Crystal Structure and Conformation of the Cyclic Dipeptide cyclo-(L-Threonyl-L-histidyl) Dihydrate

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Abstract: The crystal and molecular structures of cyclo-(L-threonyl-L-histidyl) dihydrate were determined by single crystal x-ray diffractometry. The crystals belong to the orthorhombic $P2_12_12_1$ space group with $a = 8.649 \pm 0.003$, $b = 25.557 \pm 0.009$, and $c = 6.212 \pm 0.005$ Å and $Z = 4$. The structure was solved using direct methods and refined by least-square procedures to an R factor of 0.044. The diketopiperazine ring deviates only slightly from planarity. The two functional side chains interact through a water molecule. This molecule interacts with the hydroxyl group of the threonyl side chain which is folded in a conformation. $\chi_1^1 = 53.7°$. and the $N^{\delta 1}$ atom of the histidyl residue which is stabilized in a conformation. $\chi_2^1 = -54.9°$ and $\chi_2^{2,1} = 99.1°$.

The x-ray structures of several cyclic dipeptides [cyclo-(Gly-Gly)[2] (I). cyclo-(D-Ala-L-Ala)[3a,b] (II). cyclo-(L-Ala-L-Ala)[3b,4] (III). cyclo-(Gly-L-Tyr)[5] (IV), cyclo-(L-Ser-L-Tyr)[5] (V)] and different peptides containing prolyl residues[6,7] or a epipoly sulfide[8] bridge have been established up to date.

A point of interest is the conformation of diketopiperazine (DKP) ring which involves two cis peptide bonds. In the crystalline state. this ring has been found to be planar when unsubstituted. i.e.. in 2.5-piperazinedione I and nearly planar in II and V. It is described as a "twist boat" in III and as a "boat" with quasi-axial substituents in IV. In cyclo-(L-Thr-L-His). there is found a DKP ring which deviates only slightly from planarity. In addition to the conformation of the DKP ring. the conformations of L-threonyl and L-histidyl residues are of interest. The imidazole ring of L-His is an essential constituent of the active site of several enzymes. Its tautomeric structures. pK of protonation. and preferred conformations have been widely investigated in model compounds. in peptides. and in proteins. In proteolytic enzymes such as α-chymotrypsin. a L-His residue strongly interacts with a nucleophilic L-Ser residue in the active site.[9] but never with a L-Thr residue. Then. we decided to investigate the possible particularities of interaction between L-Thr and L-His residues in a model compound. such as cyclo-(L-Thr-L-His). Our previous preliminary ^{1}H NMR study[10] of this dipeptide in solution has shown differences with the assumption of a preferred folded conformation in which the conjugate ring faces the DKP ring.[11] In the solid state. we find here that the two side chains interact through a water molecule which is hydrogen bonded to both $O^{\gamma 1}$ (L-Thr) and $N^{\delta 1}$ (L-His). These findings stimulate us to investigate further. by experimental and theoretical methods. interactions between functional side chains of neighboring peptide residues.

Experimental Section

The cyclic dipeptide cyclo-(L-Thr-L-His) was prepared as previously mentioned[10] and crystallized from basic aqueous solutions as thin colorless needles which were used directly.

Crystal Data. The crystals belong to the orthorhombic $P2_12_12_1$ space group. The cell constants were manually obtained from the measurements of ω. χ. and φ Eulerian angles for 12 reflections with a Siemens four-circle diffractometer. The refined parameters are: $a = 8.649 \pm 0.003$. $b = 25.557 \pm 0.009$. $c = 6.212 \pm 0.005$ Å: $V = 1373$ Å^3 ($Z = 4$).

The calculated density. assuming two water molecules per asymmetric unit and formula $C_{10}H_{14}N_4O_3 \cdot 2H_2O$. is 1.321 g cm^{-3}. The density measured by flotation is 1.30 g cm^{-3}.

The intensities of 1495 reflections with $\theta < 70°$. were collected by θ-2θ scans using the five-point measurement technique with Cu Kα radiation ($\bar\lambda = 1.5418$ Å). five different Ni filter thickness. and for different counting times.

