Consequences of the two chemical forms of water for hydration

(Chapter 4 part 1 of volume 2 of the Protein Primer) Lumry 11-14-2004)

Now that it has finally been established that liquid water exists in two chemical species renewed progress toward understanding its properties has become possible and in this section the basis of the Hofmeister series of aqueous mixtures is shown to be a direct manifestation of the structure-making versus structure-breaking characteristics of the two species. Structure makers both pure hydrophobes and amphiphiles become encased in cooperatively formed cages similar to the familiar clathrate ices but with relaxation times below 50 ps. The competition for water by such solvents links solute to solute forming macroscopic linkage systems complete at solute mole fractions of 0.1 and below. A multiple-equilibrium formalism rather than the classical activity-coefficient approach is required. Structure-breakers such as urea, hydrogen peroxide and hydrazine replace the two species with a strongly associated but more normal liquid. There is little free water in most aqueous mixtures. Some old puzzles are now solved. Examples in this section are protein hydration and the ionization thermodynamics of acids and bases.

Introduction-In volume 1 of the Protein Primer and related publications examination of the hydration of proteins has exploited the now established but long debated model of liquid water consisting of two chemically different species one described in terms of random connectivity all molecules independent and identical and a lower density short-lived but highly cooperative cluster. To be chemically different the latter cannot be a simple rearrangement of hydrogen bonds but must have an inductive electronic Hamiltonian in this case apparently connecting four or five water molecules for a lifetime estimated at 0.5ps at 298K The latter requirement for chemical distinguishability appears to have been first described by Benzinger in 1967. The L species begins to become dominant below about 280K so cooling water to a lower temperature and down into the supercooled region is a chemical process. However, the further association of the small clusters into clathratelike structures although very improbable in pure water is responsible for the solubility of hydrophobic and amphiphilic solutes up to at least 373 K This stabilization by such solutes has been frequently suggested as the explanation for that part of the Hofmeister table of Solubilities in water at the sulfate, aliphatic end including most amphiphiles. This idea extending the ice

clathrate concept to liquid water was first proposed by Glew but lacked general acceptance even after the two-state model for pure water was established in 1983 primarily by Walrafen et al using Raman isosbestic points. At the other end of the Hofmeister series are the strongly associating solutes such a hydrazine, hydrogen peroxide and urea because of their ability to replace water-water hydrogen bonds disrupt cluster cooperativity converting water progressively with higher solute concentrations to something resembling hydrazine, strongly associating but lacking the unique properties of liquid water. Thus the series moves from the efficient structure makers to the efficient structure breakers. The latter group is well understood in the sense that their effects on other solutes can be attributed to indirect effects

Many important errors still widely accepted have arisen from oversimplification of the nature of water, In particular cosolvent effects such as those or urea on proteins have been attributed to direct urea-protein interactions rather than the indirect consequences of changes in water produced by urea. Both effects may occur and the theory of the direct effects is well understood from the work of Timasheff and coworkers using the classical activity-coefficient framework. The indirect effect has not received an equivalent elegant explanation but now can be given a more detailed molecular description using the structure-maker and structure-breaker concepts given above and new data on clathrate structures such as those from the diffraction studies by Teeter and coworkers of the protein crambin.

Ethanol-water mixtures—First, we use Fig. 1 to generalize the clathrate explanation for amphiphile-water interactions. In 1976 Russian investigators reported the heat of mixing of water and ethanol over wide temperature and composition ranges and from the protein chemists point of view most interesting found the value zero at 354K. That temperature has emerged since Pohl's first studies of the rate of melting of mesophilic proteins as a ubiquitous property of that large class of proteins. Few mesophiles are stable even under optimum conditions above that temperature a fact establishing the dependence of stability on knot integrity. Now 354 K appears to be instead a special property of ethanol-water mixtures. Further enlightenment is found in many studies of alkanols that the ethanol data illustrate a general characteristic of the interaction of the aliphatic moiety with bulk water suggesting. That supports the conclusion that all amphiphiles share that temperature for the same reason and proteins in native states demonstrate that temperature because of the amphiphilic character of their interfaces with water.

That behavior can be traced to the Hofmeister series and from that back to the two chemical species of pure water. Data from experiments using aqueous mixtures has been interpreted to include the contribution from the water equilibrium so most deductions are incorrect to at least some degree. An

important example is the suggestion Kauzmann based on Edsall's data that the low solubility of permanent gas in water is due to the large negative entropy change and thus to the clathratelike clustering. The correct explanation is the poor reaction field between molecules with low permanent polarization and water

Even greater confusion and greater error are revealed with the proof that Benzinger's demonstration of the errors made in applying thermodynamics to processes occurring at constant temperature is correct. These complications neither yet accommodated in analysis of data from aqueous mixtures guarantee that information deduced from such experiments is quantitatively incorrect and likely to be qualitatively incorrect as well. Although it is rarely possible even to extract from the enthalpy or internal energy, entropy and volume information those parts relevant to the free-energy changes, that does not always preclude the use of such data in explaining the chemical and physical processes that establish the hydration. The errors themselves are described and correlated in Biophysical Chemistry in 2003 vol. 105, page 609 and the limited ways to work around them in a second article by Lumry on compensation in that issue

In this collection of notes I first set out the observations I now believe to contain unutilized critical information about protein hydration including major solvent dependencies. Then I will see what I can do to extract from these some reliable information about protein hydration. There are several goals that seem important at the present time. The first is updating protein hydration now possible with Teeter's icebergs, the behavior of the heat of mixing of amphiphiles deduced from the ethanol-water studies of the Russian authors (Utilities folded on the Protein Primer web site) and the updating of the proton-exchange mechanism above 55C.

