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The pH Dependence of the Conformation of Angiotensin Peptides by Nuclear Magnetic Resonance: Cis–Trans Isomerism of Proline 7†

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ABSTRACT: The pH dependence of the proton NMR spectrum of [Asn^1,Val^5]angiotensin II in aqueous solution shows the existence of one major and one minor conformation above pH 6.5, the minor conformation representing 12 ± 2% of the total peptide. A similar observation has been made for [Asn^1, Val^5]angiotensin I and Val-Tyr-Val-His-Pro-Phe. This effect is not due to the presence of angiotensin-like impurities in the peptide samples. We have shown two expected impurities, [β-Asp^1,Val^5]angiotensin II and [Asn^1,3-Bzl-Tyr^4,Val^5]-angiotensin II, to be absent, and a third impurity, [Asn^1,Val^5,D-His^6]angiotensin II, to be present at less than or equal to 2.1 mol %, too little to account for the observed amount (12 ± 2%) of minor conformation. The carbon-13 spectrum of the hexapeptide at high pH shows that the major conformation has Pro^7 in the trans form and the minor conformation has Pro^7 in the cis form.

Several models have been proposed for the solution conformation of angiotensin II (Smeby et al., 1962; Weinkam and Jorgensen, 1971; Printz et al., 1972a; Fermandjian et al., 1972). The one common feature of all of these models is a compact, ordered conformation, which was also found by Deslauriers et al. (1975) by ^{13}C NMR. However, Marshall et al. (1973) have shown that the peptide NH-C$^\alpha$H coupling constants observed for angiotensin II are inconsistent with all of the proposed models. Nevertheless, a compact conformation and the two intramolecular hydrogen bonds proposed by Printz et al. (1972b) and confirmed by Bleich et al. (1973) seem to be necessary components of the solution structure of angiotensin II.

A major difficulty in determining the solution conformation of angiotensin at high resolution is the availability of highly purified material. Interpretation of physical data which suggest different populations of conformations relies on a large supply of synthetic peptide of high, quantitatively known purity. There are several angiotensin-like contaminants known to be formed during synthesis of angiotensin by both the solution and solid phase methods. Solution synthesis of [Asp^1,Val^5]- and [Asn^1,Val^5]angiotensin II has led to the acid-catalyzed rearrangement product [β-Asp^1,Val^5]angiotensin II (Riniker, 1964). Solid phase synthesis has yielded as much as 40% [D-His^6]angiotensin (Windridge and Jorgensen, 1971; Khosla et al., 1972). In addition, acid-catalyzed rearrangement of *O*-benzyltyrosine to 3-benzyltyrosine can occur during cleavage from the resin in solid phase synthesis (Erickson and Merrifield, 1973). We intend to show here that our synthetic [Asn^1,Val^5]angiotensin II, [Asn^1,Val^5]angiotensin I, and C-terminal hexapeptide of angiotensin II are free of β-Asp^1 peptides and of 3-benzyltyrosine peptides within the limits of our analytical methods, and that these peptides contain less than 2.1 mol % of D-His^6 peptides. Therefore, the observation of the minor conformation for angiotensin II above pH 6.5, representing 12 ± 2% of the total peptide, cannot be due to the presence of any of the above angiotensin-like impurities, and is the result of trans to cis isomerization of Pro^7.

The aim of our earlier NMR study (Bleich et al., 1973) was the identification of those amide protons that had been previously observed by the tritium technique to exchange rather slowly (Printz et al., 1972b) near the pH of minimum exchange rate (pH 3–4). The assignments of all amide resonances reported in that study were subsequently confirmed by Glickson et al. (1974). The NMR study of angiotensin and analogues at and above physiological pH is beset by two problems: the base-catalyzed exchange rate of all amide protons is so large that the resulting line broadening prevents observation of the respective coupling constants, and limited solubility in this pH range requires long data accumulation time (Glickson et al., 1973). The present study by proton magnetic resonance has, therefore, been performed in deuterium oxide as solvent, and the use of Fourier transform NMR has circumvented the problem of peptide solubility.

Materials and Methods

Synthesis of Angiotensin Peptides. The tetrapeptide fragment of [Val^5]angiotensin II was obtained from material used

† From the Laboratories of the Rockefeller University, New York, New York 10021. *Received November 6, 1975.* This work was supported in part by National Institutes of Health Grants AM 02493 to Lyman C. Craig, GM 21714 to James D. Jamieson of Yale University, and RR 00639 to Jay A. Glasel of the University of Connecticut Health Center.

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TABLE I: Analytical Data for Synthetic Peptides.

