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# Unusual Chemical Properties of N-Terminal Histidine Residues of Glucagon and Vasoactive Intestinal Peptide†

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ABSTRACT: An N-terminal histidine residue of a protein or peptide has two functional groups, viz., an α-amino group and an imidazole group. A new procedure, based on the competitive labeling approach described by Duggleby and Kaplan [Duggleby, R. G., & Kaplan, H. (1975) *Biochemistry 14*, 5168–5175], has been developed by which the chemical reactivity of each functional group in such a residue can be determined as a function of pH. Only very small amounts of material are required, which makes it possible to determine the chemical properties in dilute solution or in proteins and polypeptides that can be obtained in only minute quantities. With this approach, the reactivity of the α-amino group of histidylglycine toward 1-fluoro-2,4-dinitrobenzene gave an apparent $pK_a$ value of 7.64 ± 0.07 at 37 °C, in good agreement with a value of 7.69 ± 0.02 obtained by acid–base titration. However, the reactivity of the imidazole function gave an apparent $pK_a$ value of 7.16 ± 0.07 as compared to the $pK_a$ value of 5.85 ± 0.01 obtained by acid–base titration. Similarly, in glucagon and vasoactive intestinal peptide (VIP), apparent $pK_a$ values of 7.60 ± 0.04 and 7.88 ± 0.18, respectively, were obtained for the α-amino of their N-terminal histidine, and $pK_a$ values of 7.43 ± 0.09 and 7.59 ± 0.18 were obtained for the imidazole function. Rothgeb et al. [Rothgeb, T. M., England, R. D., Jones, B. N., & Gurd, R. S. (1978) *Biochemistry 17*, 4564–4571] used proton titration and $^{13}C$ NMR to assign $pK_a$ values of 7.32 to the α-amino group and 5.32 to the imidazole function of *S*-methylglucagon at 25 °C. It is concluded that the large difference in the apparent $pK_a$ values of the imidazole moiety obtained by proton titration and chemical reactivity is an inherent property of an N-terminal histidine which results from a large decrease in the inductive effect on the imidazole moiety by the α-amino group when the latter loses its proton. While the apparent ionization behavior of the N-terminal histidine in glucagon and VIP does not differ from that in model compounds, the reactivity of the imidazole function in both is substantially greater than that in histidylglycine, and the reactivity of the α-amino group in glucagon is a factor of 2 lower. In addition, the $pK_a$ and reactivity of the solitary lysine in glucagon are very different from those of a normal ε-amino group in solution. These results indicate that both glucagon and VIP have sufficient organized structure in dilute solution ($10^{-6}$ M) to alter the chemical properties of these groups.

Most proteins have only one ionizable group at the amino-terminal residue. There are several biologically active peptides, viz., glucagon, vasoactive intestinal peptide (VIP),[1] PHI, and secretin, which have an N-terminal histidine residue and therefore have two ionizable groups at their amino-ter-

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[1] Abbreviations: VIP, vasoactive intestinal peptide; PHI, peptide with N-terminal histidine and C-terminal isoleucine amide; $N_2$phF, 1-fluoro-2,4-dinitrobenzene; $N_2$ph, 2,4-dinitrophenyl; Ala-ala, alanylalanine; imlac, L-β-imidazolyllactic acid; TLC, thin-layer chromatography.

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minal residue. This is of particular interest since there is evidence, at least in the cases of glucagon [see Epand et al. (1981) and references cited therein], VIP, and secretin (Robberecht et al., 1976), that the N-terminal histidine is essential for the biological activity of the polypeptide. Because both the $\alpha$-amino and imidazole groups potentially ionize over the physiological pH range, it is of interest to study what effects their ionizations or mutual interaction may have on the biological properties of the molecule. Rothgeb et al. (1978) have used $^{13}C$ NMR to obtain $pK_a$ values for the ionizations of the N-terminal histidine in *S*-methylglucagon. They noted, in agreement with an early study by Epand et al. (1973), that as far as proton titration behavior is concerned, these groups do not differ from those in model compounds such as histidine amide. However, neither of these studies directly evaluates the chemical properties of these groups. It has been observed that some groups in proteins with apparently normal proton titration behaviors have abnormal chemical reactivities (Hill & Davies, 1967; Garner et al., 1975; Cruickshank & Kaplan, 1975). It therefore does not follow that because a group shows normal titration behavior its properties are normal in all respects and determination of its other chemical properties is also necessary.

The competitive labeling procedure of Duggleby & Kaplan (1975) can, in principle, be used to make an unequivocal assignment of the ionization constant and chemical reactivity to any $\alpha$-amino or imidazole function in a protein or polypeptide. In the case of an N-terminal histidine, it is difficult to make such an assignment as two groups become labeled on the same amino acid residue. In this study, we describe two approaches by which the chemical reactivity of the $\alpha$-amino and imidazole functions can be studied independently of one another. This new methodology provides a means for determining how the chemical properties of one group influence the properties of the other.

## Rationale

The competitive labeling approach described by Duggleby & Kaplan (1975) consists of the following steps: (i) trace labeling of sample and standard nucleophile with $[^3H]N_2ph$-F at a series of pH values under defined solvent conditions; (ii) full reaction with $[^{14}C]N_2ph$-F under denaturing conditions; (iii) separation and purification of the $[^3H/^{14}C]N_2ph$ standard nucleophile; (iv) acid hydrolysis of the sample followed by isolation of the $[^3H/^{14}C]N_2ph$-amino acids; (v) quantification of the $^3H/^{14}C$ ratios. A protein or polypeptide with an N-terminal histidine would give the di-$N_2ph$-histidine derivative following this procedure, and the reactivity obtained would therefore be an average of the reactivities of the $\alpha$-amino and imidazole groups. In this regard, Henkart (1971) has shown that only the N′-3 $N_2ph$ derivative is formed with the imidazole ring of histidine. In order to unequivocally assign a reactivity to the $\alpha$-amino group and to the imidazole group, it is necessary to quantify the rate of reaction of each functional group independently. The outline in Figure 1 shows how this can be accomplished. After the trace labeling step, the sample is chemically heterogeneous, and the N-terminus consists of $N^{\alpha}$-$[^3H]N_2ph$-histidine, imidazolyl-$[^3H]N_2ph$-histidine, and unmodified histidine. The relative amounts of each will, of course, depend on the chemical properties, viz., $pK_a$ and reactivity, of each group. Since the object is to determine these properties, it is necessary to isolate and quantify these $[^3H]N_2ph$ derivatives without further derivatization. Therefore, in place of full reaction with $^{14}C$-labeled reagent in step ii, a mixture containing an equal number of dpm in each $N^{\alpha}$-$[^{14}C]N_2ph$-histidine, imidazolyl-$[^{14}C]N_2ph$-histidine, and

