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# Effect of alcohols on protein hydration: crystallographic analysis of hen egg-white lysozyme in the presence of alcohols

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**PDB References:** hen egg-white lysozyme, native, 1z55, r1z55sf; in the presence of ethanol, 1ykx, r1ykxsf; in the presence of 1-butanol, 1yky, r1ykysf; in the presence of 1-pentanol, 1ykz, r1ykzsf; in the presence of 2-propanol, 1yl0, r1yl0sf; in the presence of TFE, 1ykx, r1yl1sf.



Organic solvents are known to bring about dehydration of proteins, the molecular basis of which has remained uncharacterized. The dehydration effect in many cases leads to eventual unfolding of proteins through the macroscopic solvent effect. In some cases, the organic solvent molecules also bind to protein surfaces, thereby forcing local unfolding. The X-ray structure of hen egg-white lysozyme co-crystallized in the presence of alcohols with varying hydrophobicities has been studied. It was noticed that although the alcohols have very little effect on the conformation of the overall protein structure, they profoundly affect protein hydration and disorder of the bound waters. Systematic analysis of the water structure around the lysozyme molecule suggests that an increasing order of hydrophobicity of alcohols is directly proportional to the higher number of weakly bound waters in the protein. As anticipated, the water molecules in the native structure with high temperature factors ($\geq$40 Å$^2$) attain higher disorder in the presence of alcohols. It is believed that the disorder induced in the water molecules is a direct consequence of alcohol binding.

## 1. Introduction

It has long been known that hydrophobicity plays a predominant role in protein folding and stability. In aqueous solutions the interior of proteins is essentially hydrophobic, while the exterior surface is largely polar. In solvents such as alcohols, however, the behaviour of proteins is different as the overall folded state is perturbed. Alcohols destabilize the protein tertiary structures through a combination of disruption of the hydrophobic effect and alteration of ionic and hydrogen-bonding interactions. As a consequence of altered hydrogen-bonding properties, some alcohols also promote the unfolding of secondary-structure elements in proteins (Buck, 1998). Most of the work carried out to date on the effect of alcohols on secondary-structure propensities has been based on CD or NMR data (Buck *et al.*, 1993). In general, it has been observed that addition of alcohols leads to denaturation of proteins owing to the disruption of hydrophobic interactions (Buhrman *et al.*, 2003). This is corroborated by the observation that at low concentrations alcohols induce clustering of hydrophobic groups in proteins and favour a more compact structure (Calandrini *et al.*, 2000).

At the atomic level, alcohols mostly affect the protein–water interactions. As a consequence, changes in the hydration of proteins are observed. Non-polar solvents are presumed to affect the binding of protein-associated water molecules that are in constant exchange with the bulk solvent; this exchange is known to be crucial for the function of proteins (Halle, 2004). The effect of hydrophobic solvents on the behaviour and disorder of bound waters in protein structures has not been characterized however. In the present case, we have studied hen egg-white lysozyme co-crystallized with alcohols and the effect of these alcohols on protein hydration using X-ray crystallography. The effect is studied systematically for alcohols with increasing hydrophobicity.

## 2. Experimental

### 2.1. Crystallization

Crystals of hen egg-white lysozyme (HEWL) in the native form as well as in complex with various alcohols were grown in the presence

**Table 1**
Data-collection and refinement statistics.

Values in parentheses are for the last shell.

| | Native | Ethanol | 1-Butanol | 1-Pentanol | 2-Propanol | TFE |
|---|---|---|---|---|---|---|
| Unit-cell parameters | | | | | | |
| *a* (Å) | 79.21 | 79.38 | 79.29 | 79.12 | 79.26 | 79.28 |
| *c* (Å) | 37.85 | 37.82 | 37.93 | 37.79 | 37.82 | 37.95 |
| Maximum resolution (Å) | 1.9 | 1.9 | 1.8 | 1.8 | 1.8 | 1.9 |
| $R_{merge}$ | 0.11 (0.40) | 0.068 (0.274) | 0.062 (0.21) | 0.070 (0.27) | 0.069 (0.31) | 0.081 (0.24) |
| Overall completeness (%) | 99.4 (99.3) | 99.7 (100) | 99.3 (99.1) | 99.3 (100) | 98.9 (99.8) | 99.1 (100) |
| Wilson *B* (Å$^2$) | 23.10 | 24.18 | 21.67 | 24.23 | 22.22 | 23.3 |
| Final $R_{cryst}$ | 0.218 | 0.180 | 0.185 | 0.193 | 0.202 | 0.186 |
| Final $R_{free}$ | 0.247 | 0.205 | 0.223 | 0.225 | 0.243 | 0.230 |
| R.m.s. deviation (Å) | — | 0.088 | 0.094 | 0.086 | 0.168 | 0.156 |
| Ramachandran plot | | | | | | |
| Core (%) | 86.7 | 89.4 | 88.5 | 88.5 | 87.6 | 85.0 |
| Allowed (%) | 13.3 | 10.6 | 11.5 | 11.5 | 12.4 | 15.0 |
| No. of waters | 84 | 85 | 63 | 76 | 54 | 55 |
| Average *B* factor (Å$^2$) | 23.10 | 24.15 | 21.65 | 24.17 | 22.66 | 22.50 |
| Main chain | 19.79 | 20.97 | 18.68 | 20.95 | 19.70 | 20.06 |
| Side chain | 26.09 | 27.01 | 24.38 | 27.05 | 25.36 | 24.80 |
| Waters | 26.09 | 27.01 | 24.38 | 27.05 | 25.36 | 24.80 |
| Alcohols | — | 26.55 | 42.22 | 39.75 | 29.73 | 40.50 |
| PDB code | 1z55 | 1ykx | 1yky | 1ykz | 1yl0 | 1yl1 |

