

WATER STRUCTURE IN VITAMIN B₁₂ COENZYME CRYSTALS

II. Structural Characteristics of the Solvent Networks

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ABSTRACT The geometrical details of the solvent structure in vitamin B₁₂ coenzyme crystals with respect to hydrogen bonding and nonbonded contacts, are described. The individual H-bond geometries varied over wide ranges, similar to those observed in small molecule structures. Large deviations from tetrahedral coordination were found around a majority of the waters. The mutual positions and orientations of the water molecules could not be adequately explained in terms of the H-bonding relationships present in the structure. However, additional investigations, which focused on the short range nonbonded contacts around water positions in a variety of crystal hydrates, revealed several structural regularities (Savage, 1986b). These features relate to the nonbonded O...O, H...O, and H...H interactions, and give rise to a set of repulsive restrictions that are seen to be very much stronger stereochemical restraints than those associated with H-bonding. The short-range restrictions appear largely to govern the local orientational correlations and packing arrangements of the water structure within the coenzyme (and other hydrate) crystals. In more general terms, the inclusion of the nonbonding relationships as well as the attractive H-bonding interactions, leads to a significant increase in our understanding of water structure(s). The repulsive restrictions can be used as stereochemical restraints in the interpretation and refinement of solvent structures within larger hydrate systems, such as protein crystals. They may also be included in potential functions used to simulate solvent structures in aqueous solutions and hydrate systems.

INTRODUCTION

Relatively little is known about the mutual positions and orientations of water molecules with respect to water-water and biomolecular-water interactions that occur in disordered environments such as aqueous solutions, protein and DNA crystals, particularly at the molecular interfaces. The most accurate information to date has been obtained from high-resolution neutron diffraction studies (<1.0 Å) of small hydrate crystals (Ferraris and Franchini-Angela, 1972; Pederson, 1974; Chiari and Ferraris, 1982) that contain only a small number of water molecules (often less than five), and the ice polymorph structures (reviews by Whalley, 1976; Kamb, 1983). Within most of the hydrate structures, both the water oxygens and hydrogens are observed to be fully ordered and tightly bound to surrounding polar (and ionic) groups. Of the seven ice polymorph structures that have been studied by neutron diffraction, the hydrogens in only two of them are fully ordered (ices II and VIII), while in the remainder they are either partially or fully disordered.

Only a handful of larger or medium sized hydrate structures (100–300 atoms) containing more than five waters, some of which may be disordered, have been studied by neutron diffraction to atomic resolution (<1.0 Å). These include α and β cyclodextrins (containing 6 and ~12 waters, respectively, see Klar et al., 1980; Betzel et al., 1984), monoacid derivative of vitamin B₁₂ (~15 waters, see Moore et al., 1984), and coenzyme B₁₂ (~17 waters, see Savage et al., 1986); of which the latter system is discussed in this article.

In the preceding paper (Savage, 1986a), details of the examination of the solvent densities (neutron and x-ray) and formulation of possible water networks in coenzyme B₁₂ crystals were described. Here, we discuss the geometrical details of the coenzyme water structure (H-bond geometries, etc.) and more explicitly, some new features of the overall organization of water that have come to light through the examination of the intricacies of the short-range, nonbonded interactions around individual water molecules in this system.

This latter inquiry was initiated from problems that arose in understanding local water structure in terms of the conventionally accepted wisdom. For example, it was not clear why individual water molecules occupied particular

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orientations, or when the standard conventions (linear H-bonding, tetrahedral coordination, etc.) appeared to suggest otherwise. These problems prompted a more general survey on a broader basis, which involved the analysis of a large number of small hydrate and ice polymorph structures that have been studied by neutron diffraction: details of this analysis are described elsewhere (Savage, 1986b). Several nonbonded regularities were found to occur and summarized below in the section on short range structure.

In the subsequent section, the implications of the short-range regularities within the coenzyme water structure are outlined in terms of how the waters orientate themselves and pack together. It is seen that the H-bonding coordinations around the water molecules vary significantly from a tetrahedral framework: none are truly tetrahedral. The actual water geometries present depend decisively on the repulsive characteristics of the nonbonded interactions, in particular the remote H...O nonbonded contacts. The tetrahedral structure of ice I (and VIII) appears to be a special case in which all the interactions (both attractive and repulsive) are fully optimized.

SUMMARY OF SOLVENT ANALYSIS

The solvent distribution in vitamin B₁₂ coenzyme crystals was analyzed using neutron and x-ray diffraction. One set of neutron and two sets of x-ray data were measured from crystals grown in D₂O and acetone, to the following resolutions: 0.95 Å (neutron), 1.1 Å (x-ray1) and 0.94 Å (x-ray2). The experimental details are described in Savage et al. (1986). After the three respective models were refined (using least-squares and difference Fouriers) the solvent regions in each were examined in detail.

Solvent Density Analysis

The solvent regions were examined in two stages (details in Savage, 1986a). First, "main" sites were assigned to the better defined density, representing the more ordered solvent. All these assigned solvent sites were included in the least squares, except for some that were less than ~1.0 Å (the resolution of the data) apart. In the second part, "continuous" sites were assigned to represent the more disordered diffuse and elongated solvent density occurring around and between the "main" sites.

In each of the neutron, x-ray1 and x-ray2 models an acetone molecule (with a partial occupancy) was located in the same relative position. This molecule completely separates the water distribution in the crystal into two distinct regions, "pocket" and "channel" (see Fig. 1). Each asymmetric unit within the crystal is composed of a central "channel" region forming a continuous path through the crystal, and a "pocket" region that leads off from the central channel into a blind alley. In Fig. 1 the pocket is situated to the left of the acetone molecule while the channel is on the right. Between 16 and 18 water molecules

per asymmetric unit were estimated to be present from density measurements.

Water Networks

The above three models (neutron, x-ray1, x-ray2) and also the model obtained from the original structure determination (1968x-ray: Lenhert, 1968) were used to formulate water network structures from the "main" assigned sites. This was done by assigning H-bonds between polar atoms using the accepted limits criteria of H-bonding (O...O = 2.5–3.3 Å, O-D...Y angles >130°, X...O...Y angles 65°–150°, van der Waals contacts, etc. (see Savage, 1986a for details).

The networks extend throughout all the solvent regions of the crystal, and can be divided into two major groups, one with the acetone molecule present and one without. Within each of the two regions (pocket and channel) they are further divided into subgroups and also with respect to the disordered *c* side chain that is situated in the pocket region. The assigned sites within each of the networks are described in Savage, 1986a. The water within the solvent regions is seen to be partially ordered and partially disordered with, as expected, an apparent increase in order when the acetone molecule is present. The water in the channel region appears to be more disordered than in the pocket region.

When the acetone molecule is present, up to 14 or 15 water molecules can occupy each asymmetric unit, forming one main water network in the pocket region, Network *A*, and two main water networks in the channel region, Networks *B* and *C* (Fig. 11, Savage, 1986a). In the absence of the acetone molecule, a maximum of ~17 or 18 water molecules (density measurements) may be present. The networks formulated in this situation are labeled *D–J*. Local disorder around many of the "main" solvent sites was also observed, but clear unambiguous networks could not be formulated from the "continuous" sites assigned to the more diffuse regions.

The individual networks were observed to be consistent among the four models, with the assigned sites of each network occupying relatively similar positions. However, the occupancies of the individual networks were frequently seen to have different values among the four models, sometimes being highly occupied in one model but barely visible in another.

COVALENT GEOMETRIES: ACETONE AND WATER MOLECULES

The covalent bond lengths and angles for the acetone molecule located in the neutron, x-ray1 and x-ray2 models are listed in Table I. The geometries are within 2σ of the expected values found in small molecule structures.

