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Interaction of Angiotensin Peptides and of Amino Acids with *p*-Nitrophenyl Acetate†

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ABSTRACT: The interaction of *p*-nitrophenyl acetate (NphOAc) with angiotensin II (AII), seven analogs and four lower homolog peptides, was studied as a function of pH. Second-order rate constants were obtained for NphOAc reaction with the amino, imidazole, and phenoxyl groups of the peptides. Comparison with Brønsted relations obtained for amino acid and other model compounds indicated that the histidine side chain in AII is free to interact with NphOAc while the tyrosyl side chain is partially restricted. Interpretation of the data obtained for the amino groups was precluded by the large relative errors associated with these data.

The conformation of angiotensin II (AII)[1] in solution was first studied by Smeby *et al.* (1962), who proposed a helical model, and by Paiva *et al.* (1963), who favored a random coil. More recently, other models have been proposed, mainly based on data from esr spectra of spin-labeled AII homologs (Weinkam and Jorgensen, 1971), circular dichroism (Fermandjian *et al.*, 1971), hydrogen–tritium exchange (Printz *et al.*, 1972), and nuclear magnetic resonance (nmr) of protons (Fermandjian *et al.*, 1972; Bleich *et al.*, 1973; Glickson *et al.*, 1973), of ^{13}C (Zimmer *et al.*, 1972), and fluorine (Vine *et al.*, 1973). Of these models, only the β- and γ-turn structures proposed by Printz *et al.* (1972) have been clearly described in detail. However, they have not been supported by the nmr evidence obtained by Marshall *et al.* (1973).

In view of the conflicting models being proposed for AII conformation, we believe that it will be useful to obtain more information about the state of that peptide's polar side chains in aqueous solution. We have previously obtained evidence, from electrometric titrations, of interactions between the amino and carboxyl groups of the N-terminal Asp residue with the imidazole of His[6] and the C-terminal carboxyl group (Juliano and Paiva, 1974).

In order to gain further information about the reactivity of the amino, imidazole, and phenoxyl groups of AII in solution we have attempted to investigate the interaction of these groups with *p*-nitrophenyl acetate (NphOAc). This paper presents the results of an analysis of the pH dependence of the reaction of NphOAc with AII and several analog and homolog peptides (Table I). Results obtained with several amino acids and other model compounds are also presented.

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[1] Abbreviations used are: AII, angiotensin II; NphOAc, *p*-nitrophenyl acetate. Peptides were named according to the IUPAC tentative rules for naming synthetic modifications of natural peptides (1967), *Biochemistry 6,* 362.

TABLE I: Peptides Employed in this Study.

Name	Amino Acid Sequence
AII	Asp-Arg-Val-Tyr-Ile -His -Pro-Phe
[Asn^1]AII	Asn-Arg-Val-Tyr-Ile -His -Pro-Phe
[Suc^1]AII	Suc-Arg-Val-Tyr-Ile -His -Pro-Phe
[Gly^1]AII	Gly-Arg-Val-Tyr-Ile -His -Pro-Phe
[Arg^6]AII	Asp-Arg-Val-Tyr-Ile -Arg-Pro-Phe
[Leu^8]AII	Asp-Arg-Val-Tyr-Ile -His -Pro-Leu
[Pro^3,Pro^5]AII	Asp-Arg-Pro-Tyr-Pro-His -Pro-Phe
[Asn^1]AII-amide	Asn-Arg-Val-Tyr-Ile -His -Pro-Phe-NH_2
AII-(2–8)-heptapeptide	Arg-Val-Tyr-Ile -His -Pro-Phe
AII-(3–8)-hexapeptide	Val-Tyr-Ile -His -Pro-Phe
AII-(4–8)-pentapeptide	Tyr-Ile -His -Pro-Phe
AII-(5–8)-tetrapeptide	Ile -His -Pro-Phe

Materials and Methods

All the peptides used in this study were synthesized by the solid phase method (Merrifield, 1963; Stewart and Young, 1969) as described elsewhere (Paiva *et al.*, 1973, 1974). They were purified by counter current distribution and ion exchange chromatography until the following purity criteria were met: (a) the amino acid analysis of acid hydrolysates yielded a molar ratio within 3% of the theoretical value for each amino acid; (b) the peptide content determined by amino acid analysis, spectrophotometry (ξ_{275} 1375), and titration agreed within 1%; (c) only one spot was detected with Pauly, ninhydrin, and Sakaguchi reagents after thin-layer chromatography of a 0.1-μmol sample with three solvent systems and high voltage paper electrophoresis with three different buffers (pH 2.8, 4.9, and 9.9). NphOAc was prepared as described by Chattaway (1931) and recrystallized from EtOH-H_2O.

