

Reactions of Hydroxyamino Acids during Hydrochloric Acid Hydrolysis

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Chlorosubstitution reactions occur readily during HCl hydrolysis of δ - and ϵ -hydroxynorleucines (Hnle), the products of deamination of poly-L-lysine by nitrite at low pH. During amino acid analysis, chloronorleucines elute as new peaks after δ - and ϵ -Hnle. To determine if other hydroxyamino acids undergo similar changes during hydrolysis, they were subjected individually to HCl hydrolysis conditions with and without added phenol. Amino acid analyses indicated that terminal hydroxy groups on linear side chains undergo reactions during HCl hydrolysis; the products appear as new peaks which may be chloroderivatives. In contrast, no new peaks are observed in HCl hydrolysates of δ -hydroxylysine or amino acids with β -hydroxy groups (β -hydroxynorvaline, serine, and threonine). Phenol did not protect linear amino acids from reactions during HCl hydrolysis but did prevent loss of the cyclic amino acids tyrosine, hydroxyproline, and 3,4-dihydroxyphenylalanine. Although the γ -hydroxy group of homoserine would be expected to undergo reaction, HCl catalyzes its cyclization to form homoserine lactone instead.

In recent studies of the nitrous acid deamination of poly-L-lysine (1) and proteins (2) two unresolved peaks were observed during amino acid analysis of HCl hydrolysates in addition to peaks of δ - and ϵ -hydroxynorleucines (Hnle),² the deamination products of Lys. These peaks did not appear during amino acid analysis of mercaptoethanesulfonic acid (Mes) hydrolysates. After isolation by preparative TLC, the amino acids were identified by GC-MS as secondary and primary chlorinated

derivatives of δ - and ϵ -Hnle (chloronorleucines, Cnle) (2).

Inaccuracies are likely to occur in amino acid analysis of any protein which has been exposed to nitrous acid because Lys residues react readily to form δ - and ϵ -Hnle; moreover, approximately 40% of the Hnle content of a deaminated protein can be converted to Cnle during HCl hydrolysis (2). Since chlorosubstitution of Hnle occurs so readily, it was of interest to determine if other hydroxyamino acids become converted to chlorinated or other derivatives during HCl hydrolysis and if such products can lead to additional interference with accurate quantitation of the amino acid content of proteins. The investigation also tested whether the protective effect of phenol in preventing destruction of tyrosine (3) extended to other hydroxyamino acids.

MATERIALS AND METHODS

L-3,4-Dihydroxyphenylalanine (L-Dopa), L-hydroxylysine (HyL), L-Hydroxyproline (Hyp), L-Ser, L-Thr, L-Tyr, poly-L-ornithine, and poly-L-lysine were Sigma³ products. Homoserine (Hse) was purchased from Calbiochem and β -hydroxynorvaline (β -Hnva) from Aldrich. Deaminated amino acids, δ -hydroxynorvaline (δ -Hnva) and δ - and ϵ -hydroxynorleucines, were prepared from polyornithine and polylysine, respectively, by treatment with NaNO₂ in 0.1 M acetic acid, pH 3.0, followed by HCl hydrolysis to monomers, as described previously (1,2). After hydrolysis, δ - and ϵ -Hnle were isolated from the product monomers as pure components by ion-exchange chromatography with volatile buffers (4). Authentic DL- ϵ -Hnle was also synthesized (5); its physical and chemical properties were indistinguishable from those of L- ϵ -Hnle.

Hydroxyamino acids were subjected to typical hydrolysis conditions with redistilled constant-boiling HCl in

TABLE 1

Loss/Conversion of Hydroxyamino Acids during Hydrolysis with Hydrochloric Acid

Amino acid ^a	Phenol ^b	Elution time (min)	Derivative ^a	Elution time (min)	% Loss/conversion
Thr	+	17	— ^c	— ^c	15.6
Ser	+	18	— ^c	— ^c	22.2
Hse	+	21	Hsl	68	66.0
β -Hnva	+	22	— ^c	— ^c	6.8
δ -Hnva	+	21	NC ^d	46	47.8
δ -Hnle	+	21	Cnle ^e	49	14.1
ϵ -Hnle	+	27	Cnle ^e	50	34.6
Hyp	—	15	NC ^d	30	+4.9 ^f
Dopa	—	49	NC ^d	61	68.1
Tyr	—	53	— ^c	— ^c	66.5
Hyl	—	65	— ^c	— ^c	8.8

^a Cnle, chloronorleucine; Dopa, 3,4-dihydroxyphenylalanine; δ -Hnle, δ -hydroxynorleucine; ϵ -Hnle, ϵ -hydroxynorleucine; Hse, homoserine; Hsl, homoserine lactone; β -Hnva, β -hydroxynorvaline; δ -Hnva, δ -hydroxynorvaline.