Peptides	App pH of Elution[a]	Yield (g)	Yield (%)[b]	% Peptide in Product[c]	Asp	NH_3[d]	Arg	Val	Tyr[e]	3-Bzl-Tyr[f]	His	Pro	Phe	Leu
[Asn^1,Val^5]Angiotensin I	1.90	0.503	45	96	0.89	0.9	0.94	2.00	1.00		1.90	1.03	0.95	0.90
[Asn^1,(3-Bzl-Tyr)^4, Val^5]Angiotensin I	1.65	0.143	11	96	0.8	0.9	0.8	2.00	0.2	0.9	1.7	1.00	0.91	
[Asn^1,Val^5]Angiotensin II-N[g]	1.95	0.703	38	93	0.92	1.0	0.87	2.00	0.96		0.93	1.01	1.00	
[Asn^1,(3-Bzl-Tyr)^4, Val^5]Angiotensin II-N[g]	1.90	0.193	10	100	1.0	1.1	0.7	2.00	0.1	0.9	0.90	1.10	1.10	
[Asn^1,Val^5]Angiotensin II-T[h]	1.95	1.34	50	99	0.93	1.1	0.94	2.00	0.95		0.93	0.99	0.99	
[Asn^1,(3-Bzl-Tyr)^4, Val^5]Angiotensin II-T[h]	1.90	0.296	12	95	0.9	1.2	0.9	2.00	0.0	0.9	1.0	0.93	0.92	
Val-Tyr-Val-His-Pro-Phe	2.00	0.336	26	90				2.00	1.01		1.11	1.00	1.00	
Val-(3-Bzl-Tyr)-Val-His-Pro-Phe	1.70	0.082	4	94				2.00	0.0	1.0	1.0	1.0	1.0	

[a] Elution with an increasing gradient of acetic acid from the protonated form of Bio-Rex 70 (App, apparent). [b] The yield of final purified product is based on the amount of the first amino acid attached to the resin. [c] Calculated as the (weight of peptide present)/(total weight of sample), where the weight of peptide present is based on the amino acid analysis, and the peptides are presumed to be isolated as the mono-, di-, and triacetate salts for the hexapeptide, angiotensin II, and angiotensin I, respectively. [d] The ammonia contained in each peptide was determined on the amino acid analyzer by subtracting the ammonia contained in a blank hydrolysate from the amount of ammonia found to be present in each peptide sample. [e] Tyrosine was determined by acid hydrolysis in the presence of 0.2% phenol (Salnikow et al., 1973). [f] 3-Benzyltyrosine was determined after acid hydrolysis by Dr. B. W. Erickson of the Rockefeller University (Erickson and Merrifield, 1973). We are grateful to Dr. Erickson for providing us with analyses of peptides containing 3-benzyltyrosine. [g] [Asn^1,Val^5]-Angiotensin II synthesized using N^{α}-Boc-N^{G}-nitroarginine. [h] [Asn^1,Val^5]Angiotensin II synthesized using N^{α}-Boc-N^{G}-tosylarginine.

in an earlier thin-film dialysis study (Craig et al., 1964) and was not further purified. Ciba [Asn^1,Val^5]angiotensin II was purified by countercurrent distribution in the system 1-butanol–acetic acid–water (4:1:5).

[Asn^1,Val^5]Angiotensin I and II and the C-terminal hexapeptide of [Val^5]angiotensin II were prepared by solid-phase synthesis (Merrifield, 1963) using N^{α}-t-Boc-amino acids (JEM Research Products, Inc., Kensington, Md.) which were coupled to the peptide resin in methylene chloride solvent using dicyclohexylcarbodiimide. A reaction time of 2 h was employed with a threefold excess of Boc-amino acid and dicyclohexylcarbodiimide. Boc-Asn, however, was coupled as its *p*-nitrophenyl ester in dimethylformamide using a fivefold excess for 24 h. Trifunctional amino acids were coupled as Boc-His(Tos), Bos-Tyr(Bzl), and Boc-Arg(Tos). [Asn^1,Val^5]Angiotensin II was prepared twice, once using Boc-Arg(Tos) (designated as [Asn^1,Val^5]angiotensin II-T), and once using Boc-Arg(NO_2) (designated as [Asn^1,Val^5]angiotensin II-N).[1] Deprotection of α-amino groups was by 25% trifluoroacetic acid in methylene chloride for 20 min. Cleavage from the resin was with anhydrous HF–anisole (4:1, v/v) for 1 h at room temperature.

Each of the four peptides ([Asn^1,Val^5]angiotensin I, [Asn^1,Val^5]angiotensin II-T, [Asn^1,Val^5]angiotensin II-N, and the C-terminal hexapeptide of [Val^5]angiotensin II) was purified by ion-exchange chromatography on Bio-Rex 70 (H^+ form) (Bio-Rad) by eluting with a gradient of increasing concentration of acetic acid, from 1 to 50%, in analogy to the scheme used for purification of synthetic bradykinin (Guttmann et al., 1962).

Paper electrophoresis of peptides was performed in a Pherograph flat bed apparatus on Brinkmann MN paper no. 214 at 60 V/cm in pH 5.6 pyridine acetate buffer and in 50% acetic acid. Paper chromatography was on Brinkman MN paper no. 214 in the systems: 2-butanol–3% ammonia (30:11) and 2-butanol–2-propanol–water–pH 8.0 phosphate buffer (7:7:5:2) (Riniker and Schwyzer, 1964). Detection of peptides on paper was with 2% ninhydrin in acetone and with the Pauly reagent described by Stewart and Young (1969). Amino acid analyses were on a Beckman Model MS instrument and a Durrum 500 instrument.

