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Evidence for the Presence of Hydrogen-Bonded Secondary Structure in Angiotensin II in Aqueous Solution

(tritium–hydrogen exchange/thin-film dialysis/polypeptide hormone/linear peptide/conformation)

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ABSTRACT **Automated tritium–hydrogen exchange measurements have been made on the linear octapeptide Val_5-angiotensin II amide. All six amide hydrogens of the peptide backbone are observable, and are resolved into three classes according to their exchange rates. The rate of exchange of the slowest class, $t_{1/2}$ of 300 min at 0°C (pH 2.5), is compared with that of hydrogens that exchange abnormally slowly in other peptides. It is concluded that these slow hydrogens in angiotensin II are involved in secondary structure with either one or both forming stable, intramolecular hydrogen bonds. This finding demonstrates that linear peptides may have hydrogen-bonded conformations in aqueous solutions. Analysis of the pH dependence of the rate of exchange indicates that one peptide amide hydrogen, namely that of the Asn_1—Arg_2 peptide bond, is not involved in hydrogen bonding and is freely accessible to the solvent. Thus, the finding of internal hydrogen bonding, together with the assignment of the environment of one peptide bond, places major constraints on the number of allowable conformations of this linear polypeptide hormone.**

Much has been written recently about the importance of polypeptide conformation, the relation conformation bears to biological activity, and about methods to determine the conformation in aqueous solution. In spite of this only a few examples can be found in the literature where the total conformation has been reliably established, even with cyclic polypeptides in which conformational possibilities are greatly restricted. In addition, present methods have been inadequate to cope with the problems of defining the conformation of small linear polypeptides devoid of covalent crosslinks. Here, the question may be raised as to whether or not there is a unique conformation, and indeed such peptides have been frequently referred to as "random coil" types. While the synthetic polyamino acids have been much studied as linear models, showing conformation in certain solvent environments, they represent special cases and are heterogenous with respect to size. With recent advances in synthetic capability for polypeptides, together with improved methods to assess the degree of randomness by combinations of techniques such as nuclear magnetic resonance (NMR), tritium–hydrogen exchange, circular dichroism, thin-film dialysis, etc., evidence is accumulating that certain of the linear polypeptides can have a unique conformation in a given aqueous solution, or at least exhibit a highly restricted degree of randomness. Such information is essential to an understanding of the molecular basis of action of polypeptide hormones.

The linear octapeptides of angiotensin II are good models for conformational studies, as well as being of importance in their own right. Evidence was presented some years ago from thin-film dialysis (1) and optical rotatory dispersion (ORD) measurements (2) indicating the presence of a unique and compact conformation of Val_5-angiotensin II in certain aqueous environments. While another early study (3) that used ORD, titration, and hydrogen exchange was not interpreted in terms of secondary structure, all subsequent studies have been. For example, a structural change caused by high pH was first indicated by biological activity studies (4), and was supported by thin-film dialysis studies (5) and ORD measurements (6). Later, the possibility of two interchangeable forms was supported by thin-film dialysis and gel filtration studies (7). More recently, a crystalline form of angiotensin II has been reported (8).

Evidence from automated tritium–hydrogen exchange presented in this paper strongly supports the interpretation of secondary structure in angiotensin II. Tritium–hydrogen exchange measures the rate of exchange of labeled peptide amide hydrogens with hydrogens in aqueous solution. It has been demonstrated in many studies that involvement of the amide hydrogen in an intramolecular hydrogen bond (9–11) or steric shielding of the hydrogen from the solvent by folding (9, 12–14) will markedly reduce the exchange rate. Either interpretation indicates structure. The numbers of protons involved can be determined quantitatively and the rates of exchange may provide an estimate of the conformational stability of the molecule.

MATERIALS AND METHODS

Synthetic Val_5-angiotensin II amide, used for this study, was kindly supplied by Dr. R. Schwyzer, Ciba-Geigy, Basle. This synthetic preparation contains a significant quantity of the isoasparagine form of angiotensin II (15). There is no difference in the biological activities between the iso- and normal forms of the peptide. In addition, the results we obtained with this preparation were checked with a recent preparation of Val_5-angiotensin II amide (Hypertensin, Ciba) supplied by Dr. William Wagner, and insignificant differences were noted.