No correction was made either for absorption according to the small dimensions of the crystal. or for secondary extinction. Among the 1495 measured reflections. 1388 were significantly greater than their background count.

Structure Determination. The structure was solved by the multiresolutions direct method using a computer program similar to the one previously proposed by Germain et al.[12] Only the 132 reflections with a normalized structure factor. $E > 1.60$. were used in the convergence process. The origin of phases was fixed with the following reflections: 3. 1. 4. 5 ($E = 2.23$. $\varphi = 45°$). 0. 6. 7 ($E = 3.46$. $\varphi = 360°$) and 0. 25. 4 ($E = 2.86$. $\varphi = 90°$). Three other reflections chosen as symbols. with symbolic phases a. b. c. were necessary to assure the convergence process respectively: 4. 26. 2 ($E = 2.30$): 6. 1. 3 ($E = 2.51$). 0. 18. 2 ($E = 2.93$). The values 45. 135. 225. and 315° were successively attributed to them. From the corresponding 64 sets of refined phases. by using the tangent formula. emerges a group of 8 sets with similar figures of merit and nearly identical phases.

The atoms of the DKP ring and of the threonyl side chain clearly appeared on the corresponding Fourier map. The atoms of the imidazole ring and oxygens of the two water molecules have been located further on three successive difference Fourier synthesis. A first least-squares refinement of atomic coordinates with isotropic thermal factors B_i gave a reliability factor $R = 0.135$. Further refinement cycles with anisotropic factors B_{ij} gave $R = 0.075$. Hydrogen atoms were then located on difference maps and refinement was resumed.[28] The final R factor was 0.044.

Atomic parameters are listed in Tables I and II. A projection of the structure along the z axis is shown in Figure 1.

Results

(a) Distances and Angles. Intramolecular bond lengths and angles not involving hydrogen atoms are reported in Figure 2. In the same figure is listed the standard labeling of atoms and torsional angles.[13] In the DKP ring. the values of bond lengths and angles are very close to those found in other cyclic dipeptides (I. III. IV. V). The two internal angles at C^α are close to the corresponding angles in the unsubstituted DKP molecule. Thus the effect of substitution on C^α is not shown here.

In the L-Thr side chain. the C_1^α-C_1^β bond (1.54$_4$ Å) is very close to the standard bond in amino acids and peptides (1.53 Å).[14] The C_1^β-$O_1^{\gamma 1}$ bond (1.43$_4$ Å) has a normal value: the C_1^β-$C_1^{\gamma 2}$ bond (1.51$_5$ Å) is slightly shorter than the normal length (1.54 Å). but these bonds cannot be compared to those of L-threonine. the structure of which was determined with less accuracy.[15] In the linear dipeptide.

Table I. Fraction Coordinates and Anisotropic Thermal Parameters B_{ij} ($\times 10^5$)^a