Racemization of Histidine in [Asn^1,Val^5]Angiotensin I and II. The method of Manning and Moore (1968) was used to determine the extent of racemization of histidine in the three peptides obtained from totally independent syntheses. L-Glutamic acid *N*-carboxyanhydride was purchased from Chemical Dynamics Corporation, Hadley Industrial Plaza, South Plainfield, New Jersey, and was found to be of excellent quality as judged by high yield of dipeptide and a persistently clear reaction solution. For each of the peptides, [Asn^1,Val^5]angiotensin I, [Asn^1,Val^5]angiotensin II-N, and [Asn^1,Val^5]angiotensin II-T ([Asn^1,Val^5]angiotensin II-N was prepared from the C-terminal hexapeptide resin), a stock solution in 6 N HCl (about 1 mg/ml) was prepared and aliquoted into separate tubes which were evacuated, sealed, and hydrolyzed simultaneously. A tube was opened every 24 h for 144 h, the content was evaporated dry, and a tracer quantity of L-[3-^{3}H]His (New England Nuclear, 10.4 Ci/mmol) added. Less than 10^{-3} mol of L-[3-^{3}H]His was added per mol of

[1] Abbreviations used: t-Boc, *tert*-butyloxycarbonyl; Boc-His(Tos), N^{α}-Boc-N^{im}-tosylhistidine; Boc-Tyr(Bzl), N^{α}-Boc-*O*-benzyltyrosine; Boc-Arg(Tos), N^{α}-Boc-N^{G}-tosylarginine; Boc-Arg(NO_2), N^{α}-Boc-N^{G}-nitroarginine; 3-Bzl-Tyr, 3-benzyltyrosine; [Asn^1,Val^5]angiotensin II-T, angiotensin II synthesized using N^{α}-Boc-N^{G}-tosylarginine; [Asn^1,Val^5]angiotensin II-N, angiotensin II synthesized using N^{α}-Boc-N^{G}-nitroarginine; TSP, sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$; Me_4Si, tetramethylsilane; SSB, spinning-side band.

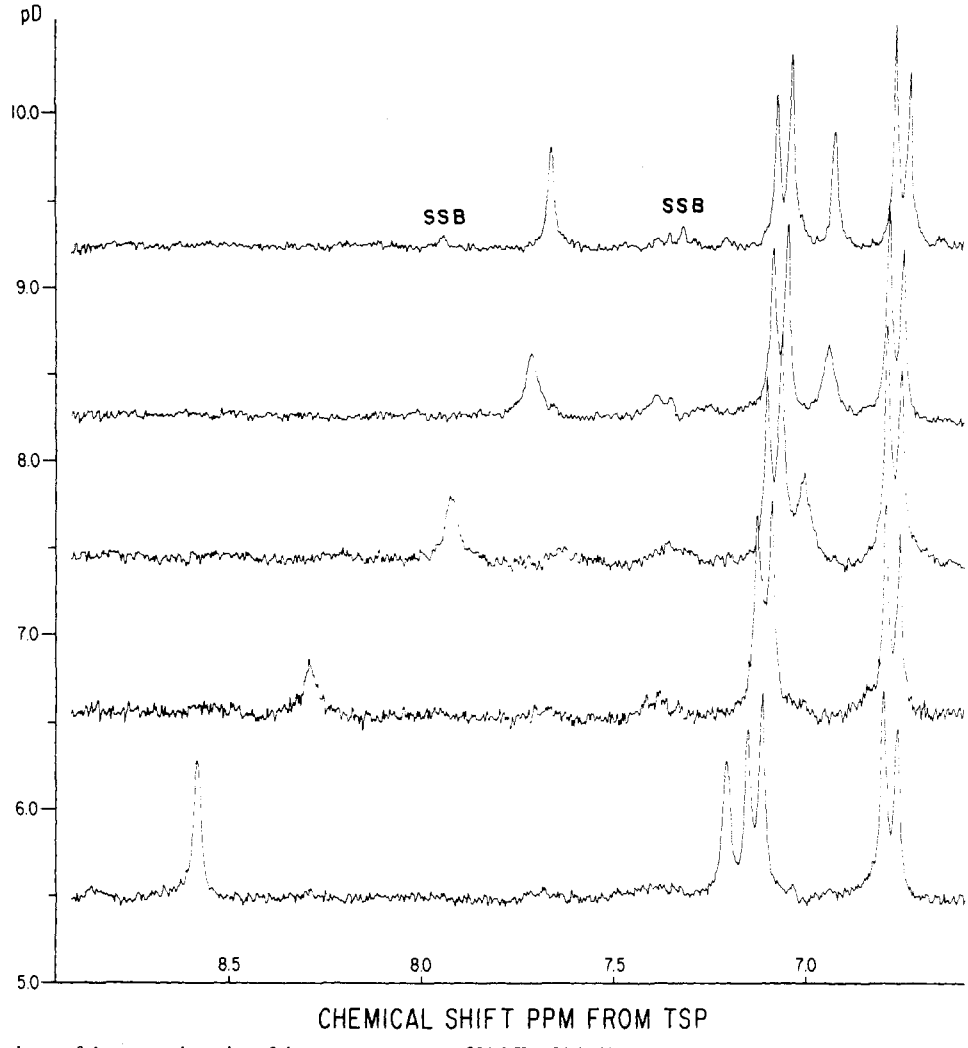

FIGURE 1: pD dependence of the aromatic region of the proton spectrum of Val-Tyr-Val-His at 17 °C. Resonances marked "SSB" are spinning side bands.