The technique of tritium–hydrogen exchange used in this study involves a newly developed, automated handling system to be described elsewhere. In general, the technique is similar to that already described (16, 17). Samples were prepared for exchange measurements by dissolving 1.3–2.0 mg of the crystalline peptide in 250–300 μl of the exchange buffer. About 5 mCi of tritiated water was added and the sample was incubated at 0°C to allow complete equilibration of the added tritium between the polypeptide and the solvent. The incubation time was 19–26 hr. Control experiments indicated that

there was no change in the observed exchange curve after extended incubation at 0°C. The exchange measurements were done with buffers consisting of 0.1 M sodium chloride–0.01 M KH_2PO_4. The concentration of peptide was determined by UV absorption, on the basis of a molar extinction coefficient at 225 nm of 11,405 M^{-1} cm^{-1}. Tritium concentrations were determined by counting in either Bray's solution or in Aquasol (New England Nuclear). The samples were counted for times sufficient to insure a standard error of less than 3% in the samples with lowest activity.

RESULTS

The exchange rates of peptide N-H protons are of sole concern in this study, since in typical aqueous solutions C-H protons do not usually exchange, while hydroxyl and amino protons exchange too rapidly to be measured. Val_5-angiotensin II amide contains eight residues; however, since one N-H residue is the N-terminus while another residue is proline, there are only six peptide hydrogens in the backbone. The asparagine residue contains two primary amide hydrogens in its side chain; however, data (Englander, S. W., personal communication) indicate that these would have observable exchange rates only around pH 4, our highest experimental pH.

The observed exchange curves for Val_5-angiotensin II amide are given in Fig. 1 for all pH values examined. The observed experimental variable in these experiments is the total number of hydrogens/mol of polypeptide remaining unexchanged at time t (H_u). This quantity is the sum of a series of independent first-order exchange reactions representing each peptide N-H. To best represent such a quantity, it is plotted on a semi-logarithmic scale. Extrapolation of the curve to the ordinate at $t = 0$ gives the total number of observable, exchangeable hydrogens. As shown in Fig. 1, we are able to observe all six backbone hydrogens at most of the pH values examined. The minimal exchange rate, pH_{min}, occurs around pH 2.5. The data are highly reproducible as evidenced by the fact that all the curves except those at pH 4.0 and pH 1.6 represent data obtained from two completely independent experiments. There are several ways to analyze a sum of exponential terms (18), and we have chosen in this study to use the method of graphical successive subtraction. Applying this technique to the data in Fig. 1, we obtain the results given in Table 1. There are two hydrogens in the slowest class, which exchange, at pH_{min}, with a half-time of 300 min. The data are further resolved into two additional classes, one of intermediate rate, $t_{1/2}$ at pH_{min} of 80 min, and one much faster. The number of hydrogens in Class I is invariant with pH. However, in Class II, and by inference in Class III, the number of hydrogens is different at pH values more acidic than pH_{min}, than at values more basic than pH_{min}, 3 versus 2. Hydrogen exchange is acid–base catalyzed, and thus the observed variation of the exchange rate with pH results from either hydrogen or hydroxyl-ion catalysis being the dominant exchange pathway (19, 20). pH_{min} represents the pH at which the two paths make equivalent contributions to the observed exchange. The variation of rates of exchange of Class I and Class II hydrogens with pH is shown in Fig. 2. Theoretically, the variation with pH on either side of pH_{min} should follow a curve with a slope of $\log_{10}$ 10/pH unit. This theoretical dependence is obeyed quite well by Class I; however, the slope of Class II is significantly less