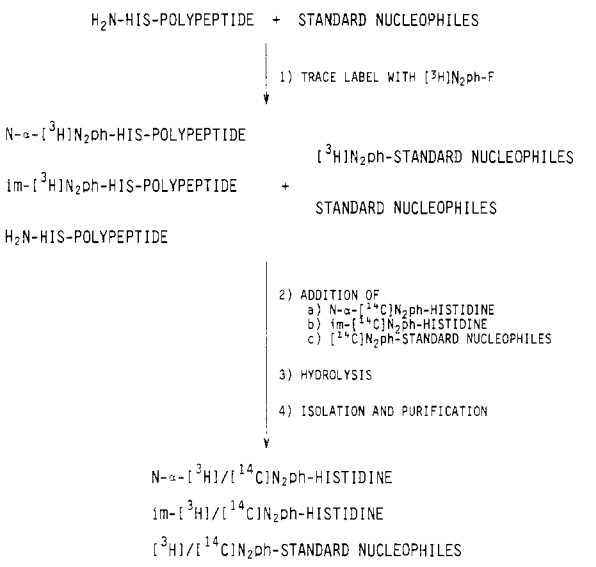


FIGURE 1: Competitive labeling procedure for determining the $pK_a$ and reactivity of the $\alpha$-amino and imidazole functions of an N-terminal histidine residue.

$[^{14}C]N_2ph$ standard nucleophile is added. The entire reaction mixture is then subjected to acid hydrolysis. Isolation of the $[^3H/^{14}C]N_2ph$ derivatives and quantification of their $^3H/^{14}C$ ratios can then be carried out. Addition of equal $^{14}C$ dpm of each mono-$N_2ph$ derivative of histidine achieves what full reaction with $[^{14}C]N_2ph$-F achieved for each solitary functional group in the procedure described by Duggleby & Kaplan (1975). Thus, the pH-dependent second-order velocity constant ($\alpha_x r$) for the reaction can be calculated from

$$\alpha_x r = \alpha_s \frac{^3H_x/^{14}C_x}{^3H_s/^{14}C_s}$$

where $\alpha_s$ is the degree of ionization of the standard nucleophile, $^3H_x$ and $^{14}C_x$ are the tritium dpm and $^{14}C$ dpm in either the $N^{\alpha}$-amino group or the imidazole group of histidine, and $^{14}C_s$ is the corresponding dpm in the standard nucleophile. From a plot of $\alpha_x r$ against pH, the values of $\alpha_x$, the degree of ionization of the functional group under study, and $r$, the second-order velocity constant relative to the standard nucleophile, obtained at high pH values where $\alpha_x = 1$ can be determined.

## Experimental Procedures

*Materials.* Porcine VIP and glucagon were supplied by Sigma Chemical Co. (St. Louis, MO) as were $N_2ph$-F, L-$\beta$-imidazolyllactic acid, histidine, $N^{\alpha}$-acetylhistidine, $N^{\alpha}$-acetyllysine, alanylalanine, and histidylglycine. Amersham Corp. (Oakville, Ontario, Canada) supplied the radioactive reagents and NEN Canada (Lachine, P.Q., Canada) the Aquasol-2 for scintillation counting. Porapak Q was supplied by Waters Associates; the thin-layer silica plates for chromatography were from Eastman Kodak. Pyrex tubes (Corning, 16 × 125 mm) were obtained from Canlab (Ottawa, Ontario, Canada). All other reagents were supplied by Fisher Scientific Limited (Ottawa, Ontario, Canada).

*Preparation of* $[^{14}C]N_2ph$ *Derivatives.* $N^{\epsilon}$-$[^{14}C]N_2ph$-lysine and imidazolyl-$[^{14}C]N_2ph$-histidine were prepared from $N^{\alpha}$-acetyllysine and $N^{\alpha}$-acetylhistidine, respectively. A sample (2 mg) of each $N^{\alpha}$-acetylated amino acid was dissolved in 1 mL of distilled water. After addition of 0.25 g of sodium bicarbonate, 125 $\mu$L of acetonitrile containing $[^{14}C]N_2ph$-F (2.38 $\mu$mol, specific radioactivity 21 mCi/mmol) was added

to each and the reaction allowed to proceed 18 h in the dark. Complete dinitrophenylation was ensured by reaction with unlabeled $N_2$ph-F (25 μL, 50% v/v in acetonitrile) for a further 18 h. The pH was then brought to 2 with concentrated HCl. Following hydrolysis in 6 M HCl (3 h, 110 °C), each sample was extracted with three 5-mL portions of diethyl ether, diluted approximately 6-fold with water, and introduced to the top of a Porapak Q column (7 mm × 30 mm) which had been preequilibrated with 95% ethanol and then washed with 0.01 M HCl. After the column was washed with 15 mL of 0.01 M HCl, the [$^{14}$C]$N_2$ph-amino acid was eluted with 80% acetone (v/v in water) and then purified by high-voltage paper electrophoresis at pH 2.1 (3500 V, 45 min). $N^{\alpha}$-[$^{14}$C]$N_2$ph-histidine and L-β-imidazolyl-[$^{14}$C]$N_2$ph-lactic acid were prepared from histidine and L-β-imidazolyllactic acid, respectively, in manner similar to that described above except that the samples were not hydrolyzed prior to ether extraction. Following elution from the Porapak Q column, L-β-imidazolyl-[$^{14}$C]$N_2$ph-lactic acid was purified by two-dimensional electrophoresis at pH 3.5 (3500 V, 75 min) and pH 2.1 (3500 V, 30 min). The $N^{\alpha}$,$N$-imidazolyl-di[$^{14}$C]$N_2$ph-histidine was dissolved in 10 mL of 20% acetone and 2% $N$-methylmorpholine (v/v in water, pH 8.0); 100 μL of β-mercaptoethanol was added and thiolysis allowed to proceed for 8 h in the dark. After evaporation of the acetone, the sample was acidified and extracted with three 10-mL portions of diethyl ether. The $N^{\alpha}$-[$^{14}$C]$N_2$ph-histidine was isolated from the aqueous phase by high-voltage paper electrophoresis at pH 2.1 (3500 V, 45 min). All derivatives were eluted from the paper with 10% acetic acid.