peptide. Each hydrolysate was chromatographed on 0.9 × 30 cm of Dowex 50-X2 according to Hirs et al. (1952) and the His fraction was located by scintillation counting, evaporated to dryness, and derivatized with L-glutamic acid *N*-carboxyanhydride. The dipeptide was analyzed on a Durrum 500 amino acid analyzer with the program: 0 min, pH 3.25, 0.2 M sodium citrate (normal first buffer); 12 min, pH 5.00, 0.2 M sodium citrate; 40 min, pH 6.0, 0.4 M sodium citrate, 1.1 M in total sodium added as sodium chloride (normal third buffer). The change to second buffer appears at 24 min and the change to third buffer at 46 min on the chromatogram.

L-Glu-L-His emerged at 33 min 45 s with an operational ninhydrin color constant equal to 0.92 that of Leu; L-Glu-D-His emerged at 28 min 22 s with an operational color constant equal to 0.85 that of Leu, under our conditions. Chromatography, evaporation, and dipeptidization of a sample of pure L-histidine revealed that no racemization occurred during these procedures.

NMR Spectroscopy. The ^{1}H NMR spectra were recorded on a Varian HR-220 spectrometer updated for Fourier transform spectroscopy by Transform Technology, Inc. The spectra were obtained at 17 °C from 8-mg samples in 0.7 ml of deuterium oxide. Sixteen to sixty-four scans were required for each spectrum, depending on the size of the peptide. The interval between scans was 5 s and the length of the 90° pulse was 25 ms. The pH was adjusted with NaOD and DCl. The pH was measured with a combination electrode at 25 °C and the pD was calculated from pD = pH reading + 0.4 (Glasoe and Long, 1960). The chemical shifts were obtained from the resonance position of a trace of chloroform in a second series of experiments. The chemical shift of chloroform was obtained separately, using a sample of deuterium oxide containing a trace of chloroform and an internal standard of sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$. Carbon-13 spectra of the hexapeptide at 30 °C were obtained by Fourier transform using broad-band proton decoupling, on a JEOL PFT-100 located at the University of Connecticut Health Center. These experiments were performed with 30 or 80 mg of peptide in 1.5 ml of deuterium oxide with external Me_4Si.

After an NMR experiment, peptide was repurified by countercurrent distribution in the system 1-butanol–acetic acid–water (4:1:5) for additional NMR experiments.

Results and Discussion

Synthesis of Angiotensin Peptides. Ion-exchange chromatography on Bio-Rex 70 of the crude peptide cleaved from the resin showed two major peaks for each of the four peptides prepared ([Asn^1,Val^5]angiotensin I, [Asn^1,Val^5]angiotensin II-T, [Asn^1,Val^5]angiotensin II-N, and the C-terminal hexapeptide of [Val^5]angiotensin II). In each case, the initial peak was found to contain the desired product, while the trailing peak contained the 3-benzyltyrosine analogue of the desired product. 3-Benzyltyrosine has been shown to result from the acid-catalyzed rearrangement of *O*-benzyltyrosine in an in-