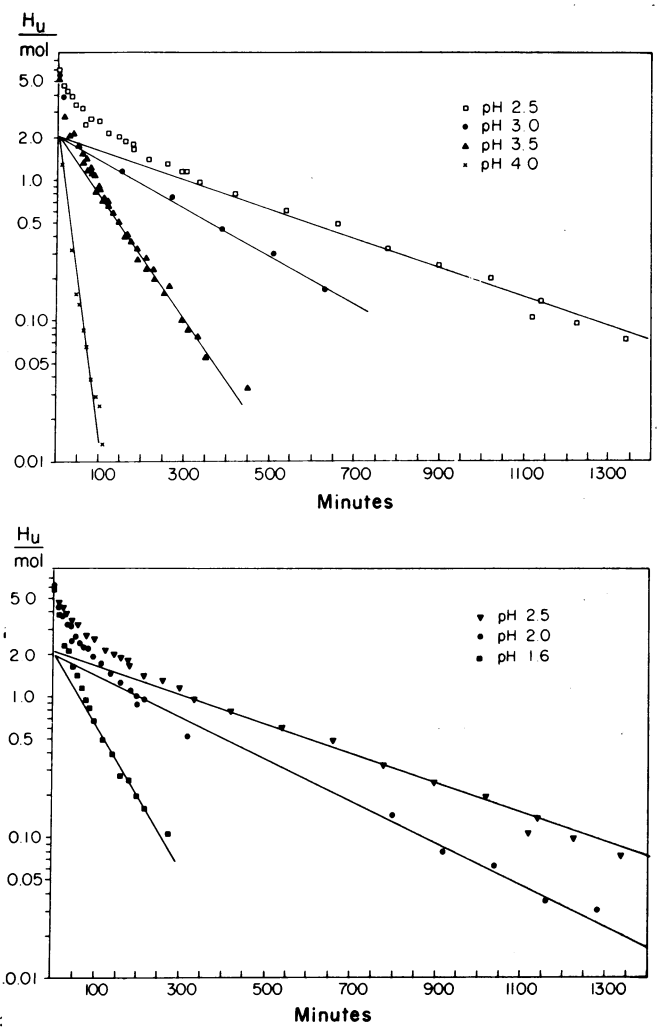

FIG. 1. Observed automated tritium–hydrogen exchange curves for Val_5-angiotensin II amide at 0°C and at various pH values.

TABLE 1. *Summary of the class analysis of the curves in Fig. 1*

pH	C_I	$t_{1/2,I}$ min	K_{obs} min^{-1} ($\times 10^3$)	C_{II}	$t_{1/2,II}$ min	K_{obs} min^{-1} ($\times 10^3$)	C_{III}
4.0	2.0	16	43.3				
3.5	2.0	73	9.5	2.1	23	30.1	2
3.0	2.0	180	3.85				
2.5	2.1	300	2.31	2.1	80	8.7	2
2.0	2.0	202	3.43	3.0	38	18.2	1
1.6	2.0	60	11.55	3.0	20	34.6	1

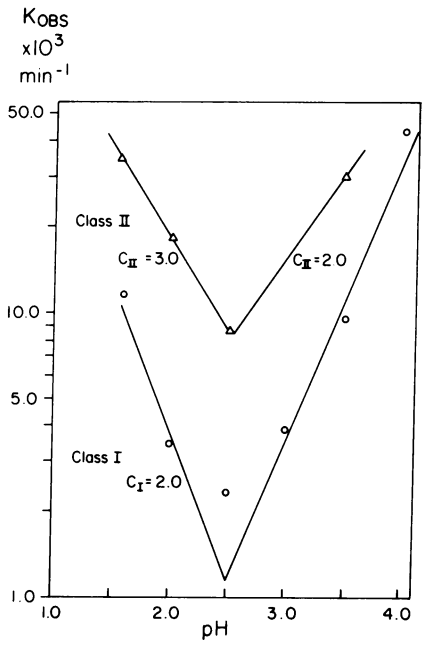

FIG. 2. pH–rate profile of Val_5-angiotensin II amide. The data plotted in this figure were obtained by graphical analysis, described in the text, of the exchange curves in Fig. 1. Class I consists of those hydrogens with the slowest exchange rate, while Class II is of intermediate exchange rate. The class sizes are given on Fig. 2.

than predicted, namely $\log_{10}$ 4/pH unit. This is presumably a result of heterogeneity in the pH dependence of the hydrogens in Class II.

Additional evidence for compactness of angiotensin II in aqueous solution

Thin-film dialysis is a technique that measures the rate at which macromolecules pass through a calibrated membrane under conditions that optimize the diffusion rate. Previous measurements of the diffusion rate of angiotensin II amide had indicated that the molecule was quite compact in aqueous solution (1). In order to exclude the possibility that charges on the membrane were, in part, responsible for the rate of dialysis of angiotensin II, measurements were repeated with membranes that were treated to remove any charge (Chen, H. C. & Craig, L. C., unpublished). In addition, the effect of temperature on the dialysis rate was determined, and is given in Table 2. In Table 2 it can be seen that the temperature coefficient for the interval 25–40°C coincides closely with that expected on the basis of no conformational or hydration change and it is due solely to free diffusion (21). This evidence, together with the lack of a salt effect (1), is in line with the concept of a compact, relatively rigid conformation. Additional measurements with angiotensin I indicated that this peptide showed a large shift between 25 and 40°C, indicating that the conformational flexibility of the decapeptide is greater than the octapeptide. This result presumably indicates that the two additional residues are in a flexible part of the molecule.