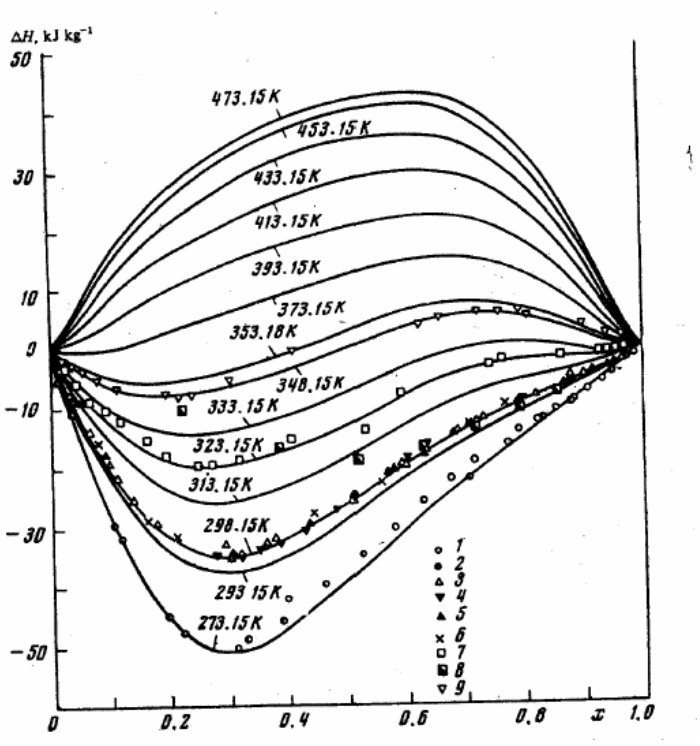


Fig.4-1-1

Heat of mixing of ethanol and water at several temperatures. (By Kessel'man and Onufriev- Russian

Journal of Physical Chemistry 50(11) (1976)2883-2887(Originally published in the Russian journal of physical chemistry in Russian) Parts of that article relevant to this chapter are copied into the Utility folder on the Protein Primer web site. The heat of mixing, excess heat capacity and volume of mixing are reported in this paper. The concentrations are mass percentage. The minimum in the enthalpy of mixing at 273.15 is -50 kj/kg and the mole fraction at that point is 0.129. The minimum moves toward smaller mole fractions as temperature rises reaching mole fraction of 0.089 at 353.15 K At 353K the heat of mixing is small at all compositions slightly negative at lower ethanol and slightly higher with excess water but zero within error at the mole fraction 0.5.

"Hydrophobic hydration"- The accommodation of amphiphiles in aqueous mixtures. . Water has two chemical species. The higher-density species, H, is probably correctly described by Pople's picture of rapidly bending hydrogen bonds each on average the same as all others of the same species. The lower-density species, L, as described by Lumry, Battistel and Jolicouer consists of clusters so four or five water molecules with estimated lifetime of 0.5 ps. The hydrogen bonds disproportionate as a consequence of the rapid electron redistributions about their oxygen atoms These free up some of the empty space in the H water making it available for the clathrate structures familiar in ice but probably just as familiar in some aqueous mixtures although in pure water the simplest clathrate, the pentagonaldodecahedron, has a very low probability of occurrence unless stabilized by a solute usually an amphiphiles or solute such as sulfate ion and H_3O^+ with the tetrahedral symmetry and bond lengths most favorable to clathrate formation. . Attempts to describe the two species have been based most often on rearrangement of hydrogen bonds without the redistribution of electrons about the oxygen atoms that makes the H-bond disproportionation possible. These changes lower the enthalpy in forming L water make a lower value of the entropy that that of H water but not that of Ih so the L species is only metastable in a glassy state below 273K. Clusters of the L building block become increasingly large as temperature drops into the subzero range but apparently no long-range order is possible and thus no crystallization although there are low-temperature ices of low stability produced with artificial and quite difficult means. Instead the rapidly changing arrays of the building block clusters may support critical behavior at the hypothetical limiting temperature of -45 C.

2. The Hofmeister series:

Structure breaking solutes such as urea, hydrazine and hydrogen peroxide unbalance the normal 2:2 ratio of hydrogen bond-donors to hydrogen-bond acceptors that for reasons nor yet well explained

prevent formation of the L clusters probably because those are formed very cooperatively .At low mole fraction of structure breakers the probability of L water cluster fluctuations is diminished (zero at about 0.25 mole fraction) but increasingly the hydrogen- bond integrity is diminished so water becomes increasing like hydrazine, a strongly associated liquid lacking possibilities for cluster formation. The fraction of broken hydrogen bonds in pure water is still a matter for debate but what structure breaking solutes break is the cooperativity of the L clusters. The highest activity coefficient for the noble gases in water at infinite dilution occurs at 328K (Benson and Krause) which is also the conditions for minimum solubility of methane and ethane (Rettich, Wilhelm and Battino) That temperature may be the one at which the normal solubility of these hydrophobic substances in pure water is just equaled by the solubility in the space made by breaking hydrogen bonds. Alternatively matrix melting fluctuations may become important at that temperature as suggested by the fact that proton-exchange at matrix sites does not depend on melting fluctuations until that temperature is reached. Thus Gregory found the onset of the exchange of matrix protons with water protons by unfolding becomes important for HEW lysozyme and ribonuclease A at 328K so above that temperature the exchange process involving migration of exchange catalysts through folded matrix is supplemented by a matrix melting step that becomes predominate at still higher temperatures.

The other end of the Hofmeister series includes sulfate ion, poly-ethylene glycol as examples of extreme structure makers consistent with their frequent use as crystallizing agents. The major explanation due to Gregory is that they force contraction of the protein as is consistent with the fact that they generally increase thermal stability. Glycerol is a classic cosolvent in that respect and serum albumen has been much used for this purpose. The use of such cosolvents is sometimes called “crowding” as a shorthand expression for the excluded volume effect but is correctly a result of the actual sequestering of water molecules by chemical association with solute. Protons have recently been confirmed to belong to the structure-making class and form an important illustration of past errors. They take up 21 water molecules one to increase the tetrahedral characters of the single water molecule and twenty to form a clathrate cage. Robinson and coworkers found a fourth-order dependence on water concentration of the rates of proton or electron attachment to liquid suggesting attachment to L clusters eventually preferred hydrogen-bonding with outershell water molecules without postulating any change in electronic Hamiltonians.