Histograms of the W-D distances and D-W-D angles in the neutron model are given in Fig. 2 *a*. The W-D distances

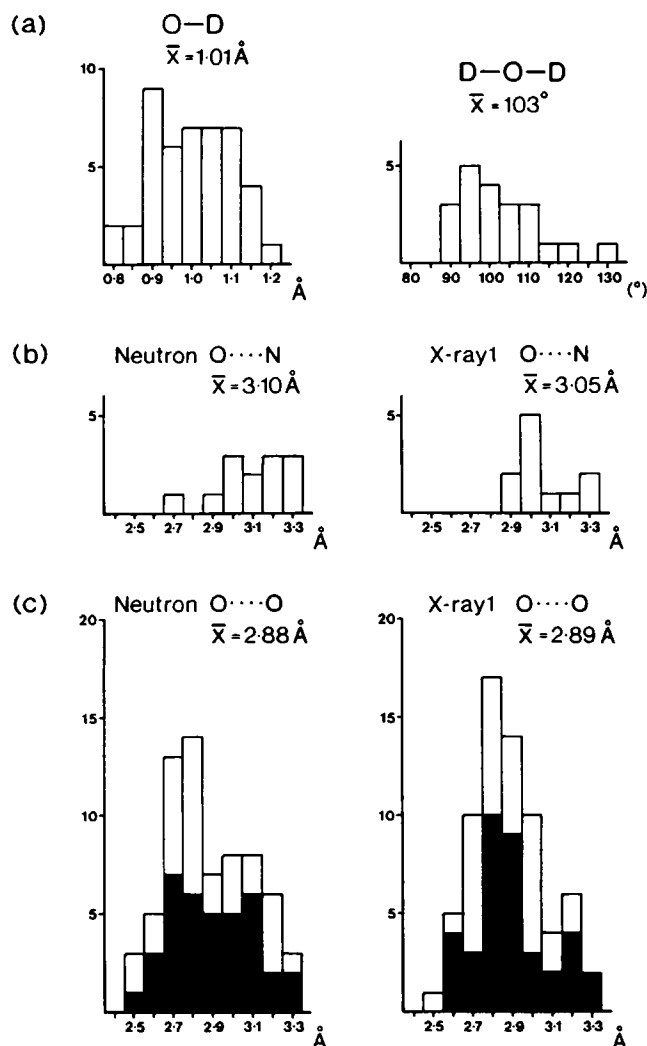


FIGURE 2 Water-water and water-coenzyme hydrogen bond geometries. (a) W-D distances and D-W-D angles from the neutron model, (b) O...N H-bond distances from the neutron and x-ray1 models and (c) O...O H-bond distances from the neutron and x-ray1 models. Shaded and unshaded areas represent the water-water and water-coenzyme geometries, respectively.

X...Y Distances

The respective distances in each of the four models are given in Table II. In the neutron and x-ray1 models the SDs of these distances range from 0.01 to 0.20 Å with the larger values corresponding to the more disordered solvent sites. Several of the solvent sites in the neutron model were seen to overlap, thus the SDs of distances involving these sites were not calculated and were estimated to be of the order of 0.1 to 0.2 Å. The SDs for the x-ray2 model are approximately twice those obtained for the x-ray1 model, while those for the 1968x-ray model were estimated (Lenhert, 1968) to be of the order of 0.06 Å or larger. Histograms of the various O...N and O...O distances for the neutron and x-ray1 models are shown in Figs. 2 b and c.

There is reasonable agreement of the corresponding X...Y distances between the neutron, x-ray1 and x-ray2

TABLE II
COENZYME-WATER AND WATER-WATER X...Y
H-BOND DISTANCES (Å) IN THE NEUTRON, X-RAY1,
X-RAY2, AND 1968X-RAY MODELS

Atoms	Neutron	X-ray1	X-ray2	1968x-ray
O33.....D-O212	2.99	2.99	2.97	—
D-O601	2.65	2.74	2.67	2.67
O39.....D-O213	2.70	2.79	2.73	2.88
O418	—	3.11	3.25	3.29
O44.....D-O223	2.90	3.03	3.04	2.75
D-O423	2.78	2.75	2.78	3.23
D-O623	2.82	2.72	2.72	—
O51.....D-O226	2.75	2.72	2.72	—
D-O227	2.78	2.92	3.01	—
D-O426	2.50	2.51	2.44	2.60
O626	—	2.98	3.09	—
O58.....D-O213	2.74	2.70	2.63	2.55
D-O423	3.27	3.18	2.98	(3.49)
O62.....D-O212	3.16	3.04	3.06	—
D-O217	2.70	2.78	2.79	—
D-O417	2.79	2.87	3.15	—
D-O604	2.77	—	2.63	2.92
O605	—	—	—	3.15
OP2.....D-O211	3.02	2.98	2.97	—
OP5.....D-O603	—	—	—	3.17
OR7-D....O216	2.87	2.70	2.74	—
O608	2.46	—	—	2.62
OR8.....D-O210	2.78	2.82	2.88	—
D-O410	2.65	2.62	2.62	—
D-O610	3.25	3.23	3.34	3.03
D-O810	3.09	2.82	2.89	3.01
O425	3.38	2.91	2.99	—
O625	—	3.05	3.35	—
OA17..D-O214	2.80	2.80	2.79	2.72
D-O414	3.23	3.05	3.38	—
D-O614	3.17	2.78	2.84	—
OA17-D..O222	2.67	2.72	2.68	2.70
OA18..D-O231	3.04	2.88	2.96	—
D-O431	3.07	2.84	2.94	—
D-O631	2.72	2.89	3.03	—
D-O831	—	3.09	3.18	3.32
N40-DO211	2.98	2.91	3.01	—
O212	3.01	2.97	3.05	—
N52-DO214	3.02	2.95	2.97	3.17
O414	3.26	3.24	2.71	—
NA3.....D-O215	3.05	2.90	2.93	3.21
D-O415	3.22	3.01	3.11	—
D-O815	2.93	3.03	3.31	—
NA10..D-O601	3.12	3.27	3.10	3.12
NA7.....D-O218	3.24	2.99	2.98	—
N59-DO211	3.20	3.06	3.13	—
O604	2.74	—	2.72	2.99
N45.....O815	3.26	3.27	3.17	—
O235	3.28	—	3.14	—
O211-D...O217	2.76	2.88	2.83	—
O213...D-O223	2.63	2.79	2.73	—
D-O623	2.61	2.78	2.72	—
O214...D-O215	2.86	2.69	2.70	2.96
O415	2.87	2.90	3.22	—
O815	2.93	2.84	2.88	—
O210	2.85	2.81	2.85	—
O410	3.06	2.88	2.90	—
O810	3.08	2.91	2.90	2.63
O215.....O228	3.29	3.16	3.28	—
O428	3.45	3.00	3.04	—

TABLE II (cont.)

