Biochemistry

 Volume 26, Number 18 September 8, 1987

Perspectives in Biochemistry

On the Rational Design of Renin Inhibitors: X-ray Studies of Aspartic Proteinases Complexed with Transition-State Analogues†

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Received April 22, 1987; Revised Manuscript Received June 8, 1987

The acceleration of the rates of specific reactions by enzymes is attributed to the stabilization of the transition state at the catalytic center. As a consequence, inhibitors that partially mimic the transition state bind more tightly than the equivalent substrate(s) (Wolfenden, 1972; Lienhard, 1973), and such transition-state analogues are being designed and tested for clinical use.

Renin, an aspartic proteinase produced in the juxtaglomerula cells of the kidneys, catalyzes removal of the decapeptide angiotensin I (AI) from the N-terminus of angiotensinogen (Figure 1). The conversion of AI to the octapeptide angiotensin II (AII) is catalyzed by a carboxydipeptidase, angiotensin converting enzyme (ACE). AII is a potent vasoconstrictor and also stimulates the secretion of aldosterone from the adrenal cortex, which increases the retention of sodium and water by the kidneys. Inhibitors of ACE are effective in lowering blood pressure, and several, for example, captopril (Ondetti & Cushman, 1980), are commercially available.

Renin is a highly specific enzyme: it cleaves only the 10–11 bond in angiotensinogen. The minimum sequence of substrate still hydrolyzed by renin at a measurable rate is the 6–13 octapeptide. However, this cleavage is sufficiently slow to enable the octapeptide to act as a weak competitive inhibitor of the enzyme in vitro, with an IC_{50} of 0.2 mM (Skeggs et al., 1964, 1980). For inhibitory activity, the minimum sequence is the 10–13 tetrapeptide in the form of its methyl ester, which has an IC_{50} of 1 mM (Kokubu et al., 1968, 1973). Replacing the scissile bond with various surrogates of the tetrahedral intermediate has yielded transition-state analogue inhibitors of high potency ($IC_{50} = 10^{-9}$–10^{-10} M) (Hofbauer & Wood, 1985).

Sequences and Structures of Renin and Other Aspartic Proteinases

Renin is a member of a homologous group of aspartic proteinases, which includes pepsin and a group of fungal enzymes such as penicillopepsin, endothiapepsin, and rhizopuspepsin. Their sequences all contain two aspartates (at residues 32 and 215 in pepsin) that are essential for catalytic activity (Hartsuck & Tang, 1972; Chen & Tang, 1972).

The crystal structures of several aspartic proteinases have been solved by X-ray diffraction, revealing a common bilobal structure with a large cleft between the N- and C-terminal domains (Andreeva et al., 1984; Bott et al., 1982; James & Sielecki, 1983, 1986; Pearl & Blundell, 1984). The two essential carboxylates of Asp-32 and Asp-215 are within hydrogen-bonding distance and are approximately coplanar due to the restraints of a hydrogen-bonding network involving residues of the two highly conserved loops that contain the two essential aspartates (Pearl & Blundell, 1984).

Modeling studies based on the homology of renin with other aspartic proteinases have shown that renins may assume tertiary structures that are similar to those of other aspartic proteinases (Akahane et al., 1985; Carlson et al., 1985; Blundell et al., 1983; Sibanda et al., 1984, 1985; Hemmings et al., 1985).

Inhibitors of Renin

The failure to trap covalently bound intermediates (Hoffmann & Fink, 1984) indicates that the aspartic proteinase

† This research was supported by SERC, MRC, Ferring, Merck Sharp & Dohme, ICI, and the Wellcome Foundation.

