THE QUANTITATION OF CARBAMINO ADDUCT FORMATION OF ANGIOTENSIN II AND BRADYKININ

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ABSTRACT The two equilibrium constants that define the extent of carbamino adduct formation with amines for all values of pH and P_{CO_2} are determined for the α-amino groups of the peptide hormones angiotensin II (AII) and bradykinin (BK) by nuclear magnetic resonance techniques. From these constants the variation of carbamino adduct formation has been calculated over the pH range 6.60–8.00 with variable P_{CO_2}, and the results are superimposed upon standard pH-bicarbonate diagrams. The mole fraction, Z, of carbamino adduct form of AII or BK shows a maximum variation in going from metabolic alkalosis, $Z \simeq 0.30$, to metabolic acidosis, $Z \simeq 0.02$, with Z near 0.2 for normal acid-base conditions. Adduct formation to hormone may alter the biological effect of the hormone (*a*) by limiting proteolysis, particularly at the amino-terminal, (*b*) by altering hormone binding affinity to specific receptors, or (*c*) by converting the hormone to an antagonist which binds to receptor but does not activate subsequent metabolic events. The requirements for any of these mechanisms to operate are examined in terms of simple equilibrium considerations, and experimental evidence of inhibition of an aminopeptidase model system is presented. These results are consistent with the hypothesis that regulation of some physiological processes through formation of carbamino adduct of peptide hormones is possible.

INTRODUCTION

Carbon dioxide produced by various metabolic reactions within cells is hydrated to carbonic acid and subsequently ionized to hydrogen and bicarbonate ions, providing the components of the major extracellular buffering system. Although levels of both hydrogen ions and carbon dioxide are carefully controlled, circulating levels of carbon dioxide are more carefully controlled than those of hydrogen ion (1). Separate effects of each for regulatory processes have been sought (2), whereas less attention has been given to their combined effects on metabolic pathways or processes through carbamino formation with molecules other than hemoglobin. Within the erythrocyte, carbon dioxide is carried in part as the carbamino adduct to the α-amino terminals of the β-subunits of hemoglobin, acting as an allosteric regulator that stabilizes the deoxy form of hemoglobin (3, 4). The extent of formation of this carbamino adduct of hemoglobin and other primary amines can be effectively quantitated by ^{13}C nuclear magnetic resonance techniques (4–8). However, any peptide unless its α-amino group is chemi-

cally or conformationally blocked can be expected to form such adducts with carbon dioxide under physiological conditions of pH and P_{CO_2} (6, 7). Whether such adducts provide a means of regulation for processes other than oxygen transport has yet to be determined. To establish the possibility of a regulatory mechanism, it is necessary to establish that such adducts do exist at physiologic pH and to quantitate the change in the relative proportions of free and adduct form under conditions of acid-base imbalance. This information may lead to a more complete description of the role of CO_2 and H^+ in biological systems.

Two vasoactive peptides, angiotensin II (AII) and bradykinin (BK), have been chosen for study. They have free α-amino groups and are interesting candidates physiologically (9–11). By controlling blood pressure they control perfusion which affects pH and P_{CO_2}, two variables that determine adduct concentration. Carbamino adducts of these hormones could bind differently to their respective receptors or may be converted into antagonists, compounds that bind but do not cause activation of subsequent metabolic processes. Alternatively, the adduct form could be more stable to degrading enzymes, particularly the aminopeptidases.

In vivo AII is acted upon by an aminopeptidase to yield des-Asp AII (AIII) with high but differing biological activity (12). Current studies are consistent with the concept that AIII is a mediator of the renin-angiotensin system at receptor sites of the adrenal cortex and the vascular smooth muscle of the renal arterioles, whereas AII appears to be the more important peptide for peripheral receptors controlling direct pressor activity (12). BK, in contrast, is formed in vivo by the action of an aminopeptidase on lysyl-BK and is degraded by a dipeptidyl carboxypeptidase to an inactive product. Both BK and lysyl-BK are potent vasodilators (9). Protection from aminopeptidase activity would clearly alter the ratio AII/AIII as well as AII(AIII)/lysyl-BK (BK) and the effects could be manifest physiologically.

In this study, we quantitate the amount of carbamino adduct formed by these peptides at hydrogen ion and carbon dioxide levels that might be encountered in various conditions of acid-base imbalance. Possible effects of adduct formation on degradation and receptor binding are discussed on the basis of simple equilibrium considerations. We present evidence for aminopeptidase inhibition using swine kidney microsomal aminopeptidase M and an artificial substrate, leucyl *p*-nitro anilide, as a simple model system. We also observe that the cleavage of the amino terminal aspartic acid residue of AII by aminopeptidase M is diminished in bicarbonate buffer as compared to phosphate buffer with otherwise identical experimental conditions. As a result of these studies, we suggest that carbamino formation may limit other extracellular aminopeptidase enzymes, including that metabolizing AII in particular and those active upon a number of other specific peptide hormones in general.