Atoms	Neutron	X-ray1	X-ray2	1968x-ray
O414	3.18	3.03	3.03	—
O614	2.81	—	2.78	—
O216-D...O217	3.01	2.88	2.97	—
O219	2.82	2.94	2.94	—
O220	2.56	2.82	2.81	—
O217-D...O218	2.68	2.58	2.50	—
O218-D...O223	3.19	3.15	3.21	—
O222-D...O228	2.83	2.78	2.76	—
O628	2.70	2.66	2.96	—
O231	2.53	2.77	2.67	—
O610	2.96	2.95	2.79	3.05
O226...D-O231	2.93	2.81	2.93	—
O227.....O431	3.05	2.75	2.84	—
O228.....O410	3.07	2.81	2.66	—
O231.....O210	2.71	2.89	2.74	—
O410	3.07	3.27	3.41	—
O410.....O431	3.30	3.16	3.26	—
O614	2.99	3.17	2.94	—
O628	—	3.08	3.05	—
O414.....O415	2.94	3.02	3.38	—
O615	2.69	2.80	2.75	—
O610	3.04	2.61	2.91	—
O418.....O623	—	3.18	3.07	—
O426.....O631	3.13	3.27	3.17	2.96
O428.....O431	—	2.60	2.66	—
O601...D-O604	2.75	—	2.83	2.61
O603-D...O604	2.71	—	2.90	2.79
O603.....O608	2.76	—	—	2.80
O618	2.83	—	—	2.91
O605.....O608	—	—	—	2.75
O610.....O810	3.08	—	—	2.98
O614.....O615	2.68	2.59	2.83	—
O631.....O626	—	2.91	2.76	—

models for the more ordered water sites of the networks *A*, *B*, and *C*. The deviations are in the order of ~ 0.2 Å (see Table VI; Savage, 1986a). For the more disordered water sites associated with the remaining networks (*D*–*J*), the average deviation is 0.4 Å. The larger discrepancies (up to 0.7 Å) mainly correspond with sites that possess relatively large temperature factors and small occupancies. The O...O and O...N distances among the four models range from 2.5 to 3.3 Å and from 2.7 to 3.3 Å, respectively. The means of these distances are 2.88 and 3.10 Å in the neutron model, and 2.89 and 3.05 Å in the x-ray1 model (Fig. 2).

D...Y Distances

Fig. 3 *a* shows a histogram of the D...Y distances for the H-bonds made by the water molecules. The distribution lies between the expected values of 1.6 and 2.4 Å for hydrogen bonding water molecules (Chiari and Ferraris, 1982).

X-D...Y Hydrogen Bond Angles

The distribution of these angles is shown in Fig. 3 *b* and range from 174° down to 130°. The majority of the

hydrogen bonds possess angles of between 150° and 170°, which is in agreement with the values generally observed in small molecule structures (Hamilton and Ibers, 1968).

X...O(W)...Y Angles

The X...W...Y angles subtended at the hydrogen-bonded water oxygens vary from 62° to 154° (Fig. 3 *c*), while the D,X...D,W...D,Y angles involving the deuterium sites range from 76° to 158°. For the waters that accept at least two H-bonds, the D...W...D angles vary between 76° and 110°, while the X...D-W-D...Y donor angles vary between 84° and 154°. The angles of the X-D...W-D...Y arrangement are between 83° and 158°. The fairly low distribution of the D...W...D angles (76°–110°) may imply that the two lone pairs of a water molecule are not well separated from each other. This is borne out in *ab initio* quantum mechanical calculations (Popkie et al., 1973; Dierksen, 1971), and in small molecule hydrate structures (Olovsson and Jönsson, 1976; Savage, 1986b). The large variations of the angles around the central water oxygen, compared with the angles of the classical tetrahedral disposition of the H-bonds around a water molecule, suggest that the lone pairs on the water oxygen exert only a weak control on the directionality of the bonds made to this region and that additional factors such as O...D (remote near neighbors) and D...D interactions should also be considered.

Discussion

Nearly all the values of the hydrogen bond parameters involving the main solvent sites fall within the accepted limits that are considered to constitute a hydrogen bond (Hamilton and Ibers, 1968; Olovsson and Jönsson, 1976). A wide spread is observed for all the individual geometries: O...O = 2.5–3.3 Å, O-D...O angles from $\sim 130^\circ$ to 174° , X...O...Y angles from 62° to 158°. These ranges are very similar to those observed in small hydrate systems (Chiari and Ferraris, 1982).

In small molecule structures an approximate correlation between the X-D...Y angle and D...Y distance is often inferred: the X-D...Y angle becomes more linear as the D...Y distance decreases (the X and Y atoms become more repulsive as they approach each other). Fig. 3 *d* shows a plot of these two parameters for the water H-bonds in the coenzyme B₁₂ hydrate. The full line in the figure shows the correlation obtained for small molecules (Olovsson and Jönsson, 1976); no clear trend can be seen, only a wide scatter.

A rationalization of the local water structure on the basis of the wide variations in the H-bond geometries observed, seemed to be somewhat impossible. However, when the local short-range nonbonded contacts were examined in closer detail, some intriguing regularities became apparent (see below).

TABLE III
COENZYME-WATER AND WATER-WATER H-BOND GEOMETRIES (X-D...Y) IN THE NEUTRON MODEL

ATOMS	O-D distances	D-O-D angle	O-D...Y angle	D...Y distance	X...Y distance	X...O...Y angle
	Å		degrees		Å	degrees
N40-D196.....O212	1.08		149	2.03	3.01	
-D197.....O211	0.99		146	2.10	2.98	
N52-D200.....O214	1.01		152	2.09	3.02	
O414	1.01		145	2.38	3.26	
N59-D136.....O211	1.00		152	2.29	3.20	
O604	1.00		149	1.83	2.74	
OR7-D206.....O216	0.94		167	1.95	2.87	
O608	0.94		130	1.75	2.46	
OA17-D208.....O222	0.87		162	1.83	2.67	
O211-D302.....O217	0.90		174	1.86	2.76	
-D303.....OP2	0.86	114	143	2.29	3.02	133
O212-D304.....O62	0.96		167	2.22	3.16	
-D305.....O33	0.93	111	133	2.27	2.99	84
O213-D306.....O39	1.05		159	1.69	2.70	
-D307.....O58	1.09	92	158	1.69	2.74	116
O214-D308.....O410	1.16		160	1.95	3.06	
D309.....OA17	1.01	103	160	1.84	2.80	120
O214-D708.....O215	0.95		163	1.94	2.86	
D309.....OA17	1.01	110	160	1.84	2.80	112
O215-D310.....NA3	1.15		164	1.93	3.05	
D311.....O214	0.96	107	163	1.91	2.86	106
O216-D312.....O217	1.13		152	1.97	3.01	
D313.....O219	0.85	104	172	1.98	2.82	123
O217-D314.....O218	0.91		148	1.87	2.68	
D315.....O62	0.93	101	155	1.82	2.70	129
O218-D316.....NA7	1.12		163	2.15	3.24	
D317.....O223	1.02	100	159	2.22	3.19	121
O222-D324.....O228	1.05		133	2.02	2.83	
D325.....O231	0.96	89	157	1.62	2.53	101
O223-D326.....O44	0.82		156	2.13	2.90	
D327.....O213	0.95	128	161	1.72	2.63	110
O226-D332.....O51	1.03		159	1.76	2.75	
-D333.....O214	0.99	103	172	(2.45)	(3.43)	86
O231-D338	1.17					
D339.....O226	0.93	89	147	2.11	2.93	
O231-D538.....OA18	1.12		172	1.93	3.04	
-D339.....O226	0.93	96	147	2.11	2.93	107
O410-D500.....OR8	0.94		150	1.79	2.65	
D501.....O231	1.12	97	156	2.02	3.07	108
O210-D300.....OR8	0.91		173	1.87	2.78	
D301.....O431	1.07	118	163	1.64	2.68	120
O414-D509.....OA17	0.91		169	2.34	3.23	
O417-D715.....O62	1.27		150	1.62	2.79	
O423-D526.....O44	0.93		165	1.88	2.78	
-D527.....O58	0.82	112	148	(2.55)	3.27	106
O623-D726.....O44	1.08		169	1.75	2.82	
D727.....O213	1.02	105	163	1.62	2.61	113
O601-D704.....O33	1.02		148	1.73	2.65	
D705.....NA10	1.04	94	170	2.10	3.12	112
O603-D702.....O604	1.12		146	1.70	2.71	
O604-D706.....O601	1.15		170	1.61	2.75	
D707.....O62	1.13	97	161	1.68	2.77	93
O810-D900.....OR8	0.98		144	2.25	3.09	
O815-D311.....O214	1.06		159	1.91	2.93	
D510.....NA3	1.05	96	147	2.00	2.93	107