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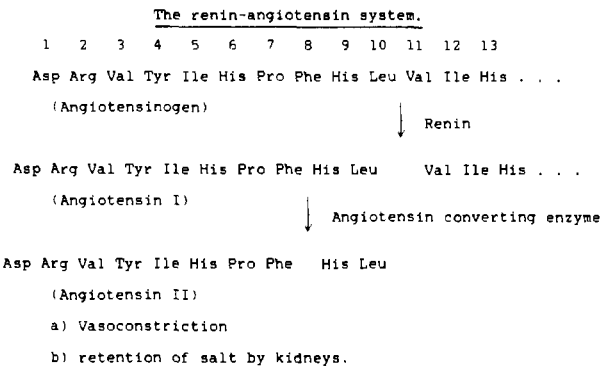
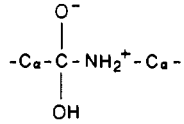


FIGURE 1: Renin–angiotensin system. Renin catalyzes the rate-limiting step of the pathway leading to the vasoactive octapeptide angiotensin II.

mechanism may not involve direct attack of the enzyme on the substrate. Current proposals implicate a water molecule as the nucleophile (James & Sielecki, 1985; Pearl & Blundell, 1984); such a scheme may involve an intermediate or transition state of the type

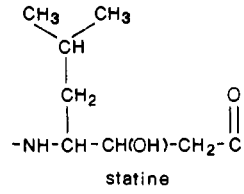


I

Hydrolysis of the scissile bond of synthetic substrates by renin may be blocked by the use of D-amino acids or phenylalanines at the cleavage site (Haber & Burton, 1979). Synthesis of the Pro^5-Phe^{10}-Phe^{11}-Lys^{14} analogue of the 5–14 polypeptide of horse angiotensinogen gave a useful inhibitor, renin inhibitory peptide (RIP), with a K_i of 2 μM for horse renin. This compound was shown to be effective in lowering blood pressure in vivo (Haber, 1984) despite its poor solubility.

The first derivative of the scissile bond to be synthesized was the reduced isostere ($-CH_2-NH-$), the most potent of this series being H142 (Figure 2) (Szelke et al., 1982). The reduced bond nitrogen may be protonated as in the transition state (I). The potency of RIP is improved by 2–3 orders of magnitude by replacing the Phe-Phe dipeptide with the reduced isostere (Szelke et al., 1982; Sawyer et al., 1985).

Pepstatins, aminoacylated pentapeptides, are naturally occurring inhibitors that contain two residues of the unusual amino acid statine [(3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid] (Umezawa et al., 1970).



statine

The central statine is essential for inhibition (Rich & Sun, 1980). The $-CH(OH)-$ group of statine may resemble the likely transition state more closely than the reduced isostere ($-CH_2-NH-$), and pepstatins are very potent inhibitors; e.g., K_i for pepsin = 5×10^{-11} M (Workman & Burkitt, 1979).

Pepstatin is a poor inhibitor of human renin (Gross et al., 1972). However, the incorporation of statine into the 6–13 octapeptide has led to several potent renin inhibitors, for example, L-363,564 (Boger, 1985) and H189 (Tree et al., 1983). Renin inhibitors containing the corresponding deoxyaminostatine residue [$-CH(NH_2)-CH_2-CO-NH-$] have also been shown to bind tightly to human renin (Jones et al., 1985; Arrowsmith et al., 1986), as have inhibitors containing the amino alcohol analogue [$-CH(OH)-CH_2-NH_2$] (Dann et al., 1986).

Several inhibitors containing keto groups ($-CO-CH_2-$) have been reported; see, for example, H272 in Figure 2 (Szelke, 1985). Although the ketone resembles the substrate more than the transition state, the conversion of the ketone to the *gem*-diol [$-C(OH)_2-CH_2-$] may be favored by the high affinity of the enzyme for the tetrahedral intermediate (Rich et al., 1982). The low tendency of ketones to hydrate may be improved by increasing the electrophilicity of the carbonyl carbon atom so that it is more susceptible to nucleophilic attack. Abeles and co-workers have synthesized protease inhibitors of the form $-CO-CF_2-$, and a fluorostatone compound was found to inhibit pepsin 20 times more potently than the equivalent statine analogue (Gelb et al., 1985).