	X	Y	Z	B_{11}	B_{22}	B_{33}	B_{12}	B_{13}	B_{23}
$O_1^{\gamma 1}$	0.7635 (4)	0.1201 (1)	0.3276 (5)	193 (5)	9 (0)	247 (9)	11 (2)	125 (13)	30 (3)
$C_1^{\gamma 2}$	0.9032 (5)	0.1744 (2)	0.0705 (8)	130 (6)	16 (1)	290 (13)	2 (3)	115 (16)	37 (5)
C_1^{β}	0.8142 (4)	0.1723 (1)	0.2805 (7)	100 (5)	11 (1)	223 (10)	-6 (3)	-4 (13)	18 (4)
C_1^{α}	0.6725 (4)	0.2091 (1)	0.2844 (6)	110 (5)	9 (0)	143 (8)	-5 (3)	-10 (12)	9 (3)
N_1	0.5678 (4)	0.1999 (1)	0.1038 (5)	106 (4)	12 (0)	127 (7)	-1 (2)	8 (10)	17 (3)
C_2	0.4219 (4)	0.1835 (1)	0.1161 (6)	103 (4)	10 (0)	136 (8)	10 (3)	-25 (11)	9 (3)
O_2	0.3381 (3)	0.1796 (1)	-0.0445 (4)	125 (4)	17 (0)	146 (6)	-5 (2)	-40 (9)	10 (3)
C_2^{α}	0.3571 (4)	0.1680 (1)	0.3314 (5)	101 (4)	9 (0)	130 (8)	1 (2)	-1 (11)	4 (3)
N_2	0.4548 (3)	0.1837 (1)	0.5140 (5)	114 (4)	10 (0)	120 (7)	-4 (2)	12 (9)	2 (3)
C_1	0.5949 (4)	0.2041 (1)	0.5022 (6)	126 (5)	8 (0)	131 (8)	-5 (3)	16 (12)	2 (3)
O_1	0.6673 (4)	0.2189 (1)	0.6631 (4)	158 (4)	16 (0)	141 (7)	-35 (3)	-15 (10)	-5 (3)
C_2^{β}	0.3277 (5)	0.1083 (1)	0.3251 (6)	149 (6)	9 (0)	171 (9)	-8 (3)	16 (14)	-4 (4)
C_2^{γ}	0.2602 (4)	0.0862 (1)	0.5288 (7)	112 (5)	8 (0)	196 (9)	-7 (3)	5 (13)	-1 (4)
$C_2^{\delta 2}$	0.1109 (5)	0.0844 (2)	0.5948 (8)	122 (6)	16 (1)	263 (13)	-7 (3)	14 (15)	21 (5)
$N_2^{\epsilon 2}$	0.1112 (4)	0.0575 (1)	0.7891 (7)	139 (5)	14 (1)	247 (10)	-13 (3)	93 (13)	19 (4)
$C_2^{\epsilon 1}$	0.2577 (5)	0.0441 (1)	0.8234 (8)	152 (6)	11 (1)	211 (11)	-8 (3)	14 (15)	10 (4)
$N_2^{\delta 1}$	0.3515 (4)	0.0605 (1)	0.6756 (6)	128 (4)	10 (0)	204 (9)	-6 (2)	-8 (12)	11 (3)
$O(W_1)$	0.6648 (3)	0.0572 (1)	0.6613 (5)	127 (4)	14 (0)	267 (9)	3 (2)	-5 (12)	37
$O(W_2)$	0.8297 (4)	0.0461 (1)	0.0260 (6)	160 (5)	13 (0)	254 (9)	-8 (2)	-5 (12)	5

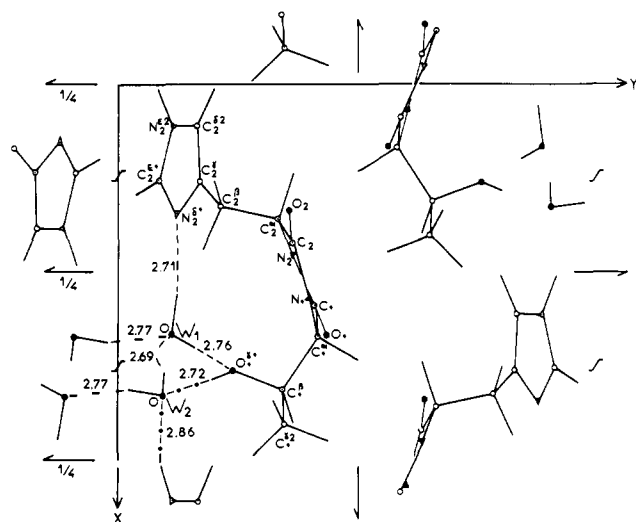^{a} Standard deviations are given in parentheses.

Figure 1. The crystal structure of cyclo-(L-Thr-L-His) projected along the Z axis. W_1 and W_2 are two molecules of water. The dashed lines indicate hydrogen bonds between: (a) W_1 and a dipeptide molecule (- - -); (b) W_2 and two different peptides molecules (- - - - -); (c) between water molecules (- - - - - -) ($\not{\cdot}\cdot\,\,^-\,\,^-\,$, $1 \rightarrow 1 + c$). The lengths (in Å) of these hydrogen bonds are given.