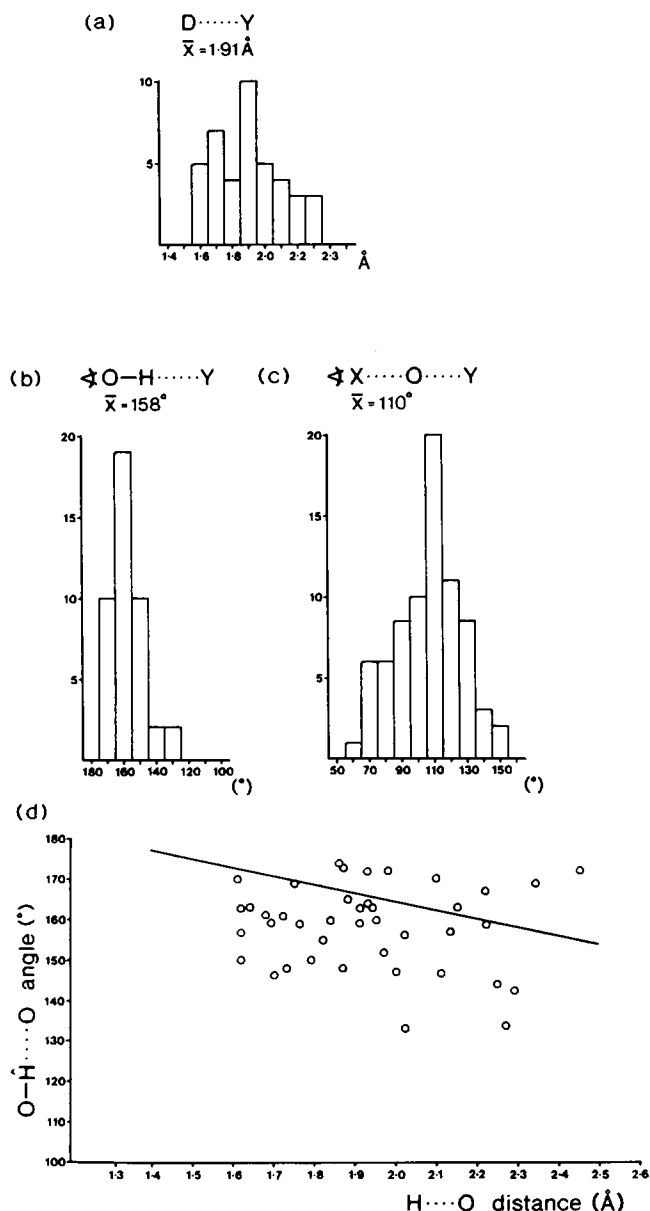


FIGURE 3 Water-water and water-coenzyme hydrogen bond geometries of the neutron model. (a) $D \cdots Y$ distances, (b) $X-D \cdots Y$ H-bond angles, (c) $X \cdots O \cdots Y$ H-bond angles, and (d) plot of $O-D \cdots Y$ H-bond angles vs. respective $D \cdots Y$ distances.

COORDINATION

Defined as the number of hydrogen bonds that a water participates in, the coordinations around the water molecules vary from 1 to 5, depending on the nature of the local environment: polar and/or apolar. The most frequent coordinations are 3 and 4. Within the polar environments, the waters are mainly 4-coordinated, but the directional influence is not seen to be specifically tetrahedral as found in ice Ih. A wide spread in $X \cdots O(W) \cdots Y$ angles is present (Fig. 3 c). Three coordinated waters occur mainly where there are apolar environments, for example, waters 212, 216, and 218 in Fig. 1 have apolar CH and CH_3 groups

(some shown) surrounding them. Several 2- and 5-coordinated waters are found, where there exists a high population of either apolar groups (2-coordinated: e.g., 227 and 423) or polar groups (5-coordinated: e.g., 211).

Approximately 60% of the waters are 3-coordinated (some very close to being trigonal planar), 30% are 4-coordinated, and the remainder are a mixture of 1, 2, and 5. Several ring structures of waters (based on $O \cdots O$ distances; some including hydroxyl groups) are present containing from 3 to 7 water molecules. However, some of these rings are not completely closed in terms of H-bonding.

SHORT RANGE STRUCTURE: NON-BONDED CONTACTS

In the above two sections, the structural characteristics of the water organization were discussed in terms of attractive H-bonding interactions. Wide variations occurred for each of the different geometries: $D \cdots Y$ distances ranged from 1.6 to 2.4 \AA and $O-D \cdots Y$ angles between 130° - 174° . Furthermore, the H-bonding coordinations around the majority of waters were found to deviate significantly from tetrahedrality with $X \cdots O \cdots Y$ angles down to 62° .

The large variations in these geometries indicate that stereochemical restraints involved with H-bonding systems are very weak or at worst, nonexistent and would thus, allow significant local disorder. However, many of the waters appeared to be held in relatively ordered orientations that could not be readily accounted for with respect to the standard concepts of H-bonding (linear bonds, $H \cdots O > 2.4 \text{ \AA}$, etc.) and nonbonded van der Waals contacts (e.g., $O \sim 1.5 \text{ \AA}$, $H \sim 1.0 \text{ \AA}$). Moreover, it was not at all clear why such large deviations in H-bond geometries should be present: some short H-bonds appeared to be very bent while some longer ones were very straight.

Many of the waters can be better positioned to optimize the accepted H-bonding and nonbonding criteria. For example water 211 forms 3 relatively weak H-bonds ($D \cdots O = 2.1, 2.3, \text{ and } 2.3 \text{ \AA}$) and one of medium strength ($D \cdots O = 1.9 \text{ \AA}$). By moving closer to the phosphate and nitrogen groups, this water can be reorientated (counter-clockwise) to strengthen the 3 weaker H-bonds. Water 217 may then move closer to 211. However, the experimental neutron data does not show this in the solvent density maps (see Fig. 3 in Savage, 1986a).

Water 217 is four coordinated, but not in a tetrahedral arrangement. It is almost trigonal planar with respect to waters 211 and 218 and the carbonyl oxygen O62, while water 216 is almost perpendicular with the trigonal plane. The H-bond between waters 216 and 217 is relatively long ($D \cdots O = 2.0 \text{ \AA}$) and the questions can be raised: why is this bond not shorter, and why is the coordination around 217 not of a tetrahedral character?

The inability to explain the mutual orientations of the experimental water positions in the coenzyme crystal led to some broader investigations to see if other interactions,

such as short-range nonbonded contacts around individual water molecules, played a significant role in determining the structure. The characteristics of the nonbonded geometries were examined in a number of small hydrate crystals (containing 1–18 waters; Savage, 1986b) that have been studied to atomic resolution by neutron diffraction. From this survey, several intriguing features of the water organization were apparent, which are of relevance to both hydrated systems and water-water interactions in general. The main structural regularities are summarized in the next section.