Unfortunately, synthesis of the transition-state analogue $-CH(OH)-NH-$ is impracticable due to the instability of this compound. Statine inhibitors, although close analogues, have the disadvantage of introducing a frame shift with respect to the active site, due to the extra main-chain atoms. To avoid this problem, hydroxyethylene [$-CH(OH)-CH_2-$] analogues have been synthesized (Szelke, 1985). A hydroxyethylene analogue has been incorporated into the human renin substrate 6–13 sequence to give an extremely potent inhibitor, H261 (IC_{50} = 0.7 nM) (Figure 2).

Sequences of the renin substrate and inhibitors													IC_{50} (μM) for human renin
Position	P_6	P_5	P_4	P_3	P_2	P_1			P_1'	P_2'	P_3'		
	(5)	(6)	(7)	(8)	(9)	(10)			(11)	(12)	(13)		
Human angiotensinogen ...	Ile	His	Pro	Phe	His	Leu	- CO - NH -		Val	Ile	His	Asn ...	
H142	Pro	His	Pro	Phe	His	Leu	- CH_2 - NH -		Val	Ile	His	Lys	0.01
L-363,564	Boc	His	Pro	Phe	His	Leu	- C(H)(OH) - CH_2 - CO - NH -		Leu	Phe	NH_2		0.01
H272		His	Pro	Phe	His	Leu	- CO - CH_2 -		Val	Ile	His		0.5
H261	Boc	His	Pro	Phe	His	Leu	- C(H)(OH) - CH_2 -		Val	Ile	His		0.0007

FIGURE 2: Sequences and binding constants for selected transition-state analogue renin inhibitors.

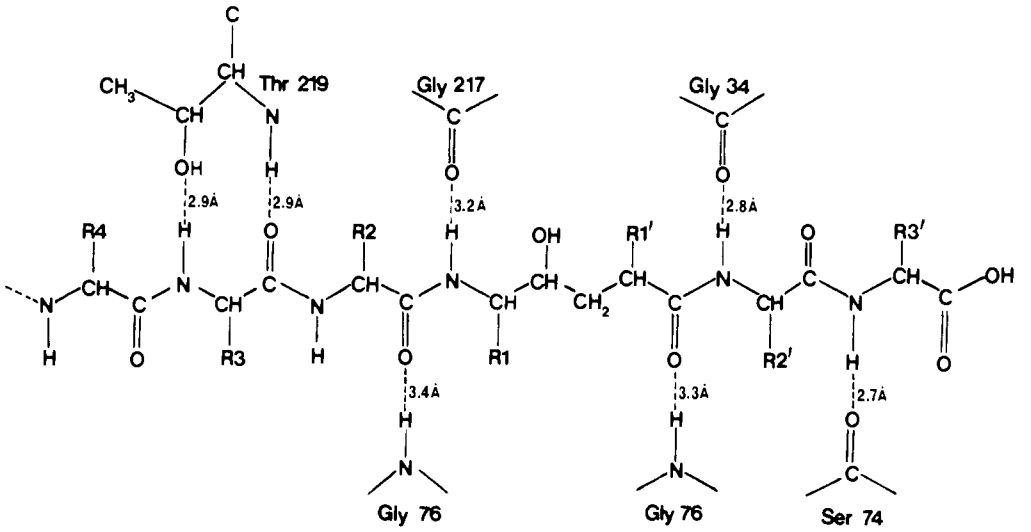


FIGURE 3: Schematic representation of the hydrogen bonds formed between endothiapepsin and the hydroxyethylene inhibitor H261. The distances between acceptor and donor atoms are shown.