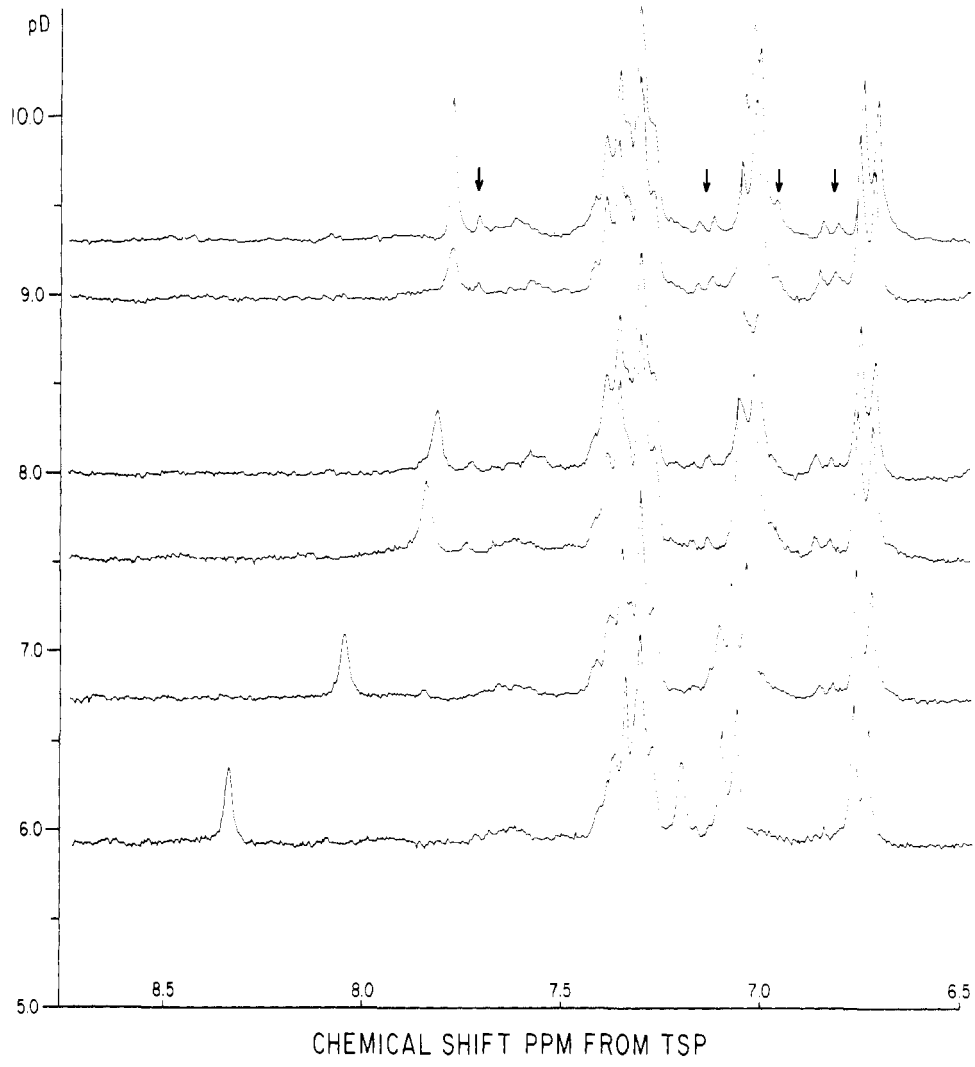

FIGURE 2: pD dependence of the aromatic region of the proton spectrum of Val-Tyr-Val-His-Pro-Phe at 17 °C. In this and the following two figures, arrows indicate the positions of resonances attributable to the observed minor peptide component.

tramolecular reaction (Erickson and Merrifield, 1973). The yields and amino acid analyses of the eight angiotensin peptides isolated are shown in Table I.

None of the peptides, [Asn^1,Val^5]angiotensin I, [Asn^1,Val^5]angiotensin II-T, [Asn^1,Val^5]angiotensin II-N, and the C-terminal hexapeptide of [Val^5]angiotensin II, was detectably cross-contaminated by its 3-benzyltyrosine analogue. Each 3-benzyltyrosine peptide ran slightly behind its parent peptide on paper electrophoresis and ahead of its parent on paper chromatography. Each of the four parent peptides was homogeneous in the two paper electrophoresis and two paper chromatography systems. [Asn^1,Val^5]Angiotensin I had a mobility in each system identical with that of authentic [Asn^1,Val^5]angiotensin I (Ciba). [Asn^1,Val^5]Angiotensin II-T and [Asn^1,Val^5]angiotensin II-N had mobilities in each system identical with each other and identical with that of purified Ciba [Asn^1,Val^5]angiotensin II. Each of the peptides [Asn^1,Val^5]angiotensin I, [Asn^1,Val^5]angiotensin II-T, and [Asn^1,Val^5]angiotensin II-N released the correct molar ratios of all amino acids from Asn^1 to Val^5 upon digestion with aminopeptidase M. The electrophoretic results rule out the presence of β-Asp analogues of these peptides. The aminopeptidase digestion results rule out the presence of gross racemization of amino acid residues from Asn ^{1}to Val^5.

[Asn^1,Val^5]Angiotensin I, [Asn^1,Val^5]angiotensin II-T, [Asn^1,Val^5]angiotensin II-N, and the C-terminal hexapeptide of [Val^5]angiotensin II were also found to be homogeneous upon countercurrent distribution in the system 1-butanol-acetic acid-water (4:1:5) (100 transfers). The 220-MHz ^{1}H NMR spectra confirmed the identity and purity of all of the parent peptides and of Val(3-Bzl-Tyr)Val-His-Pro-Phe.

The synthetic results clearly demonstrate the disadvantages of benzyl ether protection of the side chain of tyrosine since, for each of the four peptides synthesized, about 20% of the isolated peptide products consisted of the 3-benzyltyrosine analogue. The use of 2,6-dichlorobenzyl ether protection of the tyrosine side chain, as suggested by Erickson and Merrifield (1973), should help to eliminate this undesirable side product.

Racemization of Histidine in [Asn^1,Val^5]Angiotensin I and II. Racemization of free L-His was found to be 0.15% per h, or 3.3% in 22 h, compared with 3.1% in 22 h found by Manning (James Manning, The Rockefeller University, personal communication), and 2.8% found by Lin et al. (1972) for free L-histidine treated with hydrogen fluoride and then acid hydrolyzed. Extrapolation to zero time of our graphs of racemization vs. hydrolysis time gave 1.9, 2.1, and 2.1% D-His present respectively for purified [Asn^1,Val^5]angiotensin I, [Asn^1,Val^5]angiotensin II-T, and [Asn^1,Val^5]angiotensin II-N. The amount of D-histidine in Boc-His(Tos) was, unfortunately, not determined prior to synthesis of our angiotensin peptides.

