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MECHANISM OF SOLUTE EXCLUSIOS FROM CELLS: THE ROLE OF PROTEIN-WATER IXTERACTION

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• *Studies of native and denatured proteins and rwo synthetic polymers, polyvinylpyrrolidone and poly(ethylene oxide), indicate that arrays of fully extended protein chains with their NH and CO groups directly exposed to bulk water reduce the solubility of Na⁻, sucrose, and glycine within that water. These findings suggest that the exclusions of Na⁻, sucrose, and amino acids seen in living cells have a common mechanism—the dynamic ordering of water by certain extended intracellular protein chains.*

INTRODUCTION

A fundamental requirement of life is that its basic unit, the living cell, be separate irom yet maintain a finely tuned continuity with its environment. This continuity is shown by the presence within the cells of ions, sugars, and other essential ingredients of life derived from the external medium but in some way held internally to lower or higher levels than those in that medium.

To explain the low levels of permeant solutes like Na– and the high levels of other solutes like K^+ in living cells, proponents of the membrane theory postulated specific energy-consuming pumps.[1,2] In contrast, the association-induction hypothesis (AI hppothesis) attributes the high level of K^+ to specific adsorption on β- and y-carboxyl groups carried on cell proteins and the low levels of Na^+ and other solutes to a single cause; i.e., reduced solubility within cell water in a state of polarized multilay-

Many believe that overwhelming evidence exists in support of the pump hypothesis. They cite, for example, the observation that Na/K-activated ATPase, when incorporated into phospholipid vesicles and supplied with ATP as an energy source, can apparently transport K^+ and Na^+ in opposite directions across the vesicular membrane and against concentration gradients[7-9] (for critique, see ref. 10). Others, however, believe there is evidence decisively in support of the AI hypothesis[11-15] (for critique, see refs. 16, 17).

One way to help resolve these conflicting views is to carry out stringent tests of the so-basic postulations of each. For example, according to the AI hypothesis, cell proteins maintain the polarized multilayered state of cell water, and in this state water has reduced solubility for Na^+, sugars, and free amino acids. The question then arising is: Can proteins *outside* living cells create such major changes in solvent properties of *bulk phase* water?

In the present report, we present results of experimental efforts designed to answer that question.

The AI hypothesis assumes that virtually all of the cell water is polarized in multilayers primarily by the exposed NH and CO groups of certain as yet unidentified intracellular proteins. In this state, water has decreasing solubility for hydrated ioas and molecules of increasing size and complexity on both an enthalpic and an entropic basis.[5,6,18] The concept differs from that of

"bound water" in that water molecules in the state of polarized multilayers are quite free to exchange or evaporate; it also differs from the concept of "non-solvent water" since no water in the system is considered to be categorically non-solvent to all solutes.

It is further assumed under the AI hypothesis that to exercise long-range effects on the physical state of water, proteins must be extended, with the alternating sequence of positively charged NH groups and negatively charged CO groups directly exposed to that water. In such conformation, the protein chain constitutes part of a regular matrix of parallel extended protein chains between which most cell water is found. H-bonding of the NHCO groups either to the protein's own CONH groups (e.g., a helix) or to CONH groups of other proteins (e.g., interchain H bonds) annuls their abilities to polarize water in multilayers. Therefore, most native proteins with their CONH groups locked in α-helical and other intra- and intermolecular H bonds would have little or no long-range effect on water structure.

EXPERIMENTAL AND RESULTS

Native Globular Proteins Have No Effect, but Gelatin and Two Synthetic Polymers Do Have Effect, on Water Solvency

To test the foregoing theoretical deductions. we filled ¼-inch dialysis tubing with solutions of proteins (or melted gel) at concentrations similar to those found in cells (15 to 25%). The sacs were tied at both ends and incubated at 25°C in 1.5 M Na_2SO_4 solution containing radioactive ^{22}Na for from 2 to 3 days. a period of time much longer than that required to reach equilibrium of Na_2SO_4 between the inside and the outside of the sac (40 to 60 min). The equilibrium Na^+ concentration in the water within the sac was then assayed, and the result was divided by the Na^+ concentration value for the external solution. This ratio, called the *apparent* equilibrium distribution coefficient, or ρ-value, is to be distinguished from the q-value, or the *true* equilibrium distribution coefficient, which refers only to solute dissolved in the water within the sac.[5,6,18] If any part of the solute is adsorbed to the protein, the ρ-value will be larger than the q-value.