A single proton in a cooperative cage of 20 water molecules with short lifetime makes the hydration more like a fast amorphous solid than a true liquid. The sequestering extends at least to the boiling point thus well above the stability of L water indicates considerable stability in well-organized arrangements of first-shell L clusters. Although such large structures have been suggested to be the

explanation for the peculiar low enthalpy change in ionization of aliphatic carboxylic acids and protonated primary amines, that explanation now appears to be established as a consequence of hydration of the ejected proton. Since electron trapping by water also involves a change in charge, a similar explanation may be required to explain that much-studied phenomenon. In any event the Eigen-DeMaeyer description of the hydrated proton as a cluster of four water molecules considerable underestimates the true structure and changes in proton hydration common in reactions of many kinds requires reconsideration of structure in terms of the thermodynamic changes.

Stoichiometry and the first-shell localization of the interaction suggest a pentagonal dodecahedron structure for glycerol and smaller hydrophobic molecules and amphiphiles but a very dynamic one. That structure was previously well known as the smallest of the ice clathrates and now in water is general supposed to be the smallest of the "icebergs" of Frank and Evans. That appropriate name is sometimes taken too literally as suggesting ice Ih rather than the ice clathrate structures.. Tetrahedral solutes such as sulfate, bisulfate and perchlorate ion are very effective in forming icebergs and PEG though much larger favors water structures that form a tube along the polymer axis thus explaining the unique properties such as the great decrease in flow viscosity so important to firemen. .

As illustrated by Robinsons cluster model for the hydrated proton, most attempts to explain these structures have involved rearrangement of the normal hydrogen bonds found in the higher density species of water but Benzinger's adding clarity to Carnot's arguments about the use of heat to produce free energy showed that there must be a change in electronic Hamiltonian on conversion of the H form to the lower-density L form from which the clathrate structures are formed. Frank and Wen had earlier proposed inductive coupling for charge rearrangement in small water clusters. These "flickering clusters" as a result of the polymoleculer electron rearrangements are obviously formed cooperatively. Subsequently enthalpy and heat-capacity data of Arnett and coworkers on amphiphile-water mixtures revealed extrema in enthalpy and entropy clearly indicating high cooperativity They noticed unexpected enthalpy changes in amphiphile solution varying in size rapidly with mole fraction of amphiphiles as illustrated for ethanol in Fig. 1. The heat of mixing is negative at low ethanol mole fractions with largest absolute value found at 270K and 0.09 ethanol mole fractions. The extremum mole fraction does not change rapidly with temperature but the heat of mixing itself does decreasing to zero at 354K At still higher temperature it becomes positive rising rapidly with increasing ethanol mole fraction and temperature. Extensive studies of alkanols, both straight-chain and branched chain, most abundantly by Wadso et al generalized the ethanol behavior as a general characteristic of the interaction of bulk water with alkanols. The effects have been found even larger with primary amines.

As noted in a previous section, it appears necessary to conclude that appearance of the 354K temperature in protein melting must be attributed to the amphiphilic groups of the proteins as well a to an intrinsic feature of polypeptides. A major immediate goal for protein chemists is to untangle this apparent coincidence. With the usual reluctance to give evolution much credit for biological mechanisms the inclination is to credit the coincidence to D'Arcy Thompson's "fitness of the environment" an explanation seemingly equally improbable. Is that the case or are the two closely coupled by success in evolution? In any event the entire phenomenon of protein melting must be reexamined.

An important feature of Fig. 1 is the small mole fraction at which the enthalpy of mixing is most negative. This is also a feature of some other observable notably the viscosity of ethanol-water mixtures. It was called the "magic mole fraction" for ethanol by Lumry and Rajender and they reported similar characteristic magic mole fractions for other alkanol-water mixtures. Ethylene glycol, glycerol and amphiphiles of no greater size have similar magic mole fractions. Methanol has 0.11, 1-propanol lower than 0.08 and t-butanol 0.045 consistent with the idea that they are solvated by incorporation in water cages with size depending on solute size. That relationship is common in the ice clathrates which vary with guest size from pentagonal dodecahedron up to larger, often low-symmetry structures with large cavities from many small cavities of the H water species. Hydroxyl groups of alkanols and sugars often substitute for first-shell water molecules and primary amines are even more effective in stimulating iceberg formation presumably because the tetrahedral structure of the amine head is an additional source of enhancement.

3. The "magic mole fractions".

Once the two-species model of water was established it became possible to explain the magic mole fractions as a general manifestation of the chemical changes produced by structure makers. At low solute mole fraction there is enough free water to form complete cages for all solute molecules but at higher solute mole fraction the solutes compete for water molecules. At still higher solute mole fractions the fraction of bare molecules becomes large and the fraction of water-water hydrogen bonds small. The magic mole fraction is the statistical mole fraction at which the number of water molecules per solute molecule is a maximum. The changes in enthalpy and heat capacity are very abrupt at the magic mole fraction indicating a large degree of cooperativity among water molecules at those special mole fractions. That behavior was discussed for many years but the latter feature which is central to its explanation was not fully explained although Frank and Evans in their seminal papers of 1945 on what Frank called "icebergs" made the necessary first step. What was missing was proof that water actually forms two

different chemical species. The modern era begins with rediscovery of the magic mole fractions by Arnette and McKelvey and has been completed with proof for the two-species model of water and the proposal by Glew that water forms clathrates to accommodate non-polar parts of solute molecules for which the well-known ice clathrates of water ices have proved to be a good model. A well-studied example is provide by ethanol water mixtures.

The ethanol-water results (Fig. 1) seem to establish that at low solute mole fractions each solute molecule has its full quota of the first water shell forming a complete clathrate cage. As solute mole fraction increases this condition is less and less satisfied because water is increasingly limited. Below 354K the association process is exothermic; above endothermic. The magic mole fraction for ethanol at 298 K is slightly lower than 0.09 and does not much change as the temperature rises. Below 354K the association process is cooperative perhaps weakly. This is the message of the deep enthalpy minimum at the magic mole fraction which becomes positive above 354K.. Given the size of clathrate cage, the magic mole fraction and simple models for the cooperativity average statistical parameters can be easily estimated. Above the magic mole fraction the cages become less and less complete and cooperative. They may not form but experiments of (D.. F.) Evans et al on micelle formation in water at temperatures as high as 335K suggest that they do. Micelle formation depends on the poor solubility of the participating surfactants but also tales place in hydrazine, hydrazine-water mixtures and even in fused salts and in fact wherever the structure depends on near neighbors Water is thus a micelle-former but a special one requiring a special explanation. Micelle formation both normal and inverted can add complications as a response to the strong hydrogen bonds rather than clathrate formation so water and hydrazine are about equally effective in favoring micellization.