*Procedures for VIP and Glucagon.* (*A*) *Sample Preparation.* Stock solutions containing equimolar ($10^{-6}$ M) L-β-imidazolyllactic acid, alanylalanine, and either glucagon or VIP were prepared in 5 mM $N$-methylmorpholine/5 mM sodium borate/5 mM acetic acid/0.1 M KCl.

(*B*) *$^3$H-Trace Labeling.* Aliquots (3.0 mL) of the stock solution containing L-β-imidazolyllactic acid, alanylalanine, and either glucagon or VIP were temperature equilibrated at 37 °C in a thermostated water jacket and adjusted to the desired pH by using either 1 M NaOH or 1 M HCl. An aliquot (50 μL) of acetonitrile containing [$^3$H]$N_2$ph-F (2.08 nmol, specific radioactivity 16.6 Ci/nmol) was added to each sample and the reaction allowed to proceed 18 h in the dark. Concentrated HCl was added to bring the pH to 2.0 (Figure 1, step 1).

(*C*) *Addition of [$^{14}$C]$N_2$pH-amino Acids.* The following was added to each trace-labeled glucagon sample in 2.0 mL of a solution containing 1% $N$-methylmorpholine/25% acetone (v/v in water): imidazolyl-[$^{14}$C]$N_2$ph-histidine (2500 dpm), L-β-imidazolyl-[$^{14}$C]$N_2$ph-lactic acid (2500 dpm), $N^{\alpha}$-[$^{14}$C]$N_2$ph-alanylalanine (2500 dpm), $N^{\epsilon}$-[$^{14}$C]$N_2$ph-lysine (2200 dpm). The solution also contained 0.03 mg of each unlabeled $N_2$ph derivative as carrier. For VIP, the $^{14}$C derivatives were added in 175 μL of solution which contained 12 500 dpm of each of the above derivatives. An aliquot (500 μL) of another solution containing 0.1 mg of each unlabeled derivative as carrier was added (Figure 1, step 2). After removal of the acetone by partial evaporation, an equal volume of 12 M HCl was added to each sample (5.00 mL in the case of glucagon samples and 3.68 mL in the case of VIP samples). Each sample was hydrolyzed 18 h at 110 °C (Figure 1, step 3).

(*D*) *Isolation and Purification of $^3$H/$^{14}$C-Labeled Derivatives.* Each hydrolyzed sample was extracted with three 5-mL aliquots of diethyl ether. The ether layer was evaporated; the sample was redissolved in chloroform saturated with dibasic phosphate solution (1 g of $Na_2HPO_4$ in 100 mL of water) and applied to a silica G column (7 mm × 30 mm). The column was washed with 20 mL of basic chloroform to remove the dinitrophenol (Steven, 1962). The $N^{\alpha}$-[$^3$H/$^{14}$C]$N_2$ph-alanine was eluted by using 80% acetone (v/v in water) and purified by two-dimensional TLC of silica plates (first dimension, chloroform/benzyl alcohol/glacial acetic acid, 70/30/3; second dimension, benzene/pyridine/glacial acetic acid, 80/20/2) according to the overflow technique of Brenner & Niederwiesser (1961).

The acid phase was lyophilized, redissolved in 0.01 M HCl, and applied to a Porapak Q column. After the column was washed with 15 mL of distilled water, the [$^3$H/$^{14}$C]$N_2$ph-amino acids were eluted with 80% acetone (v/v in water) and subjected to high-voltage electrophoresis at pH 2.1 (3500 V, 65 min). Imidazolyl-[$^3$H/$^{14}$C]$N_2$ph-histidine and L-β-imidazolyl-[$^3$H/$^{14}$C]$N_2$ph-lactic acid bands were further purified by electrophoresis at pH 3.5 (3500 V, 30 min). A band containing $N^{\epsilon}$-[$^3$H/$^{14}$C]$N_2$ph-lysine and $N^{\alpha}$-[$^3$H/$^{14}$C]$N_2$ph-histidine was cut and subjected to electrophoresis at pH 6.5 (3500 V, 120 min). The $N^{\alpha}$-[$^3$H/$^{14}$C]$N_2$ph-histidine isolated from this electrophoresis was then purified by electrophoresis at pH 3.5 (3500 V, 30 min).

*Procedures for Histidylglycine.* (*A*) *Sample Preparation.* A stock solution containing equimolar ($10^{-3}$ M) L-β-imidazolyllactic acid, alanylalanine, and histidylglycine was prepared in 5 mM $N$-methylmorpholine/5 mM sodium borate/5 mM acetic acid/0.1 M KCl.

(*B*) *$^3$H-Trace Labeling.* Aliquots (5.0 mL) of the stock solution were temperature equilibrated and adjusted to the desired pH as described above. To each sample was added 50 μL of acetonitrile containing [$^3$H]$N_2$ph-F (0.95 nmol, specific radioactivity 26 Ci/nmol) and the reaction allowed to proceed 18 h in the dark. Concentrated HCl was added to bring the pH to 2. The sample was then divided in half. Half was used to quantify the reactivity of the imidazole function and the other half to quantify the reactivity of the α-amino group.

*Determination of the Reactivity of the Imidazole Function of Histidylglycine.* (*A*) *Acetylation.* The first half of each $^3$H-trace-labeled sample was adjusted to pH 9.0 with 5 M NaOH. The sample was fully acetylated with unlabeled acetic anhydride (60 μL) at 22 °C. After the addition of 0.5 g of $NaHCO_3$ and 2.5 g of urea, each acetylated sample was fully dinitrophenylated with unlabeled $N_2$ph-F (500 μL, 50% v/v in acetonitrile) for a further 12 h at 22 °C in the dark.