Table I (A) shows that all 13 globular proteins studied exhibit ρ-values of Na^+ equal or close to unity, indicating that under this particular set of conditions there was little or no water with Na^+ solubility different from that of normal liquid water. Table I (B) shows, in sharp contrast, the ability of water in the gelatin-water system to exclude Na^+; the ρ-value for Na^+ was only 0.537 ± 0.013 in a total of 37 determinations in 8 separate experiments on 4 varieties of commercial gelatins. (Indeed, the ability of gelatin to exclude solutes has been known since the work of Hollemann, Bungenberg de Jong, and Moddermann in 1934.[19] The phenomenon was cited by Troschin in his theory of solute exclusion on the basis of the existence of gelatin and living cells in the state of colloidal coacervates.[20])

Why is gelatin an exception in relation to the other proteins? It is well known that repeating sequences of glycine-proline-hydroxyproline in gelatin prevent it from assuming the a-helical conformation. Also, the denaturation process used in preparing gelatin disrupts a major portion of the chain-to-chain H bonds present in native collagen.[21] As a result, in a gelatin-water system portions of the polypeptide chains exist in an extended conformation with exposed NHCO groups, thereby satisfying the requirement of the AI hypothesis for long-range effects on water structure.

While the data of Table I (A and B) agree with the theoretical expectations, one may argue that gelatin differs from the 13 globular proteins in that it alone exists as a

vency for Na_2SO_4; the ρ-value for Na^+ at 25°C is only 0.239 ± 0.005. Since PVP does not contain proton-donating groups, these findings establish that the properly spaced oxygen atoms are the primary sites of water interaction, in full accord with the conclusion drawn from Wolfenden's vapor-phase analysis of the amide-water system that the peptide oxygen is the major site of water interaction.[26]

The data presented thus far correlate the water solubility effect with the exposure of NHCO or simply CO groups. However, to ensure that this effect is not due to the presence of the pyrrole rings in both gelatin and PVP, we studied yet another linear polymer, poly(ethylene oxide) (PEO).[27] Unlike gelatin and PVP, PEO contains no side chains. Being a chain of oxygen atoms interspersed between pairs of ethylene groups, it is probably the simplest model available for the role of the water-polarizing intracellular proteins as visualized in the AI hypothesis. Table I (D) shows that PEO. like PVP, does indeed have strong effects on the ρ-value of Na^+ in the form of citrate. Even niore dramatically, PEO demonstrates that the basic requirement for bulk-phase water polarization and solvency is a properly spaced sequence of exposed oxygen atoms, be it in the form of carbonyl or ether oxygen.

Protein Denaturation Effect on Bulk-Phase Water Solvency

Further verification of the theoretical linkage between exposure of SHCO groups and solvency change of bulk-phase water was achieved by altering the conformations of native globular proteins through denaturation. That such conformation change does in fact alter the solvency property of water lends strong support to another main theme of the AI hypothesis: i.e., reversible changes of cell water between polarized multilayered state and a more random state are the basis for many physiological manifestations of the living cell.[3,28]

In the experimentation, to guard against possible interference from electric charge effects, we chose one neutral and another effectively neutral probe molecule, sucrose and glycine respectively, in an environment of 0.1 M Na_2SO_4. Sucrose and glycine, like Na^+, are as a rule excluded from water in living cells.[3,6,18,29,30]

Four denaturants were chosen: urea (9 M), guanidine HCl (6 M), sodium dodecyl sulfate (SDS) (0.1 M), and n-propanol (2 M). The first two are well known for their ability to unravel the NHCO bonds maintaining the secondary structure of protein~;:~'-the last two are now believed to have little or no effect on the secondary structure and may even promote α-helix formation, but they disrupt and unravel tertiary and quaternary structure.[33-38] We expected, therefore, that the ρ-values for sucrose and glycine should decrease as a result of denaturation by urea and guanidine HCl but not as a result of denaturation by SDS and n-propanol.