Gly-L-Thr. the corresponding bonds are abnormally long, according to the literature.[16]

As far as the L-His side chain is concerned, the distances are not significantly different from those found in other molecules containing an unprotonated histidyl residue.[17-20,22]

(b) Molecular Conformation. The conformation of the molecule is defined by the two sets of ϕ, ψ, and ω angles for the DKP ring and the χ_1^1 (L-Thr). χ_2^1, and $\chi_2^{2,1}$ (L-His) angles for the side chains (Figure 2). The conformation of the molecule viewed as a projection on the DKP ring mean plane is shown in Figure 3. The values of torsional angles are reported in Figure 2.

As compared with dipeptides III and IV, the puckering of the central DKP ring found here is small. The deviation from planarity corresponds to a "flagpole boat" form. slightly twisted as revealed by ω values. +5.1 and 4.6° ($\omega = 0$ for planar cis peptide bond).

The conformations of the two side chains are mainly determined by their interactions with a water molecule W_1 which establishes a bridge over the DKP ring. between the

Table II. Fractional Coordinates and Isotropic Temperature Factors B_i (in Å^2) for Hydrogen Atoms

	X	Y	Z	B_i
$H_1^{\gamma 1}$	0.7876	0.0982	0.1986	4.4
$H_1^{\gamma 2}$	0.9894	0.1412	0.1165	4.4
$H_1^{\gamma 2}$	0.8409	0.1615	-0.0722	3.6
$H_1^{\gamma 2}$	0.9501	0.2191	0.0452	3.6
H_1^{β}	0.8986	0.1840	0.4026	3.2
H_1^{α}	0.7338	0.2506	0.2636	2.6
H_1	0.6009	0.2114	-0.0514	2.7
H_2^{α}	0.2380	0.1832	0.3342	2.7
H_2	0.4004	0.1814	0.6644	2.7
$H_2^{\beta 2}$	0.2593	0.1010	0.1794	3.5
$H_2^{\beta 1}$	0.4413	0.0885	0.3023	3.5
$H_2^{\delta 2}$	-0.0017	0.9639	0.5408	4.1
$H_2^{\epsilon 2}$	0.0152	0.0431	0.8722	3.7
$H_2^{\epsilon 1}$	0.2990	0.0184	0.9625	3.3
$H_1(W_1)$	0.5565	0.0622	0.6897	3.6
$H_2(W_1)$	0.7007	0.0800	0.5407	3.6
$H_1(W_2)$	0.8127	0.0107	0.1127	3.8
$H_2(W_2)$	0.7703	0.0501	-0.1055	3.8

hydroxyl group of L-Thr and the imidazole ring of L-His (Figure 1). The W_1 molecule is proton donor to $O^{\gamma 1}$ and to $N^{\delta 1}$. For L-Thr residue. this intramolecular interaction is compatible with the existence of a stable staggered rotamer since $\chi_1^1 = 53.7°$. It is sufficiently interesting to note that this rotamer was found to be very stable in aqueous solutions of cyclo-(L-Thr-L-His) at neutral pH.[10]