MATERIALS

1-Asp-5-Ile angiotensin II (AII) was obtained from Beckman Instruments Inc. (Mountainside, N.J.). Bradykinin (BK) was purchased from Bachem (Torrance, Calif.). Sodium bicarbonate enriched to 87.6% in ^{13}C was supplied by Bio-Rad Laboratories (Richmond, Calif.). Reagent

grade sucrose at ^{13}C natural abundance was used as the internal standard for the angiotensin studies. Aminopeptidase M, purchased from Rohm and Haas Co. (Philadelphia, Pa.) (Henley and Co.), and from Sigma Chemical Co. (St. Louis, Mo.), was desalted on a P-2 gel column and lyophilized before use. The enzyme was reconstituted to the desired activity with distilled water, and aliquots were frozen separately to avoid repeated thawing and freezing. Enzymic activity was tested on a myoglobin peptide fragment (residues 140–153) which was a gift from Dr. Barry N. Jones (Indiana University, Bloomington, Ind.). Leucyl-*p*-nitro anilide (LpNA) was purchased from Sigma Chemical and recrystallized twice from methanol. *P*-nitro aniline (pNA) was purchased from Matheson, Coleman & Bell (East Rutherford, N.J.) and recrystallized from ethanol. Other chemicals were reagent grade.

THEORY AND METHOD

Three simultaneous equilibria necessary for describing the interaction of CO_2 with amines are as follows:

$$RNH_3^+ \overset{K_z}{\rightleftharpoons} RNH_2 + H^+ \tag{1}$$

$$RNH_2 + CO_2 \overset{K_c}{\rightleftharpoons} RNHCO_2^- + H^+ \tag{2}$$

$$CO_2 + H_2O \overset{K_1'}{\rightleftharpoons} HCO_3^- + H^+. \tag{3}$$

In the above K_z is the acid dissociation constant for the amino group in question and K_c is the association constant for the carbamino adduct expressed to include the disassociation of the relatively strong carbamic acid ($pK_a < 5$). K_1' is the overall constant for the hydration of CO_2 and subsequent disassociation to bicarbonate and has a value of 6.02×10^{-7} (4). The disassociation to carbonate which occurs at pH >9 can be neglected here. At low values of pH the concentration of the unprotonated form of the amine will limit carbamino formation, whereas at higher pH values the concentration of dissolved CO_2 will be limiting (6).

Eqs. 1 and 2, along with the equation for total amine, TA ($TA = [RNH_2] + [RNH_3^+] + [RNHCO_2^-]$), can be solved to give an expression for the mole fraction (Z) of carbamino adduct (4), where mole fraction of adduct is defined as $RNHCO_2^-/TA$

$$Z = \frac{K_cK_z(CO_2)}{K_cK_z(CO_2) + K_z(H^+) + (H^+)^2}. \tag{4}$$

With Eq. 3 and the equation for total carbonates, TC($TC = [CO_2] + [HCO_3^-] + [RNHCO_2^-]$), Eq. 4 can then be expressed in terms of the more easily measurable total carbonates rather than free CO_2. This result is expressed below and for the purpose of this work is solved for K_c.

$$K_c = \frac{Z[K_zK_1' + (K_1' + K_z)(H^+) + (H^+)^2]}{K_z[Z^2(TA) - Z(TC + TA) + (TC)]}. \tag{5}$$

This equation defines the experimental protocol.

The quantities that appear on the right-hand side of Eq. 5 were determined as follows: hydrogen ion concentrations were measured on a Radiometer PHM-4 meter (Radiometer Co., Copenhagen, Denmark) with a combination electrode and standardized against Radiometer buffers at pH 7.42 and 9.18. Total carbonates were measured on a Natelson microgasometer, model 600. Values of K_z for both BK and AII were determined from the pH dependencies of appropriate resonances (Asp-1-C^{α} and Asp-1-C^{β} for AII and Arg-1-C^{β} for BK) in the natural abundance proton-decoupled ^{13}C nuclear magnetic resonance (NMR) spectra of these compounds. Chemical shift values were referenced to CS_2. Total amine concentration was determined from amino acid analysis using a Beckman 121 B analyzer (Beckman Instruments, Inc. (Fullerton, Calif.) BK was dissolved in water. The AII sample contained 0.15 M NaCl for solubilization. Chemical shift and pH data were fit to the Henderson-Hasselbalch expression by least squares analysis. Values of Z for each peptide were obtained at five different pH values over the range of 7.10 to 8.60 from undecoupled ^{13}C NMR spectra. Each peptide sample was equilibrated with a ^{13}C enriched bicarbonate buffer at a total carbonate concentration of 44–68 mM in a sealed NMR tube (6, 8).