^b +, 0.05% phenol added to HCl before hydrolysis; —, no phenol added.

^c Not observed.

^d Not characterized.

^e Cnles were isolated as an unresolvable mixture and identified by GC-MS as secondary and primary Cnles (2). The 49-min peak is produced only from δ -Hnle and the 50-min peak only from ϵ -Hnle.

^f Increase in area due to coelution of hydrolysis product(s) with Hyp.

sealed, evacuated tubes at 110°C for 24 h. To confirm that additional peaks arose only via reaction with HCl, δ - and ϵ -Hnle and δ -Hnva were also hydrolyzed with Mes (Pierce). All amino acid analyses were performed on a Beckman 119CL single column analyzer. Two recorder pens monitored absorbance at 440 nm (λ_{\max} for ninhydrin adducts of Pro and Hyp) and the sum of absorbances at 440 and at 570 nm (λ_{\max} for ninhydrin adducts of all other amino acids); the summed absorbance values also appeared in the output of a Hewlett-Packard 3390A integrator attached to the analyzer. An internal standard, norleucine (Nle) or Arg, was added to each sample. Phenol (0.05%) was added to some HCl hydrolyses. Calculations of losses during hydrolysis were based on ninhydrin color constants determined before and after each analysis for the standard amino acids in Bec-Mix (Beckman) and for δ - and ϵ -Hnle (2) or on differences in integrated peak areas of hydroxyamino acids before and after hydrolysis.

RESULTS

Table 1 shows the extent of losses observed for individual hydroxyamino acids during HCl hydrolysis. Losses of Thr and Ser in the presence of phenol were in the range usually observed in protein hydrolysates, and no new peaks appeared during the 90-min analyzer runs. Loss of β -Hnva during HCl hydrolysis was minimal. In

contrast, an additional peak was observed in the amino acid analysis of an HCl hydrolysate of δ -Hnva (phenol present) (Fig. 1) but not in an Mes hydrolysate. HCl hydrolysates of pure δ - and ϵ -Hnle (phenol present) produced extra peaks at 49 and 50 min, respectively, of secondary and primary Cnle (2). These peaks were missing from Mes hydrolysates, as noted. Losses during HCl treatment of δ -Hyl were minimal in both the presence and the absence of phenol.

Phenol prevented loss of both Tyr and Dopa during HCl hydrolysis. When phenol was absent significant losses occurred in both the Tyr and the Dopa peaks. No additional peaks were observed from Tyr, but a new peak

