics of the inactivation of the enzyme at various ionic strengths. Arrhenius plots of the apparent first-order rate constants of inactivation in 3 M NaCl indicated that at 23 C the enzyme exhibited maximal stability, and increased inactivation was obtained both above and *below* this temperature, showing "cold sensitivity" (16). The spontaneous inactivation of the enzyme was, as mentioned in an earlier section, much more rapid in the absence of salt than at high concentrations of NaCl. Without added salt, however, the biphasic Arrhenius plot was not obtained. It was suggested, therefore, that the protective effect of high NaCl concentrations consists of encouraging the formation of new hydrophobic bonds which, under these conditions, become the principal barriers to unfolding. Brandts (16, 17) considered the temperature of maximal stability to be indicative of the extent of hydrophobic stabilization of proteins. Most proteins show stability maxima below 10 C, and some below 0 C. The proximity of the value for halophilic menadione reductase to Brandt's suggested maximal figure of 25 to 30 C indicates that at high NaCl concentrations this enzyme is rich in such interactions. Analysis of the slopes of the Arrhenius plots under these conditions yielded very large negative enthalpies and entropies of inactivation, consistent with the breaking of 15 to 20 hydrophobic bonds.

On the basis of the above evidence Lanyi and Stevenson (79) suggested that the effect of salt on menadione reductase is divided into two components: (i) charge screening at low salt concentrations (<0.5 M), which could also be accomplished by lower concentrations of multivalent cations, and (ii) stabilization of hydrophobic interactions at higher salt concentrations. In order to account for these effects, as well as the inactivation kinetics observed, a scheme was proposed, which suggested that the enzyme can exist in various states of folding (Fig. 5). Physical evidence for the presence of these states has yet to be obtained, however.

The two proposed effects of salt, charge screening and salting-out, could be separated into distinct components for the cytochrome oxidase of *H. cutirubrum* (83). The dependence of this enzyme's activity on salt concentration showed two plateaus (Fig. 6), the first reaching completion at 0.6 M, the second near 4 M NaCl. The first increase of enzyme activity, apparently brought about by charge screening, was cation-nonspecific and could be obtained also upon addition of $MgCl_2$ or spermine. The second, larger, component of enzyme activity, on the other hand, showed a marked preference toward salting-out-type salts. Also, this portion of enzyme activity was found to be particularly sensitive to denaturation by short-chain alcohols. Thus, it appeared that in cytochrome oxidase a larger part of the enzyme activity was due to folding dependent on hydrophobic interactions. Despite the known complexity of the *H. cutirubrum* cytochrome oxidase (28, 29), spectroscopic studies failed to reveal any selective effects, at low or high salt concentration, on the different heme groups present.

The suggested effect of salts on hydrophobic interactions is consistent with previously observed anion effects. For example, Baxter (5) found that lactic dehydrogenase from *H. salinarium* showed a preference for anions in the following order: $Cl^- > Br^- > NO_3^-$. Similarly, Mochstein et al. and Dalton (54) reported that the enzyme activity of the NADH oxidase system from a halophile was supported by Cl^- and $SO_4^=$ but not by Br^-, NO_3^-, or I^-. In the latter case, $MgCl_2$ was found to be inhibitory at higher concentrations, whereas $MgSO_4$ was not, as might be expected on the basis of the salting-in properties of these Mg salts. The attachment of some membrane proteins, particularly the fla-