For the L-His residue. it was previously proposed that. by analogy with aromatic residues. Phe. Tyr. or Trp. the imidazole ring faced the DKP ring in a folded conformation of the dipeptide.[11] The fundamental role of hydration on the conformation of this residue is clearly demonstrated. cyclo-(L-Thr-L-His) was crystallized from aqueous solution at pH ~8. for which the imidazole ring is not protonated. In solution. it was demonstrated that the $N^{\epsilon 2}$-$H^{\epsilon 2}$ tautomeric form is predominant.[21] Only the $N^{\delta 1}$ atom or the $N^{\delta 1}$-$H^{\delta 1}$ group (depending on the tautomeric form) can be involved in intramolecular short interactions with the peptide backbone or with the side chain of neighboring residue. The insertion of a water molecule W_1 between the hydroxyl group of L-Thr and the imidazole ring of L-His is easily realized when this molecule donates hydrogen to $O^{\gamma 1}$ and to $N^{\delta 1}$: then the $N^{\epsilon 2}$-$H^{\epsilon 2}$ form is stabilized. The intramolecular water bridge stabilizes a conformation of the L-His residue defined by $\chi_2^1 = -54.9°$ and $\chi_2^{2,1} = +99.1°$. Note. as we
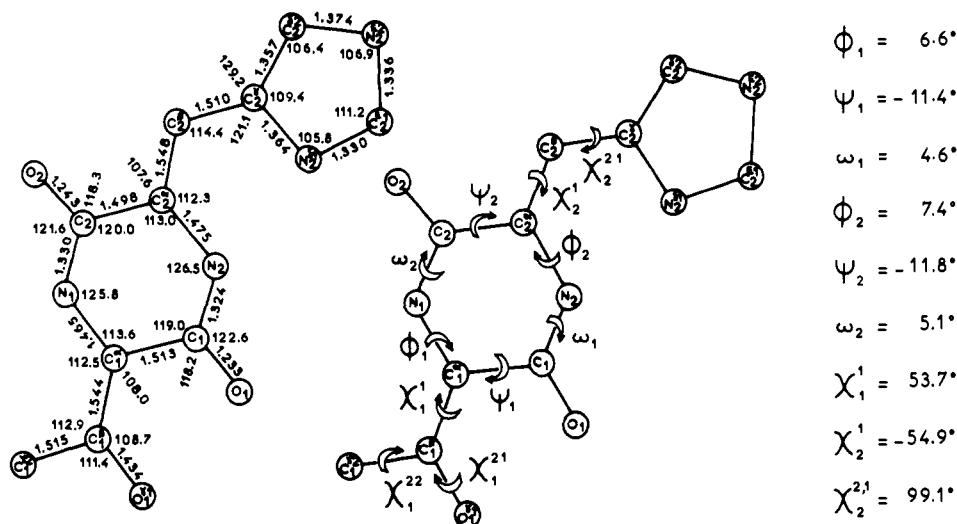
Figure 2. On the left are shown bond lengths and bond angles of *cyclo*-(L-Thr-L-His). The standard deviations for the distances range from 0.005 to 0.007 Å, while the standard deviations for the angles range from 0.32 to 0.43°. On the right, the standard labeling of atoms and torsional angles is listed (see ref 13), and the values (in degrees) of these angles are reported.
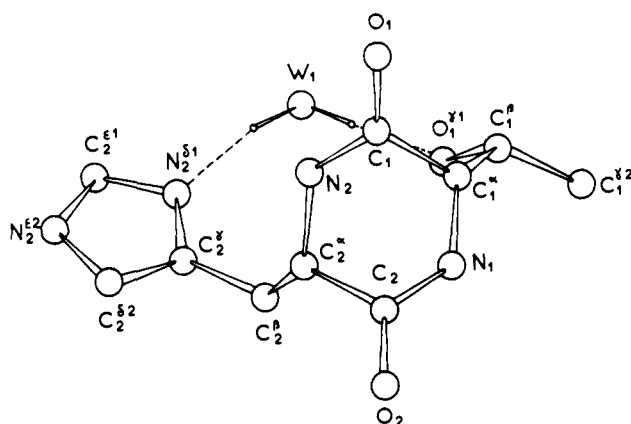

Figure 3. Conformation of the *cyclo*-(L-Thr-L-His) molecule viewed as a projection on the DKP mean plane. The water molecule W_1 is hydrogen bonded to the two His and Thr side chains. The deviations from the mean plane defined by the $C_1{}^\alpha$, N_1, $C_2{}^\alpha$, N_2 atoms are (in Å): $C_1{}^\alpha$ (−0.05), N_1 (+0.04), C_1 (+0.08), O_1 (+0.23), $C_2{}^\alpha$, (−0.05), N_2 (+0.03), C_2 (+0.10), O_2 (+0.25).

