Reports

Structure of Pressinoic Acid: The Cyclic Moiety of Vasopressin

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Arginine vasopressin consists of a 20-membered, disulfide-linked macrocyclic ring system called pressinoic acid to which is attached a COOH-terminal tripeptide. The molecular conformation of pressinoic acid has been determined from single crystal x-ray diffraction data. The 20-membered macrocyclic ring, stabilized by two intramolecular hydrogen bonds, has a type I β-bend centered on Gln^4 and Asn^5 and a highly distorted type II′ bend centered on Phe^3 and Gln^4. In vasopressin the Asn^5 side chain extends away from the macrocyclic ring system and hydrogen bonds to the terminal tripeptide, but in pressinoic acid the Asn^5 side chain lies over the molecule and forms a strong hydrogen bond to the nitrogen of Tyr^2. The absence of pressor activity in pressinoic acid may be a result of both the loss of the COOH-terminal tripeptide and the incorrect orientation of the Asn^5 side chain. Whether this class of hormones has pressor or oxytocic activity is determined by the orientation of the Tyr^2 side chain, that is, whether it is extended away from or over the ring system, respectively. In pressinoic acid, the Tyr^2 side chain is in the expected "pressor conformation," that is, extended away from the ring system, and is stabilized through a hydrophobic interaction with the Phe^3 side chain. Thus, the conformation of the pressinoic acid molecule partly explains the activity of vasopressin-like hormones.

THE NEUROHYPOPHYSEAL HORMONES, vasopressin and oxytocin, have been widely studied since 1953 when the structure of oxytocin was proven through synthesis (*1*). The amino acid sequence of vasopressin differs from that of oxytocin at two positions (Fig. 1). Vasopressin has no oxytocic activity but is a potent antidiuretic and vasoconstrictor. Nuclear magnetic resonance (NMR) studies suggest that the cyclic moiety of oxytocin consists of an antiparallel β-pleated sheet with a type II β-turn involving Tyr^2-Ile^3-Gln^4-Asn^5 (*2*). Residues called "active elements" (*3, 4*) in arginine vasopressin (the side chains of Asn^5, Tyr^2, and Phe^3) interact with the receptor and are proposed to be directed away from the center of the 20-membered ring. In contrast, the tyrosine side chain is thought to lie over the ring moiety of the oxytocin molecule. This hypothesis has led to the design and synthesis of a conformationally restricted analogue of vasopressin that antagonizes antidiuretic activity (*5*).

Although it has not been possible to obtain suitable crystals of vasopressin or oxytocin for diffraction, we have obtained the structure of pressinoic acid (*6*), the inactive cyclic moiety of vasopressin. Pressinoic acid lacks the Pro^7-Arg^8-Gly^9-NH_2 terminal tripeptide of vasopressin, which is important for activity but not for binding to its neurophysin (*7, 8*). The macrocyclic ring moiety is involved in binding to both its receptor and its storage-transport protein neurophysin; therefore, the relative positions of the side chains should provide insight into interactions with these two recognition surfaces. The conformation of the pressinoic acid macrocycle may be useful for the structure activity analyses of known analogues, for the design of additional analogues with specific properties, and as an initial model for the computational simulation of the dynamic flexibility of the molecule (*9*).

Oxytocin

Cys^1-Tyr^2-Ile^3-Gln^4-Asn^5-Cys^6-Pro^7-Leu^8-Gly^9-NH_2
S——S

Vasopressin

Cys^1-Tyr^2-Phe^3-Gln^4-Asn^5-Cys^6-Pro^7-Arg^8-Gly^9-NH_2
S——S

Fig. 1. Amino acid sequence for oxytocin and vasopressin.