The partial molar volumes for ethanol and t-butanol measured by Johnson and Franks shown in Fig. 2 reveal that there is additional important detail in the water-ethanol interaction data. Well below the magic mole fraction there now emerges a second minimum, point of inflection and new maximum. However, of even more interest is the sharpness of the magic mole fraction of t-butanol. Its value of 0.04 288 K and about half of that at 323 K indicate a high degree of cooperativity. The average number of sequestered water molecule is near 20 water molecules per solute molecules suggests a complete pentagonal dodecahedron.

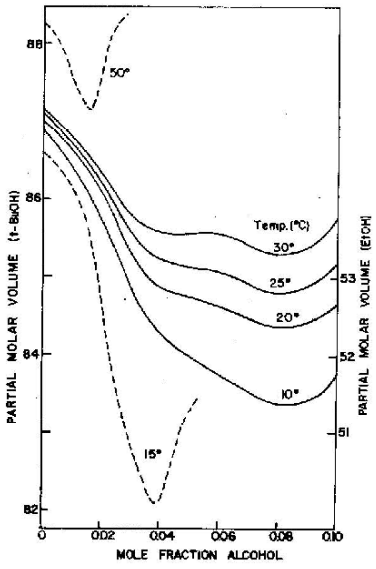


Fig.5-1 2 from Johnson and Franks (Trans. Faraday Soc. 58,(1962), 656) **reveals the magic mole fraction through the partial molar volume. This variable shows that the magic mole fraction as typified by ethanol-water mixtures has at least two minima.**

The simple model can become complicated not only because of lower association enthalpy but also because of solute variations. The division of the Hofmeister series into structure-making and structure breaking branches leaves an unhappy middle group forced to awkward compromises. Guanidine sulfate gets there by independent combination and ethylene glycol has a structure-breaker side and a structure-maker side.. Glycerol and other polyhydroxy solutes on the other hand have the best of both sides since their hydroxyl groups' substitute for water molecules in the cage. However, as shown for example by Franks and coworkers, some geometries enhance cage formation more than others; sugars with differences in equatorial versus axial favor orientation of hydroxyl groups favor different cage probabilities and probably different geometries. Engberts' group has carried out many studies of compensation behavior mostly in chemical reactions but in solubility studies they found 1-4 dioxane and 1-3 dioxane to have such different thermodynamics in water solubility as to indicate totally different accommodation by L water. Glycerol is another interesting example since large industries are based on mixtures which especially useful physical features not at all similar to those of pure water at ordinary temperatures At 30% by volume the fluidity is reduced and the hygroscopicity altered in ways favorable to many uses as in skin creams. PEG with the unique properties of its mixtures with water has water cages arranged along the polymer length forcing long parallel tubes with very low viscosity in the tube direction.

Structure breakers such as hydrazine, hydrogen peroxide and urea lower the probability of cage formation by lowering the probability of the formation of the lower-temperature clusters forming the

lower-temperature species of pure water, the L species. What one concludes from such examples is that there are few aqueous mixtures with much normal water character. With many amphiphiles, even the butanols, extreme dilution and special experimentation is required to explore highly aqueous regions historically neglected. Since it has been based on water as a homogeneous fluid, a large part of the chemistry in textbooks must be incorrect

Frank's proposal for clathrate stability in liquid water

Frank explained the unique features of the cages even before the existence of the L species of water was established. Despite what is still generally believed Kauzmann' suggestion that the low solubility of non-polar moieties is due to icebergs Frank suggested that instead it is due to the poor reaction field between substances with low permanent polarization and water. This conjecture was first proved by Lumry, Battistel and Jolicoeur using his suggestion that hydrazine is "inhibited water", that is, water without the special solubility properties of real water. This deduction was placed on Frank's collected data showing the great similarity despite the large difference in enthalpy and entropy changes in hydrophobic hydration without major free-energy differences.. Thus the latter were likely to be due to the "iceberg" formation possible in water but not in hydrazine. After subtracting the contributions from the clathrate structure to their solubility it was found that the remaining positive enthalpy in "hydrophobic hydration" explains their poor solubility. The much increased solubility in cold water as compared with that in cold hydrazine is then due to the icebergs. They have low probability in pure water hot or cold but by improving the reaction field between low polarization solutes and water they favor formation of cavities for the low-polarization parts of solute from free volume released by conversion of H to L water. In water there is a small net reduction in the unfavorable enthalpy contribution resulting in an improved reaction field as the first-shell water molecules draw back into the high dielectric constant of bulk water. Estimates of the inner shell expansion put it as 0.05Å thus below the experimental error of x-ray diffraction for the ice clathrates (personal communication to Lumry from Jeffries). The improvement in free energy produced in this way is described by Fig. 4-1-3.