Table I: Kinetic Constants for Inhibition of Several Aspartic Proteinases by H142 and H261 (Determined at pH 3.1)[a]

enzyme	H142 K_i (nM)	H261 K_i (nM)
human pepsin	40000	
human gastricsin	15000	
human cathepsin D	40000	
human renin	10[b]	0.7
endothiapepsin	160	<1.0[c]
penicillopepsin	24000	

[a] Hallett et al., 1985; J. Kay, personal communication. [b] Indicates an IC_{50} determined at pH 7.0. [c] Indicates that K_i is too low to measure.

X-RAY STUDIES OF INHIBITORS OF RENIN AND OTHER ASPARTIC PROTEINASES

X-ray crystallographic studies of the cocrystallized complexes of fungal aspartic proteinases with pepstatin provided the first information on how an inhibitor might bind (Bott et al., 1982; James et al., 1982; Bott & Davies, 1983). The 3(*S*)-hydroxyl of the statine residue is within hydrogen-bonding distance of both essential carboxyl groups and replaces a tightly bound water molecule or ion in the native structure. 3(*R*)-Statine, which may not displace this water molecule, binds 10^2-fold less tightly (Liu et al., 1979). It is uncertain if the hydroxyl group of statine represents the position of the attacking nucleophilic water molecule or the carbonyl oxygen of the scissile bond. However, the structural results indicate that one oxygen atom of the transition state will bind tightly to the aspartate pair. As shown in Table I, endothiapepsin binds the human renin inhibitor H142 substantially more tightly than other fungal or mammalian aspartic proteinases. This indicates that the active sites of renin and endothiapepsin may be rather similar and encouraged us to cocrystallize several inhibitors with endothiapepsin, the structure of which has been solved at 2.1 Å (R = 0.19) (Pearl & Blundell, 1984; Blundell et al., 1985).

Our first studies concerned the complex of human renin inhibitor H142 (Hallett et al., 1985). This has now been refined at 2.1 Å to an agreement value of 0.19 (Foundling, 1986; Foundling et al., 1987; Cooper et al., 1987). The inhibitor binds in an extended conformation along the active-site cleft with the reduced bond close to both essential aspartate residues, as anticipated. The structure of the complex of endothiapepsin with L-363,564 (Boger et al., 1983), a statine-containing inhibitor, at 2.2 Å (R = 0.18) (Foundling et al., 1987), has been solved, revealing a binding mode that is generally similar to that of H142. Statine contains two more main-chain atoms than a single amino acid but only one less than a dipeptide. Hence it has been suggested that statine may behave as a dipeptide analogue. This is confirmed by the pattern of hydrogen bonds between L-363,564 and endothiapepsin. Despite the absence of a P1′ side chain (Figure 2), this pocket is not completely empty since the histidine at P2 takes up a different orientation from that of H142 and partly fills the S1′ specificity pocket (Foundling et al., 1987).

The structure of a hydroxyethylene inhibitor complex should give some insight into the conformation of the tetrahedral intermediate and the residues of the enzyme that stabilize the transition state. An X-ray analysis of the complex of H261 with endothiapepsin at 2.6-Å resolution shows that the inhibitor binds to the enzyme via hydrophobic contacts and hydrogen bonds that are generally the same as those identified in the other complexes (Figure 3).

In all complexes the main chain of P3 phenylalanine makes two hydrogen bonds with threonine-219; the hydroxyl of the threonine accepts a hydrogen bond from the amino group of P3, and the carbonyl group of this residue accepts another hydrogen bond from the peptide nitrogen of the threonine. In all complexes the carbonyls of glycines at 217 and 34 accept hydrogen bonds from the P1 and P2′ amide nitrogens, respectively. The active-site flap makes two hydrogen bonds with the inhibitor between the carbonyls of Ser-74 and the amide of P3′ and another between the carbonyl at P1′ and the amide nitrogen of Gly-76. The hydrogen bonds to H261 are shown schematically in Figure 3. In this complex the hydroxyl of the transition-state isostere displaces the water that lies between the carboxyls of aspartates-32 and -215 and binds symmetrically to both groups via hydrogen bonds of equal length, within the error of measurement.