A single crystal of pressinoic acid grown by the slow cooling of an ethanol solution provided a total of 2808 intensity measurements recorded with a Picker FACS-1 diffractometer operating in a θ-2θ scan mode. The scan speed was 1.0 degree per minute; background radiation was measured for 40 seconds at each scan extreme. Intensities were corrected for Lorentz and polarization effects but not for absorption. A total of 2547 measurements were detected that were above the background radiation levels and had intensities greater than their estimated standard deviations. The space group was $P2_1$ with lattice parameters a = 10.18 Å, b = 16.77 Å, c = 12.09 Å, and β = 91.30°.

The crystal structure of pressinoic acid, $C_{33}H_{42}S_2N_8O_{10} \cdot 4.5\ H_2O$, was determined by direct methods procedures (*10*). An E map based upon the phases of the 315 largest $|E|$ values revealed three chemically interpretable molecular fragments comprising 30 of the 53 nonhydrogen atoms of the molecule. Fourier refinement provided the positions for the remaining atoms of the peptide molecule and five water molecules designated OW1 to OW5. However, the distance between OW4 and OW5 was found to be unreasonably short and a difference map clearly showed that the electron density in the vicinity of OW4 was shaped like a dumbbell. One lobe made reasonable hydrogen-bonded geometry with OW5, which suggested that the other lobe represented a second disordered position for OW4 in the absence of the OW5 molecule. Because the peak densities of the disordered positions were equivalent, the occupation factors of OW5 and the two OW4's were changed to 0.5; this resulted in the equivalent of 4.5 H_2O molecules per asymmetric unit. The structure was refined by full-matrix least-squares analysis, treating the vibration of all full-occupancy, nonhydrogen atoms anisotropically. Idealized positions for the conformationally determined hydrogen atoms were determined from the geometry of the peptide molecule. The contributions of these hydrogen atoms were included in the final three cycles of least-squares refinement, the positions being recalculated at the end of each cycle. The final difference electron density map did not disclose unequivocal positions for the hydrogen atoms of the water structure. The refinement converged at R = 0.128 and R_w = 0.159 for the 2547 measurements used in the refinement; the standard deviation of an observation of unit weight was calculated to be 3.41 (*11*). Since it is difficult to obtain good quality peptide crystals, it is not uncommon to observe agreement indices larger than 0.10 for the structures of this class of compounds, particularly in the presence of disordered solvent.

Mean values for the N–C, N–C^α, C^α–C, and C–O (*12*) bond lengths are 1.35 ± 0.03 Å, 1.48 ± 0.02 Å, 1.49 ± 0.04 Å, and 1.25 ± 0.02 Å, respectively (n = 6 in each

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Table 1. Torsion angles in degrees. Estimated standard deviations are given in parentheses.

Residue	ϕ	ψ	ω	χ^1	$\chi^{2,1}$	$\chi^{3,1}$
Cys^1		164(1)	180(2)	79(2)	93(1)	
Tyr^2	−143(2)	110(2)	176(2)	−172(2)	78(2)	
Phe^3	55(2)	−115(1)	−165(1)	−64(2)	86(2)	
Gln^4	−66(2)	−20(2)	177(1)	−42(2)	90(2)	−33(3)
Asn^5	−86(2)	3(2)	178(1)	70(2)	21(2)	
Cys^6	−76(2)	174(2)		−61(2)	−70(1)	

case). These values deviate by less than 1.5 standard deviations from their expected values (*13*). All peptide linkages are *trans* and the torsion angle at the disulfide bond is 95°. The fact that the two C–O bond lengths, at the carboxyl terminus, 1.24(2) and 1.26(2) (estimated standard deviation in parentheses) differ by only one standard deviation indicates that the hormone is a zwitterion in this crystalline modification. Torsion angles describing the conformation of the entire molecule are given in Table 1.