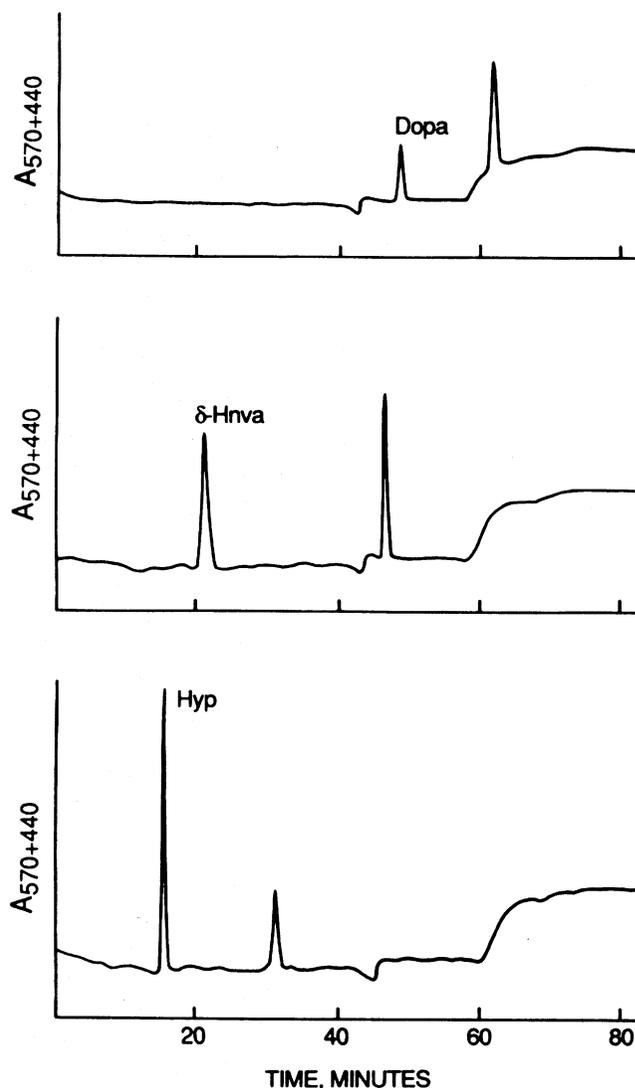


FIG. 1. Appearance of new amino acid analysis peaks in HCl hydrolysates of Dopa (top), δ -Hnva (center), and Hyp (bottom). Phenol was added to the hydrolysis sample of δ -Hnva. Dopa and Hyp were hydrolyzed in the absence of phenol; additional peaks were not observed when 0.05% phenol was present. Peaks of internal standards (Nle or Arg) are not shown.

appeared during amino acid analysis of Dopa hydrolysates (Fig. 1). In the case of Hse, the substantial loss of the 21-min peak after HCl hydrolysis can be accounted for by the formation of homoserine lactone (Hsl), as expected. Although an additional condensation product, identified by GC-MS as a diketopiperazine (Malin and Piotrowski, unpublished), can be formed from Hse in dilute HCl (0.05 N) at 100°C, it was not observed after HCl hydrolysis.

Hyp results were anomalous. In the presence of phenol losses were minimal, but when phenol was absent the peak area of Hyp increased and a new peak was present (Fig. 1). The increase probably resulted from coelution of an acyclic Hyp hydrolysis product with the parent amino acid, an interpretation based on comparing the heights of the 440-nm and 570 + 440-nm peaks. In samples hydrolyzed without phenol, the (570 + 440)/440 ratio increased by 4.2%, suggesting that the coeluted product contained an α -amino group rather than the imino group of Hyp.

DISCUSSION

Although losses of Ser and Thr during hydrolysis with hydrochloric acid make quantitation of these amino acids in proteins more difficult, products arising during their hydrolysis do not interfere with quantitation of other amino acids. The protective effect of phenol in preventing loss of Tyr (3), Dopa, and Hyp was clearly demonstrated, but this effect did not extend to the straight-chain hydroxyamino acids. As noted, the increase in the ratio of absorbance traces on the chromatogram of Hyp hydrolyzed without phenol strongly suggests that phenol prevents formation of an acyclic amino acid product coeluting with Hyp. No product other than Hse and Hsl was seen during amino acid analysis of Hse hydrolyzed with HCl. If a diketopiperazine forms initially during hydrolysis, it is ultimately converted to the lactone.

Of the hydroxyamino acids which produced additional peaks after HCl hydrolysis, only the Hyp and Hse products had elution times which could interfere with amino acid analysis of the standard protein amino acids. The Hyp product formed during HCl hydrolysis in the absence of phenol elutes at the same time as Gly. This means that addition of 0.05% phenol is essential for hydrolysis of any protein which may contain Hyp, regardless of the Tyr content. Hsl elutes about 0.5 min before Lys. Since Hsl would normally be present only in the hydrolysates of large peptides generated by cyanogen bromide cleavage of proteins at Met residues, its presence would be expected and appropriate corrections made.

Mechanisms have not been established for the reactions observed in this work which lead either to destruc-

tion or to formation of products which elute during routine amino acid analysis. In chlorination of Hnle S_N2 substitution is expected for a primary hydroxy group, whereas for secondary hydroxy groups elimination-addition reactions should predominate. The same concepts would be applicable if the additional peak observed after HCl hydrolysis of δ -Hnva is due to formation of a chloro-norvaline.

In amino acids with β -hydroxy substituents, the proximity of the highly charged α -carbon presumably inhibits substitution reactions at the β -carbon. Destructive reactions obviously occur in Ser and Thr but not in β -Hnva, perhaps indicating a stabilizing effect of the longer carbon chain. A similar situation appears to be present in the case of Hyl; thus the ϵ -amino group can also inhibit reactions at the adjacent δ -carbon. The only amino acid with a γ -hydroxy group, Hse, is driven to lactone formation although substitution or formation of other products should be possible.

Structural studies of membrane proteins have acquired an added dimension with the discovery that many are linked at their carboxyl termini to phosphatidylinositol (PI) of the lipid bilayer through amino sugar units (6) and can be released from the membrane by PI-specific phospholipase C. The common structural features of the units linking proteins to PI include several ethanolamine connections which can be cleaved by nitrous acid deamination at low pH to yield free protein (6-8). Since formation of Hnle occurs readily in proteins deaminated under these conditions, subsequent HCl hydrolysis could result in the appearance of both Hnles and Cnles in the amino acid analysis profile.

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