(*B*) *$^{14}$C Labeling.* A portion of the stock solution containing 0.9 μmol each of histidylglycine, L-β-imidazolyllactic acid, and alanylalanine was lyophilized and then redissolved in 3 mL of an 8 M urea solution containing 2.5 g of $NaHCO_3$. The pH was adjusted to 9 and the sample fully acetylated by using 40 μL of unlabeled acetic anhydride and then dinitrophenylated with 2.98 μmol of [$^{14}$C]$N_2$ph-F (specific radioactivity 21 mCi/mmol) by reacting 18 h at 22 °C in the dark. The final volume was adjusted to 20.0 mL with 8 M urea solution. An aliquot (1.0 mL) of this solution was added to each of the above $^3$H-trace-labeled samples. After addition of 0.5 mg of $N^{\alpha}$-acetylimidazolyl-$N_2$ph-histidine and 0.5 mg of imidazolyl-$N_2$ph-lactic acid to each sample (as carrier), the pH was adjusted to 2 with concentrated HCl.

(*C*) *Isolation and Purification of $^3$H/$^{14}$C-Labeled Derivatives.* Each sample was extracted 3 times with a 5-mL aliquot of diethyl ether. The aqueous phase was passed over a Dowex 50W column (7 mm × 30 mm) which had been preequilib-

Table I: Summary of the Chemical Properties of Amino and Imidazole Groups[a]

| functional group | $pK_a$ | | | reactivity | |
|---|---|---|---|---|---|
| | acid–base titration | competitive labeling | | | |
| | | Ala-ala[b] | im-lac[b] | Ala-ala[b] | im-lac[b] |
| alanylalanine | | | | | |
| α-amino | 8.03 ± 0.008 | | 8.02 ± 0.03 | | 25.4 ± 0.5 |
| histidylglycine | | | | | |
| α-amino | 7.69 ± 0.02 | 7.64 ± 0.07 | 7.49 ± 0.06 | 0.413 ± 0.017 | 10.75 ± 0.03 |
| imidazole | 5.85 ± 0.01 | | 7.16 ± 0.07 | 0.041[d] | 1.06 ± 0.03 |
| glucagon | | | | | |
| α-amino (His-1) | 7.23[c] | | 7.60 ± 0.04 | 0.202[d] | 5.13 ± 0.11 |
| imidazole (His-1) | 5.23[c] | | 7.43 ± 0.09 | 0.080[d] | 2.02 ± 0.08 |
| ε-amino (Lys-12) | | | 8.49 ± 0.09 | 0.338[d] | 9.66 ± 0.52 |
| VIP | | | | | |
| α-amino (His-1) | | 7.88 ± 0.18 | | 0.42 ± 0.04 | 10.16[d] |
| imidazole (His-1) | | | 7.52 ± 0.18 | 0.111[d] | 2.81 ± 0.22 |
| ε-amino (average of four) | | 8.63 ± 0.06 | | 2.4 ± 0.1 | 60.96[d] |

[a] The values of $pK_a$ and reactivity and their standard errors were calculated by fitting the data to theoretical titration curves using a nonlinear least-squares regression procedure. [b] Internal standard nucleophile used. [c] Value obtained by Rothgeb et al. (1978). [d] Value calculated on the basis of a reactivity of 25.4 ± 0.5 for alanylalanine relative to L-β-imidazolyllactic acid.

rated at pH 2 with 0.1 M HCl. The column was washed with 15 mL of water to remove the urea. L-β-Imidazolyl-[$^3$H/$^{14}$C]$N_2$ph-lactic acid and $N^\alpha$-acetylimidazolyl-[$^3$H/$^{14}$C]$N_2$ph-histidylglycine were eluted with a 2% ammonia solution. After lyophilization, the sample was dissolved in 1.0 mL of 6 M HCl and hydrolyzed 18 h at 110 °C. L-β-Imidazolyl-$N_2$ph-lactic acid was separated from imidazolyl-$N_2$ph-histidine by paper electrophoresis at pH 2.1 (3500 V, 45 min). The derivatives were eluted from the paper with 10% acetic acid.

*Determination of the Reactivity of the α-Amino Group of Histidylglycine.* (*A*) *Dinitrophenylation.* To the other half of each $^3$H-trace-labeled sample were added 0.5 g of $NaHCO_3$ and 2.5 g of urea and the samples dinitrophenylated by using the procedure described above without prior acetylation.

(*B*) *$^{14}$C Labeling.* A portion of the stock solution containing 0.9 mmol each of histidylglycine, L-β-imidazolyllactic acid, and alanylalanine was lyophilized and then redissolved in 2.0 mL of 8 M urea containing 2.5 g of $NaHCO_3$. The sample was dinitrophenylated by using 2.98 μmol of [$^{14}$C]$N_2$ph-F (specific radioactivity 21 mCi/mmol). The final volume was adjusted to 20.0 mL with 8 M urea solution. An aliquot (1.0 mL) of this solution was added to each of the above $^3$H-trace-labeled samples. In this case, 0.5 mg of di-$N_2$ph-histidine, 0.5 mg of L-β-imidazolyl-$N_2$ph-lactic acid, and 0.5 mg of $N^\alpha$-$N_2$ph-alanylalanine were added as carrier, and the pH was adjusted to 2.

(*C*) *Isolation and Purification of $^3$H/$^{14}$C-Labeled Derivatives.* Each sample was extracted with three 5-mL aliquots of diethyl ether. The aqueous phase was passed over a Dowex column and hydrolyzed as described for the first half of the solution. L-β-Imidazolyl-[$^3$H/$^{14}$C]$N_2$ph-lactic acid was separated from di-[$^3$H/$^{14}$C]$N_2$ph-histidine by paper electrophoresis at pH 2.1 (3500 V, 45 min). $N^\alpha$-[$^3$H/$^{14}$C]$N_2$ph-histidine was prepared from di-[$^3$H/$^{14}$C]$N_2$ph-histidine by thiolysis at 22 °C for 8 h in 2% *N*-methylmorpholine and 20% acetone at pH 8.0 using 30 μL of β-mercaptoethanol. After reaction, the solution was brought to pH 2 by using concentrated HCl, the acetone was evaporated, and the aqueous phase was extracted several times with diethyl ether to remove *S*-$N_2$ph-β-mercaptoethanol. The $N^\alpha$-[$^3$H/$^{14}$C]$N_2$ph histidine was purified by two-dimensional thin-layer chromatography (first dimension; toluene/pyridine/2-chloroethanol/0.8 M ammonia, 10/3/6/6; second dimension, benzene/pyridine/glacial acetic acid, 80/20/2) followed by paper electrophoresis at pH 2.1 (3500 V, 40 min).