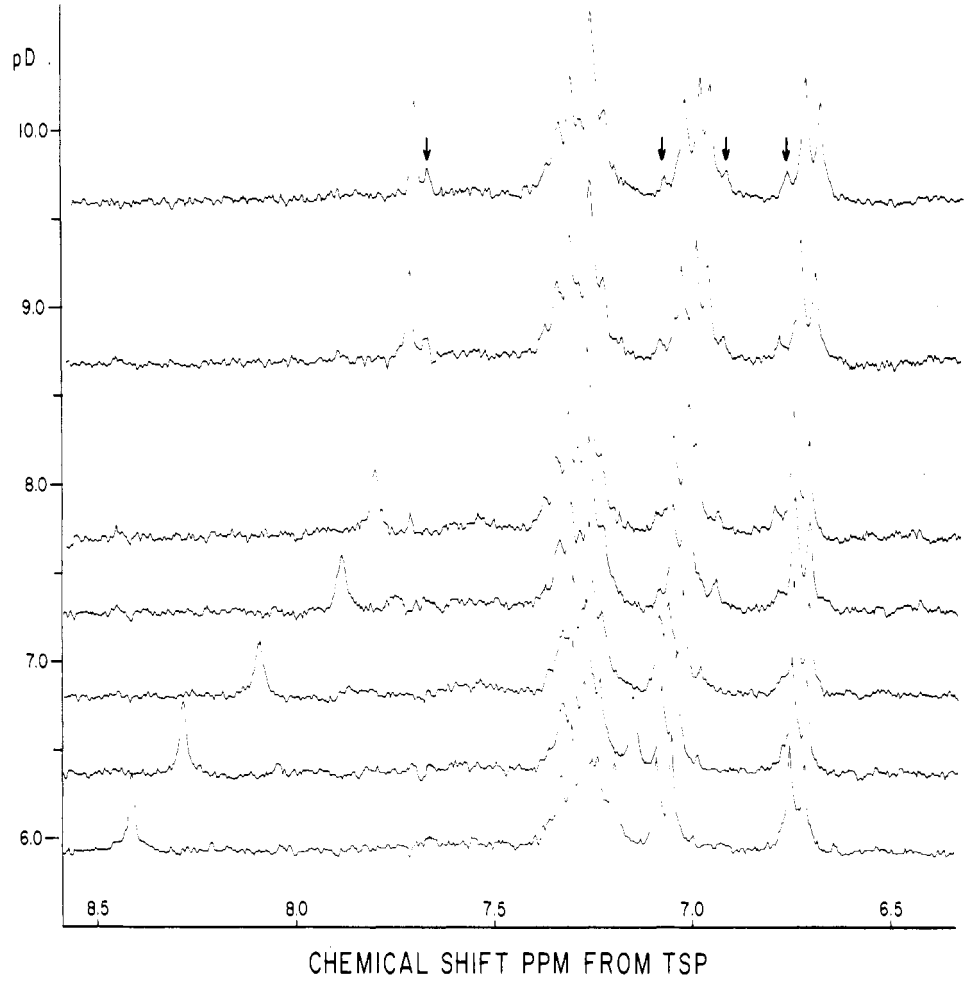

FIGURE 3: pD dependence of the aromatic region of the proton spectrum of [Asn^1,Val^5]angiotensin II at 17 °C.

The extent of racemization of histidine in Ciba [Asn^1,Val^5]-angiotensins I and II was not investigated. This small amount of racemization of L-His during solid-phase synthesis is in contrast to the results of Windridge and Jorgensen (1971) and Khosla et al. (1972) who found up to 40% racemization of L-His in angiotensin peptides using benzyl masking of the histidine side chain, and 15–20% racemization using tosyl masking of the histidine side chain. Lin et al. (1972) found 0.3 and 0.7% racemization of tosylhistidine in two synthetic ribonuclease peptides.

NMR Spectroscopy. Figure 1 shows the pD dependence of the aromatic region of the proton spectrum of the tetrapeptide. At low pD the low-field resonance of the C_2 proton and the high-field resonance of the C_4 proton of His^6 are observed to titrate while at high pD the aromatic resonances of Tyr^4 can be seen to change. Smaller resonances are due to spinning side bands, as can be demonstrated by changing the sample spinning rate. Figure 2 shows a similar pattern for the hexapeptide, which includes the additional resonances of Phe^8. As the pD is increased, a small resonance upfield from the C_2 resonance of His^6 appears at pD 6.7, as do small resonances downfield from the Tyr^4 resonances. These small resonances are indicated in this and the following figure by arrows. To ascertain that these resonances are real, the spinning rate was varied from 40 to 70 Hz and identical spectra were obtained. Therefore, two conformations that interconvert slowly on the NMR time scale (determined by the line width of 1–10 Hz) are observed.