TABLE 2. *Temperature-dependent thin-film dialysis measurements*

Temperature (°C)	Val_5-angiotensin II amide $t_{1/2}$ (min)	Val_5-angiotensin I $t_{1/2}$ (min)
25	38	75
40	25	34
Observed ratio		
$t_{1/2}$ 25/$t_{1/2}$ 40	1.52	2.2
Theoretical ratio (22)	1.52	1.52

The measurements recorded in this table were made in a size 18 seamless Visking tubing that had been treated with glycine amide according to the method of Chen and Craig (unpublished) to remove all fixed charges. It was calibrated at 25°C in 0.1 M NaCl against bacitracin A; $t_{1/2}$ = 85 min. The value of 38 min found for Val_5-angiotensin II amide is in accord with its compact size (1). Likewise, the measurement for Val_5-angiotensin I is in agreement with previous findings.

DISCUSSION

Evidence for hydrogen-bonded backbone hydrogens

It is clear from the data in Fig. 1 that all six backbone peptide hydrogens are observable by automated ^3H–^1H exchange at 0°C. Class analysis indicated that two of the six hydrogens exchange very slowly compared to the other four. The rate of exchange of these two slow protons with the exchange rates of protons in other polypeptides are compared in Table 3. Poly(D,L-alanine), an example of a neutral polypeptide without hydrogen bonds, has an exchange rate at pH_{min} of 82

TABLE 3. *Rate constants and class sizes for various polypeptides*

Peptide	Class size	Half-time for exchange at pH_{min}	Ref.
Angiotensin II amide			
Class I	2	300	
Class II	2–3*	80	
Class III	2–1*	15	
Bacitracin A			
Class I	1	325	26
Class II	4	158	
Class III	6	41	
Gramicidin-SA			
Class I	2	2772	9
Class II	2	385	
Class III	4	96	
Polymyxin B_1			
Class I	1	215	27
Class II	2–3*	57	
Class III	8–7*	14	
Bradykinin			
Class I	3	80	23
Adrenocorticotropin, β^{1-24}	2–4†	190–260	
Poly(D,L-alanine)	1	82	28
Poly(D,L-lysine)	1	192	28

Tabulation of the class sizes and rate constants for various polypeptides.

* Denotes that the class size is different on the base-catalyzed side of pH_{min} than on the acid-catalyzed side.

† Denotes that insufficient data have been collected to obtain an accurate class analysis.

min. Poly(D,L-lysine) is 2.5- times slower (192 min), although it too is not hydrogen bonded. However, evidence has been presented to indicate that it may have a relatively rigid conformation in solution when it is fully ionized (22). This conformation, we believe, may result in some steric shielding of the peptide backbone from the solvent, with a concomitantly reduced exchange rate of the peptide protons. In all of the naturally occurring polypeptides listed in Table 3, there are several classes of exchanging hydrogens, and, in addition, Class I is substantially slower than Class II. In general, the exchange rates of Class II hydrogens in those peptides approximates that of poly(D,L-alanine). It is generally agreed that gramicidin-SA contains four hydrogen bonds, and we have shown that four of the amide hydrogens are very slowly exchangeable. Two of these four slowly exchangeable hydrogens have rates similar to those of the other Class I hydrogens listed in Table 3.

The Class II hydrogens in Val_5-angiotensin II amide ($t_{1/2}$ = 80 min) fall in range *A*, and are probably freely accessible to the solvent and not involved in any internal structure. However, the exchange rate for its Class I hydrogens ($t_{1/2}$ = 300 min) falls well within range *B* of Table 4. It is clear that this exchange rate is substantially slower than that found in model unstructured polypeptides. We believe that these two hydrogens are abnormally slow to exchange because of their involvement in secondary structure. To support this interpretation, three additional observations must be considered. First, we have shown that bradykinin, a nonapeptide, does not have any slowly exchanging hydrogens (i.e., $t_{1/2}$ 100 min) (23). This finding is consistent with a lack of hydrogen-bonded structure, presumably attributable to the presence of three proline residues out of nine total amino acids. Second, on the basis of exchange measurements with simple amides, Molday has predicted a theoretical exchange curve for an unstructured model of Val_5-angiotensin II amide that contains no hydrogen bonds (Molday, R., personal communication). His calculations indicate that there are no hydrogens that, over a pH range of 2–4, should have exchange rates as slow as 300 min. Third, many studies have demonstrated that involvement of an exchangeable hydrogen in a stable hydrogen bond markedly reduces its exchange rate. Since the process of exchange must involve hydrogen-bond formation to the solvent, the exchange rate must be directly proportional to the fraction of time the hydrogen is bonding to the solvent. Thus, involvement in a hydrogen bond or steric shielding by the polypeptide chain would be expected to proportionately reduce the exchange rate of that hydrogen. The two slow hydrogens in Val_5-angiotensin II amide may either both be hydrogen bonded, or one may be hydrogen bonded while the second is sterically shielded by the folded polypeptide chain. Since the peptide is very small and has no covalent crosslinks, such as -S-S-bridging, it is unlikely that the backbone would fold into such a compact conformation as to make two hydrogens inaccessible to the solvent. Thus, we postulate that at least one, and possibly two, of the backbone peptide hydrogens in Val_5-angiotensin II amide are involved in intramolecular hydrogen bonding.