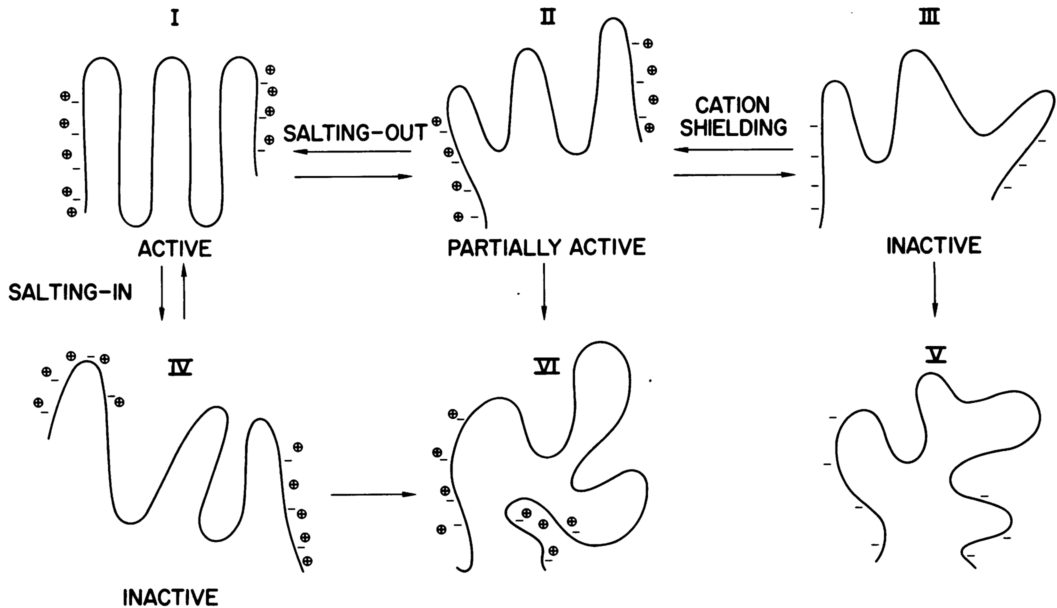

FIG. 5. *Schematic representation of the proposed effect of salts on H. cutirubrum menadione reductase (on the basis of data in ref. 77). The enzyme is assumed to be optimally folded in state I, which is in equilibrium with a partially active, unstable species, conformation II. The latter shows increased K_m for menadione. State II is in equilibrium with an inactive form, state III, which is more labile. Low concentrations of monovalent and particularly divalent cations encourage the III → II transition, a result of charge screening, whereas high concentrations of salting-out-type salts, such as NaCl, encourage the II → I transition, which involves the formation of hydrophobic bonds. Adding salting-out-type salts, such as NaBr or $NaNO_3$, caused inactivation without a change in K_m, hence a state IV was proposed, which contains screened charges and unstabilized hydrophobic interactions.*

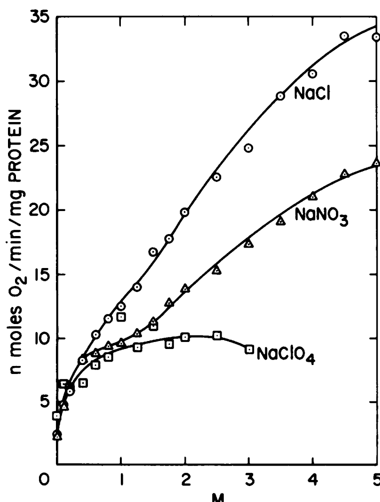

FIG. 6. *Salt dependence and anion specificity of cytochrome oxidase from H. cutirubrum. Reprinted with permission from M. M. Lieberman and J. K. Lanyi (1971), Biochim. Biophys. Acta* **245**:*21 (ref. 83).*

voproteins, to the cell envelopes of *H. cutirubrum* was also shown to be influenced by the salting-out character of the salts added (77). The general significance of the salting-out effect on halophilic enzymes has not yet been fully explored, however. Perhaps it is significant that cold sensitivity was observed in halophilic threonine deaminase (84) and aspartate transcarbamylase (96) as well. Interestingly, the free energy change of inactivation for menadione reductase (79) and threonine deaminase (84) was raised by the same amount, 3.5 kcal/mol, on addition of 3 M NaCl.