The kinetics of reactions with NphOAc were studied at several pH values. The buffer solutions used were: 0.1 M potassium phosphate in the pH range 6.0–7.8; 0.1 M Tris in the pH range 7.8–8.8; 0.03 M borate in the pH range 8.8–9.5. The ionic strength of all buffer solutions was brought up to 0.2 with KCl.

The peptides or amino acids were dissolved in the appropriate buffer and, after equilibration at 25.0° (±0.1°) in a Forma-Temp bath, the solution pH was adjusted to within 0.005 pH unit of the desired value with 1 N KOH or HCl. The pH was measured with a Radiometer Model 4 pH meter calibrated with phosphate and phthalate or borate buffers (Bates, 1954). Peptide concentrations ranged between 2×10^{-3} and 6×10^{-4} M and the amino acid concentrations varied between 1.1×10^{-2} and 10^{-3} M. Peptide concentrations were checked, for all the peptides containing tyrosine, by the absorption at 275 nm (ξ 1375).

Two milliliters of the peptide or amino acid solution was placed in a 1-cm quartz cell, in the thermostated (25.0°) cell compartment of a Shimadzu QV-50 photometer. The reaction was initiated by the addition of 0.025 ml of a solution of NphOAc in EtOH. The EtOH concentration in the reaction mixture was always 0.8% (v/v) and the NphOAc molar concentration ranged from 10 to 100 times less than that of peptide or amino acid. The appearance of *p*-nitrophenolate was measured at 400 nm, for at least 80% of the reaction course when the pH was 7.8 or above, and for at least 40% when the pH was lower. In these conditions Lambert–Beer's law was found to apply to *p*-nitrophenolate, and pseudo-first-order kinetics was always observed. The first-order rate constant (k_1) was obtained from

$$\ln [(A_\infty - A_t)/(A_\infty - A_0)] = - k_1 t \qquad (1)$$

where A_0, A_t, and A_∞ are, respectively the absorbances at times zero (obtained by extrapolation), t, and at completion of the reaction. A_∞ was obtained from the absorbance of tenfold dilution of the reaction mixture with 1N KOH, by the equation

$$A_\infty = A_{max}(10^{pH-7.10}/(1 + 10^{pH-7.10})) \qquad (2)$$

where A_{max} is the absorbance in 1N KOH. A_∞ values obtained in this way were frequently checked by absorbance measurements made in the reaction mixture after more than 15 half-lives, with very good agreement.

The second-order rate constants (k_2) were obtained from

$$k_2 = (k_1 - k_w)/c \qquad (3)$$

where c is the peptide or amino acid concentration and k_w is the rate constant for the reaction measured when $c = 0$. A blank for obtaining k_w was run simultaneously with each k_1 determination and the largest value obtained for k_w (at pH 9.2) did not exceed 50% of the corresponding k_1.

When only one nucleophilic group was present in the molecule, k_2 values were obtained in at least four pH's. When more than one nucleophilic group was present, data were obtained in at least 15 pH values in the range 6–9.5. These data were fitted, by a least-squares method, to a multiple linear correlation of the form

$$k_2 = k_2^a\alpha^a + k_2^b\alpha^b + k_2^c\alpha^c \qquad (4)$$

where the superscripts a, b, and c refer to the imidazole, amino, and phenoxyl groups, respectively, and α indicates the fraction of the deprotonated form of each group. To calculate α for the different groups in the peptides, the pK_a values determined by electrometric titrations (Juliano and Paiva, 1974) were employed.