of 100 m*M* sodium acetate buffer pH 4.6, 1–2.5 *M* NaCl and co-crystallized with an appropriate quantity of alcohols (20% ethanol, 24% 1-butanol, 24% 1-pentanol, 20% 2-propanol and 16% trifluoroethanol) using the hanging-drop method at room temperature (Wilson *et al.*, 1991). Crystals grown in the highest possible concentration of organic solvent were used for data collection.

### 2.2. Data collection and processing

Diffraction data for HEWL complexed with ethanol, 1-butanol, 1-pentanol and TFE were collected using a MAR345 imaging plate mounted on a Rigaku rotating-anode X-ray generator and data for the native and the complex with 2-propanol were collected on a MAR345dtb. Diffraction data for the HEWL complexes with ethanol, 1-butanol, 1-pentanol and TFE were processed using the *HKL* suite of programs and scaled using *SCALEPACK* (Otwinowski & Minor, 1997). The diffraction data for the native and the 2-propanol complex were processed using *MOSFLM* and scaled using *SCALA* from the *CCP*4 suite (Leslie, 1990). All crystals belonged to the tetragonal space group $P4_32_12$ (Table 1).

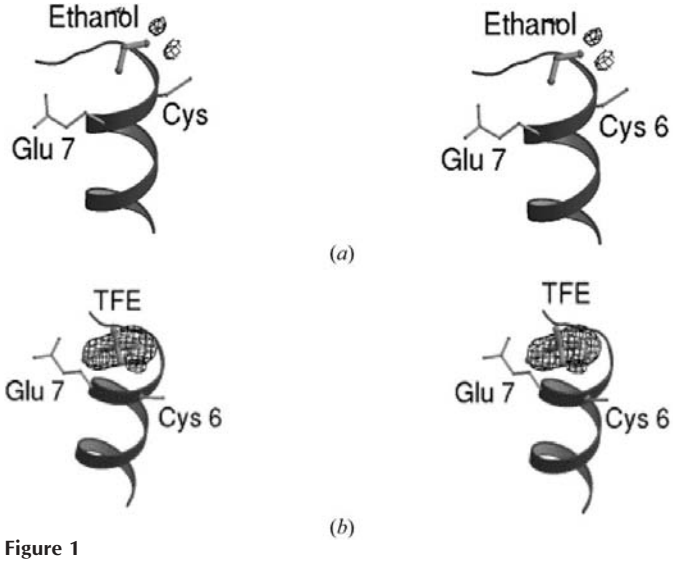


**Figure 1**
(*a*) Stereo figures of the N-terminal binding site for TFE, showing the difference density for fluorines. Electron-density maps for a representative solvent molecule, TFE, at the N-terminal site. The ($|F_o| - |F_c|$) map was calculated by modeling an ethanol molecule rather than TFE and hence peaks at 2.5$\sigma$ are observed at the fluorine sites. (*b*) Stereo figures of the N-terminal binding site. Modelling of TFE molecule in the electron-density maps at the N-terminal site. ($|F_o| - |F_c|$) maps were contoured at 3.0$\sigma$. Figures were produced using *BOBSCRIPT* (Esnouf, 1999) and rendered using *RASTER3D* (Merritt & Murphy, 1994)