For quantitation, AII samples contained 82 mM sucrose, which gives a single narrow resonance in undecoupled spectra at 89.3 ppm due to the single nonprotonated C-2 carbon of the fructose moiety (13). Because of the higher solubility of BK, the envelope of resonance due to the 9 peptide carbonyls centered at 25 ppm was adequate for quantitation of Z values (4). Spectra were recorded on 25.2 or 15.1 MHz instruments using a pulse repetition rate of 6 s and a 60° flip angle to avoid saturation. Areas of the carbamino and reference resonances were determined by computer integration of the digitized spectra. After the NMR experiment, the mole fraction of $^{13}CO_2$, $\chi^{13}CO_2$, was determined on a Varian Mat CH7 mass spectrometer (Varian Associates, Palo Alto, Calif.). The values for pH and total carbonate were separately determined. Z is then computed from Eq. 6 (4)

$$Z = \frac{(\text{area of carbamate resonance})(\text{concentration of reference})\ 1.1 \times 10^{-2}}{(\text{area of reference resonance})(\text{concentration of peptide})\ \chi^{13}CO_2}. \quad (6)$$

The values of Z are then applied to Eq. 5 to calculate K_c, which was averaged for the Z determinations at different pH values.

The use of Eq. 6 to relate resonance areas to concentrations requires either that the spectra are fully relaxed or under conditions of partial saturation so that the T_1 values of the pertinent resonances are approximately equal. As was stated above, in these experiments care was taken to avoid saturation. However, in the event that these precautions were insufficient, model compound studies indicate that the T_1 values of the resonances of interest are similar enough (6, 7) so that the variations in Z due to saturation effects give rise to pK_c values within the estimated uncertainties reported in Table I.

TABLE I

EXPERIMENTAL VALUES OF THE CONSTANTS AND THEIR STANDARD DEVIATIONS DETERMINING THE QUANTITY OF CARBAMINO ADDUCT FORMATION

Peptide	pK_z	K_z	pK_c	K_c
AII*	7.55 ± 0.05	2.8×10^{-8}	4.80 ± 0.10	1.6×10^{-5}
BK‡	7.14 ± 0.05	7.2×10^{-8}	5.02 ± 0.10	9.6×10^{-6}

*1-Asp-5-Ile-angiotensin II.

‡Bradykinin.

Enzyme Studies

Enzyme assays using the artificial substrate LpNA were performed after a modified version of the procedure of Wachsmuth et al. (14). Molar extinction coefficients for substrate LpNA and product *p*-nitroaniline were determined to be 30 and 8,630 $M^{-1}cm^{-1}$, respectively, at 410 nm. Appearance of product, *p*-nitroaniline, was followed spectrophotometrically at 410 nm for 4 min at 37°C. Each assay mixture had a total volume of 3 ml. The reaction was initiated by adding enzyme. Enzyme activity in buffer systems with varying ratios of bicarbonate and phosphate and ionic strength 0.2–0.3 was measured at pH 7.35–7.40. For a given ionic strength and ratio of total phosphate to total carbonates the amounts of NaH_2PO_4, Na_2HPO_4, and $NaHCO_3$ required by the Henderson-Hasselbalch equation at pH 7.4 were used. The solution was subsequently titrated to pH 7.4 with gaseous CO_2. Separate determinations of inhibition in the complete absence of phosphate and with ionic strengths controlled by the addition of KCl were made.

Aminopeptidase M cleavage of AII in phosphate or bicarbonate buffer was measured at pH 7.40 ± 0.02, 37°C, and ionic strength of 0.155 by measuring the sequential appearance of aspartic acid in the assay system using the amino acid analyzer.

pK_z Determination

The NMR tritations of AII and BK are shown in Figs. 1 and 2, respectively, and the constants are given in Table I. For AII the determination of pK_z from the pH dependence of the chemical shifts of either Asp-1-C^{β} or Asp-1-C^{α} could be obtained. These give the same result within experimental error. The value of pK_z for BK is somewhat lower than that for AII as seen in Table I.

pK_c Determination

Figs. 3 and 4 show typical undecoupled ^{13}C NMR spectra of the compounds equilibrated with $^{13}CO_2$. In Fig. 3 peak 1 (29.7 ppm) corresponds to the ^{13}C-enriched AII carbamino adduct, peak 2 (32.8 ppm) is ^{13}C-enriched HCO_3^-, peak 3 is that of $^{13}CO_2$, and peak 4 is the reference peak of sucrose. In Fig. 4, the envelope of resonances labeled 1 and centered at 21 ppm is used as the internal reference and is due to the nine backbone carbonyls of BK. Peak 2 (29.5 ppm) is the ^{13}C-enriched BK carbamino resonance, peak 3 (32.8 ppm) is ^{13}C-enriched HCO_3^-, and the remaining resonance at higher field is one of the natural abundance ^{13}C resonances of BK.

Examination of these results reveals a number of significant points with respect to adduct formation. The pK_z values for each compound, as derived from Figs. 1 and 2 and given in Table I, are near the extracellular pH of 7.4. Inasmuch as it is the unprotonated form of the peptide that binds the CO_2, small changes in pH in the normal range consequently change the amount of carbamino formed at any level of Pco_2. That this is the case may be noted from examination of Fig. 3, peak 1, and Fig. 4, peak 2, both of which increase with respect to the reference peaks as pH rises above the value of pK and the amino group loses its proton. At pH values higher than those shown the amount of adduct again falls so that a bell-shaped curve of adduct con-