(*D*) *Histidylglycine and Alanylalanine Titrations and pH Measurements.* A 10-mL solution containing 20 μmol of either histidylglycine or alanylalanine in 0.1 M KCl was titrated at 37 °C under nitrogen with 0.2 M NaOH. The titrant was addded from an Agla syringe apparatus. Blank corrections were made by using a 10-mL solution of 0.1 M KCl titrated under the same conditions. A Radiometer 26 pH meter fitted with a type GK 232/C glass electrode was used for pH measurements.

(*E*) *Liquid Scintillation Counting.* All samples were dissolved in 100 μL of 0.01 M HCl and added to 10 mL of Aquasol-2. Scintillation counting was performed on a programmable LKB 1215 Rack Beta scintillation counter equipped with automatic quench correction and a disintegrations per minute converter.

## Results and Discussion

*Histidylglycine.* As expected, the $pK_a$ values for the α-amino group of alanylalanine determined by conventional acid–base titration and competitive labeling are the same within the limits of experimental error (Table I). Titration data for histidylglycine give two ionizations which are separated by approximately 2 p*K* units (Table I). However, the reactivity of this N-terminal histidine residue as determined by the isolation of di-$N_2$ph-histidine after competitive labeling gives an excellent fit to a single titration with a $pK_a$ value of 7.44 ± 0.02 (Figure 2A), corresponding to the higher $pK_a$ value obtained by acid–base titration. There is no indication of a second $pK_a$ value corresponding to the lower value obtained by acid–base titration. When the reactivities of these two groups are separated (Figure 2B,C), the imidzole group has approximately the same apparent $pK_a$ value as the α-amino group and not the low value expected from acid–base titration. There is, however, a large difference in the reactivities of these two groups (Table I): the α-amino group is an order of magnitude more reactive than the imidazole. The average reactivity of the two groups relative to L-β-imidazolyllactic acid from the data in Figure 2A is 5.98 ± 0.08, which is in excellent agreement with an average value of 5.90 calculated from the data for the individual groups in Table I. When alanylalanine is used as the internal standard, isolation of di-$N_2$ph-histidine gives a single ionization with a $pK_a$ value of 7.49 ± 0.04. The reactivity obtained is 0.235 ± 0.005 relative to alanylalanine which also is in excellent agreement with the average value of 0.242 calculated from the data in Table I.

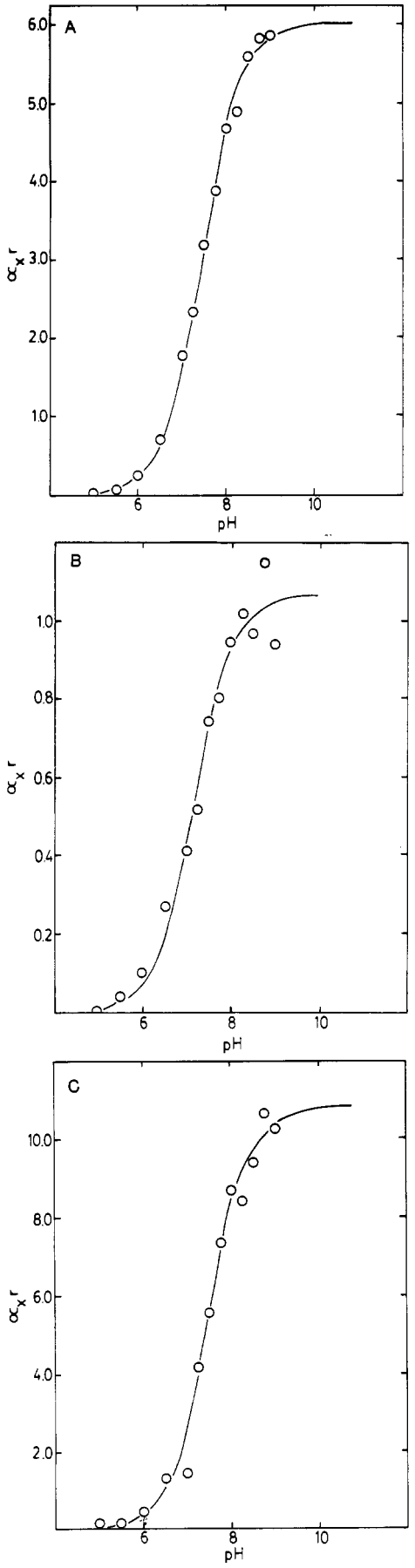


FIGURE 2: Reactivity–pH profiles of the N-terminal histidine residue of histidylglycine. The solid lines are theoretical titration curves with the following $pK_a$ and $r$ values relative to L-$\beta$-imidazolyllactic acid: (A) di-$N_2$ph-histidine, $pK_a$ = 7.44 and $r$ = 5.98; (B) imidazolyl-$N_2$ph-histidine, $pK_a$ = 7.16 and $r$ = 1.06; (C) $N^{\alpha}$-$N_2$ph-histidine, $pK_a$ = 7.49 and $r$ = 10.75.

An imidazole group of a histidine in the interior of a polypeptide chain has a $pK_a$ value of approximately 6.5 (Cohn & Edsall, 1943). When the imidazole function is on an N-terminal histidine, acid–base titration gives a $pK_a$ value that is lowered by approximately 1 $pK$ unit (Table I; Greenstein, 1933; Rothgeb et al., 1978). This observation can be explained by the inductive effect of the protonated $\alpha$-amino group on the imidazole moiety. In previous studies (Chruickshank & Kaplan, 1975), the chemical reactivity for the imidazole groups yielded $pK_a$ values which were close to those expected from titration data. In the present study, however, the $pK_a$ value obtained by competitive labeling is substantially higher than that obtained by titration. It appears from the data in Figure 2 that the apparent ionization of the imidazole moiety obtained by reactivity is close to that of the $\alpha$-amino group.