An important characteristic of some halophilic enzymes is thus shown to be an inability to maintain their structure without the critical involvement of hydrophobic interactions that are ordinarily not supported by water. The requirement for salt may be to overcome unfavorable steric conditions which prevent the close packing of the non-polar groups. Alternatively, the need for salt may reflect the lack of an adequate number of nonpolar residues and the requirement to increase the hydrophobic character, through the salting-out effect, of borderline nonpolar residues, such as the side chains of serine and threonine, and of the polypeptide backbone. Table 1 contains indices of hydrophobicity and polarity for various halophilic and nonhalophilic proteins. The data

clearly show that halophilic proteins, particularly those of the cell envelope which are responsive to salt are, on the average, poorer in residues capable of hydrophobic interactions and richer in polar groups. This deficit is evidently a consequence of the fact that in these proteins the number of acidic groups is increased, at least partially, at the expense of the nonpolar residues. The structural implications of such a nonpolar residue deficit is that the polypeptide chain in the interior of the halophilic proteins may not fold tightly enough to bring about the conformation required for the functional state of the molecule. The participation of the borderline nonpolar residues in hydrophobic interactions, encouraged at high salt concentration, may thus help these enzymes assume their optimal conformation. This conformation is, naturally, different for different proteins. Given a sufficiently large collection of proteins, however, it might be expected that some statistical correlations in the amino acid compositions can be observed. Fig. 7 shows the molar percentage of excess acidic amino acids, of strongly hydrophobic amino acids (valine, leucine, isoleucine, and phenylalanine), or borderline hydrophobic amino acids (serine and threonine), and of amino acids with short side chains (glycine and alanine), determined for 14 proteins in the S-1 fraction of *H. cutirubrum* 50s ribosomal particles (53, 122). As indicated in Table 1, these proteins are dependent on high KCl concentrations for attachment to the ribosome. Also shown in Fig. 7 are the same kind of data for 26 proteins of the *E. coli* 50*S* ribosomes (66, 114). The acidity of the *H. cutirubrum* ribosomal proteins analyzed is evident from Fig. 7. A more subtle effect is that the lowered frequency of hydrophobic amino acids in the halophilic system appears to be counterbalanced by a higher average frequency of borderline hydrophobic residues. Means and standard deviations were calculated for the amino acid mole fractions in Fig. 7 for the two populations of proteins. Applying *t*-test statistics, the probabilities of the means being the same for the proteins of *H. cutirubrum* and *E. coli* ribosomes were found to be <1% for the strongly hydrophobic amino acids, <5% for the weakly hydrophobic amino acids, and 60 to 70% for the short side-chain amino acids.

The case for the involvement of weakly hydrophobic residues in the structure of halophilic proteins is perhaps best supported by the amino acid analyses of Louis and Fitt of the *H. cutirubrum* DNA-dependent and RNA-dependent RNA polymerases (89, 90). The data, reproduced in Table 3, show that these enzymes are not unusually acidic, but contain very low amounts of hydrophobic residues, as compared