Short-Range Repulsive Limitations

The nonbonded O...O, H...O, and H...H contacts in a variety of neutron hydrate structures were analyzed and four different structural regularities formulated in terms of repulsive restraints: these are defined in Fig. 4 *a*. The minimum van der Waals contacts around water molecules appear to be angular dependent (Figs. 4 *b* and *c*). The main characteristics of the four short-range components are as follows:

RR (Repulsive Restrictions)1: O...O Repulsions of H-bonds. As H-bonds increase in length, they can bend to a significant degree, and standard plots of the O-H...O H-bond angles vs. the H...O and O...O bond lengths for accurate small H-bonding structures tend to show this effect. Fig. 5 *a* shows such a plot for H-bond geometries of waters examined. A correlation line can be drawn (not shown) through the points representing a mean decrease in the angle as the length increases. However, at longer H-bond lengths a larger scatter in the values is observed: some bonds are very bent and some almost linear. A cutoff line can be drawn that represents the limits of H-bonding geometries, the H-bond bending limit curve (full line in Fig. 5 *a*), due to the repulsion between the oxygens. This line effectively divides the geometrical conformations into an excluded region below the curve and an accessible area above. The actual angles and distances H-bonds may have, depend mainly on the local packing arrangements determined by the surrounding short range contacts: RR2-RR4.

RR2: O...O Nonbonded Contacts. Values used for the van der Waals radius for a water oxygen range from ~1.4 to 1.7 Å, and some confusion arises as to what the acceptable minimum nonbonded O...O contact may be (2.8–3.4 Å). In addition to this, H-bonds are often assigned between oxygen positions (in x-ray structures) that are up to 3.4 Å apart. In the hydrates examined, the minimum water-water nonbonded O...O contact in the survey was found to be of the order of 3.1–3.2 Å. Furthermore, the water oxygens appeared to have an anisotropic repulsive core depending on the orientation of the water molecule. Fig. 5 *b* shows the distribution of minimum contact distances of <3.5 Å observed in small hydrates with respect to

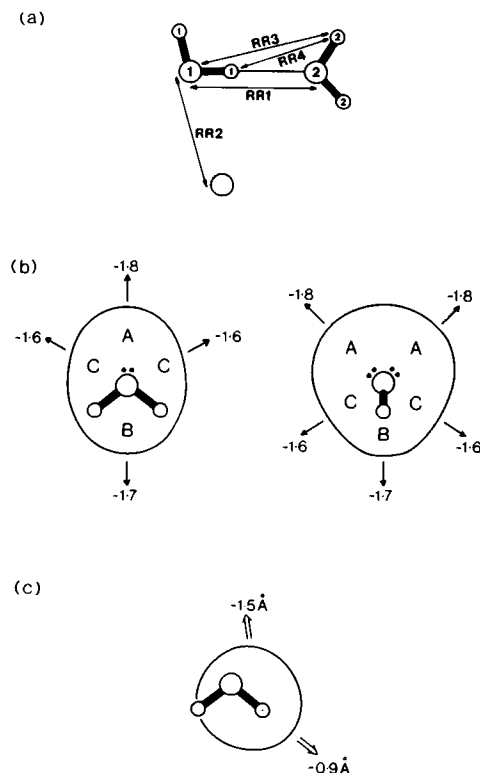


FIGURE 4 Schematic diagrams showing (a) the four RR nonbonded contacts: RR1, O...O H-bond repulsion; RR2, O...O nonbonded repulsion; RR3, H...O remote nonbonded neighbor contacts; RR4, H...H contacts, and (b) and (c) proposed angular dependencies of the van der Waals radii for water *b*, oxygen and *c*, hydrogen atoms.

two orientational angles, θ_1 and θ_2 , defined for the water molecule (see inset motif in Fig. 5 *b*). Three regions may be approximately assigned with the following van der Waals radii: (region A) ~1.8 Å over the lone pairs region, (region B) ~1.7 Å between the hydrogens, and (region C) ~1.6 Å between the lone pairs and the hydrogens.

RR3: H...O Remote Neighbor Interactions.

The nonbonded H2...O1 contacts of the configuration H2-O2...H1-O1, were seen to have a minimum contact distance of ~3.0 Å in all the high-resolution neutron structures examined: the O-H covalent distances were set to 0.8 Å to represent a possible center of interaction. In Fig. 6 the H2...O1 contacts around the water molecules of networks A and B in the coenzyme hydrate are shown. The minimum H2...O1 contact of ~3.0 Å appears to be retained (within experimental errors) in the different H-bonding configurations. When the H2-O2...O1 angle (ϕ), of the H2-O2...H1-O1 configuration, is plotted against the O1...O2 H-bond distance (Fig. 7 *a*), an approximate limiting line (full line) may be drawn dividing the space into accessible and excluded regions. As the O1...O2 H-bond distance decreases, the ϕ angle increases to maintain the minimum H2...O1 contact distance of ~3.0 Å (see Fig. 8 *a*).