The linear regression equations for the Brønsted relations were obtained by the least-squares method. Linear and multiple correlations were calculated and plotted with a Hewlett-Packard 9100A calculator with 9101A extended memory and 9125A plotter.

Results

Amino Acids. A Brønsted plot of the nucleophilic reactions of the amino groups of L-amino acids with NphOAc is shown in Figure 1. Our data for glycine and Gly-Gly agree with those previously reported by Koltun *et al.* (1960) and fit well into the regression equation obtained for the Brønsted relation from data for glycine and nine glycyl peptides (Koltun *et al.*, 1963)

$$\log k_2 = 0.744pK_a - 5.079 \qquad (r = 0.988,\ s = 0.103) \qquad (5)$$

where r is the correlation coefficient and s is the standard error of the estimate. The Brønsted plot for the other amino acids, although parallel to that for the glycine peptides, is given by a different equation

$$\log k_2 = 0.726pK_a - 5.692 \qquad (r = 0.957,\ s = 0.098) \qquad (6)$$

The results shown on Figure 1 do not include data for tyrosine, cysteine, and lysine because our analysis of the ki-

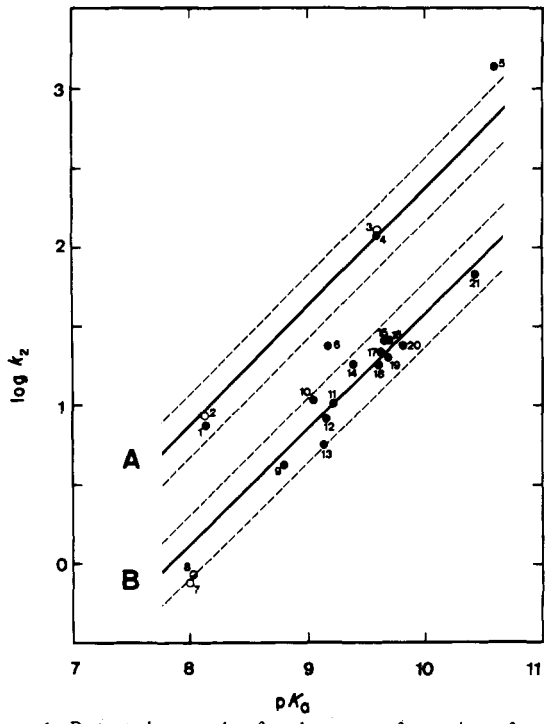

FIGURE 1: Brønsted type plot for the rates of reaction of L-amino acids with NphOAc as a function of the amino group pK_a: (●) present paper; (○) from Koltun *et al.* (1963); (1 and 2) Gly-Gly; (3 and 4) glycine; (5) proline; (6) histidine; (7) Val-Gly; (8) Val-Ala; (9) asparagine; (10) arginine; (11) methionine; (12) serine; (13) phenylalanine; (14) tryptophan; (15) alanine; (16) glutamic acid; (17) valine; (18) leucine; (19) isoleucine; (20) aspartic acid; (21) threonine. Solid lines represent eq 5 (A) and 6 (B). Dashed lines indicate 95% fiducial limits.

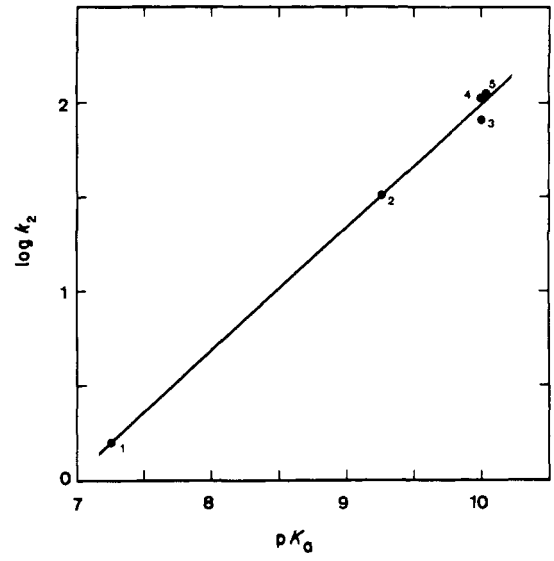

FIGURE 2: Brønsted type plot for the rates of reaction of phenolic groups with NphOAc: (1) vanillin (present paper); (2) *p*-bromophenol (present paper); (3) *N*-acetyltyrosine (Koltun *et al.*, 1963); (4) phenol (Jencks and Carriuolo, 1960); (5) Gly-Tyr (Koltun *et al.*, 1963).

netic data would not allow the individualization of the microscopically different ionized species present in the pH range studied (Benesch and Benesch, 1955; Martin *et al.*, 1958).

