The energy of interaction between a unique conformation of the protein and a single water molecule was found to be a valuable description of solvent space, when presented as a contour map in three dimensions. The zero-energy contour surface separates protein and solvent space and may be considered to define an effective protein surface. Solvent space within the zero-energy contour consists of a single network of channels and spaces of various sizes and shapes plus one isolated low-energy volume that contains one water molecule. All but one of the 47 crystallographically-located water molecules are within the zero-energy contour, and many are in volumes of quite low energy.

A Monte Carlo simulation was performed to sample the equilibrium ensemble of protein-solvent configurations. Translation/rotation of solvent and internal rotation of side chains provided motion. Provision was made for small motions to obtain rapid local equilibration, and for large motions to obtain proper distribution of solvent. Simulated solvent structure is found to be highly ordered: all water molecules in a first, and part of those in a second layer at the protein surface maintain a unique hydrogen-bonded network, that may be considered "anchored" to the protein at low-energy positions of solvent space. The network contains twice as many ordered water molecules as have been located by x-ray crystallography in the crystal of trypsin inhibitor. Extent and order of simulated water are more similar to solvent structure determined by x-ray crystallographic refinement of another small protein, rubredoxin (2). Possible reasons for the differences include high molarity of salt in the crystals, incomplete freedom of motion in the simulated crystal, insufficient size of the simulated statistical sample, use of a model for water-water interactions (3) that exaggerates local order, and incomplete crystallographic refinement of solvent structure.

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WATER-PROTEIN INTERACTIONS

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The interaction of macromolecules with solvent water is an important determinant of their properties, but this relationship has not yet been described satisfactorily. The following experiments focus on the process of protein hydration—the addition of water to dry protein to obtain the solution state. A detailed description of the sequence of hydration events is

expected to produce a fuller understanding of the protein in dilute solution. This approach is analogous to the use of studies of protein unfolding for understanding the folded state.

Heat capacity measurements (1) are of particular interest. These can be carried out over the full range of system composition, from dry protein to the dilute solution, and they serve to correlate information obtained by other techniques that can be applied only to solution or solid state but not both. The dependence of the heat capacity on water activity defines stages in the hydration process and suggests the following simple picture of it: water at the lowest activity, 0–0.07 *h* (g of water/g of protein), is bound principally to ionizable groups; in the mid-range of water activity, 0.07–0.25 *h*, surface clusters form, probably centered on polar surface elements; these clusters are mobile, with heat capacity greater than bulk water, and grow with increased water activity until after the polar sites are saturated at 0.25 *h* there is a condensation of water over the most weakly interacting portions of the surface, resulting in completion of a water monolayer containing ~300 molecules. From this hydration level, 0.38 *h*, to the dilute solution there are no changes in thermal properties of the protein. This picture accomodates results of other static measurements, such as infrared spectroscopy (2) and sorption isotherms.

Electron spin resonance (ESR) measurements were made on powders of a lysozyme-TEMPONE mixture (50:1) that had been partially hydrated by equilibration at appropriate water activities. There are stepwise changes in the properties of the bound TEMPONE that parallel those in the heat capacity: the ESR spectrum changes with hydration only above 0.07 *h*; the rotational correlation time, estimated by spectrum simulation, changes only above 0.25 *h*. Apparently kinetic properties can be interpreted within the framework of the picture derived from static measurements. The ESR properties continue to change slightly with hydration above 0.38 *h*, the level at which static properties have the solution values, and thus dynamic measurements reflect more than a monolayer of water about the protein. It is particularly significant that the principal change in motional properties of the bound TEMPONE comes with the proposed condensation event at 0.25 *h* that leads to completion of the monolayer shell.

Enzymatic activity was measured for powders of the lysozyme: $(GlcNAc)_6$ complex (1:1), using product analysis after reaction at a desired hydration level. Enzymatic activity was observed to develop with the condensation event at 0.25 *h*, which is also the hydration level at which TEMPONE gains motional freedom. This behavior is consistent with the properties of the enzymatic reaction in solution in that there is a change in the geometry of the enzyme-substrate complex in the rate-determining step (3).

The following questions are among those that remain unresolved. Is there a characteristic structure of the water monolayer? The following evidence suggests that there is: 300 molecules of water would be sufficient for covering the surface of a protein the size of lysozyme only if the arrangement is different than in bulk water; the polar groups distributed about the surface apparently contribute the dominant water-protein interactions; the monolayer shell must mesh simply with the bulk water for there to be no change in static properties above 0.4 *h*. If there are special water arrangements on the surface, these must be mobile, as for bulk water. Does the conformation of the dry protein differ from that in solution? Some experiments indicate that it does not, perhaps because, in the absence of water, changes in conformation are inhibited. In this connection, interaction between spin centers of lysozyme covalently labeled with a TEMPONE derivative changed little with change in hydration level from 0.02–0.6 *h*.

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SOLVENT AND TEMPERATURE EFFECTS ON CRAMBIN, A HYDROPHOBIC PROTEIN

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Crambin, a 5,000-mol. wt. water-insoluble protein found in *crambe abyssinica* seeds (1) is presently being studied by x-ray diffraction to 0.9 Å resolution (2) and ^{1}H-nuclear magnetic resonance (NMR) spectroscopy. Preliminary ^{1}H-NMR data at 250 and 600 MHz have suggested that this hydrophobic protein retains a similar globular conformation in both glacial acetic acid (AA), a Brønsted acid, and dimethylformamide (DMF), a Lewis base (3). These observations suggest that the globular conformation observed in these organic solvents is most likely the native structure present in the crystalline state. At 22–29°C, however, the DMF spectrum is significantly broadened due to aggregation, which is broken at higher temperatures. In contrast, the AA spectra are of relatively high resolution. Despite its exaggerated structural stability, requiring ~20d for the amide ^{1}H resonances to disappear in deuterated glacial AA, the protein unfolds in dimethylsulfoxide (DMSO) and in trifluoroacetic acid. We have now found that once the protein has been pretreated with AA, it can be dissolved in DMF to yield narrow-line aromatic and methyl spectra essentially identical to that of the untreated protein. The disaggregation promoted by glacial AA appears to be kinetically irreversible and to preserve the native structural features.

DMSO titration of the pretreated protein in DMF solution shows little effect below 10% DMSO/90% DMF and appearance of 100% DMSO-type spectra at a 50% solvent mixture. As DMSO is added, the transition is gradual, involving broadening (i.e., conformational exchange) and shift (to the random coil position) of tyrosyl, phenyl alanyl, and methyl peaks.

Most interestingly, the overall trend of the solvent titration spectra parallels the sequence of spectral changes caused by temperature. Heat causes unfolding, which is still incomplete up to 105°C in DMF and 85°C in AA. One of the two tyrosyl residues, Tyr_{II}, yields a "doubled" aromatic spectrum, probably because of its occurrence in a rigid conformational situation; however, its lines neither broaden nor become equivalent at the higher temperatures, suggesting a rather high interconversion barrier. Despite this, the spectra in the two solvents are indicative of increased internal mobility for side-chain groups as the temperature is raised, as can be deduced from signals broadening or sharpening according to the internal dynamics of motion as viewed by the NMR frequency window. Thus, several methyl group resonances, contributing to the broad background of the lower temperature spectra, narrow and become discernible above ~55°C, growing "out of nothing". As suggested by the high intrinsic resolution of the crystallographic x-ray diffraction pattern, and demonstrated by the NMR data, crambin is a very rigid protein. Work is in progress to assign the ^{1}H-resonances