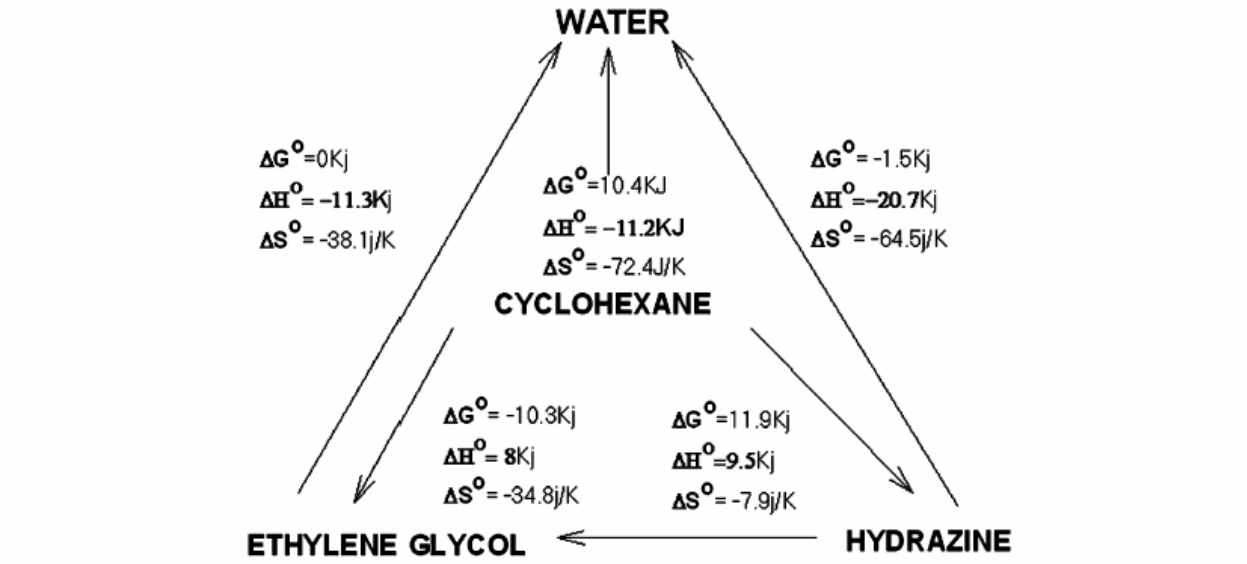


Fig. 1. Solubility of argon in several solvents. 'Hydrophobic hydration' is illustrated by the transfer from cyclohexane to water. 'Inhibited hydrophobic hydration' a la Frank is illustrated by transfer from cyclohexane to hydrazine and the path on the right is transfer from inhibited water to normal water Frank proposed (see text).

For many amphiphiles the partial molar volume in water can be estimated within a few percent by adding 0.05 Å to the van der Waals radii of the aliphatic parts. Because the electron Hamiltonians changes in cage formation about amphiphiles is a change in electronic Hamiltonian, it is a chemical process aptly named "chemical hydration". The "temperature factors" from x-ray-diffraction studies because they measure atom free volume show that increment is a good estimate of the packing compactness of proteins, much smaller than generally appreciated and with major implications for changes in electronic Hamiltonians. Any given residue and interaction with solvent will contribute to folding and function in somewhat different quantitative way depending on its neighbors in its specific position in the protein. Thus for example the structural and thermodynamic implications of a serine depend on its neighbors.

The versatility of the water-water hydrogen bond provides the basis for the two states of pure water and the formation of clathrate cages about amphiphilic and hydrophobic solutes but the critical uniqueness of water for L and H forms is the fact that the tetrahedral angle in ice Ih and H water is only one degree different from the O-O bond in the water pentagon; the difference with the hexagon is only slightly more.

Protein hydration revisited-Crystallization prevents cold denaturation but not the subtle change in enzymes. We are now in position to understand Teeter's elegant studies of the hydration of the

small, non-enzymic protein crambin.(Teeter, Yamano, Stec and Mohanty, PNAS 98 (2002) 11242) In a second paper in the same journal in 2002 her group examined the cooperative transition that occurs on cooling crystals below about 200K. The protein appears to be the native, fully folded species. However there are some puzzles about temperatures below 273K. In particular in free solution proteins, that is, freely dissolved in supercooled water, there is considerable evidence that denaturation occurs. Following Biltonen and Shiao early work on "cold denaturation" Franks and Hadley extended Brandts' van't Hoff plot for melting into the supercooled region and found normal melting for several proteins complete within no more than twenty degrees below 273K. Studies of crystals give a different result since few if any x-ray-diffraction studies on cooling to 200K and lower are consistent with cold denaturation in the supercooled region. The systematic diffraction studies of ribonuclease A down to 80K by Tilton et al is particularly convincing and Teeter and coworkers found only good crystallinity with crambin at least down to 150K. The experimental results are often complicated by the use of cosolvents to preserve native structure of structures on cooling. Glycerol and methanol as efficient structure makers are very effective in this use. The most probable conclusion from such studies is that crystal confinement prevents cold denaturation but does not prevent the phaselike behavior of crambin and many other proteins crystals at temperatures near 180-200K That latter much-studied process has been discussed in detail by Gregory in interpreting the changes in free-volume distribution in the process obtained using positron annihilation methods. He cites a number of major references that have not caught the attention of most protein crystallographers important because they reveal a source of major error in protein diffraction work.

The Subtle change process

This process was defined for proteins as one with large enthalpy and entropy changes but small heat-capacity changes to contrast it with the normal thermal denaturation process. It is discussed in Chapter. @ of volume 1 of the Protein Primer with additional information added here. Its occurrence was first detected by Brandts using a variety of conformation-sensitive methods of which optical rotation proved to be the most informative. His results with this variable are shown below as Fig. 4. (Brandts and Lumry, J. Am.Chem.Soc. 83,(1964).4290) and the data were analyzed using the Moffatt equation the parameter a_o is dominated by peptide bands near 200nm and b_o by bands in near ultraviolet. The effects are very large; Parker, Biltonen, Madison and Rosenberg later computed molar ellipticities from their ORD data for proteins in the chymotrypsin family to be about 6000 ° near 206nm, the center of the maxima on the Cotton effects, and changes in ellipticities on most acyl-enzyme formation were of the same order. The pH dependency is large only for unliganded chymotrypsin. The sizes reflect the cooperation of many peptide groups in two separate processes. The largest is the folding from unstructured bubble into the

native form with expanded matrices. The second occurs on matrix contraction sometimes with a small contribution from further knot contraction. In conformation of Brandts' report thirty years earlier, Akasaka et al found the peptide-group frequencies responsible for the ellipticities lie around 206nm varying only slightly but indicating participation of more than one of the peptide absorbances near 200nm. These effects occur only in folded proteins and provide provide a sensitive though still little exploited measure of the folding in the two common folding processes. Comparison of the values of the two Moffatt parameters for chymotrypsinogen (on the left) and chymotrypsin (on the right) (Fig. 4-1-4) give a better description of the differences between the zymogen and the active enzyme than is available from most other methods. The melting transition temperature is indicated by the arrow.