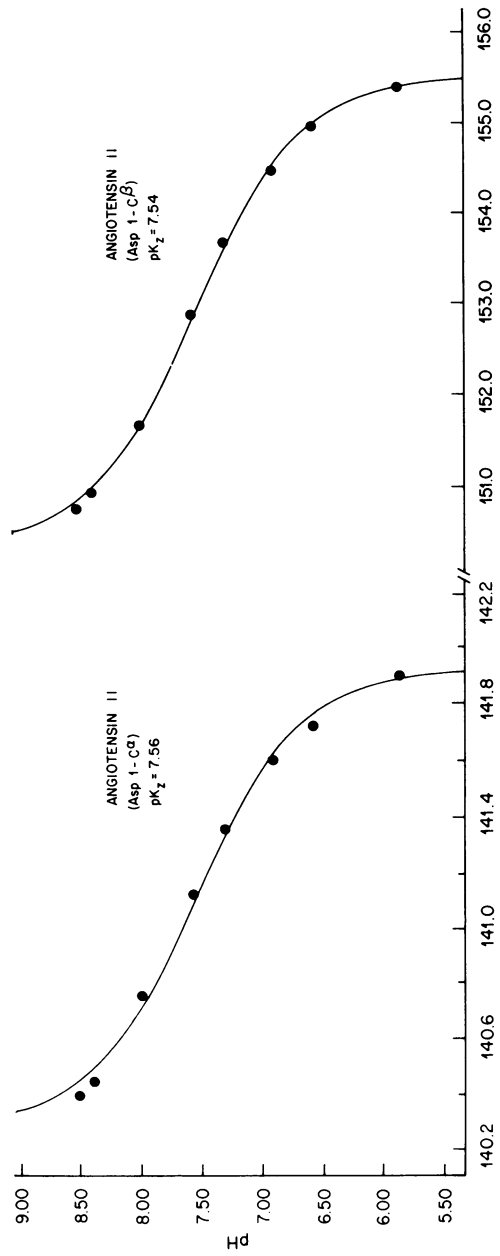

FIGURE 1 ^{13}C NMR titration curves for Asp-1-C^{α} and Asp-1-C^{β} of AII.

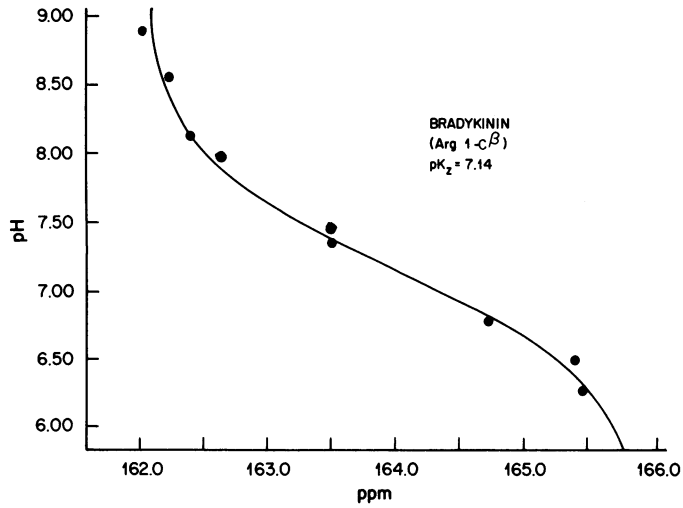

FIGURE 2 ^{13}C NMR titration for Arg-1-C^{β} of BK.

centration with pH is described at any given P_{CO_2}. This relationship is to be expected from a consideration of Eqs. 1, 2, and 3 (6).

From the intensties of these peaks and the determined enrichment of CO_2 the values of K_c, the association constant for CO_2, are determined. As seen in Table I, the K_c values for AII and BK are also similar. These two constants, K_c, the association constant for CO_2, and K_z, the dissociation constant for the proton of the amine define the affinity of that amine for CO_2 and protons, respectively (Eqs. 1 and 2). Carbamino formation is favored if both pK_c and pK_z are low. BK, which has a pK_z less than AII, has a pK_c higher than AII. This is understandable chemically because both K_c and K_z reflect broadly the nucleophilicity of the amine nitrogen for the partially positive charge of the CO_2 carbon or the positively charged proton. On a comparative basis if pK_c is found to be smaller, indicating a stronger nucleophile, then pK_z is usually expected to be larger, also indicating a stronger nucleophile.

Carbamino Adduct Variation

When pH and P_{CO_2} are varied, Z, the mole fraction of carbamino adduct, is defined by a series of curves, as illustrated in Figs. 5 and 6. The curves are drawn to include the pH of extracellular fluid (pH 7.4) and extend toward more acid values characteristic of intracellular pH values. They are conveniently superimposed on standard pH-bicarbonate-P_{CO_2} plots (16). The general characteristics of the web plots for AII and BK are similar because of the compensation between pK_c and pK_z. At pH values below the normal extracellular pH of 7.4 the Z_{AII} curves are steeper because the pK_z for AII is somewhat >7.4, whereas the pK_z for BK is somewhat <7.4.