The k_2 value found for the imidazole group of histidine was 2.56 M^{-1} min^{-1}, in good agreement with the equation obtained for several imidazole derivatives and histidine-containing peptides and proteins (Koltun *et al.*, 1963).

$$\log k_2 = 0.541pK_a - 2.678 \qquad (r = 0.946, \quad s = 0.115) \qquad (7)$$

For the phenolic group, the k_2 values available in the literature, to our knowledge, are those for phenol (Jencks and Carriuolo, 1960), *N*-acetyltyrosine, and glycyltyrosine (Koltun *et al.*; 1963). We have determined the values for *p*-bromophenol (k_2 = 32.20 M^{-1} min^{-1}; pK_a = 9.25) and for vanillin (k_2 = 1.56 M^{-1} min^{-1}; pK_a = 7.26). The Brønsted relation for the reaction of NphOAc with the phenolic groups in all the five above mentioned compounds (Figure 2) is given by

$$\log k_2 = 0.653pK_a - 4.545 \qquad (r = 0.998, \quad s = 0.056) \qquad (8)$$

Angiotensin Peptides. We have studied the pH dependence of the reaction of NphOAc with AII and 11 analog and homolog peptides. A typical result is illustrated in Figure 3, which shows the fit of the experimental points to the curve representing eq 4 with the k_2 values for the imidazole, amino, and phenol groups obtained by a method of least squares. Another way of obtaining separate k_2 values for the imidazole and phenolic groups is shown in Figure 4, where the overall second-order rate constants are plotted as a function of the degree of ionization of either the imidazole (A) or the phenolic (B) groups. The k_2 values obtained from these plots did not differ significantly from those obtained with the multiple linear correlation method. However, the latter method is more precise because it takes into account the contribution of each term of eq 4 at all the pH values studied and does not depend on the choice of points for the linear plot to be extrapolated. For this reason, the least-squares fit to eq 4 was used to obtain the data for the 12 peptides shown on Table II. In this table, the k_2 value for each group of each peptide is compared with the 95% fiducial limits calculated from the linear regression of the Brønsted relations for amino acids and other model compounds (k_2^{calcd}). In order to determine whether the experimental values differ significantly from the calculated range it is necessary to take into consideration the errors associ-

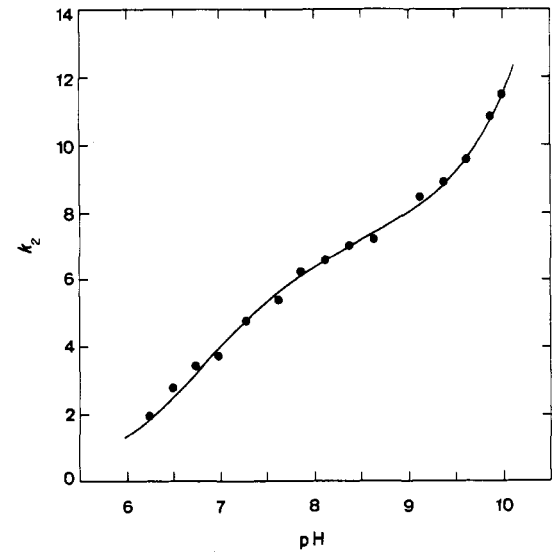

FIGURE 3: Rate of reaction of AII with NphOAc as a function of pH. The points represent experimental data and the curve was obtained from eq 4, with k_2^a = 6.00 M^{-1} min^{-1}, k_2^b = 1.70 M^{-1} min^{-1}, k_2^c = 41.92 M^{-1} min^{-1}. α values were calculated from the following pK_a values: imidazole, 6.47; amino, 7.60; phenol, 10.09.