Figure 3 shows the spectrum of [Asn^1,Val^5]angiotensin II as a function of pD. The behavior is similar to that of the hexapeptide: above pD 6.5 two components are observed, the minor one accounting for 12 ± 2% of the resonance intensity. At pD 7.5 the temperature was varied from 17 to 45 °C. With increasing temperature, the resonances of His^6 were observed to broaden, but no drastic change was recorded in the ratio of the two conformations. At pD 7.5 and 17 °C the ionic strength was increased by the addition of 20 mg of sodium chloride and no spectral changes were observed. The appearance of the minor conformation at high pD was completely reversed upon returning to low pD.

[Asn^1,Val^5]Angiotensin I also appeared to show a minor conformation at high pD, based on observation of additional resonances near the Tyr^4 aromatic protons. However, these spectra were less clear than the case of [Asn^1,Val^5]angiotensin II, and are not shown.

The reported observation of two conformations of [Asn^1,Val^5]angiotensin II cannot be the result of contaminating angiotensin-like impurities containing either isoaspartic acid, 3-benzyltyrosine, or D-histidine since the former two have been shown to be absent and the D-histidine analogue present in ≤2.1 mol %, too little to account for the observed mole fraction of minor conformation (12 ± 2%).

The observation of only one conformation for the tetrapeptide and the absence of proline in this analogue suggest the possibility of cis–trans isomerization of Pro^7. The ^{13}C spectrum of the hexapeptide was, therefore, recorded as a function of pD. The hexapeptide is more soluble at high pH than angiotensin I and II and also lacks the arginine residue whose β-carbon and γ-carbon resonances obscure the corresponding resonances of

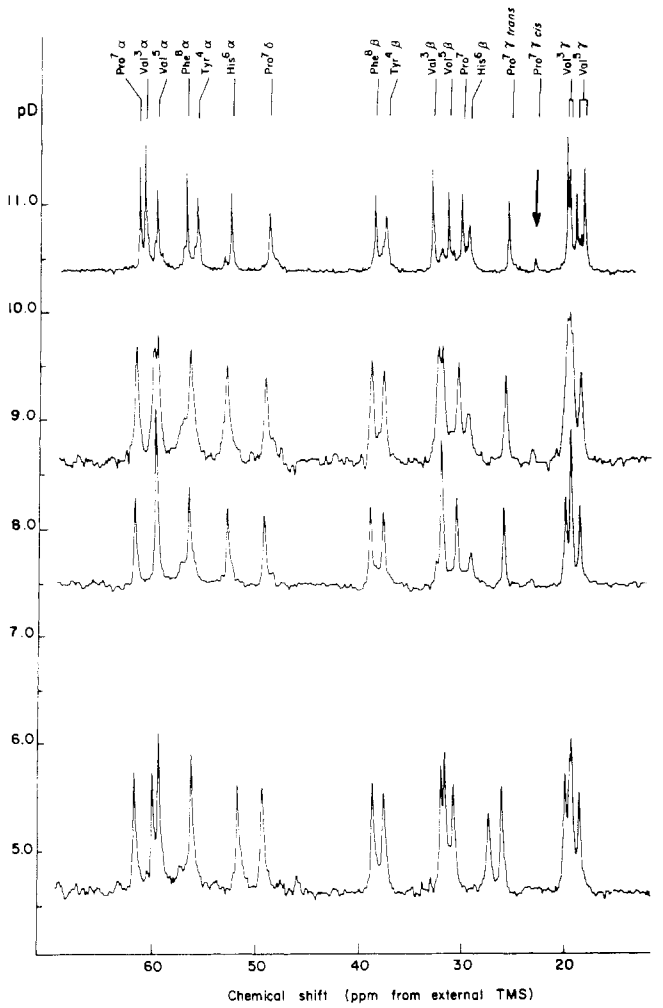

FIGURE 4: pD dependence of the aliphatic side chain region of the ^{13}C spectrum of Val-Tyr-Val-His-Pro-Phe at 25.15 MHz and 30 °C. The pD 10.4 spectrum was with 80 mg of peptide and 16K memory; the remaining spectra were with 30 mg of peptide and 8K memory.

proline. Figure 4 shows the aliphatic side chain region of the ^{13}C spectrum of the hexapeptide as a function of pD at 30 °C. The cis (minor) and trans (major) isomers are clearly visible for the γ-carbon resonances of proline at high pD, while only the trans is visible at low pD. The changes in the proton spectra in Figures 2 and 3 as a function of pD presumably reflect this trans to cis isomerization of Pro^7, although the conformational change observed by NMR may involve other secondary and tertiary structure in addition to isomerization of proline. We estimate 16% *cis*-proline peptide in the hexapeptide at pH 10.4 by comparing peak heights in Figure 4. This estimate is in good agreement with 12 ± 2% cis peptide found for [Asn^1,Val^5]-angiotensin II above pH 6.5 by integrating the proton spectra in Figure 3. Deslauriers et al. (1973b) found 14% *cis*-proline peptide in thyrotropin releasing hormone.