Urea has a propensity to decompose at the elevated temperatures necessary for sample drying. Hence the experiments were carried out using three different procedures: (1) prolonged drying at 80°C, (2) labeling water with tritiated water, and (3) careful measurement of total weight changes and subtraction of other components determined chemically or with isotopes. These procedures ultimately provided consistent results. Urea substantially increased the time for probe molecule equilibration. To ensure accuracy, we conducted dual sets of experiments in which the radioactive labels of sucrose and glycine were initially added either outside or inside the sac; agreement of results denionstrated equilibrium. The details of the extensive data regarding sucrose and glycine distribution in 10 to 15 proteins studied will be presented elsewhere.

We present here one specific example, sucrose distribution in a bovine serum albumin-water system (Fig. 1A), and the averages of all data on sucrose distribution for the 10 to 15 proteins (Fig. 1C).

The ρ-value for sucrose in a native bovine serum albumin (bsa) solution containing 78.1 $\pm$ 2.25% (mean $\pm$ SE) water is 0.947 $\pm$ 0.012 (n = 24) while the ρ-values of urea- and guanidine-HCl-denatured bsa were reduced to 0.697 $\pm$ 0.010 (n = 24) and 0.679 $\pm$ 0.034 (n = d), respectively, with p far below 0.001 (t-test). On the other hand, neither SDS nor n-propanol prodused a significant fall of the $\rho_{sucrose}$. Figure 1C shows that essentially the same results occur with other proteins studied in regard to sucrose distribution. Although space does not permit a presentation of the less complete glycine data, it may be stated that they