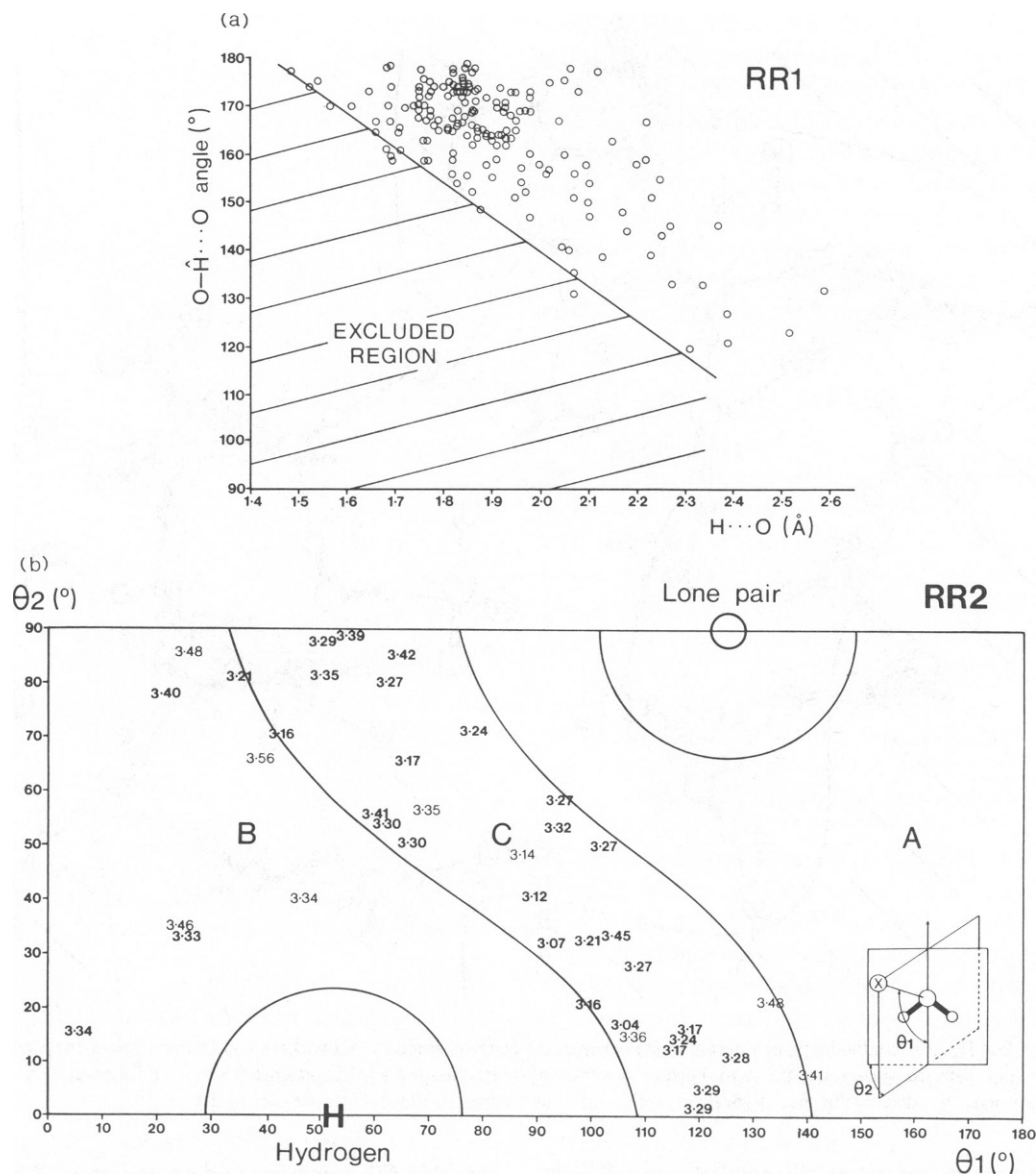


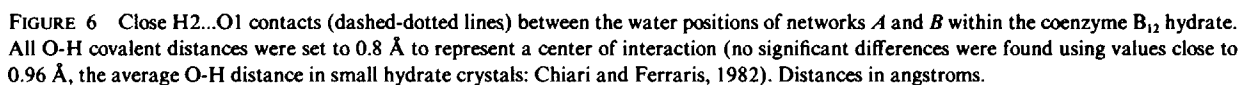
FIGURE 5 (a) Plot of O-H...O angle vs. H...O distance for water H-bonds in small crystal hydrates (neutron structures). The solid curve approximately represents minimum allowed values; H-bonding configurations that lie significantly below the curve (excluded region) are not allowed due to repulsion between the oxygens. (b) θ_1/θ_2 orientational angle (defined in the inset motif) plot for nonbonded O...O,C contacts (in angstroms) of <3.5 Å around four coordinated water molecules. Bold numbers are O(W)...O contacts and lighter numbers are O(W)...C contacts. The contours approximately divide the plot into three areas of minimum contacts: (A) (over the lone pairs) ~ 3.5 Å, (B) (between the hydrogens) ~ 3.3 Å, and (C) (between the lone pair and hydrogen) ~ 3.1 – 3.2 Å.

RR4: H...H Repulsions. The majority of the closest contacts between water hydrogens lie between 2.3 and 2.4 Å, but some down to ~ 2.1 Å are present. Plotted in Fig. 7 b are the H...H contacts between waters as a function of the sum (χ) of the angles ($\alpha + \beta$) subtended at each of the hydrogens (defined in the inset motif). As χ increases, the H...H contacts tend to decrease and again a dividing line representing the limiting values can be drawn (full line).

The RR1 restraint effectively compels the H-bonds to be as straight as possible. However, this is not possible in many environments as a consequence of local packing

forces that prevent the donor and acceptor groups from occupying appropriate positions. In these cases the H-bonds may bend (to within the limits of the H-bond bending curve in Fig. 5 a) to (a) form as many H-bonding interactions as possible, and (b) relieve the local repulsive interactions of RR2-RR4. With respect to the latter, the relatively large number of RR3 H2...O1 contacts is seen to greatly influence the individual H-bond geometries around the water molecules.

For RR2, minimum O...O contacts of between 3.1 and 3.6 Å (Fig. 4 b) occur, with longer contacts around the lone-pairs region. The RR3 and RR4 interactions can be



When the above restraints are considered in addition to H-bonding, many of the specific characteristics of water structure can be readily explained, both in hydrates and in its pure phase. The short-range restrictions appear largely to control the final orientations of individual water molecules. The basic tetrahedral characteristics can be related to a minimization of the repulsive interactions (especially the remote H2...O1 contacts) and a maximization of the number (four) and strengths (2.7–2.8 Å) of H-bonds formed. Formation of shorter H-bonds, down to ~2.5 Å, requires the acceptor water to adopt a conformation that tends toward being trigonal planar to reduce the strain of

The H-bonding coordinations within hydrate structures range from 2 and 5 (or 6), with values of 3 and 4 being the most common. There is a distinct lack of directional control by the lone pairs on hydrogen bonding, and this is reflected in the wide ranges in the $X...O(W)...Y$ angles observed in small molecule hydrate crystal structures (Chiari and Ferraris, 1982). The actual coordination values depend on the local repulsive restrictions (RR1-RR4, H-bond lengths, etc.) and the presence of polar and apolar groups.

The variations present in the H-bond angles and distances appear to result from an optimization of the attraction and repulsive interactions, whereby the maximum number of possible H-bonds are formed, while all the local repulsive interactions (RR1-RR4) are minimized. The repulsive interactions, however, clearly control the final positionings and mutual orientations of adjacent water molecules.

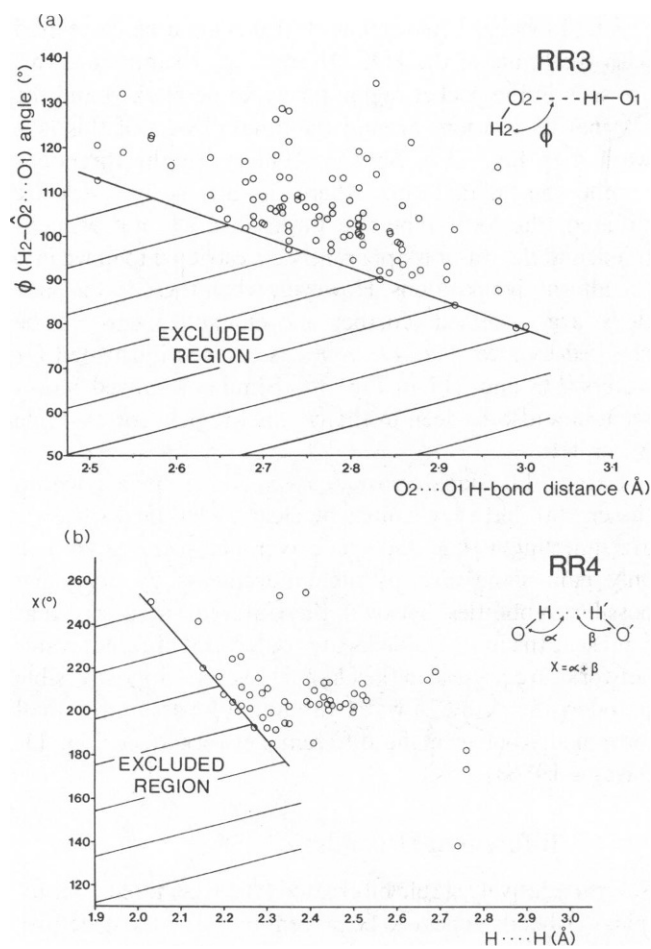


FIGURE 7 (a) Plot of $H_2-O_2 \cdots O_1$ angles (ϕ) vs. $O_2 \cdots O_1$ H-bond distances for waters involved in hydrogen bonds within crystal hydrates (neutron structures). The solid line represents the minimum allowed values. Configurations are not found that lie significantly below this approximate cutoff line. (b) Plot of water-water $H \cdots H$ contacts vs. χ , the sum of the angles α and β subtended at the hydrogen atoms (see inset motif).

In summary, the short-range repulsive restrictions approximately divide the mutual geometrical configurations between water molecules into two groups: those that are excluded, corresponding to the areas below the curves in Figs. 5a and 7, and those that are accessible (lying above the curves). Within the accessible volume, water molecules may, under thermal fluctuations, move into alternative positions. Several cases of this phenomenon are present around main sites in the coenzyme hydrate (Fig. 11, Savage, 1986a). A more detailed discussion of the short-range structural regularities and their implications is given in Savage, 1986b.

WATER ORGANIZATION

Here we discuss some of the details of the water structure in coenzyme crystals with respect to both H-bonding and the limitations imposed by the repulsive restraints discussed above.