The reactivity–pH profile of a nucleophile should parallel that of a titration curve as the reactivity of the protonated nucleophile is negligible in comparison to that of the deprotonated nucleophile. In this regard, the imidazole moiety of histidylglycine appears to be anomalous since the reactivity–pH profile seems indicative of an ionization with a much higher $pK_a$ value than that observed by titration. It can only be concluded that there is another factor influencing the reactivity of this group to such an extent that it completely masks the effects of the protonation–deprotonation reaction. An obvious clue as to what this factor may be is the observation that the reactivity of the imidazole moiety very closely parallels that of the $\alpha$-amino group (Figure 2B,C). Since the protonated $\alpha$-amino group has such a large inductive effect on the imidazole group, it follows that the nucleophilicity of the imidazole moiety, and hence its chemical reactivity, will be substantially reduced. This explains the lack of reactivity observed in the pH range where the imidazole group is ionizing. It also follows that deprotonation of the $\alpha$-amino group will remove this inductive effect and the reactivity of the imidazole moiety should increase and reflect the degree of ionization of the $\alpha$-amino group. When the $\alpha$-amino group is completely deprotonated, it is expected that, as observed in Figure 2B, the reactivity of the imidazole moiety of histidylglycine is virtually identical with that of L-$\beta$-imidazolyllactic acid.

The reactivity data obtained in this study demonstrate that if a protein or polypeptide has an N-terminal histidine, the imidazole moiety will have chemical properties which are dependent on the state of ionization of the $\alpha$-amino group. There are several naturally occurring polypeptides such as glucagon and VIP which have an N-terminal histidine which is essential for their biological activity. It is therefore of interest to determine the chemical properties of these groups in order to determine whether or not the special properties of an N-terminal histidine are related to their biological activities and mechanism(s) of action.

*Glucagon and VIP.* In the case of histidylglycine, the reactivity of the imidazole moiety could be separated from that of the $\alpha$-amino group by acetylating the $\alpha$-amino group in one half of the trace-labeled sample, isolating the $N^{\alpha}$-acetyl-imidazolyl-$N_2$ph-histidine after full derivatization as described under Experimental Procedures, and quantifying its $^3H/^{14}C$ ratio. The reactivity of the $\alpha$-amino group was determined by fully derivatizing the other half of the sample with $N_2$ph-F (to form the di-$N_2$ph-histidine derivative), removing the $N_2$ph group from the imidazole moiety by thiolysis, isolating the $\alpha$-$N_2$ph-histidine, and quantifying its $^3H/^{14}C$ ratio. In principle, the same strategy could be applied to the N-terminal histidine of glucagon and VIP. There are, however, two considerations which make the same approach impractical.

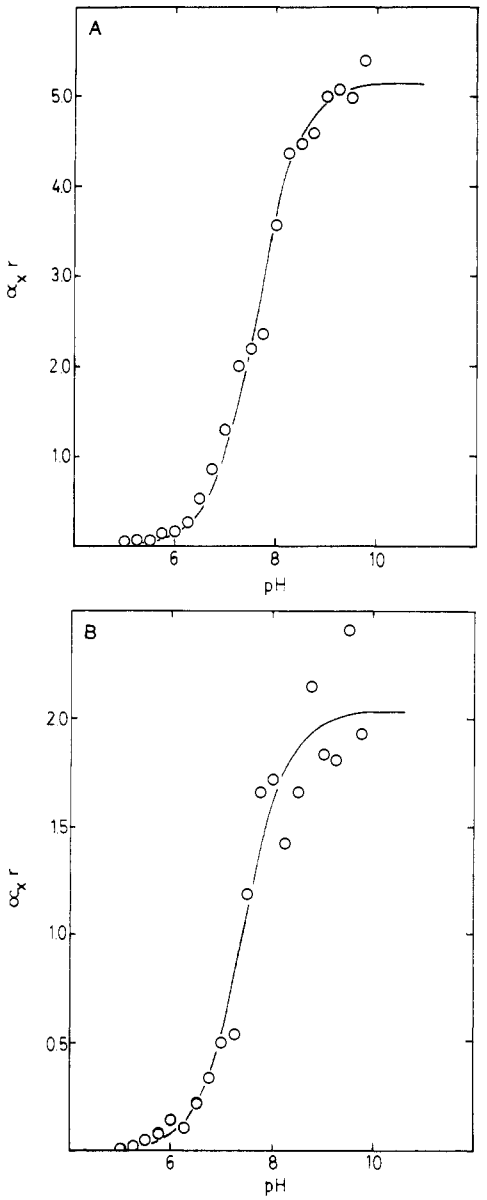


FIGURE 3: Reactivity–pH profiles of the N-terminal histidine residue of glucagon. The solid lines are theoretical titration curves with the following $pK_a$ and $r$ values relative to L-$\beta$-imidazolyllactic acid: (A) $\alpha$-amino group, $pK_a$ = 7.60 and $r$ = 5.13; (B) imidazole group, $pK_a$ = 7.43 and $r$ = 2.02.

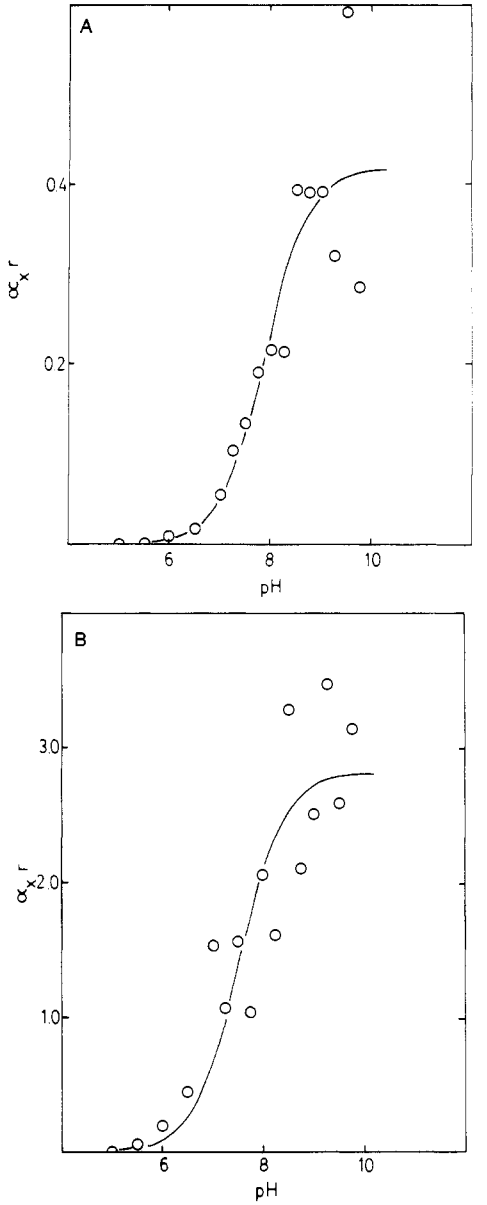


FIGURE 4: Reactivity–pH profiles of the N-terminal histidine residue of VIP. The solid lines are theoretical titration curves with the following $pK_a$ and $r$ values: (A) $\alpha$-amino group, $pK_a$ = 7.88 and $r$ = 0.42 (relative to alanylalanine); (B) imidazole group, $pK_a$ = 7.52 and $r$ = 2.81 (relative to L-$\beta$-imidazolyllactic acid).