The conformation of the backbone of the molecule (Fig. 2) consists of two β-bends. The first bend occurs at residues Tyr^2-Phe^3-Gln^4-Asn^5 and can be best classified as a very distorted type II′ β-bend (*14*). Since both torsion angles of Phe^3 differ by only 5° from the ideal values for ϕ and ψ (that is, 60°, −120°, −80°, and 0°), the distortion in this bend arises primarily in Gln^4, for which the deviations are 14° and 20°, respectively. In addition, the −165° ω torsion angle of Phe^3 is significantly different from 180° and from the average of the absolute value of the other four ω torsion angles (178°). As a result of these distortions, the carbonyl oxygen of Tyr^2 is too far removed (3.59 Å) from the amino nitrogen of Asn^5 to form a hydrogen bond.

A second β-bend involves residues Phe^3-Gln^4-Asn^5-Cys^6 and is a type I bend (*14*). In this case, a hydrogen bond does exist between Cys^6 and Phe^3 (N_6–O_3, 3.11 Å) and the torsion angles deviate by 6°, 10°, 4°, and 3°, respectively, from the torsion angles of an ideal type I β-bend, which are −60°, −30°, −90°, and 0°. Two additional intramolecular hydrogen bonds involve the side chains of Gln^4 and Asn^5. The first of these (N_4–$O_4^{\epsilon 1}$, 2.84 Å) is an intraresidue hydrogen bond and stabilizes only the glutamine side chain conformation. The second hydrogen bond exists between the amide oxygen of the side chain of Asn^5 and the nitrogen of Tyr^2 (N_2–$O_5^{\delta 1}$, 2.83 Å) and provides considerable conformational stability to the entire molecule. This requires the Asn^5 side chain to lie over the macrocyclic ring moiety, in contrast to the position of this side chain proposed for oxytocin and vasopressin on the basis of NMR spectroscopy. This may, however, be a consequence of the loss of the COOH-terminal tripeptide of the parent molecule because, in vasopressin, the Asn^5 side chain may interact with the side chain of the amino acid in position 8 (*3, 4*). The aromatic rings of Tyr^2 and Phe^3 are directed away from the macrocyclic ring and lie over one another but are not parallel (*9, 15*). Instead, the planes of the two rings are inclined to each other by 72° and the geometric centers are separated by 4.78 Å, in agreement with the packing of aromatic rings in proteins (*16*).

The two chain reversals or β-bends in this structure are of interest from several points of view. A type II′ β-bend normally possesses a glycine or a D-amino acid in the $i + 1$ position ($\phi = 60°$, $\psi = -120°$). Thus, it is very unusual to find L-phenylalanine in the conformation observed in this structure. As noted earlier, the torsion angles of the $i + 1$ and $i + 2$ residues in this turn (Phe^3, Gln^4) are significantly distorted from that normally observed. The deviation of the ω torsion angle from *trans* may be an attempt to relieve the stereochemical strain that results from the unusual conformation of Phe^3. Gln^4 lies at a corner in each of the two β-bends. This would normally occur only in type III β-bends for which the torsion angles of each residue are the same and a 3_{10}-helical conformation would result. However, the pressinoic acid molecule has adopted a conformation in which the second β-bend (Gln^4, Asn^5), stabilized by a hydrogen bond, deviates only slightly from ideal at the expense of the conformation of the first bend.

An extensive three-dimensional network of hydrogen-bonded interactions exists in the crystal structure; four hydrogen bonds occur between a pressinoic acid molecule and four of its nearest neighbors, and eight hydrogen bonds link a pressinoic acid molecule to the water of crystallization. With the exception of the amino nitrogen of Asn^5, all potential hydrogen bond donors are involved in at least one inter- or intramolecular hydrogen bond. The nitrogen of Asn^5 does make two intramolecular contacts of 3.13 Å and 3.08 Å to O_3 and $O_5^{\delta 1}$, respectively, but the geometry precludes hydrogen