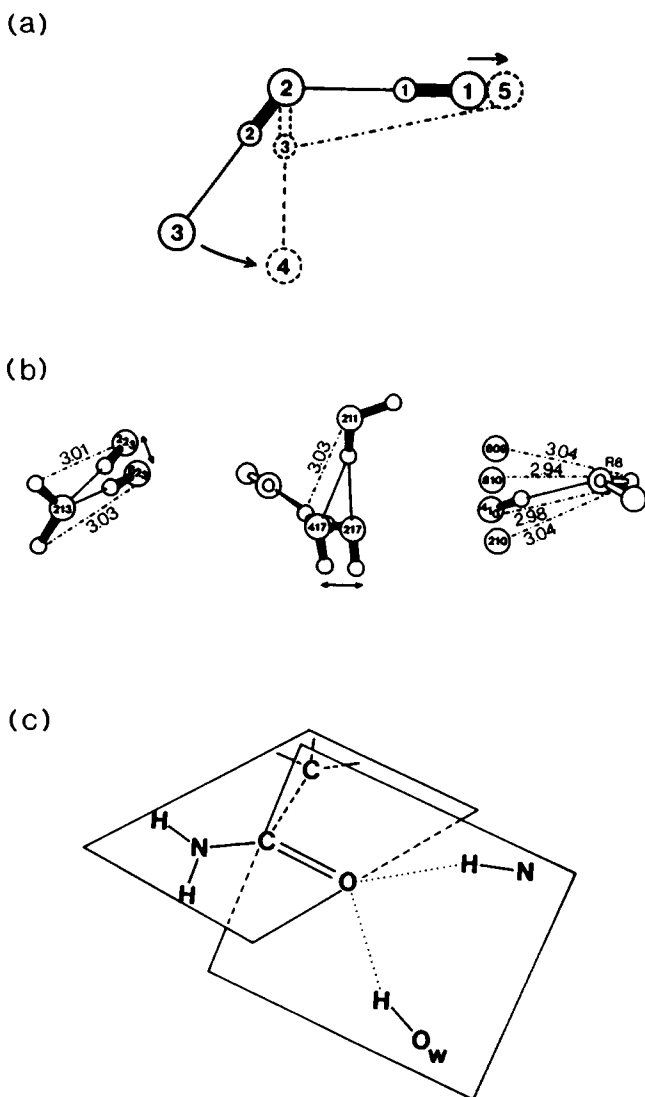


FIGURE 8 (a) The operation of the RR3 repulsive restraint in the decrease of $O \cdots O \cdots O$ H-bond angles. As O_3 moves to O_4 , that is the $O \cdots O \cdots O$ angle decreases, the $O_1 \cdots O_2$ H-bond distance has to increase (O_1 moves to O_5) to maintain the minimum $H_2 \cdots O_1$ contact distance of ~ 3.0 Å ($O-H$ distances fixed to 0.8 Å). (b) $H_2 \cdots O_1$ contacts involved in the local disorder around the main water sites 223, 217, and 410 of networks *A* and *B*. (c) Carbonyl plane: the H-bonded deuteriums of the amide and water and the $C=O$ atoms of the carbonyl group lie very close to a plane for the majority of carbonyl groups.

Basic Structure

The hydrogen bonds undoubtedly play a major role in orientating the waters toward one another and to the coenzyme polar groups through the electrostatic interactions. However, the repulsive contacts play a very significant part in determining the final positions and orientations of the individual water molecules. The most dominant repulsive component appears to be the RR3 ($H_2 \cdots O_1$) interactions, for which a larger number is present: around a central water in a tetrahedral lattice, there are 4 RR1, 12 RR3, and 8 RR4 close contacts.

If the preferred organization of water in hydrate crystals was one of tetrahedral coordination, then only a small percentage of the solvent accessible polar groups of the coenzyme molecule would be H-bonded to the waters, and many potential bonds would remain unmade. This is because the accessible polar groups are not spaced at the required distance (~ 4.5 Å) for a tetrahedral lattice. Therefore, to facilitate the formation of as many H-bonds as sterically possible, some (or all) of the $X\cdots O\cdots Y$ H-bond angles involving the waters must deviate from 109° . In doing this, the repulsive restraints come into operation in determining the actual bond lengths and orientations involving the polar groups.

To transform a tetrahedral water structure, in which all the $O\cdots O$ H-bond distances are assumed to be 2.76 Å (e.g., ice Ih), to a more compact or irregular form, some of the $O\cdots O\cdots O$ H-bond angles must decrease. When doing this, the remote RR3 $H2\cdots O1$ neighbor contacts must retain their minimum contacts of ~ 3.0 Å. Thus, one or both of the $O\cdots O$ H-bond distances must lengthen to accomplish this. Fig. 8 *a* illustrates the operation of RR3 in the decrement of the $O1\cdots O2\cdots O3$ H-bond angle. The orientations of waters 216, 217, and 218 show this effect: the $O\cdots O$ distance between 217 and 216 is relatively long, ~ 3.0 Å, thus the $O\cdots O\cdots O$ angle between the three waters ($218\cdots 217\cdots 216$) is able to decrease while the $H2\cdots O1$ minimum contact between the hydrogen of the $217\cdots 218$ H-bond and the oxygen of 216 is retained (Figs. 1 and 5).

The RR3 limitation is one of the main reasons why we see such a large variation in the water H-bond geometries. Very short H-bonds of 2.5–2.6 Å, are allowed in conformations that are close to being trigonal (to relieve minimum $H2\cdots O1$ contacts). An example of this is seen for the short H-bond between waters 217 and 218. The coordination around water 217 is almost trigonal planar with respect to waters 211 and 218, and carbonyl O62 (Fig. 1) and a shorter H-bond of ~ 2.6 Å is present. Water 216 is bonded to 217 at a position that is almost perpendicular to the trigonal plane. This is allowed because the H-bond between these waters is relatively long, which in turn allows relatively small $O\cdots O\cdots O$ angles (see above).

The remote $H2\cdots O1$ contacts influence the possible ring structures of water molecules that may be formed. Five membered rings (predominant in water clathrates) represent the smallest rings in which normal H-bonds (2.7–2.8 Å) are present with the $H2\cdots O1$ contacts at their minimum before they become highly strained. To go to four membered rings, some of the $O\cdots O$ H-bond lengths must increase to maintain the minimum $H2\cdots O1$ contacts (Fig. 8 *a*).

Disorder and Possible Movements of Waters

There appears to be two categories into which the movement of waters can be separated: (*a*) local disorder and (*b*) larger coupled (diffusional) movements.

Small localized movements (~ 1.0 Å) can be correlated with the limits of the $H2\cdots O1$ contacts. Examples of this are seen in the pocket region for water network *A* and the alternative positions around the “main” sites of this network (see Fig. 11 *b*, Savage, 1986*a*). In the directions around the “main” sites where the alternative sites are situated, the local repulsive limitations are not encountered, and the water is apparently unrestricted to move into the alternative positions. However, when these latter positions are occupied, further movement appears to be checked by local $H2\cdots O1$ contacts. This is illustrated for waters 223 and 217 in Fig. 8 *b*. Similar localized movement can also be seen in the channel region, for example water 410.

Larger diffusional movements are also present within the crystal, but they cannot be clearly identified since we are sampling a time and space averaged picture. We can only gain some idea of such movements by analyzing possible mobilities between the different networks that represent the more stable locations. Several different water networks are present in the channel region and it is possible to follow the path of a water through it by assuming small movements between the different networks (see Fig. 11, Savage, 1986*a*).

Bifurcated H-bonds

Several relatively stable bifurcated (three centered interactions) H-bonds appear to be present in the water structure. Water 211 forms a bifurcated arrangement with the oxygens OP2 (phosphate, $D\cdots O = 2.29$ Å) and OR7 (ribose, $D\cdots O = 2.47$ Å), while water 216 hydrogen bonds to waters 217 ($D\cdots O = 1.97$ Å) and 218 ($D\cdots O = 2.45$ Å), see Fig. 1. The relative stability of these interactions can be rationalized in terms of the hydrogen atoms (of the bifurcated bond) being held in position by the local nonbonded remote $H2\cdots O1$ contacts, the RR3 restraints.