A striking feature of the web plots (Figs. 5 and 6) is that the variation in Z will be greatest where metabolically induced perturbations of acid-base balance occur. For either AII or BK web plots, the points B and C represent values on the grid that might

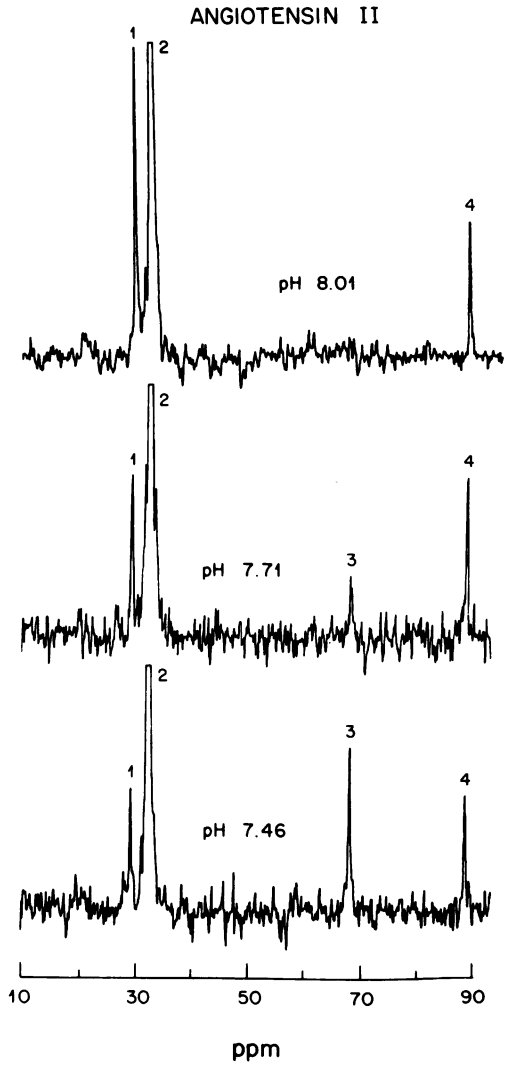

FIGURE 3

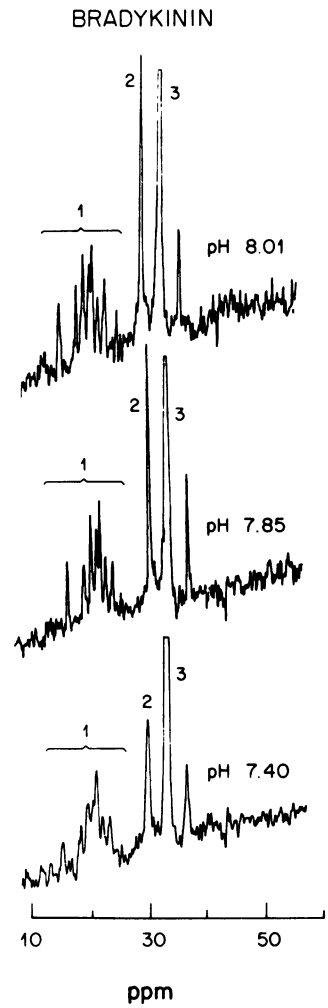

FIGURE 4

FIGURE 3 Undecoupled ^{13}C NMR spectra of AII equilibrated with $^{13}CO_2$ at various pH values. Chemical shifts are referenced to CS_2.

FIGURE 4 Undecoupled ^{13}C NMR spectra of BK equilibrated with $^{13}CO_2$ at various pH values. Chemical shifts are referenced to CS_2.

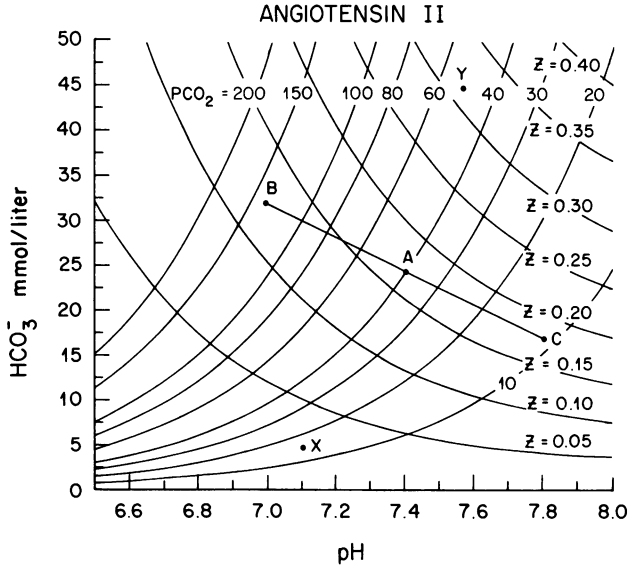

FIGURE 5 Web plot for AII based on pK_c and pK_z values from Table I. The values used for the acid disassociation constant, pK'_1, and the Henry's law constant for CO_2 are 6.20 and 0.0301 mm Hg, respectively (15).