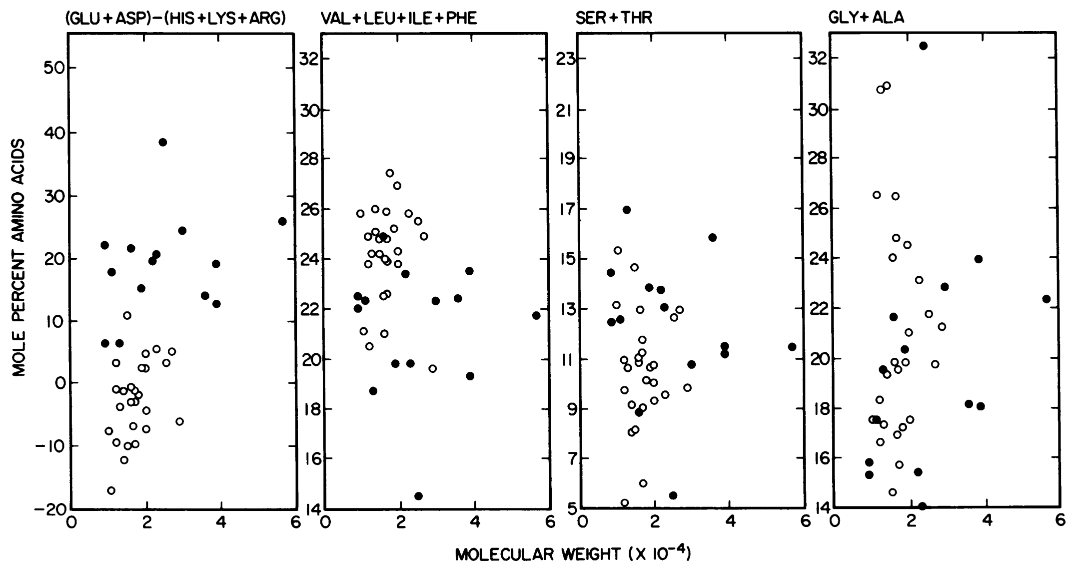

FIG. 7. *Composition of 14 H. cutirubrum ribosomal proteins (fraction S-1 in ref. 53, closed circles) and 26 E. coli ribosomal proteins (from 50S particle in ref. 66 and 114, open circles) arranged in selected groups of amino acids and plotted as a function of molecular weight. From left to right: excess acidic amino acids, glutamic and aspartic acid minus histidine, lysine and arginine; strongly hydrophobic amino acids, valine, leucine, isoleucine, and phenylalanine; borderline hydrophobic amino acids, serine and threonine; short side chain amino acids, glycine and alanine.*

TABLE 3. *Amino acid analyses of nucleic acid polymerases from H. cutirubrum and E. coli*[a]

Amino acid	*H. cutirubrum* RNA-dependent RNA polymerase	*H. cutirubrum* DNA-dependent RNA polymerase		*E. coli* DNA-dependent RNA polymerase
		α subunit	β subunit	
Asp	8.2[b]	7.0	6.3	10.2
Thr	6.7	5.3	5.0	5.3
Ser	6.9	15.9	16:1	6.7
Glu	11.0	14.2	13.4	13.6
Pro	3.7	4.2	4.1	3.4
Gly	17.6	15.6	17.8	7.4
Ala	9.9	8.0	8.2	8.8
Val	4.5	3.6	3.6	6.7
Met	0.8	0.6	0.5	2.9
Ile	2.5	1.8	2.2	5.6
Leu	4.6	3.1	4.0	10.9
Tyr	2.0	1.8	1.9	2.6
Phe	2.1	1.4	1.8	2.8
Lys	12.0	11.1	9.0	5.4
His	3.8	3.1	3.4	1.6
Arg	3.6	3.2	2.7	5.8

[a] Reprinted with permission from Louis and Fitt (1972), Biochem. J. **127**:69 and **128**:755 (ref. 89 and 90).

[b] Mole %.

with the *E. coli* enzyme. In the subunits of the DNA-dependent enzyme, but not in the RNA-dependent enzyme, the hydrophobic deficit is accompanied by the presence of unusually large amounts of serine. Considering that the activity of the DNA-dependent enzyme is absolutely dependent (89) on 2 M NaCl or KCl (for bacterial and phage DNA templates), whereas the RNA-dependent enzyme is little affected by salt, the salt-induced participation of serine in the structure of the salt-responsive enzyme is a possibility.

CONCLUSIONS

The requirement of unusually high concentrations of NaCl or KCl for maintaining the optimally folded structure of halophilic proteins raises the possibility that these macromolecules are put together according to different rules that are nonhalophilic systems. This is probably not the case. It is necessary merely to assume that some of the interactions, which are ordinarily involved in stabilizing protein structure, are weaker in halophilic systems, whereas others are stronger because of the presence of salt. The complexity of protein structure precludes any single explanation for the salt dependencies observed. Some generalities have begun to emerge, however. Electrostatic shielding of charged groups probably predominates at salt concentrations below 0.5 M. Such shielding decreases the stabilities of charge interactions and hydrogen bonding. Nevertheless, owing to the high negative charge densities of the halophilic proteins, the overall effect of salt is structural stabilization. At higher concentrations of salt, new hydrophobic interactions are formed, which had insufficient stability in water, and the molecule assumes a more tightly folded conformation. Finally, local residues appear to be influenced by salt, both through changes in the overall structure and through direct effects on the residues. The relative importance of these effects appears to be different for the various halophilic proteins studied.

The rationale for investigating halophilic systems is that they can be expected to contain identifiable structural alterations, adapted to a drastically different environment, yet they are capable of carrying out the same functions as nonhalophiles. Comparative studies of halophiles and nonhalophiles, therefore, may help to uncover the details of structure and function in proteins and to understand their responses to external perturbation.

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