### 2.3. Refinement

The atomic coordinates of the previously solved structure of lysozyme at 2.0 Å resolution (PDB code 2lym; Kundrot & Richards, 1987) were used as a starting model. The structures were refined by simulated annealing using the maximum-likelihood target as implemented in the program *CNS* (Brünger *et al.*, 1998). 5% of the total data were set aside for calculation of $R_{free}$ for monitoring the progress of refinement. $\sigma_A$-weighted electron-density maps ($|F_o| - |F_c|$) and ($2|F_o| - |F_c|$) were calculated after each cycle of refinement and visualized using the program *O* (Jones *et al.*, 1991). Water molecules were identified in the difference densities ($|F_o| - |F_c|$) contoured at 2.0$\sigma$, with at least one contact with a protein atom. The waters were identified from a peak list that was generated after calculating an ($|F_o| - |F_c|$) map. The identified water molecule was omitted from further refinement cycles if its temperature factor exceeded 80 Å$^2$ or if it appeared in negative density in the ($|F_o| - |F_c|$) map. These criteria were used to define waters in all complexes. The alcohol molecules were modelled at very late stages of refinement in the ($|F_o| - |F_c|$) difference maps contoured at 2.0$\sigma$ where the density deviated significantly from a spherical shape. The topology and parameter files for all the alcohols were obtained from the HIC-Up database (Kleywegt & Jones, 1998). The quality of the refined structure was determined using *PROCHECK* (Laskowski *et al.*, 1993).

## 3. Results and discussion

### 3.1. Crystallographic refinement and model validation

One standard protocol for the identification of waters was followed in all the complexes. The alcohol molecules were modelled in the late stages of refinement if the difference density at the 2.0$\sigma$ level deviated significantly from a spherical shape and appeared in the form of an extended shape. The difference maps were examined at all

**Table 2**
Contacts of protein with the ligand in the sugar-binding cleft of the enzyme (C site).

| | |
|---|---|
| Native | Asn59 N (contacts with water present in the ligand-binding site) |
| Ethanol | Asn59 N |
| 2-Propanol | Asn59 N |
| 1-Butanol | Gln57 O, Asn59 N |
| 1-Pentanol | Gln57 O, Asn59 N, Val 109 N |
| TFE | Gln57 O, Asn59 N, Ala 107 O, Trp108 $C^{\varepsilon 2}$, Trp108 $C^{\delta 2}$ |

stages of refinement, with particular attention to any negative density appearing on the existing water or alcohol molecules. The final $R_{\rm cryst}$ and $R_{\rm free}$ values of all the structures are listed in Table 1. The X-ray data and coordinates have been deposited in the Protein Data Bank. R.m.s. deviations in $C^{\alpha}$ positions, as shown in Table 1, indicate that binding of alcohols does not affect the overall structure of the enzyme.

### 3.2. Binding sites for the alcohol molecules

We have observed two binding sites for all the alcohols, namely the C site, the sugar-binding cleft of the enzyme, the most common for all the ligands reported so far, and an N-terminal site near Cys6 and Glu7 (Fig. 1). In the latter binding site, the hydrophobic part of the ligand interacts with Cys6 and the alcohol group with the side chain of Glu7. Binding of the ligand replaces one water molecule in the C site and two water molecules in the N-terminal region. We also notice that an increase in the chain length of the alcohols increases the interactions of the alcohol with the protein in the C site (Table 2). Interestingly, it is known that an increase in the alkyl chain length has an inhibitory effect on the activity of lysozyme. Moreover, the temperature of denaturation of lysozyme also decreases with the increasing alkyl chain length (Velicelebi & Sturtevant, 1979). Thus, our observation that alkyl chains interact intimately with the protein supports the above observations.

### 3.3. Interactions with water molecules

Keeping the criterion of protein–water distance to <3.6Å, the number of waters in the primary water shell which surround the protein was found to be maximum in the native and the ethanol complex (Table 1). In comparison, the complexes of the alcohols with higher hydrophobicity, such as TFE, show a smaller number of water molecules in the primary shell. Nearly half the water molecules in all the complexes had only one interaction with the protein molecule, while almost a quarter of them had two contacts and less than a quarter had three contacts. Defining water bridges as those water molecules which hydrogen bond to two or more amino-acid residues, the maximum number of water bridges was found in the 1-pentanol complex.

### 3.4. Study of disordered waters

Upon studying water molecules with *B* factors in excess of 40 Å$^2$ of the native structure and comparing them with the corresponding waters in the complexes, we find that out of a total number of 84 waters in the native structure there are 25 water molecules with a high *B* factor and that these waters become more poorly bound as the hydrophobicity of the solvent increases (Table 3). It is to be noted that the interactions of these 25 water molecules are with residues with temperature factors less than 40 Å$^2$.