Assignment of one unstructured residue in the angiotensin molecule

As detailed above, we found that the size of Class I was invariant with pH, and that the exchange rate varied with pH in a theoretically predicted manner. However, Class II showed both a variation in size with pH and an apparent diminished dependence of the rate with pH change. There were two hydrogens in Class II on the base-catalyzed limb and three on the acid-catalyzed limb of the pH–rate profile. Thus, one proton (in Class III) that exchanged very rapidly via base catalysis became slower exchanging when the dominant exchange mechanism became specific acid catalysis. All amide protons should theoretically have the same dependence on hydrogen or hydroxyl ion. Thus, the number of hydrogens in any class should be constant both on the acidic and basic sides of pH_{min}. There are four possible reasons for the class size to change. First, there may be changes in the state of ionization of side chains of the residues. This has been shown to result, in some instances, in altered inductive effects on the amide and possible concomitant changes in its acidity or basicity (24). However, titration studies (3) indicate that the only group that is titrated over the range of pH 4.0–1.6 is the carboxyl group on the C-terminal phenylalanine. The pK of this group at 0°C is about 3.5. Thus, a minimal contribution would be expected at pH 2.5 or lower. In addition, we observe a shift from a fast-exchanging class (at high pH) to a slower-exchanging class at low pH, the opposite of what would be predicted. Therefore, it is unlikely that the shift in class size is due to a titration event. Second, a conformational change may occur that would shift exchangeable hydrogens from a structured state to a fully accessible form, or vice versa. However, there is no evidence in the literature to indicate that any conformational change occurs in angiotensin II around pH 2.5. Furthermore, the rate of exchange of the hydrogen affected would indicate that it is always in an unstructured part of the molecule. Third, there may be peptide degradation that results in a markedly altered primary structure. However, it is very unlikely that any degradation would have occurred over the time or at the temperature of the experiment. Fourth, the effective concentration of hydrogen or hydroxyl ion in the immediate vicinity of the peptide backbone would be influenced by the presence of charged side chains. Thus, the presence of two adjacent, similarly charged side chains might greatly alter the ease of catalysis of exchange of the intervening amide proton by hydrogen or hydroxyl ion. We believe that this fourth explanation is the most convincing, and that the change in class size due to a shift of one proton from Class III to Class II results from a change of that amide

TABLE 4. *Relationship between exchange half-time and conformation*

Range	Half-time for exchange at 0°C and pH_{min} (min)	Conformational classification
A	≤100	Not hydrogen bonded, freely accessible
B	200–400	Hydrogen bonded *or* partly shielded from the solvent
C	>400	Hydrogen bonded and/or shielded from the solvent

Classification of the exchange rates of backbone peptide hydrogens in terms of a generalized conformational description. This table would be applicable to small polypeptides or proteins but not necessarily to high molecular weight proteins or polypeptides. The exchange half-time would be in a completely aqueous solution.

proton from a state of facilitated hydroxyl-ion catalysis to a state of normal or retarded hydrogen-ion catalysis. Since this would occur if that peptide N-H were in the immediate environment of two positively charged side chains, we believe that it must be localized to the peptide bond between asparagine and arginine. Furthermore, if this is the case, we are able to assign this peptide bond to a freely accessible part of the molecule that is not hydrogen bonded. This assignment is consistent with structure–activity relationships found with angiotensin analogs (25).

Constraints to be applied to any model of angiotensin II

The evidence presented in this paper may be summarized as follows:

(*a*) The side chain of asparagine is freely accessible to the solvent.

(*b*) Two of the six backbone peptide hydrogens are involved in secondary structure.

(*c*) At least one of these two hydrogens is involved in an intramolecular hydrogen bond.

(*d*) The peptide NH of the asparagine–arginine peptide bond is not involved in any secondary structure, but is freely accessible to the solvent.

These findings place major constraints on possible conformations of Val_5-angiotensin II amide, and further indicate that this hormone has a unique conformation in aqueous solution formed by the chain folding back upon itself. Based on these observations, we have developed models for this polypeptide that will be reported elsewhere.

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