First, in order to assure sufficient $^{14}C$ counts for accurate quantification of the $^3H/^{14}C$ ratio, relatively large amounts of material would be required, making the experimentation costly. Second, the problem of assuring sufficient amounts of the di-$N_2$ph-histidine derivative for thiolysis and subsequent isolation of the mono-$N^{\alpha}$-$N_2$ph-histidine derivative would add significantly to the amounts of material required. An alternate strategy, which requires only that very small amounts of polypeptide be used in the trace-labeling step, was employed (Figure 1). In this prodecure, the $N^{\alpha}$-[$^{14}C$]$N_2$ph-histidine and the imidazolyl-[$^{14}C$]$N_2$ph-histidine derivatives are added to the reaction mixture directly after the trace labeling and prior to acid hydrolysis so that no further derivatization is necessary. Quantification of the $^3H/^{14}C$ ratio for each derivative permits an unequivocal assignment of parameters to the $\alpha$-amino and imidazole groups. A further technical advantage is that acid hydrolysis can be carried out directly on the reaction mixture, thus eliminating several operations required in the procedure described by Duggleby & Kaplan (1975). Much more efficient use is made of the $^{14}C$-labeled reagent which gives an additional cost savings to the method. The chemical properties of lysine, tyrosine, and cysteine residues, if they are present, can also be determined by this procedure by adding the appropriate [$^{14}C$]$N_2$ph derivative to the reaction mixture. In the present study, $N^{\epsilon}$-[$^{14}C$]$N_2$ph-lysine was added so that the reactivity of the solitary lysine at position 12 in glucagon could be quantified and the average reactivity of the four lysines in VIP obtained.

Figure 3A,B shows the fit to theoretical titration curves of the reactivity data obtained for the $\alpha$-amino group and the imidazole moiety of the histidine in glucagon. It is clear that the reactivities of these functional groups in glucagon resemble those of the corresponding groups in histidylglycine in that the reactivity of the imidazole function parallels that of the $\alpha$-amino group. The results with VIP (Figure 4A,B) are similar to those of glucagon and histidylglycine.

The difference in the apparent $pK_a$ value of 7.43 obtained in this study for the imidazole moiety of glucagon and that of 5.23 obtained by Rothgeb et al. (1978) for *S*-methyl-

glucagon can be explained by the same inductive effect observed in histidylglycine. The approach of Rothgeb et al. (1978) reflects changes in the state of ionization of this group whereas the present approach reflects changes in the chemical properties. For most groups, these changes occur simultaneously, but as we have shown, an incomplete picture of the chemical properties of the imidazole group of an N-terminal histidine residue is obtained if one ignores subsequent chemical changes due to the ionization of the $\alpha$-amino group. This may be especially relevant if, as most evidence indicates, the histidine residue is essential for biological activity [see Epand et al. (1981) and references cited therein]. However, Sueiras-Diaz et al., (1984) have provided evidence to the contrary.

The present results show that the similarity of the reactivity–pH profiles of the $\alpha$-amino group and imidazole function in glucagon and VIP is a special property of an N-terminal histidine residue. In the case of histidylglycine, the reactivity of the imidazole function is not detectable when the $\alpha$-amino group is protonated. However, when the $\alpha$-amino is deprotonated, the imidazole group's reactivity is the same as that in L-$\beta$-imidazolyllactic acid. In the cases of glucagon and VIP, the reactivity of the imidazole is also undetectable when the $\alpha$-amino is protonated, but when the $\alpha$-amino is deprotonated, the imidazole groups have a significantly higher than expected reactivity: approximately 2 times higher in the case of glucagon and 3 times higher in VIP. The $\alpha$-amino groups in histidylglycine and VIP have the expected reactivity based on a Bronsted relationship (Kaplan et al. 1973; Chan et al., 1981) whereas the $\alpha$-amino group in glucagon has approximately half the expected reactivity.

The most obvious explanation for the enhanced reactivity of the imidazole moiety in glucagon and VIP is that this reactivity is reflecting some organized structure in the N-terminal region of the molecule. The evidence from circular dichroism studies for glucagon in a dilute situation shows that there is no substantial amount of repeating structure [see Blundell & Wood (1982) and references cited therein]. On the other hand, there is evidence from NMR studies (Boesch et al., 1978; Wagman et al., 1980; Jardelzky & Roberts, 1981) that stable localized structures exist in glucagon in solution. Such a local structure could be the presence of a hydrogen bond involving the deprotonated imidazole group. This would account for the increased reactivity observed in the present study. An increased reactivity due to hydrogen bonding has been observed previously in the case of the active-center histidine of chymotrypsin (Cruickshank & Kaplan, 1975). We cannot, at present, rule out the possibility that a localized hydrophobic environment exists in the vicinity of the imidazole group which preferentially attracts $N_2$ph-F. This also would lead to an increased reactivity. Nevertheless, each of these explanations requires that the N-terminal portion of the molecule be part of a structured region. The solitary $\epsilon$-amino group of glucagon has an unusually low p$K_a$ value of approximately 8.5 and one-sixth the reactivity expected on the basis of a Bronsted relationship (Chan et al., 1981). Again, this is indicative of a localized structure.