The hydrophobicity of alcohols appears to have a remarkable effect on protein hydration. TFE, the most hydrophobic solvent used in the present study, shows the presence of only 55 water molecules. We refer to water molecules that are present in one structure but

**Table 3**
List of water molecules with *B* factors higher than 40 Å$^2$.

The numbers in parentheses represent water molecules which correspond to those in the native structure with a *B* factor greater than 40 Å$^2$.

| Lysozyme–alcohol complexes | No. of waters with *B* factor $\geq$ 40 Å$^2$ |
|---|---|
| Native | 25 |
| Ethanol complex | 34 (9) |
| 1-Butanol complex | 12 (8) |
| 1-Pentanol complex | 21 (7) |
| 2-Propanol complex | 9 (4) |
| TFE complex | 6 (3) |

absent in the rest of the structures as unique waters. The numbers of unique waters are thus at a maximum in the native structure. With increasing hydrophobicity of the alcohols, the number of unique water molecules falls. This is partly owing to the fact that some of the water molecules that are present in the ligand-binding site are displaced upon binding of the ligand. However, the effect of the hydrophobicity of alcohols is most prominently seen in the secondary hydration shell. The waters that belong to the secondary hydration shell are weakly bound in response to the hydrophobicity of the alcohols. Thus, hydrophobicity of solvents has an overall dehydration effect on the protein structure.

It is a well established fact that the hydrophobicity of the short-chain aliphatic alcohols increases with increasing chain length (McKarns *et al.*, 1997). The series of alcohols studied here are in increasing order of hydrophobicity, *i.e.* ethanol, 2-propanol, 1-butanol, 1-pentanol and TFE in that order. Proteins in hydrophobic solvents are thought to retain their native structure as a result of kinetic trapping, which results in a more rigid structure in the absence of water. Polar solvents can easily strip water from the protein and compete with hydrogen bonds between the protein atoms. Following this mechanism, solvents such as DMSO, dimethylformamide (DMF), urea *etc*. usually denature the protein by unfolding (Pike & Acharya, 1994; Mande & Sobhia, 2000; Mattos & Ringe, 2001). On the other hand, alcohols disrupt the tertiary structure and do not disturb the secondary-structure interactions (Liepinsh & Otting, 1997; Mattos & Ringe, 2001). The molecular mechanism of protein structure disruption by alcohols has, however, remained unknown.

We have observed that although the numbers of water molecules are the same in the native structure as well as in the ethanol complex, the numbers of waters in the ethanol complex with *B* factor > 40 Å$^2$ are much higher in number. We believe that this effect is a consequence of the property of water and ethanol to form an azeotropic mixture, owing to which the waters in the ethanol complex possibly become disordered.

The effect of solvent hydrophobicity on poorly ordered waters becomes more obvious as we move from the complexes of ethanol, 1-butanol, 2-propanol and 1-pentanol to TFE. We observe that compared with the 25 common waters that are in the weakly bound state in the native molecule, 16 are not seen in the ($|F_{\rm o}| - |F_{\rm c}|$) density in the ethanol complex, 17 in the 1-butanol, 18 in the 1-pentanol and 21 and 22 in the isopropanol and TFE complexes, respectively. This supports our view that as the hydrophobicity increases, the number of waters that become disordered increases and this number would probably increase with increasing concentration of the solvent. Thus, the hydrophobicity of the solvent plays an important role in protein dehydration.

The reduction in the number of bound waters in the presence of alcohols that we have observed in the present case was also observed earlier in crystals soaked in very high concentrations of alcohols, where the soaking with alcohol had an altered effect on the affinity of

organic molecules (Imoto *et al.*, 1972). In the studies carried out on Ras crystals in the presence of TFE and 2-propanol (Buhrman *et al.*, 2003), authors have reported drastic reduction in the number of waters in the presence of TFE and no effect on the numbers of waters in the 2-propanol complex. It is notable that in the Ras–2-propanol complex, whereas the number of waters is almost equal to that in the aqueous solution structure, the number of waters with high temperature factors is greater in the 2-propanol complex. These observations are comparable to our results with the ethanol–lysozyme complex. We have noted a similar trend in the lysozyme structures at low humidity (Kodandapani *et al.*, 1990), with an acetonitrile–water mixture (Wang *et al.*, 1998), crystals grown under microgravity (Vaney *et al*., 1996) and the thermal stability mutants of lysozyme (Shih *et al.*, 1995). The observations made corroborate our point of view that the water molecules become weakly bound with increasing hydrophobicity of the bound solvent.

In conclusion, we have obtained a glimpse into the effect of alcohols on protein hydration. We observe that as the hydrophobicity of solvent increases HEWL becomes serially dehydrated. Water molecules become intrinsically disordered in response to hydrophobicity of co-solvents. Secondary-shell waters are those that become disordered first with the addition of hydrophobic solvent.

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