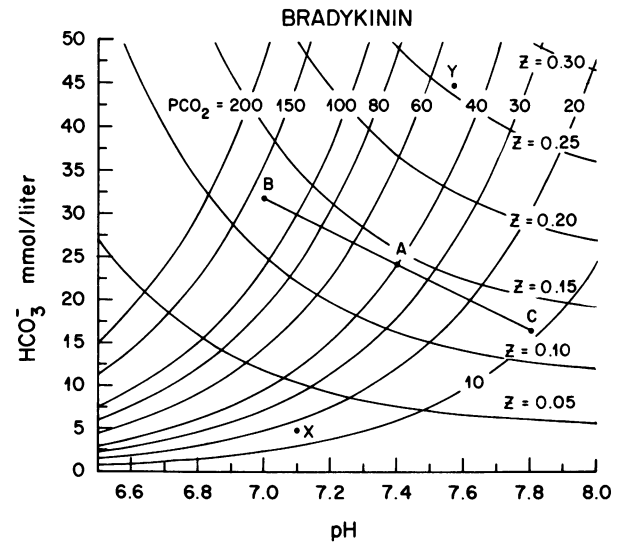

FIGURE 6 Web plot for BK based on pK_c and pK_z values from Table I. The values used for the acid disassociation constant, pK'_1, and the Henry's law constant for CO_2 are 6.20 and 0.0301 mm Hg, respectively (15).

be encountered in respiratory acidosis and alkalosis, respectively. Z values at B and C differ only minimally. In contrast are points X and Y, values for metabolic acidosis and alkalosis, respectively. As a consequence of the fact that the P_{CO_2}, isobars and the Z curves intersect as illustrated, the Z values differ drastically at points X and Y for both AII and BK.

Values of Z for AII and BK typical of those to be found in the normal state of acid-base balance as well as values of Z that might be found in respiratory and metabolic acidosis and alkalosis are included in Table II. In the normal case, about 15% of either AII or BK is seen to exist as the carbamino adduct. Z may be reexpressed as the ratio of free to adduct form of either AII or BK. This ratio is seen to vary somewhat during respiratory-induced pH changes. However, the ratio of free to adduct form varies considerably from normal with variables of pH, HCO_3^-, and P_{CO_2} typical of metabolic changes.

The envelope of resonances in Fig. 4 at natural abundance ^{13}C of the peptide is attributable to individual carbonyl carbons of the peptide backbone. The individual resonances are clearly distinguishable and are invariant with the experimental conditions employed in this study. More detailed studies of the aromatic regions of chemical shift (spectra not shown) show invariance of the characteristic chemical shifts of these nuclei over the pH region where carbamino adduct formation occurs, indicating that no gross conformational alteration of the peptide structure attends the formation of the carbamino adduct.

The artificial substrate, LpNA, was also shown to form carbamine adducts with CO_2 to a similar extent as the hormones AII and BK.

TABLE II

SUMMARY OF POSSIBLE ACID-BASE PERTURBATIONS AND THE ASSOCIATED ALTERATION IN CARBAMINO ADDUCT FORMED

Uncompensated condition	pH	P_{CO_2}	HCO_3^-	Z* angiotensin II	Free AII/ carbamino AII‡	Z* bradykinin	Free BK/ carbamino BK§	Free AII‡/ free BK§
		mmHg	*mM*					
Normal	7.4	40	24	0.17	4.9	0.16	5.3	0.92
Respiratory acidosis	7.0	115	27	.11	8.1	.12	7.3	1.1
Respiratory alkalosis	7.8	13	22	.21	3.8	.16	5.3	0.71
Metabolic acidosis	7.1	15	4	.02	49.0‖	.03	32.0	1.5
Metabolic alkalosis	7.55	55	42	.32	2.1	.29	2.4	0.88

*Mole fraction of compound found in the carbamino adduct form.

‡1-Asp-5-Ile-angiotensin II.

§Bradykinin.

‖This value is large but the exact value will be dependent upon the number of significant figures of Z used in its calculation.

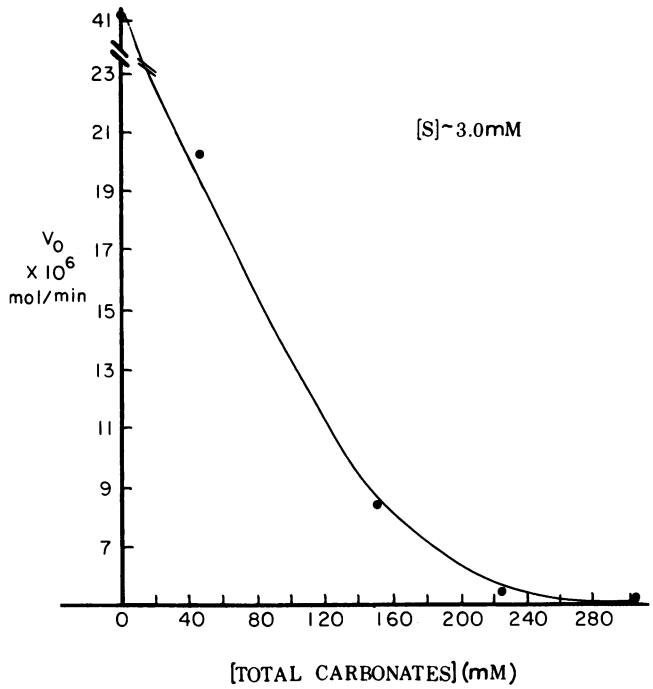

FIGURE 7 The inhibition of aminopeptidase M with increasing total carbonates. The initial velocity V_0, is for the hydrolysis of LpNA at a substrate concentration, S, near 3.0 mM, 37°C, pH 7.4, in the presence of phosphate buffer at ionic strength 0.200–0.300. Further details are given in the text.