In the hexapeptide the resonances of the γ carbon of *cis*- and *trans*-proline occur at 23.08 and 25.68 ppm, respectively. In thyrotropin releasing hormone they occur at 22.86 and 25.73 ppm and in [Asn^1,Val^5]angiotensin II at 22.94 and 25.63 ppm (Deslauriers et al., 1973a). Our observations are in excellent agreement with those made by Deslauriers et al. and similar to those made for the model compounds glycylproline, alanylproline, and valylproline (Thomas and Williams, 1972). The observed cis resonance of the γ-carbon resonance of proline is conspicuously close to the expected resonance position of the methyl carbon of acetate which could be present in the peptide samples. However, the corresponding carbonyl resonance of acetate was not observed, and a sample of sodium acetate at pD 10.4 shows the methyl carbon resonance to be located 1.48 ppm downfield from the cis resonance of the γ carbon of proline. Deslauriers et al. (1973b) have reported on the observation of cis–trans isomerization of proline in [Asn^1,Val^5]angiotensin II ("less than 20%" *cis*-proline). However, their experiments were performed at pH 4.5, and they did not exclude the presence of a D-His peptide. The present study indicates that such an effect is only observed above pH 6.5, and that this effect is not due to the presence of [Asn^1,Val^5,D-His^6]angiotensin II. The possibility of cis–trans isomerism of proline in [Asn^1,Val^5]angiotensin II had also been suggested earlier by Printz et al. (1972a) and Fermandjian et al. (1971).

Vine et al. (1973) have reported on the observation by ^{19}F magnetic resonance of two conformations at high pH in the analogue [4-F-Phe^8]angiotensin II. The spectra published by these authors indicate a higher concentration of the minor component than we observe in [Asn^1,Val^5]angiotensin II. These observations and the present report on the existence of two conformations for [Asn^1,Val^5]angiotensin II above pH 6.5 may be relevant to the nature of its biological activities. A correlation of thin-film dialysis studies (Craig et al., 1964), NMR studies, and the observation of increasing biological activity with increasing pH (Paladini et al., 1963; Needleman et al., 1972) appears to be justified. The observation of increased biological activity at high pH may be the result of the appearance of the *cis*-proline isomer, which may be a more biologically active form of [Asn^1,Val^5]angiotensin II. A similar correlation of the effect of salt on the solution conformation of [Asn^1,Val^5]angiotensin II with the reported increase in biological activity (Schaechtelin et al., 1974) could provide a firmer foundation for the relevance of conformational studies to the understanding of the function of peptide hormones.

Acceptable models for a rigid conformation of [Asn^1,Val^5]angiotensin II in aqueous solution will have to allow for the observation of cis–trans isomerism. In addition, experimental evidence for models proposing minor populations of conformational isomers must be obtained from peptides of high quantitatively known purity to rule out minor populations of angiotensin-like impurities.

Acknowledgments

We express our gratitude to Dr. Paul Fletcher of Yale University for amino acid analyses, and to Dr. James D. Jamieson of Yale University for financial support.

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Association of Methanol and Ethanol with Heme Proteins†

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ABSTRACT: The behavior of ferrihemoglobin and ferrimyoglobin in widely varying concentrations of the lowest four alcohols has been studied by optical and electron paramagnetic resonance absorption spectroscopy. Methanol and ethanol, at concentrations too low to cause general conformational destabilization of the protein, produce both optical and electron paramagnetic resonance absorption spectral changes in ferrihemoglobin. These changes arise from equilibrium associations, characterized by dissociation constants at 25 °C of about 40 and 200 mM, respectively, for the methanol-ferrihemoglobin and ethanol-ferrihemoglobin complexes so formed. Other optical spectral changes appear when the methanol concentration exceeds 3.5 M and the ethanol, 1.0 M. At concentrations lower than 0.5 M, 1- and 2-propanol produce spectral changes of this second kind. At room temperature no optical evidence has been found that the propanols associate with ferrihemoglobin in the manner of methanol and ethanol. Methanol and ethanol at low concentration have specific effects, characterized by electron paramagnetic resonance spectral differences, upon ferric α_{SH} chains. All four alcohols, over a wide range of concentrations, reduce the symmetry of electron paramagnetic resonance spectra from frozen solutions of ferrihemoglobin; even at the high end of this concentration range, none of the alcohols reduces the symmetry of electron paramagnetic resonance spectra from frozen ferrimyoglobin. Ferrimyoglobin and catalase association with methanol is measureable optically; the binding is about five and sixty times weaker, respectively, for these two proteins as compared with ferrihemoglobin.

There is considerable information available on the effects of alcohols on protein stability and reactivity obtained under experimental conditions designed to alter the structure of water and weaken hydrophobic bonds (Kaminsky and Davison, 1969; Herskovits et al., 1970; Tan and Lovrien, 1972; Anusiem and Lumry, 1973). With regard to denaturation, for example, an increase in the length of the hydrocarbon chain increases the effectiveness of the alcohol, and branching decreases it; methanol, ethanol, 2-propanol, and 1-propanol at concentrations of about 12, 6, 4, and 3 M, respectively, are required for half-denaturation (based upon Soret absorbance) of myoglobin and cytochrome *c* (in acetate, 0.1 M, pH 5.7, 25 °C) (Herskovits et al., 1970). In other experimental systems, effects produced by alcohols can be attributed to complex formation. Thus, the influence of alcohols on the thermal transition of ribonuclease has been interpreted in terms of the hydrophobic

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