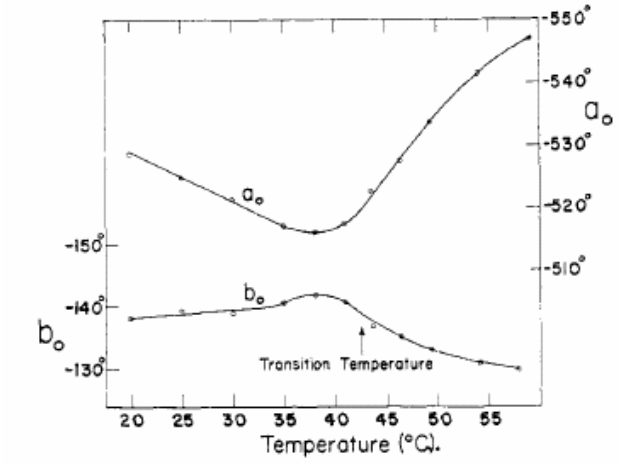


Fig. 1.—Changes in rotatory dispersion constants of chymotrypsinogen during thermal denaturation at pH 2.0. The system was completely reversible from 58° back to 20°.

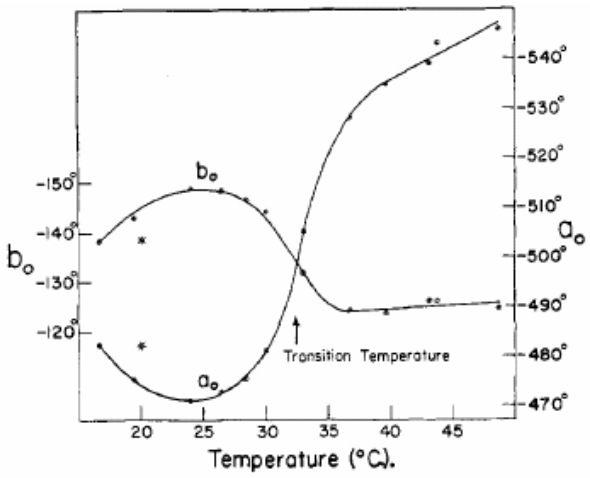


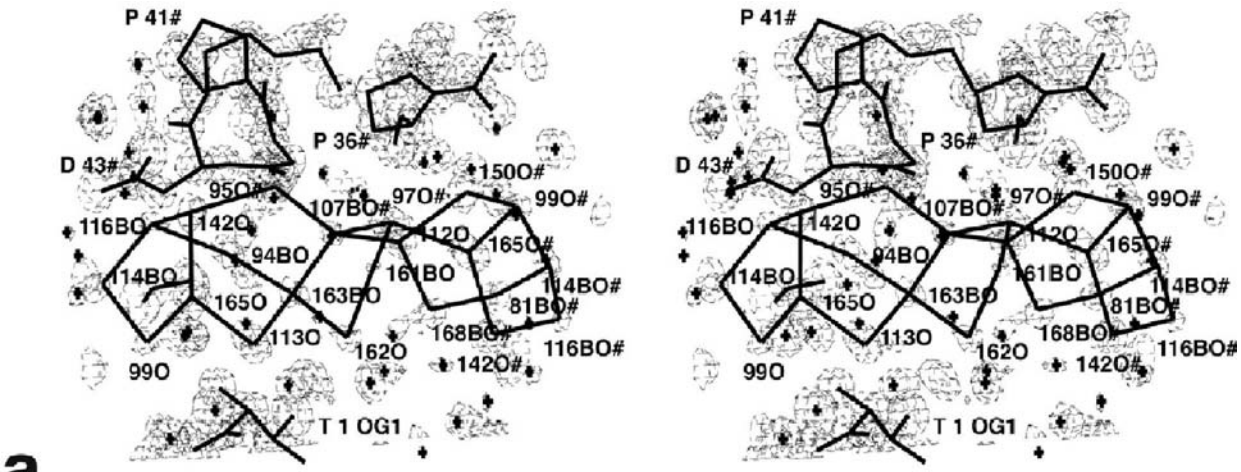
Fig. 2.—Changes in rotatory dispersion constants of chymotrypsin during thermal denaturation at pH 2.0. Starred points show reversal back to 20° from 49°.

A problem in low-temperature studies is that cosolvents such as methanol to keep the supporting solution from freezing favor matrix contraction. That has been established for glycerol at room temperatures by Gregory. Huber and coworkers studied the contraction process around 200K as a function of methanol concentration.

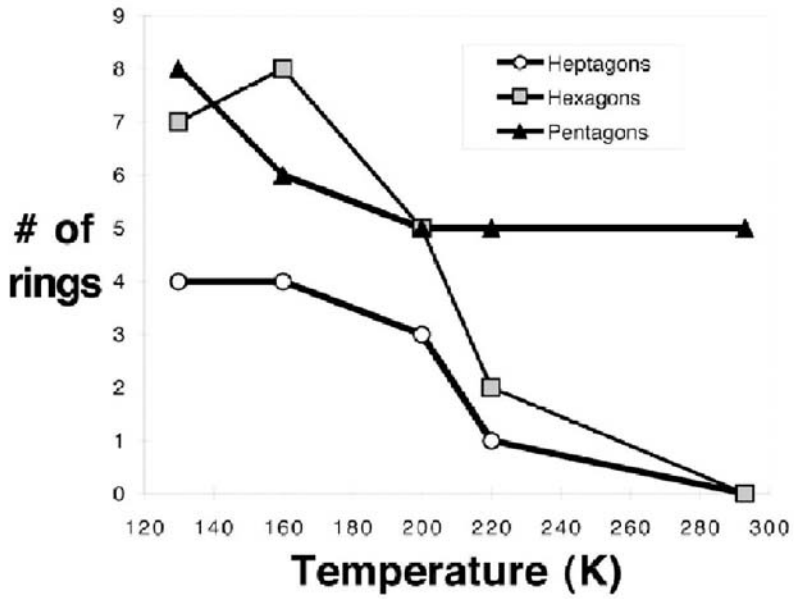
Contraction was favored by lower temperature and as well by increasing methanol mole fraction. Gregory related these changes to change in hydration apparently as they alter matrix properties since cooling and dehydration reduce free volume without melting. Lüscher et al studied hydration directly starting with the dry protein in two different states. The first was the wild type chymotrypsin free of specific ligands. The second was tosyl chymotrypsin which Parker found to have very high ellipticity near 206nm indicative of extensive matrix contraction. The two forms of chymotrypsin demonstrated very different hydration patterns that could be interpreted using their high-precision enthalpy-entropy compensation patterns (Lumry, Biophysical Chemistry, 2002). The compensation temperature of the expanded species was that