Enzyme Inhibition

Fig. 7 describes the marked inhibition of the initial velocity of the cleavage of the artificial substrate LpNA by aminopeptidase M when total carbonates are increased at constant pH and controlled ionic strength. Additional control studies (data not included) indicate that the effect is not due to phosphate or to ionic strength variations. In the studies reported here we did not distinguish whether the inhibition resulted from adduct formation to the substrate or the enzyme, nor have we clearly ascribed the effect to CO_2 itself rather than to some other existing species of the bicarbonate buffer system. Cleavage of the amino terminal aspartic acid of AII by aminopeptidase M was also diminished when bicarbonate buffer was substituted for the phosphate buffer as described above. At pH 7.4, I = 0.155 M, 37°, the rate of release of aspartic acid from AII in bicarbonate buffer was approximately one-half that observed in the analogous phosphate buffer system.

DISCUSSION

Carbamino adduct formation with hemoglobin and its allosteric effect on that molecule have been recently investigated by techniques similar to those used here (4, 5, 8), and the possibility that adduct formation with other primary amines, notably hor-

mones, may exert a regulatory influence has been suggested (7). Whether the reversible post-translational modification of such compounds in vivo is biologically significant depends ultimately upon (*a*) the quantitation of the extent of adduct formation, (*b*) the demonstration that significant changes in the concentration of adduct occur over physiologically or pathologically relevant ranges of P_{CO_2} and pH, (*c*) the perturbation of subsequent biochemical processes, and finally (*d*) the reflection of this biochemical perturbation in an altered physiological response.

We have shown that the first two conditions above are fulfilled for AII and BK and that similar changes can produce biochemical changes in appropriate model systems. Figs. 1 and 2 define the K_z values for these compounds. The value of pK_z is near extracellular pH so that higher pH values than pK_z yield more of the unprotonated peptide, that form required for binding CO_2, and conversely.

Figs. 3 and 4 demonstrate unequivocally the existence of the adduct at various pH values and permit the calculation of K_c from which the mole fraction, Z, of carbamino adduct over a wide range of pH and P_{CO_2} may be calculated.

The magnitude of Z over a physiologically important range is indicated by the web plots of Figs. 5 and 6 corresponding to extracellular values of pH and $[HCO_3^-]$. The variation in mole fraction of adduct form and hence in the ratio of free to adduct form in metabolic and respiratory forms of acidosis and alkalosis is extensive as shown in Table II. Maximum variation occurs on going from metabolic acidosis to metabolic alkalosis and is much greater than the variation which occurs with respiratory-induced acidosis and alkalosis. Variation in mole fraction of adduct may be seen to be greater at extracellular pH values than at the lower pH values typical of intracellular conditions.

Among metabolic effects to be considered is the potential for altered binding of the adduct form to extracellular degrading enzymes or hormone-specific plasma membrane receptors. This may be illustrated by the scheme below where A is the hormone amine and B is the binding species, either an enzyme or a receptor.

$$\begin{array}{ccccc} & & CO_2 & & \\ & & + & & \\ B + & & A & \overset{K_b}{\rightleftharpoons} & BA \\ & \lambda & \Downarrow & & \\ & & ACO_2 & & \end{array}$$

In this diagram K_b is the association constant for the free amine whereas λ is the pH-dependent binding constant of the hormone for CO_2 ($\lambda = (ACO_2)/(A)(CO_2)$). The value of λ is related to K_c and K_z (Eqs. 1 and 2) by $\lambda = K_cK_z/[K_z(H^+) + (H^{+2})]$ and at pH 7.4 has a value of 160 M^{-1} for BK or 170 M^{-1} for AII as calculated from values given in Table I.

Typically, the values for K_b are $<10^5$ M^{-1} for the binding of peptides to degrading enzymes (11). K_b is much larger for the binding of hormone to receptors, and in the range 10^{10} to 10^{12} M^{-1} (17). From a consideration of the equilibria involved and appropriate estimates of the concentrations of CO_2, hormones, enzymes, and recep-

tors, it is possible to show that a change in CO_2 concentration within physiological expectations cannot perturb the ratio of amine containing CO_2 to amine bound to receptor and that receptor binding of hormone will predominate. An exception would apply to the case in which receptor binding of amine and of adduct are equally strong. With small peptide hormones such as AII and BK, the comparably strong binding of both native hormone and adduct hormone would seem improbable on the basis of bulk or charge, but for somewhat larger molecules such binding should not be overlooked.

In contrast to the above, quantitative consideration of the equilibria involved when B represents a degrading enzyme indicates that the relative proportions of the two forms, BA and ACO_2, will be sensitive to the amount of CO_2 present and the pH. If the enzyme is less active toward the adduct form, ACO_2, and this adduct form is present in significant amounts, then the rate of degradation of substrate will be less.