Figure 4. The crystal structure of *cyclo*-(L-Thr-L-His) projected along the Y axis. Hydrogen bonds between the DKP rings are shown (. . . .) and their lengths are given. A water molecule W_1 which is hydrogen bonded (- - -) to the $O_1{}^{\gamma1}$ and $N_2{}^{\delta1}$ atoms is also represented. (- · · · · · -) represents an hydrogen bond between W_1 and W_2 water molecules.

emphasized in another paper, that for the five known crystal structures of histidine or histidyl residue in dipeptides with unprotonated imidazole ring, the χ^1 angle is close either to 180 or to 300°, the $\chi^{2,1}$ angle being generally close to 60°.[22] In *cyclo*-(L-Thr-L-His), the conformation of L-His residue and the $N^{\epsilon2}$-$H^{\epsilon2}$ tautomeric form of the imidazole ring are also stabilized by an intermolecular hydrogen bond involving the $N^{\epsilon2}$-$H^{\epsilon2}$ group and the oxygen of a second water molecule W_2 (Figure 1).

(c) Hydrogen Bonds. Every proton-donor or proton-acceptor group of the dihydrated molecule in the crystal is engaged at least in an hydrogen bond as generally observed.[22,23] Hydrogen bonds are listed in Table III and shown in Figures 1 and 4.

The hydrogen-bond network is rather complicated and involves three classes of interaction.

(1) Two intermolecularly quite linear and very similar hydrogen bonds -NH···O=C- connect the DKP rings into chains parallel to the Z axis. Both are of mean strength[25] according to distances and angles.

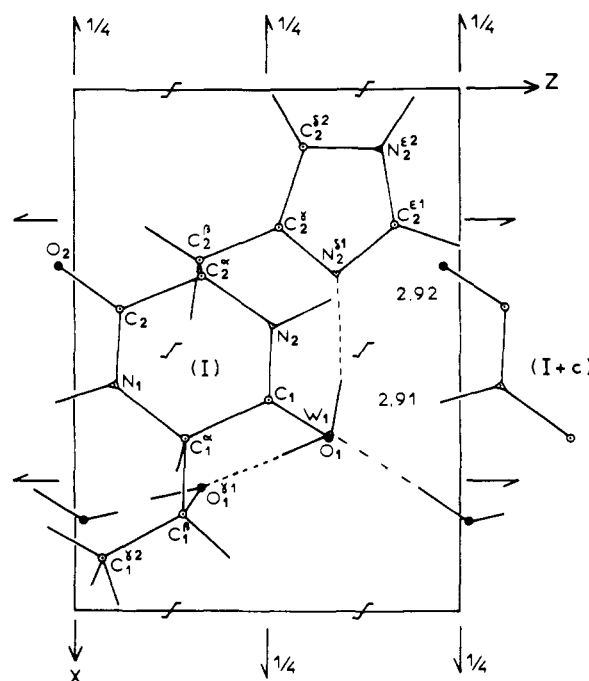(2) Each water molecule is tetrahedrally coordinated. For the intramolecular molecule W_1, the two hydrogen

atoms are engaged in bonds with the peptide side chains and the two oxygen orbitals are engaged in bonds with two neighboring water molecules W_2.

(3) For the intermolecular water molecule W_2, the two hydrogen atoms are engaged in bonds with neighboring W_1 molecules: the two oxygen orbitals are involved in bonds respectively with the threonyl OH group of one dipeptide molecule and with the imidazole $N^{\epsilon2}$-$H^{\epsilon2}$ group of another dipeptide molecule. The $(W_2)O$···HO(Thr) bond is rather weak.[25] while the $(W_2)O$···HN(His) bond is of normal strength.[24]

Water molecules form channels along Z axis and this network makes the cohesion between the chains of dipeptide molecules.