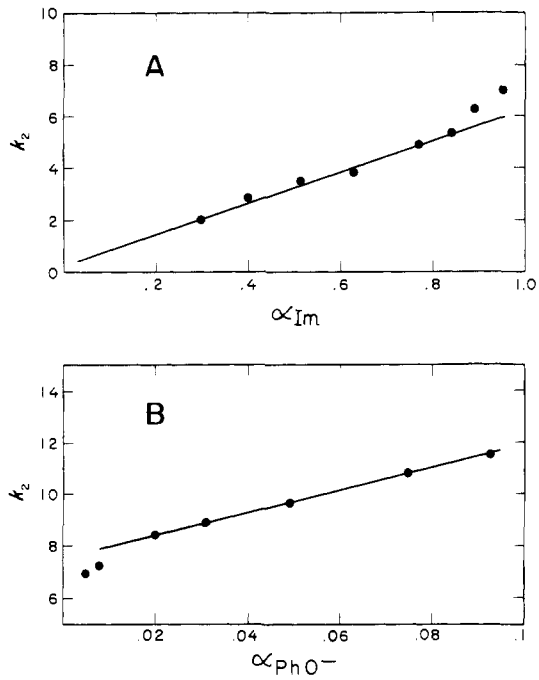

FIGURE 4: Rate of reaction of the imidazole (A) and phenolic (B) groups of AII with NphOAc as a function of the degree of ionization of each group. The k_2 values obtained from the slope of the linear plots were 5.99 M^{-1} min^{-1} for the imidazole and 42.57 M^{-1} min^{-1} for the phenolic group. The k_2 value for the amino group was obtained by subtracting the imidazole k_2 from the ordinate intercept of the linear plot in B and found to be 1.56 M^{-1} min^{-1}.

ated with the k_2 values. The best assessment of these errors that could be obtained from our analysis is the standard error of the estimate (s) calculated for the multiple linear regression for each peptide. Since one value for s was obtained for the entire fit, the relative error was much greater for the amino groups (for which relatively small k_2 values were found) than for the other groups. For this reason, only the k_2 values for the amino groups of AII, [Asn^1]AII, and [Asn^1]AII-amide might be considered significantly higher than the k_2^{calcd} range.

The k_2 values for the imidazole groups of the 12 peptides studied were all within the 95% fiducial limits of k_2^{calcd}.

The phenoxyl groups of AII and of most of the other peptides reacted with NphOAc at significantly slower rate than the calculated ones. The exceptions were [Arg^6]AII and [Pro^3,Pro^5]AII, for which the observed k_2 values fell within the k_2^{calcd} range.

Discussion

Although the reactivity of a wide variety of nucleophiles toward NphOAc has been studied (Jencks, 1969) no data are available in the literature for most amino acids. Koltun *et al.* (1963) found that k_2 values for glycine and several glycyl peptides closely obeyed the Brønsted relation but Val-Ala and Val-Gly were much less reactive than predicted by that relation. They attributed this to a steric factor due to the valine side chain. Our data (Figure 1) show that valine, the two above mentioned dipeptides, and most of the other amino acids with substitution on the carbon atom bearing the amino group also obey the Brønsted relation, but with k_2 values about four times smaller than those for the glycine compounds. The k_2 value found for the amino group of histidine (Figure 1) was significantly higher than expected from its pK_a value, possibly because of a cooperative effect with the imidazole group.

The k_2 value that we observed for proline (1371 ± 129 M^{-1} min^{-1}) is high when compared with the other amino acids on a Brønsted plot (Figure 1). However, this value falls within the 95% fiducial limits of the Brønsted relation obtained from the data of Jencks and Gilchrist (1968) on four secondary amines (eq 9).

$$\log k_2 = 0.82pK_a - 5.76 \qquad (r = 0.998, \quad s = 0.144) \qquad (9)$$

The analysis of the data obtained with the angiotensin peptides indicates that the imidazole groups in these peptides had the same reactivity toward NphOAc as in histidine and other simple imidazole derivatives. This indicates that the histidine side chain in AII and the other peptides studied is not involved in intramolecular interactions. An al-

TABLE II: Reaction of NphOAc with the Amino, Imidazole, and Phenolic Groups of Angiotensin Peptides.