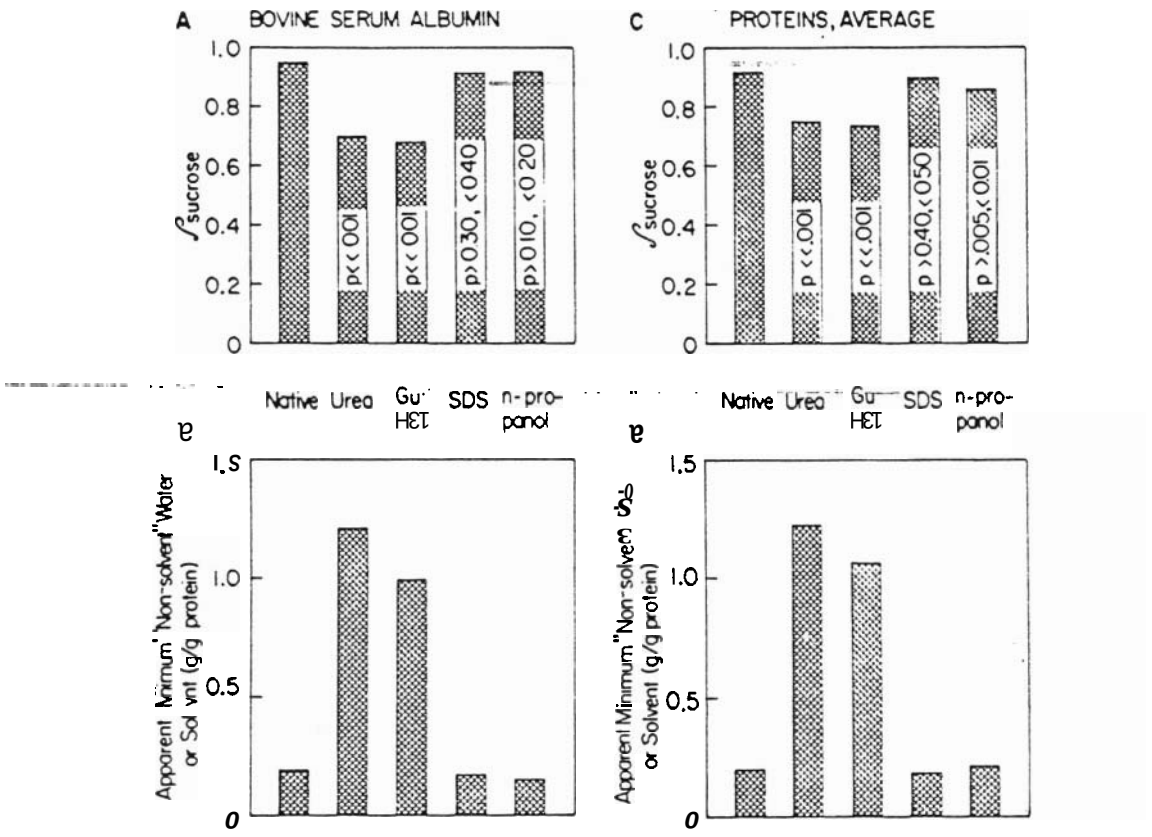

FIGURE 1. The ρ-values of sucrose (A and C) and the apparent minimum "non-solvent" water (B and D) of native and denatured proteins. C and D represent the averages of 15 proteins studied: actin, albumin (bovine), albumin (egg), chondroitin sulfate, α-chymotrypsinogen, edestin, fibrinogen, γ-globulin, hemoglobin, β-lactoglobulin, lysozyme, myosin, trypsin, trypsin inhibitor, and histone. Values for the native and urea-denatured states were determined from all 15 proteins, guanidine HCl values from proteins 2-11, 13, 14, SDS and n-propanol values from proteins 2-11. No significant ρ-value difference was observed in the native protein value whether it was determined from 15, 12, or 10 proteins. Incubating solutions contained Na_2SO_4 (100 mM), glycine (10 mM), sucrose (10 mM), and $MgCl_2$ (10 mM). In addition, urea (9 M) and guanidine HCl (6 M), sodium dodecyl sulfate (0.1 M), and n-propanol (2 M) were present as indicated. Incubation at 25 $\pm$ 1°C lasted from 28 to 96 hours, a sufficient time to establish equilibrium. The test tubes were shaken (30 excursions per min, each excursion measuring $\frac{3}{4}$ inch). Water contents were assayed by 3 different methods (see text): sucrose was labeled with ^{14}C or ^{3}H; extracts were assayed with a β-scintillation counter.

are qualitatively similar to the results obtained for sucrose. It should be mentioned that the ρ-value of sucrose and glycine in urea-denatured proteins can be calculated on the basis of sucrose and glycine distribution. either in water alone in the protein-water-urea system or in water plus urea, with essentially the same results, since the ρ-value for urea itself is close to unity (0.991 $\pm$ 0.0055, from all 15 sets of data).

Together, these data suggest that the criterion for reducing the solvency for the probe molecules is indeed the presence of extended polypeptide chains with the NHCO groups free to interact with water, independent of the means by which the chain extension is brought about.

The data given in Fig. 1 also permit an estimate of the minimum amount of water affected by the extended protein chains. To facilitate this assessment, we introduced an artificial device—the separation of water into (1) a normal category, with normal" solvent properties, and (2) a totally "non-solvent" portion, given in grams of H_2O per gram of dry protein—thereby providing an index of the apparent minimum amount of water affected by the proteins (AMINOW). Thus if $\rho_{sucrose}$ for a protein-water system containing 18% proteins and 80% water is 0.45, the AMINOW is $0.80 \times (1 - 0.45)/0.18 = 2.44$ g H_2O/g protein. Since the part of the water affected is not likely to be totally non-solvent, this figure is a minimal estimate of a larger amount of water actually affected.