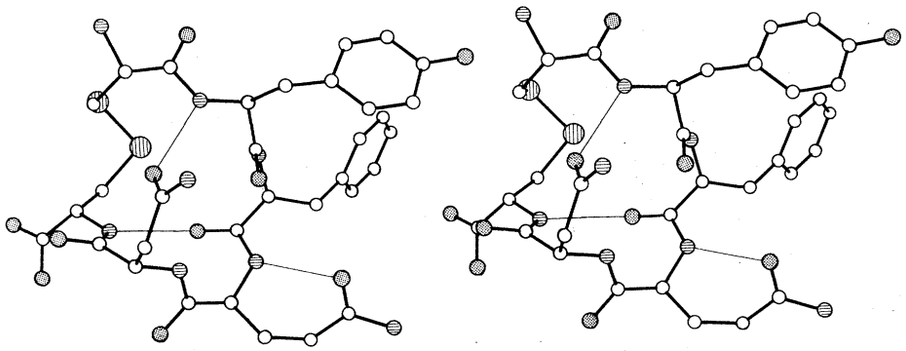

Fig. 2. Stereo drawing of the pressinoic acid molecule. Oxygen atoms are illustrated with stippling, nitrogen atoms with horizontal lines, and sulfur atoms with vertical lines.

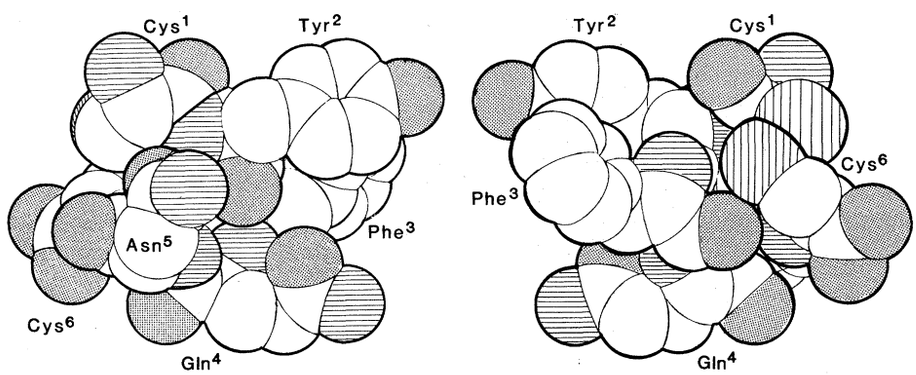

Fig. 3. Space-filling drawings showing (left) the hydrophilic surface on the tyrosine side of the molecule and (right) the hydrophobic surface on the phenylalanine side of the molecule. The heavy lines delineate the boundaries of each of the amino acid residues; oxygen atoms are illustrated with stippling, nitrogen atoms with horizontal lines, and sulfur atoms with vertical lines.

bond formation. Because of the folding of the backbone of the molecule and the placement of the Asn^5 side chain over the one surface of the molecule, N_5 is effectively shielded from the surroundings and makes no contact of less than 3.5 Å with a neighboring peptide or water molecule.

The overall conformation of the molecule results in a structure with a large hydrophilic surface (Fig. 3, left) consisting of the terminal amino and carboxyl groups, the entire Asn^5 residue, portions of Gln^4, and the carbonyl oxygen of Phe^3. A hydrophobic surface, composed primarily of the Tyr^2 and the Phe^3 aromatic side chains (Fig. 3, right), lies on an edge of the hormone while the opposite edge of the molecule is occupied by the two sulfur atoms of the disulfide link. The entire molecule assumes a very compact shape because the Gln^4 and Asn^5 side chains are folded back over the ring moiety. Additional stability for these side chain conformations is provided by hydrogen bonds to the peptide backbone. The orientation of the Asn^5 side chain over one face of the pressinoic acid molecule is in contrast to that which has been proposed on the basis of spectral studies for both oxytocin and vasopressin (*2–4*). It has been suggested that not only is the Asn^5 side chain directed away from the macrocyclic ring, where it interacts with the COOH-terminal tripeptide, but also that it is one of the "active elements" of both hormones. Thus, pressinoic acid is inactive because the COOH-terminal tripeptide is absent and the Asn^5 side chain is in the wrong orientation.