characteristic of protein matrices; on tessellation it was 298K indicating that the hydration occurs without free-energy change. Tosylation also increased the total amount of water at saturation vapor pressures. Like Gregory's study of the free-volume contraction in HEW lysozyme produced by changes in the hydration, the Lüscher results showed that hydration of matrices catalyses conformational relaxation in the expanded protein.

Clathrate formation in protein hydration. **T**he recent studies by Teeter et al on crambin hydration add much more to the use of hydration observations.



a.



b.

Figure 4-1-5. Top:Water partial cages about exposed parts of structure makers in crambin: Bottom: Numbers of partial water polygons Teeter et al found in x-ray-diffraction data for crambin at 150K Copied from Teeter, (cf. new refs.)

Teeter et al found that crambin in frozen crystals at 150K was in its contracted state and had many partial Frank-Evans pentagonal dodecahedral icebergs formed about the exposed parts of oily residues and apparently in inter-domain cavities not clearly distinguished because crambin is not an enzyme. The term domain is used throughout protein research reports usually referring to clusters of oily side chains with little specific detail. Such uses of the term of which there are several pervading current use should not to be confused with either functional domains or knots as used in the Protein Primer. The water clusters despite the large coordinate errors in even very high resolution in protein diffraction studies can be distinguished in the reports of Teeter et al as water pentagons and hexagons. That is an immediate consequence of the fact that water clathrates such as the pentagonal dodecahedra are constructed from pentagons plus a few hexagons. The large size of their structures apparently limited only by local geometry and accessible surface area identifies the clusters with the smaller Frank-Evans icebergs from pentagonal dodecahedron on up to sizes familiar from the ice clathrates. The new data confirm and extend the less rigorous deductions from other kinds of data. The pentagons and larger species in water as in ice are another consequence of the very special characteristic of the the water molecule that the O-HO angle in tetrahedral structures is only 1 degree larger than that angle in the pentagon: 109.2 degrees for ice 1h and H water and 108 degrees for the pentagon. The small differences appears to be entirely consistent and strong support for the changes required to convert H water into the L building blocks and then into the clathrate structures. In 1952 in a once-famous paper Claussen and Poleglase suggested the number of water polygons in liquid clathrates for alkanes up to n-butane (J. Am. Chem. Soc. 74(1952)4517). This extended the “iceberg” hypothesis of Frank and Evans which originally emphasized the pentagonal dodecahedron but also led Frank to conclude that these large water polygons have low probability of occurrence in pure water either in L and H species. Rather they are composed of the ordered cluster of the L but stable only as they accommodate to hydrophobes and amphiphiles including PEG, sulfate, perchlorate and phosphate species. Cations re usually structure breakers but acid forms of carboxylate and ammonium groups are structure makers as can be confirmed by the enthalpy and entropy changes in conversion from their ionized forms.

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The clathrate hydration water of proteins is quite specific as opposed to the remaining bulk water which is chemically different subject to thermodynamic considerations as a separate phase. Although the clusters scattered about the protein surface fit the description of the A-shell hydration advanced in several studies, they apparently have short lifetimes though less so than the subpicosecond times of the H and L water in which they are embedded. Lichtenstein et al primarily with EPR data have given the characteristic relaxation times and Liischer and coworkers have shown the A-shell water to be held with much larger negative free-energy, enthalpy and entropy changes than the B-shell water. The interaction of the shell water with the protein is nearly isoergonic. Sulfate and hydrosulfate ions are often bound tightly in protein surfaces and clefts where the most obvious use is to induce clathrate formation just as do the crambin hydrophobic tails. But the importance of that interaction has not been clearly understood. Now the heat-of-mixing data for the ethanol-water solutions (Fig. @) provide a better basis for exploring protein hydration than has been available. The enthalpy changes on mixing amphiphiles and hydrophobic molecules such as the noble gases first notice by Arnett and coworkers and then illustrated over a wide range of temperatures in Fig.1 indicate the clathrate shells form with considerable cooperativity subject to containment and temperature. That would be consistent with a large contribution from the surface partial clathrates and the other interactions of the protein with water to the thermodynamic stability of the native species. Positron annihilation experiments (Gregory and Chai) and neutron diffraction experiments especially those of Parcuaribu, Cinneli and Onori (Biophysics J. 83(2002) 1157) show major dependence of conformation characteristics, (free volume, proton. exchange parameters, volume , stiffness, permeability, young's modulus, transconformation rates and activation parameters) It is noted above that the 354K temperature central to conformational behavior of mesophiles may be attributable to supporting media.