Peptide	r^a	s^b	Amino		Imidazole		Phenoxyl	
			k_2^c	k_2^{calcd} [d]	k_2^c	k_2^{calcd} [d]	k_2^c	k_2^{calcd} [d]
AII	1.00	0.28	1.70	0.43–1.05	6.00	3.91–11.28	41.92	85.46–143.14
[Asn^1]AII	0.99	0.20	1.72	0.12–0.29	4.85	3.16–9.13	50.63	82.93–138.90
[Suc^1]AII	0.97	0.89			6.62	4.49–12.94	56.28	89.40–149.75
[Gly^1]AII	0.99	0.54	5.02	3.41–10.28[e]	7.30	3.81–11.00	63.66	78.09–130.79
[Arg^6]AII	0.98	0.60	1.20	0.36–0.89			110.00	78.09–130.79
[Leu^5]AII	0.99	0.14	1.03	0.41–1.02	6.64	3.81–11.00	44.85	84.19–141.01
[Pro^3,Pro^5]AII	1.00	0.26	0.54	0.46–1.12	7.34	4.27–12.31	86.38	66.19–110.86
[Asn^1]AII-amide	0.99	0.34	0.94	0.11–0.26	3.79	2.59–7.48	61.72	74.64–125.03
AII-(2–8)-heptapeptide	1.00	0.11	0.10	0.27–0.67	6.93	3.50–10.08	60.71	82.93–138.90
AII-(3–8)-hexapeptide	0.99	0.65	2.43	0.55–1.35	5.98	3.50–10.08	71.84	88.07–147.51
AII-(4–8)-pentapeptide	0.92	1.85	0.08	0.31–0.75	6.10	3.41–9.83	84.05	102.36–171.45
AII-(5–8)-tetrapeptide	0.99	0.08	1.73	0.61–1.49	3.95	3.16–9.13		

[a] Correlation coefficient found for the fit of the data to eq 4. [b] Standard error of the estimate obtained for the least-squares fit of the data to eq 4. [c] k_2 for each group, in M^{-1} min^{-1}, was obtained from the best fit of the data to eq 4. [d] The range of values shown for k_2^{calcd} represent the 95% fiducial limits calculated from eq 6, 7, and 8 for the amino, imidazole, and phenoxyl groups, respectively. [e] Obtained from eq 5.

tered reactivity of the histidine side chain would be expected if a carboxylate-imidazole interaction (Weinkam and Jorgensen, 1971) were present. Our results also do not support the possibility of a hydrogen bond between the *pros*-nitrogen of imidazole and the fifth amide nitrogen, similar to that proposed for Gln-His-Pro-NH_2 (Grant *et al.*, 1972).

The data for the amino groups (Table II) indicate that these groups are more reactive in AII, [Asn^1]AII, and [Asn^1]AII-amide, but it is quite possible that similarly higher reactivities in some of the other peptides may have been masked by the large relative errors associated with the k_2 values for the amino groups. For this reason, further discussion about the data for the amino groups does not seem justified.

In the case of the phenoxyl groups, however, the k_2 values are much larger, and the relative errors are small. Our results, in this case, indicate that the tyrosyl side chain in all the peptides, except [Arg^6]AII and [Pro^3,Pro^5]AII, is not entirely free to react with NphOAc. This is in partial agreement with our conclusion, from titration data, that the tyrosine side chain in AII is not totally free to interact with the solvent (Juliano and Paiva, 1974). This restriction to the freedom of the Tyr^4 side chain has not been demonstrated before, but evidence in its favor has also been obtained by ^{13}C spin-lattice relaxation time (R. Deslauriers, I. C. P. Smith, and A. C. M. Paiva, in preparation).

Our results indicate that models for AII conformation, whether as a unique structure or an equilibrium of various conformations, should include the following features: (a) the N-terminal amino group and the C-terminal carboxyl should be close enough to allow for electrostatic interaction (Juliano and Paiva, 1974); (b) the imidazole side chain of His^6 is not involved in intramolecular interactions; (c) the phenolic side chain of Tyr^4 has restricted freedom.

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