A comparison of the structures of H261 and H142 (Figure 4) clearly shows that despite similar main-chain conformations there are some remarkable differences in the orientations of the side chains at P5 and P2. There appear to be small differences in the positions of the inner residues (P1 and P1′) of H261 and H142 (<0.5 Å), which may be due to the different chemical structures of the transition-state analogues. In H261 the hydroxyl is in the plane defined by the aspartate carboxyls whereas in H142 the amino group of the reduced isostere ($-CH_2-NH_2^+-$), which may dominate the interaction with the carboxyls via a salt link, appears closer to the plane of the carboxyls. On the other hand, the P3 residues of both H142

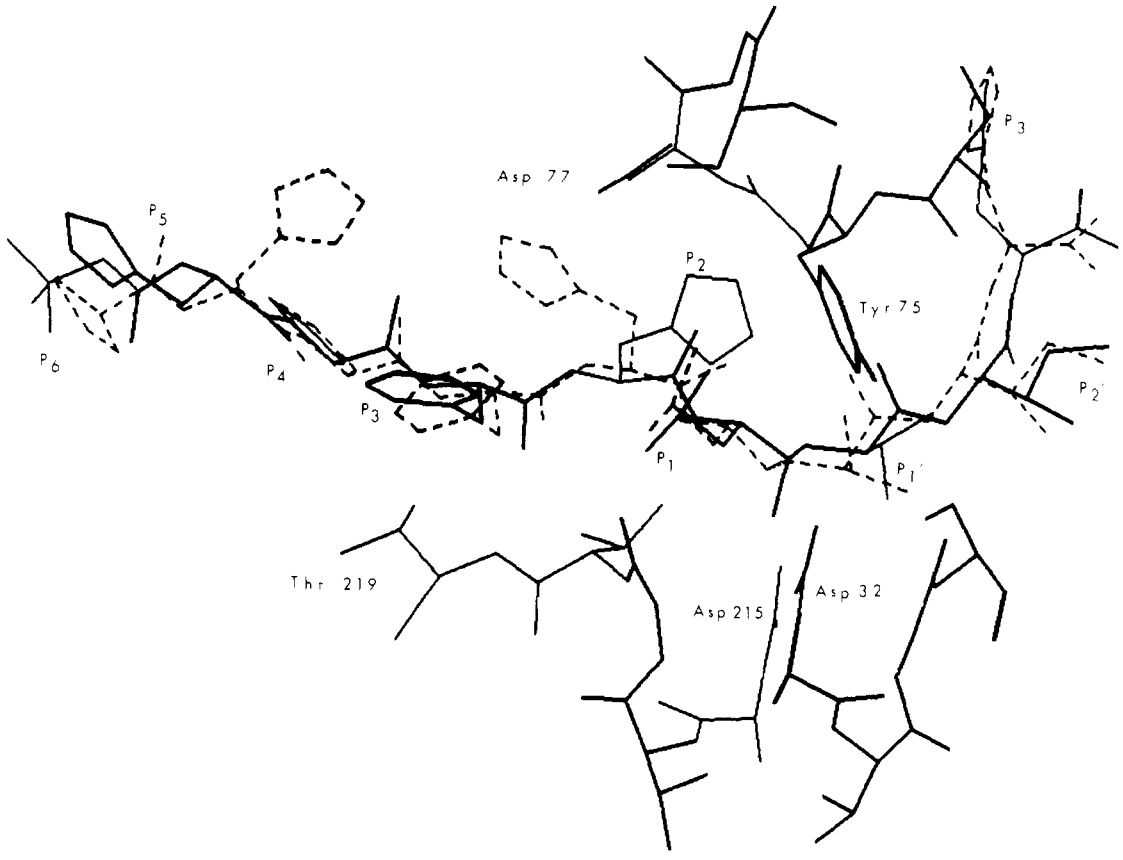


FIGURE 4: Bound conformations of H261 (solid) and H142 (dashed), the active-site flap (above), and the essential aspartic acid residues (below).

and H261 are held in similar positions by the two main-chain hydrogen bonds involving this residue (Figure 3).