Temperature variation patterns of protein hydration- The heat capacity of native forms increases with temperature but the heat capacity of activation for the melting rate does not so that of the transition state is the same as that of the native-state... Morozov and Morozov measure the Youngs modulus for the native state and found the force required to produce a given distortion decreased steadily reaching a negligible value at 354K the characteristic temperature at which mesophiles cease to be stable. Their experiments establish that the distortion produced by application of a force from outside depends on the integrity of the knots. It is a whole-molecule distortion and can be correlated with the standard heat capacity of the native state but the source of the latter is uncertain. It is probably matrix melting or the loss of Teeter's amphiphile hydration or both.. Probably both participate but most of the melting-rate information has been obtained by proton-exchange experiments in which matix and knot identification

utilized data obtained below 328K where as Gregory has shown, exchange at matix sites does not depend on matrix melting. As already discussed, 328K is the temperature at which hydrophobic groups as measured by the noble gases and the smallest alkanes have the largest activity coefficient in pure water. The exchange mechanism begins to depend on transient matrix melting without knot melting. And as the native state has an increase probability the native state relieved of the stress from the matrices should become increasingly stable at increased temperature melts off the icebergs. Since that does not occur nor has any third alternative been established, it is necessary to draw the conclusion at least tentatively that the icebergs contribute to native-state stability. The icebergs contribute to the stability of the native state essentially by shielding the water from the exposed hydrophobic groups in the protein surface and they may not melt in the usual way real icebergs melt. Instead like ethanol and water in Fig. 1 the negative enthalpy of mixing with water first goes to 354K and then becomes positive to be increasingly unfavorable to that state. That change does not alter the activation quantities for the protein melting rate because the changes in geometry in formatting that transition state are very small. It appears true that matrix proton exchange with bulk water on real but partial unfolding above 328K but the unfolding events are so rare the fraction of the total time in which they exist is negligible. We can add important details using the results reported by Lüscher et al from hydration studies (summarized above).

In contrast the hard relatively stiff knots the matrices expand and contract in a spontaneous process occurring nearly inorganically. When totally dry, the matrices become stiff approaching the glassy character of the knots. Normal denaturation to the bubble produce cannot occur but the phase-like melting in forming the transition state has large thermodynamic consequences but very small geometric ones just as when the protein is dry. Gekko and Timasheff using chymotrypsinogen found that glycerol as cosolvent produced the same effects as physical drying at temperatures below 301K, the compensation temperature for glycerol concentration variation. The standard enthalpy and entropy are reduced so below that temperature thermal stability is increased. That cosolvent is very effective in stabilizing proteins in solution but that effect is apparently due more to the increase in activation free energy for melting rather than the standard free-energy change. The latter behavior is to be expected from Gregory's finding that glycerol in the lower-temperature range forces contraction even in knots with decreased proton-exchange rates These observations are consistent with the hydration results on chymotrypsin of Lüscher et al here given above. Contraction produced by tosylation essentially froze the matrix so that water is transferred from bulk to protein without free energy change I contrast to the free enzyme in which the matrices relax into their normal degree of contraction as water is taken up. The compensation temperature was 470K as expected for matrices. Glycerol, tosylated and physical drying produce the same changes. Those changes

are divided among three types of hydration: internal, clathrate cages at hydrophobic sites and the remainder of surface hydration primarily that of the hydrophilic groups either structure breakers when ionized of structure makers when unionized.. In the latter category we include the ionizing or ionizable acid and base groups mostly near the protein surfaces. However since ionization involves change in the proton concentration in bulk water, those changes are changes in hydrophobic hydration as defined here in terms of clathrate formation just as are the changes in hydration of bound sulfate ions and exposed aliphatic tails..

Relaxation times for major conformational process were reported by Likhtenshtein and coworkers who used EPR data and recently by ultra-speed laser kinetic measurements Zewali et al. Thus Pal, Peon and Zewail (PNAS 99(2002) 15297 found rapid water fluctuations between the knots in expanded species of chymotrypsin and tight packing of water in the contracted form in substantial agreement with the results from hydration studies reported by Liischer et al (vide infra). Kamal, Zhao and Zewail measured hydration kinetics for human serum albumin, a protein with a unusually large number of acid and base groups. The bulk water process had a relaxation time of 0.8 ps at 273K a value in good agreement with the previous estimate for the L-H relaxation at 298K as 0.6 ps at 298K The enthalpy change in that process is 2.5 kcal per mole of the cooperative unit consisting of four or five water molecules. Relaxation of the hydration of the urea and guanidinium chloride denatured species was 13 ps to be compared with native protein hydration from 30 to 60 ps. The last is probably from clathrate relaxation in which case it indicates somewhat higher cooperativity in clathrate formation than suggested above. This possibility can be checked using dielectric relaxation data for water-amphiphile solution and the EPR results reported by Likhtenshtein et al.

All of those hydration changes alter the matrix- process and thus the physiological functions and the transmission of information and free energy between a protein and the and proteins in its immediate environment. There is a wealth of information on such phenomena in the literature of which the behavior of the susceptibility of heme iron in cytochrome C as a function of hydration provides especially useful information. Dehydration and rehydration produced large change in susceptibility reflecting change in electron spins of the iron ion but also produced several first-order phase transitions reflected by the susceptibility. The purpose of the study in 1960 was to determine the extent to which the electronic properties of the heme group and especially the iron atom can be controlled by conformation changes. Paper by Lumry, Sollbakken, Sullivan and Reyerson is copied into the Utility folder in the Protein Primer web page.

The purpose of these considerations of protein hydration is to complete a thermodynamically reliable description of the nutcracker mechanism by which enzymes function. The major remaining uncertainty is the entropy change in the contraction process due to changes in hydration. It now appears likely in view of the high efficiency in rate and specificity of enzymic catalysis has been perfected in evolution by finding conformations that make the nutcracker mechanism entirely isoergonic. Despite any irreversibility arising from the restoration of the protein in its initial state by irreversible chemical conversion of substrate to produce in the decay from the true transition state, the minimization of heat production makes perfect cycling of protein itself possible. Then the only heat production occurs in the substrate-to-product process itself. This is as close to true reversibility possible in a catalytic process and yields the maximum catalytic rate possible using a nutcracker mechanism. This extraordinary accomplishment does not appear to be impossible given unlimited possibilities for mutational experiments. If so, these ultimate mechanisms perhaps for all enzymes can be said to be "perfect" at some level improving further only as enzymes are evolved into the multi-enzyme systems making the cooperative units able to support life. Further discussion of the isoergonic possibility and its dependence on entropy considerations and thus apparently on protein hydration are given in part 3 of this chapter.