Table III. Parameters of Hydrogen Bonds A–H···B in the Crystal[a]

A–H		B	d_{A-B}	d_{H-B}	α_{A-H-B}
N_2 (I)	$-H_2$ (I)	—O_2 (I + c)	2.925	1.892	170.1
N_1 (I + c)	$-H_1$ (I + c)	—O_1 (I)	2.910	1.874	173.6
$N_2^{\epsilon 2}$ (I)	$-H_2^{\epsilon 2}$ (I)	—O (W_2, 1 – a + c)	2.863	1.832	169.6
$O_1^{\gamma 1}$ (I)	$-H_1^{\gamma 1}$ (I)	—O (W_2, I)	2.724	1.773	164.5
O (W_1, I)	$-H_1$ (W_1, I)	—$N_2^{\delta 1}$ (I)	2.712	1.774	163.7
O (W_1, I)	$-H_2$ (W_1, I)	—$O_1^{\gamma 1}$ (I)	2.762	1.790	179.9
O (W_2, I)	$-H_1$ (W_2, I)	—O (W_1, II + a – c)	2.770	1.853	156.1
O (W_2, I)	$-H_1$ (W_2, I)	—O (W_1, I + c)	2.689	1.717	179.8

a II: $1/2 - x, \bar{y}, 1/2 + z$.

Discussion

Structure correlation previously drawn from the studies of cyclic dipeptides containing an aromatic residue[5] cannot be extended to cyclo-(L-Thr-L-His). In this last dipeptide, hydration plays a fundamental role in the crystal structure and in the molecular conformation. An intramolecular water molecule precludes the formation of a direct hydrogen bond between the imidazole ring of L-His and the hydroxyl group of L-Thr. It precludes also the existence of a folded form in which the imidazole ring faces the DKP ring. Several different possibilities exist for the insertion of a water molecule between the two side chains of cyclo-(L-Thr-L-His). This molecule can be proton donor or proton acceptor. In the structure described here, it is proton donating, and its right insertion requires only slight distortion of the DKP ring.[26] The packing does not impose important constraints on the dipeptide molecules since no singularities were detected in the intermolecular contacts and in the molecular conformation. From our previous ^{1}H NMR study,[10] it was concluded that the hydroxyl group of L-Thr points above the DKP ring and that the folded form of L-His was not predominant. This suggests an important effect of hydration on the conformation of cyclo-(L-Thr-L-His) in solution.

The interaction between the hydroxylated side chain of threonyl and seryl residues and the imidazole ring of histidyl residue, eventually involving a water molecule, is of special interest by reference to the structure of the catalytic site of proteolytic enzymes. In α-chrymotrypsin, the interaction between Ser(195) and His(57) involves a distorted hydrogen bond between $N^{\epsilon 2}$(57) and O^{γ}(195) and also a water molecule which is apparently hydrogen bonded to both $N^{\epsilon 2}$(57) and O^{γ}(195)[9] (His(57) is also interacting with Asp(102)). Wang[27] has discussed the possible role of hydrogen bonds in the transfer of proton in a "charge relay" system assuming an important effect of the bending of these bonds. Then, it should be interesting to investigate the properties of a family of small peptides including Asp, His, Ser, and also Thr residues in order to compare the interactions between neighboring side chains in peptides and their constrained interactions in the active site determined by the macromolecular conformation.

Conclusion

As compared with already known structures of cyclic dipeptides, the crystal structure of cyclo-(L-Thr-L-His) is characterized by the presence of two water molecules which mainly participate in the molecular conformation and in the crystal cohesion. Hydration may greatly influence the con-

formation of this dipeptide in solution and we are still investigating this point in more details. It shall be interesting to compare the prediction of theoretical calculations of the conformation of the molecule in absence of the solvent.[26]

Cyclic dipeptides enable the investigation of the possible specific interactions between functional residues, such as Ser, His, and Asp (and Thr and Glu for comparison), namely with regard to the formation of hydrogen bonds and the role of water. We are continuing our studies in this field.

References and Notes

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(28) The C–H, N–H, and O–H distances underestimated from x-ray data have been fixed respectively to 1.09, 1.04, and 0.96 Å, according to neutron diffraction data and refinement resumed only with C, N, and O atoms and B_i factors of H atoms.