The interactions at the primary specificity pockets (S1 and S1′) are largely conserved in H142 and H261. At S1 the leucine side chain of H261 interacts with the hydrophobic side chain of Tyr-75, Phe-111, and Leu-120 as well as Asp-30, which is probably protonated. At P1′ the valine side chain interacts with isoleucines-213, -297, -299, and -301 and Phe-189.

The differences in side-chain orientations at P5 and P2 may be due to the slightly different positions of the inhibitor main chains and the permissiveness of the S2 and S5 binding pockets. For example, at P2 the histidine of H261 is oriented toward and partially fills the S1′ pocket (as in the L-363,564 complex) and interacts with a number of residues from the hairpin loop that covers the active site. However, in H142 the histidine adopts a different orientation in which the imidazole interacts only with Asp-77 of the active-site loop.

At S3 the Phe residue is in van der Waals contact with a number of hydrophobic residues (Ile-7, Ala-13, Ile-117, and Leu-120) as well as three aspartic acid residues (Asp-12, Asp-30, and Asp-114), which are presumably protonated. In human renin the corresponding residues are threonine, valine, and alanine, providing a larger and less polar pocket which accounts for the affinity of this subsite for aromatic residues and its importance in potent inhibition of the enzyme (Boger, 1985; Plattner et al., 1986).

Beyond P1′ the main chains of each inhibitor curl out of the active-site cleft. The P2′ side chain (Ile) is sandwiched between the main chain of the active-site flap and Leu-128 while the P3′ histidine makes no obvious interactions with the enzyme and is exposed to solvent.

Modeling the Transition State

Given the structures of several transition-state analogues,

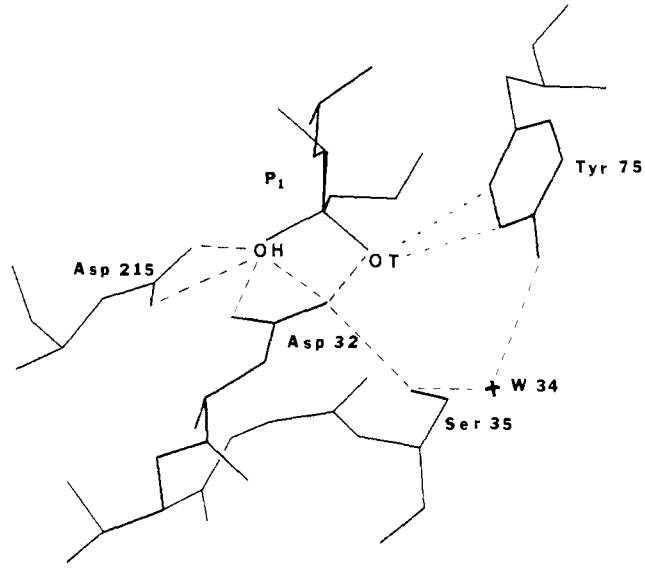


FIGURE 5: Model of the transition state showing one oxygen (OH) hydrogen bonded to both aspartate carboxyls and the other oxygen (OT), where T represents a negative charge or a proton, close to the edge of Tyr-75.

an attempt was made to model the tetrahedral intermediate of the reaction pathway. A distance geometry algorithm devised by Dr. I. Haneef (unpublished results) was used to average the structures of the molecules. With use of the averaged framework, a model of the transition state was built and the interactions it makes with the enzyme were examined (Figure 5).