The vasoactive peptides studied form carbamino adducts to nearly equivalent extent and variability, but, as pointed out, the hormones have opposing apparent physiological effects. Clearly adduct formation will produce no different biological response unless the adduct form is subsequently sensed by enzyme or receptor in a different way. Because in vivo AII is converted by aminopeptidases to a derivative with a different spectrum of activity whereas BK activity is not altered by aminopeptidase activity, an effect upon the aminopeptidase system in vivo would effectively alter AII/AIII as well as AII(AIII)/BK activity, and a physiological response could easily ensue. Enzyme studies carried out using either LpNA or AII as a substrate for the aminopeptidase M model system show that under conditions where CO_2 adduct formation exists, enzymic activity is diminished.

CONCLUSION

Carbamino adducts to vasoactive peptides AII and BK have been demonstrated to exist and their quantity to vary over a physiologically pertinent pH range. Simple equilibrium considerations lead us to suggest that adduct formation may reasonably be expected to cause a biochemical difference for selective enzyme binding rather than for receptor binding. Enzymes that convert AII to AIII differ from those that inactivate BK, and the products of each differ in activity. An in vitro aminopeptidase model system is inhibited in the presence of the bicarbonate buffer system and is consistent with, but not conclusive for, carbamino adduct formation with substrate as the responsible factor. Further studies on these and other peptide hormone systems are warranted to elucidate the potential regulatory role of carbamino compounds in physiological processes.

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REFERENCES

1. HASTINGS, A. B. 1970. A biochemist's anabasis. *Annu. Rev. Biochem.* **39**:1–24.
2. NAHAS, G., and K. E. SCHAEFER, editors. 1974. Carbon Dioxide and Metabolic Regulations. Springer-Verlag Inc., New York.
3. ARNONE, A. 1974. X-ray studies of the interaction of CO_2 with human deoxyhemoglobin. *Nature (Lond.).* **247**:143–145.
4. MATTHEW, J. B., J. S. MORROW, R. J. WITTEBORT, and F. R. N. GURD. 1977. Quantitative determination of carbamino adducts of α and β chains in human adult hemoglobin in presence and absence of carbon monoxide and 2,3-diphosphoglycerate. *J. Biol. Chem.* **252**:2234–2244.
5. MORROW, J. S., and F. R. N. GURD. 1975. Nuclear magnetic resonance studies on hemoglobin: functional state correlations and isotopic enrichment strategies. *CRC Crit. Rev. Biochem.* **3**:221–284.
6. MORROW, J. S., P. KEIM, and F. R. N. GURD. 1974. CO_2 adducts of certain amino acids, peptides and sperm whale myoglobin studied by carbon 13 and proton magnetic resonance. *J. Biol. Chem.* **249**: 7484–7494.
7. MORROW, J. S., R. S. GURD, and F. R. N. GURD. 1974. The chemical basis and possible role of carbamino homeostatic mechanisms. *In* Peptides, Polypeptides and Proteins. E. R. Blout, F. A. Bovey, M. Goodman, and N. Lotan, editors. John Wiley & Sons, New York. 594–604.
8. MORROW, J. S., J. B. MATTHEW, R. J. WITTEBORT, and F. R. N. GURD. 1976. Carbon 13 resonance of $^{13}CO_2$ carbamino adducts of α and β chains in human adult hemoglobin. *J. Biol. Chem.* **251**:477–484.
9. COLMAN, R. W. 1974. Formation of human plasma kinin. *N. Engl. J. Med.* **291**:509–515.
10. OPARIL, S., and E. HABER. 1974. The renin-angiotensin system. *N. Engl. J. Med.* **291**:389–401.
11. PEACH, M. J. 1977. Renin-angiotensin system: biochemistry and mechanism of action. *Physiol. Rev.* **57**:313–370.
12. FREEMAN, R. H., J. O. DAVIS, T. E. LOHMEIER, and W. S. SPIELMAN. 1977. [Des-Asp^1] angiotensin II: mediator of the renin-angiotensin system? *Feb. Proc.* **36**:1766–1770.
13. OLDFIELD, E., R. S. NORTON, and A. ALLERHAND. 1975. Studies of individual carbon sites of proteins in solution by natural abundance carbon 13 nuclear magnetic resonance spectroscopy. *J. Biol. Chem.* **250**:6368–6380.
14. WACHSMUTH, E. D., I. FRITZE, and G. PFLEIDERER. 1966. An aminopeptidase occurring in pig kidney. II. A study on the mechanism of hydrolysis. *Biochemistry.* **5**:175–182.
15. VAN SLYKE, D. D., and J. S. SENDROY. 1928. Studies on gas and electrolyte equilibria in blood. *J. Biol. Chem.* **78**:781–798.
16. DAVENPORT, H. W. 1974. The ABC of Acid-Base Chemistry. The University of Chicago Press, Chicago.
17. CUATRECASAS, P., and M. D. HOLLENBERG. 1976. Membrane receptors and hormone action. *Adv. Protein Chem.* **30**:252–428.