Figures 1B and 1D present, in terms of AMINOW, the diverse effects of the four denaturants as well as the AMINOW in the native proteins. In the specific case of bsa. as well as in the average of all the 10 to 15 proteins studied, AMINOW rises in response to urea and guanidine HCI. On the other hand. as predicted. destruction of the tertiary and quaternary structures by high concentration of SDS and n-propanol produced little or no change in AMINOW.

Let us now compare these AMINOW values of the extended protein chains produced by urea and guanidine HCl with the value that would theoretically be expected to operate in living cells. The ρ-values for different probe molecules vary considerably from tissue to tissue. For safety, we shall choose skeletal muscle as an example, since it tends to have a low q-value and hence a high AMINOW. In this tissue the q-value for sucrose is 0.18[29] and for glycine about 0.30;[30] total tissue-protein content is about 20%. If all the proteins participate in the interaction with cell water, the AMINOW needed would be between 2.5 and 3.0, which is 2 to 3 times higher than that in ,urea-denatured proteins. In fact, not all intracellular proteins could be in an extended state. The required AMINOW would be still higher.

DISCUSSION

Several factors are likely to reduce the water-orienting property of proteins under the conditions of our experiments. First. proteins in solution are not likely to be themselves oriented in an orderly way, but maximal water polarization will occur only when there is a more or less parallel orientation of the protein chains. This was demonstrated in an experiment that compared the exclusion of Na^+ from sacs of PVP solution that remained quiescent (Q) and from similar sacs that were constantly stirred (S). Since to-and-fro stirring of a linear polymer solution tends to align the chains in parallel (as witnessed by the phenomenon of flow birefringence), and since agitation would also tend to diminish interchain adhesion, we expect an increase of the solvent effect of estended polypeptide chains with agitation. As shown in Table I (E), this was indeed what we observed: the ρ-value of Na^- is consistently lower in agitated samples. In a prior report. Woessner and Snowden de-

scribed a long-range ordering effect on water by a bacterial polysaccharide, Kelzan®. This ordering effect was also enhanced by mechanical stirring,[39] although just the opposite might be expected.

A second factor that can alter the water-orienting properties of extended protein chains is the number of available NHCO groups. It is unlikely that urea or guanidine HCl will release all NHCO groups; for example. witness the inability of urea to denature poly-L-alanine[40] and pepsin.[41,42]

A third factor is the tendency of denatured protein chains to aggregate and clump together by forming side-chain to side-chain hydrophobic bonds, salt linkages. etc. rather than distribute themselves away from each other at regular intervals.[31,43]

However, all three factors preventing water polarization can be expected to be reduced when water contains the simple polymer PEO, because (1) PEO possesses one side chain and therefore cannot undergo side-chain to side-chain interaction, (2) PEO possesses no H-donating group and therefore cannot form strong inter- or intracellular H bonds, and (3) PEO readily aligns itself in parallel, as witnessed by the remarkable property of a drop of PEO water to be pulled into an ever-lengthening, ever-thinning thread in a quiescent atmosphere;

As expected, the data in Table I (D) of PEO in 0.5 M Na-citrate yield an AMINOW value of 4.00, a value approaching or equalling that envisaged for living cells.[34]

The data in Table I (D) also permit calculation of the minimal average number of water molecules under the influence of each oxygen atom of PEO. At an external Na_2SO_4 concentration of 0.5 M, this number is 14; clearly multilayers of water molecules must be involved. Years ago. Brunauer, Emmett. and Teller[44] showed that charged sites can influence more than one layer of adsorbed molecules only if those molecules possess a large permanent dipole moment. as is the case with water ($\mu = 1.834 \times 10^{-18}$ e.s.u.). In other words, oxygen atoms in PEO and other polymers can influence distant water molecules only by a mechanism of propagated electrical polarization involving both induced and permanent dipole moments of the "target" as well as intervening water molecules. This is just another way of saying that these water molecules exist in the state of polarized multilayers.

To conclude, our data indicate strongly that a search for the specific cellular proteins involved in the polarization of cell water and a delineation of the factors acting to enhance this phenomenon are likely to be fruitful.

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