The extra oxygen of the transition state, not represented in the inhibitors studied so far, would be buried by the active-site flap (Trp-71–Asp-82) and may lie between 3 and 3.5 Å from the edge of the phenol ring of Tyr-75. It has been shown that

edgewise contact between an aromatic ring and a buried oxygen is the most stable and can theoretically contribute as much as 5 kcal mol^{-1} to the interaction energy (Thomas et al., 1982). Hence an interaction of this sort may be energetically equivalent to a hydrogen bond. This suggests that Tyr-75 plays an important role in stabilizing the transition state. The function of the tyrosyl hydroxyl appears to be to stabilize this arrangement through hydrogen bonds with Trp-39. Accordingly, Tyr-75 is absolutely conserved among the 16 known aspartic proteinase sequences.

Design of Transition-State Isostere Inhibitors

Peptide inhibitors of renin containing transition-state analogues have the advantages of potency and, in some cases, species and enzyme specificity (Szelke et al., 1982; Hallett et al., 1985). Animal and clinical trials have shown that they lower blood pressure as significantly as captopril (Leckie, 1985). However, they are inefficiently absorbed from the gut, and the drop in blood pressure is transient even when the peptides are infused. This is due to rapid removal from the bloodstream, and although the effects may be prolonged by addition of blocking groups to the N- and C-termini (Wood et al., 1985), the problem of rapid biliary excretion is harder to solve and may require that the inhibitors be smaller. Retention of at least some of the inner core of hydrogen bonds, identified by the X-ray analysis, would be advantageous for a nonpeptide inhibitor.

One common feature of the inhibitors studied so far is that they bind in an extended conformation; i.e., alternate side chains are on the same side of the molecule. The binding pockets for these alternate side chains are contiguous, e.g., S2 and S1′ or S3 and S1. Cross-linking alternate side chains should not, therefore, interfere with binding to the enzyme and may increase the potency of the inhibitor by locking it in the bound conformation, hence reducing the loss of entropy that occurs on binding. it may also be possible to further stabilize the interactions of the transition-state isostere with the aromatic ring.

Acknowledgments

We are grateful to Drs. L. Pearl, J. Kay, and J. Boger for useful discussion and especially to Dr. I. Haneef for the distance geometry calculations.

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Accelerated Publications

Molecular Recognition between Oligopeptides and Nucleic Acids. Monocationic Imidazole Lexitropsins That Display Enhanced GC Sequence Dependent DNA Binding†

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Received May 19, 1987; Revised Manuscript Received June 24, 1987

ABSTRACT: A series of monocationic lexitropsins, or information-reading oligopeptides, were synthesized to minimize and offset the AT bias for doubly cationic ligands bound in the minor groove of DNA. The compounds possess an *N*-formyl group in place of the guanidinium moiety normally present in netropsin. By systematic replacement of the *N*-methylpyrrole groups of the dipeptide with *N*-methylimidazole, a remarkably high degree of sequence specificity was obtained. One of the compounds having two *N*-methylimidazole residues was found to exhibit dramatically altered specificity when compared with netropsin and preferred to bind to the sequence $^{5'\text{-CCGT-}3'}_{3'\text{-GGCA-}5'}$. The structural elements underlying sequence recognition in terms of the model for the netropsin-DNA interaction are presented and discussed.

The combination of synthetic organic chemistry with DNA footprinting methodology is a powerful way to uncover the structural elements important in ligand-DNA sequence recognition. The antiviral agent netropsin (**1**) is particularly well suited for this approach since the compound has been extensively modified and the structure of its DNA complex at the atomic resolution level is known. A single crystal X-ray analysis of the compound bound to a dodecanucleotide showed that it interacts with four A·T base pairs by displacing the spine of hydration located in the minor groove of double-stranded DNA (Kopka et al., 1985). The drug lies in the center of the groove with its three amide hydrogen atoms engaged in bifurcated hydrogen bonds with O-2 of thymine and N-3 of

† This investigation was supported by grants (to J.W.L.) from the National Cancer Institute of Canada and the Biotechnology Strategic Grants Programme of the Natural Sciences and Engineering Research Council of Canada and by Grant GM 31895 (to J.C.D.) from the National Institutes of Health.
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0006-2960/87/0426-5590$01.50/0