Directionality of Certain Interactions

As discussed above, the local repulsive interactions around water molecules play a significant part in the bending of hydrogen bonds. As the $X\cdots Y$ distance increases the $X-D\cdots Y$ bond can, when necessary, bend to within the limiting line of Fig. 5 *a*. The majority of H-bonds tend toward being as linear as possible, but deviations are imposed to relieve the local stereochemical packing restraints (asymmetric repulsive cores of the water oxygens and hydrogens).

The lone pairs of carbonyl groups appear to impose some directionality on the groups H-bonding to them. It was observed in the coenzyme structure that when an amide N-D group H-bonds to one of the lone pair regions of a carbonyl oxygen and is displaced from the amide plane, then a water molecule H-bonding to the other lone pair region is also displaced from the plane, but in the opposite direction (provided that the water is fully accessible to the

C=O). The two H-bonded deuteriums (of the amide and water) and the C=O atoms of the carbonyl group itself lie very close to the plane defined by these atoms (<0.05 Å). These carbonyl planes are also rotated about the C=O carbonyl double bond with respect to the amide plane (see Fig. 8 c).

Geometries Around Apolar Groups

The contacts between water oxygens and carbons present are mainly larger than the expected van der Waals contact of ~ 3.5 Å (C-H) = 2.0 Å, and O = 1.5 Å). For a few cases however, short contacts down to ~ 3.2 Å were observed, for example water 0423...C53 (methyl) = 3.23 Å. The shorter O(W)...C contacts can be rationalized in terms of the anisotropic repulsive core associated with the water oxygen, ~ 1.5 – 1.8 Å (Fig. 4 b). Two main interactions appear to be present: O(W)...C and O(W)...H, which depend on the relative orientations of the CH_x group and the water molecule. When the water is situated in a position between the hydrogens of CH_x as in Fig. 9 a, then the main interaction is between the oxygen and carbon. The O...C contact distance also depends on how the water molecule is orientated, that is, whether region A, B, or C (Fig. 4 b) interacts with the apolar group. With the lone pairs pointing away from the carbon, distances down to ~ 3.2 Å are permitted, but when they point toward the carbon, distances of 3.5 Å or greater are generally observed.

For waters located in positions that are almost linear with the C-H vector (Fig. 9 b), the main interaction is between the hydrogen of the CH_x and the water. Here, an opposite effect relative to the O...C contacts is apparent:

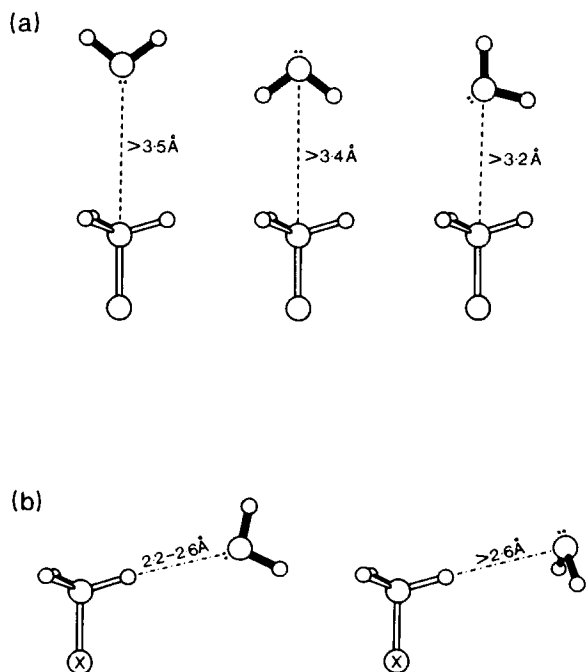


FIGURE 9 Water geometries around apolar groups. (a) away from CH hydrogens and (b) in line with CH hydrogens.

when the lone pairs point away from the hydrogen, the closest O(W)...H contacts lie between 2.6–2.7 Å; however, when the lone pairs point toward the hydrogen, the O(W)...H distances are very often <2.6 Å. The values observed depend on the electronegativity of the adjacent groups attached to the CH_x. In several cases O(W)...H distances down to 2.2–2.3 Å occur. For example, C-H hydrogens point towards one of the lone pairs of waters 213 and 222; in the former case, the C-H (CR5) is bonded to a hydroxyl while in the latter the C-H (CB7) is part of a benzene ring. Where the CH_x groups are attached to saturated carbons, O(W)...H contacts of >2.4 Å are present. These shorter contacts (<2.6 Å) probably correspond to weak C-H...O hydrogen bonding interactions (Green, 1974).

DISCUSSION

The water structure in coenzyme crystals can be rationalized on the basis that the attractive H-bonding interactions and the repulsive short-range interactions each play a prominent role in controlling the water organization. The former hold the molecules together, while the latter are crucial in determining the relative positions and orientations of the individual molecules.

Initially, the use of standard notions of H-bonding could not adequately explain, in a consistent manner, why certain water molecules occupied specific orientations within the coenzyme crystal. Wide variations of the individual H-bonding geometries were present and at best, such ranges could only be used as relatively weak restraints or guidelines (compared with covalent geometries) in understanding water structure. In contrast, however, a more comprehensible picture is obtainable when details of the local nonbonded relationships around the water molecules are taken into consideration. Four main short-range stereochemical constraints appear to be evident, which are much stronger than any that maybe associated with H-bonding. They are labeled as - RR1: O...O repulsion of H-bonds, RR2: O...O nonbonded interactions, RR3: remote H2...O1 nonbonded contacts, and RR4: H...H contacts. In more general terms they can be rationalized with respect to both the water oxygen and hydrogen atoms possessing anisotropic repulsive cores. The local repulsive limitations determine an accessible environment within which a wide range of H-bonding configurations between the water molecules are allowed.

The water molecules within the coenzyme hydrate pack together in such a way that the H-bonding interactions are maximized in terms of forming as many bonds as possible (usually 3 or 4), while at the same time, all the local short range contacts are minimized. These criteria are fulfilled in a tetrahedral water structure. However, when deviations from tetrahedrality are required within the local structure (which appears to be the case in almost all water structures except ices I and VIII), the repulsive restraints, RR, largely control the overall orientational correlations

between the waters. Of the four repulsive restraints, the RR3 remote H2...O1 contacts appear to be the most dominant in determining the immediate local orientational and H-bond geometries. Where necessary, the H-bond angles may bend to within the limits governed by the O...O H-bond repulsions (RR1: Fig. 5 a), to accommodate the RR2, RR3, and RR4 packing limitations.

Consideration of the short-range nonbonded interactions in terms of structural restraints gives us a much firmer framework on which to base the organization of water assemblies in both the coenzyme hydrate and other crystalline solids. Hopefully, the nonbonded interactions within potential functions presently utilized in computer simulations of water and aqueous solutions, can be revised to take into account the anisotropic effects that appear to be present.

Thanks are expressed to Paul Barnes, Jeremy Baum, Guy Dodson, Julia Goodfellow, Peter Lindley, Walt Stevens, Francoise Vovelle, Alexander Wlodawer, and Giuseppe Zaccai for many useful discussions and interesting comments. The continued interest and encouragement of John Finney and Dorothy Hodgkin have been greatly appreciated throughout the course of this project.

Support from the Science and Engineering Research Council, United Kingdom and the National Institutes of Health is gratefully acknowledged.

Received for publication 30 April 1986.

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