The observed conformation of the Tyr^2 side chain, extended away from the center of the macrocyclic ring, is stabilized in this orientation by a nearly perpendicular Phe^3 aromatic ring. Similar orientations are the most commonly observed interactions between aromatic rings in proteins (*16*). The Tyr^2 side chain has been proposed as an additional "active element" in oxytocin and vasopressin and its orientation determines oxytocic or antidiuretic activity (*3*). Antidiuretic and vasoconstrictor activity has been proposed to result from the orientation of the Tyr^2 side chain away from the 20-membered ring where it is stabilized by a parallel π-π interaction with the Phe^3. However, in oxytocin, Phe^3 is replaced by Ile^3, and this interaction can no longer exist. As a result, the Tyr^2 side chain may be rotated to a position over the macrocyclic ring, imparting oxytocic activity to the hormone. The results of our crystal structure analysis of pressinoic acid strongly support this proposal, but show that the Tyr^2 ring is in the commonly observed perpendicular orientation relative to the Phe^3 ring rather than in a parallel orientation.

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Plant Glutamine Synthetase Complements a *glnA* Mutation in *Escherichia coli*

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A glutamine synthetase gene from alfalfa *(Medicago sativa)* has been expressed in *Escherichia coli* after fusion of bacterial transcription and translation signals to a complete alfalfa glutamine synthetase coding sequence. Synthesis of the alfalfa glutamine synthetase enzyme in *Escherichia coli* was demonstrated by functional genetic complementation of a glutamine synthetase–deficient mutant and by immunoblotting analysis. These results should facilitate protein engineering and structure-function analysis of the plant enzyme.

THE APPLICATION OF MOLECULAR genetics to protein engineering and structural analysis has been amply demonstrated, especially for *Escherichia coli* proteins (*1–5*). The demonstration of expression, assembly, and activity of certain foreign proteins in *E. coli* (*6*) suggests that similar molecular genetic studies on plant proteins in bacteria is feasible, particularly when a genetic complementation scheme can be devised. As a step toward the molecular genetic analysis of a plant enzyme, we describe here the complementation of an *E. coli glnA* mutation by glutamine synthetase (GS) from alfalfa.

GS is an interesting model enzyme for protein engineering and structure-function studies. In plants, GS is an enzyme of central importance in nitrogen metabolism that, together with glutamate synthase, carries out the assimilation of ammonia resulting from nitrate reduction, catabolism, and nitrogen fixation (*7, 8*). The plant GS enzyme is octameric and is usually composed of a single polypeptide species. It appears to be regulated both genetically and allosterically.

Our interest in GS resulted from the isolation of alfalfa suspension cell lines resistant to a herbicide, L-phosphinothricin, which is an irreversible inhibitor of GS (*9–11*). The mode of resistance in these lines is by increased GS enzyme activity resulting from the amplification of a GS gene. Recently, we have cloned and sequenced this amplified GS gene and the corresponding complementary DNA (cDNA) (*10*). The gene is about 4 kb long, contains 11 introns, and encodes a 1400-nucleotide-long transcript and a 39,000 molecular weight polypeptide.

For the synthesis of alfalfa GS in *E. coli*, fusion of appropriate bacterial transcription and translation signals to a complete and uninterrupted GS coding DNA sequence is required. The longest GS cDNA clone isolated in our previous work was 1.3 kb and lacked 44 nucleotides of coding sequence at the 5′ end (*10*). To reconstruct a complete GS coding sequence devoid of introns, we joined the 5′-terminal 70 bp of GS coding sequence present in a clone of a GS nuclear gene to the 3′-terminal 1000 bp of GS coding sequence present in the cDNA. The resulting construct, pGS100, was cleaved

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