Korn & Ottensmeyer (1983) have reviewed and summarized the evidence in the literature regarding the structure of glucagon in solution and have come to the conclusion that a compact, well-defined structure exists in dilute solution. They have proposed a model based, in part, on the available experimental data and, in part, on theoretical considerations of intramolecular interactions. One of their major conclusions is that the imidazole moiety of the N-terminal histidine is hydrogen bonded to glutamine-20 and is the terminal residue in a hydrogen bonding network involving five residues. Such a structure would give rise to the enhanced reactivity we observe. They have also suggested that this hydrogen bonding network may provide the basis of an allosteric mechanism by which adenylate cyclase is activated. Unfortunately, they have not considered the fact that the chemical properties, and hence the hydrogen bonding properties, of the imidazole group will depend on the ionization state of the $\alpha$-amino group. Since the $\alpha$-amino group has a p$K_a$ value of 7.6, less than half of the imidazole groups, on average, could take part in hydrogen bonding at normal, physiological pH values. It is possible that on binding to the receptor the p$K_a$ value of the $\alpha$-amino group is lowered by this new microenvironment with a concomitant increased nucleophilicity, and hence hydrogen bonding capacity, for the imidazole group. In such an event, our results would lend support to their hypothesis that this structural feature is important for the activity of glucagon.

Korn & Ottensmeyer (1983) also predict that the aliphatic chain of lysine-12 is involved in a hydrophobic interaction with leucine-14. If this is the case, one would predict a lowered p$K_a$ value due to the hydrophobic environment and a lowered reactivity due to steric hindrance. This is what we have observed, and while such an exact agreement may be fortuitous, it does give additional support to the view that glucagon has a well-defined and ordered structure in dilute solution.

In summary, we have shown that the N-terminal histidine of a polypeptide has special properties in which the nucleophilicity of the imidazole group is dependent on the state of ionization of the $\alpha$-amino group. Evidence has also been provided in the cases of glucagon and VIP that an ordered structure exists in the vicinity of the N-terminal residue in which the deprotonated imidazole function is possibly involved in hydrogen bonding. A localized structure in the vicinity of the solitary lysine of glucagon is also indicated by the data.

**Registry No.** VIP, 37221-79-7; histidylglycine, 2578-58-7; glucagon, 9007-92-5; histidine, 71-00-1.

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# Comparison of the Transient Folding Intermediates in Lysozyme and α-Lactalbumin†

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ABSTRACT: Refolding kinetics of two homologous proteins, lysozyme and α-lactalbumin, were studied by following the time-dependent changes in the circular dichroism spectra in the aromatic and the peptide regions. The refolding was initiated by 20-fold dilution of the protein solutions originally unfolded at 6 M guanidine hydrochloride, at pH 1.5 for lysozyme and pH 7.0 for α-lactalbumin at 4.5 °C. In the aromatic region, almost full changes in ellipticity that were expected from the equilibrium differences in the spectra between the native and unfolded proteins were observed kinetically. The major fast phase of lysozyme folding has a decay time of 15 s. The decay time of α-lactalbumin depends on the presence or absence of bound $Ca^{2+}$: 10 s for the holoprotein and 100 s for the apoprotein. In the peptide region, however, most of the ellipticity changes of the two proteins occur within the dead time (<3 s) of the present measurements. This demonstrates existence of an early folding intermediate which is still unfolded when measured by the aromatic bands but has folded secondary structure as measured by the peptide bands. Extrapolation of the ellipticity changes to zero time at various wavelengths gives a spectrum of the folding intermediate. Curve fitting of the peptide spectra to estimate the secondary structure fractions has shown that the two proteins assume a similar structure at an early stage of folding and that the intermediate has a structure similar to that of partially unfolded species produced by heat and, for α-lactalbumin, also by acid and a moderate concentration of guanidine hydrochloride. Thus, in spite of their known difference in equilibrium unfolding behavior and the difference of their biological functions, folding of the two proteins occurs via a similar structural intermediate. The thermal transition of the secondary structure in the equilibrium intermediate of α-lactalbumin was also investigated. The relevance of the present results to the equilibrium unfolding behavior of the proteins is described.

Lysozyme and α-lactalbumin are homologous proteins which have similar amino acid sequences (Brew et al., 1970; Imoto et al., 1972; Shewale et al., 1984; Qasba & Safaya, 1984), but their apparent physicochemical properties are markedly different. The unfolding equilibrium of lysozyme in the transition region when treated with guanidine hydrochloride (Gdn·HCl)[1] is expressed as a highly cooperative two-state reaction (Tanford et al., 1966; Aune & Tanford, 1969; Tanford, 1968, 1970). The unfolding of α-lactalbumin is less coopertive and shows a stable intermediate state at a moderate concentration of Gdn·HCl, and a similar conformational state exists also at acid pH (Kuwajima et al., 1976, 1981; Nozaka et al., 1978); in the following, each of these states is termed the A state. There are many comparative studies of the two proteins which have suggested similarity in their tertiary structures (Browne et al., 1969; Warme et al., 1974; Takase et al., 1978; Hill & Brew, 1975, and other references cited therein), but some authors have also reported nontrivial differences especially in their dynamic properties and have suggested a more flexible structure of α-lactalbumin than of lysozyme (Lin, 1970; Barman & Bagshaw, 1972; Iyer & Klee, 1973; Takesada et al., 1973; Pfeil, 1981a). The recent discovery that α-lactalbumin is a $Ca^{2+}$-binding protein and that it requires $Ca^{2+}$ for structural stabilization in spite of low affinity of lysozyme to $Ca^{2+}$ gave a new light for understanding the differences between the two proteins (Hiraoka et al., 1980; Permyakov et al., 1981; Murakami et al., 1982; Segawa & Sugai, 1983; Kronman & Bratcher, 1983) [see also Imoto et al. (1981) for lysozyme's affinity to $Ca^{2+}$].

Nevertheless, it has been postulated that not only the native structure but also the folding pathway of a protein are determined by its unique amino acid sequence (Anfinsen & Scheraga, 1975; Kim & Baldwin, 1982; Ghélis & Yon, 1982). It is also suggested that the pathways of folding of homologous proteins have been conserved during evolutional divergence (Ptitsyn & Finkelstein, 1980; Rossmann & Argos, 1981; Hollecker & Creighton, 1983; Krebs et al., 1983). Otherwise, a very large number of different mechanisms of folding must

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[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; CD, circular dichroism; UV, ultraviolet; RMS, root mean square.

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