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Conversion of prohistidine decarboxylase to histidine decarboxylase: Peptide chain cleavage by nonhydrolytic serinolysis

(acyl transfer/α,β-elimination/pyruvoyl proteins/^{18}O-labeled proteins/[^{18}O]serine)

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ABSTRACT **Unlabeled prohistidine decarboxylase and prohistidine decarboxylase containing L-[*carboxyl*-^{18}O]serine or L-[*hydroxyl*-^{18}O]serine were isolated in homogeneous form from mutant 3 of *Lactobacillus* 30a grown with the appropriately labeled serine. There was no randomization or redistribution of label during growth, isolation of the protein, or enzymatic hydrolysis and reisolation of the labeled amino acids. These proteins were used to show that during proenzyme activation, in which individual π subunits of the proenzyme are converted to α and β subunits of the active enzyme**

$$\underset{\pi\text{ subunit}}{\overset{1}{\text{Ser-}}\ .\ .\ .\overset{81}{\text{-Ser-}}\overset{82}{\text{Ser-}}.\ .\ .\text{-Tyr}} \xrightarrow[NH_3]{H_2O} \underset{\beta\text{ subunit}}{\overset{1}{\text{Ser-}}.\ .\ .\overset{81}{\text{-Ser-O}^-}} + \underset{\alpha\text{ subunit}}{\overset{82}{\text{Prv-}}.\ .\ .\text{-Tyr}}$$

(in which π, α, and β subunits have the partial structures shown and Prv designates a pyruvoyl group), no ^{18}O from $H_2{}^{18}O$ is incorporated into the newly formed carboxyl terminus (Ser-81) of the β chain, although no labilization of ^{18}O from proenzyme labeled with L-[*carboxyl*-^{18}O]serine occurred when the proenzyme was activated in $H_2{}^{16}O$ by the same procedures. The additional oxygen atom present in the carboxyl group of Ser-81 of the β subunit is transferred from the hydroxyl group of Ser-82 of the proenzyme during the activation reaction. The same result was obtained with wild-type enzyme formed intracellularly. Peptide bond cleavage during activation of the proenzyme thus proceeds by a hitherto unobserved direct or indirect "serinolysis" coupled to α,β-elimination at Ser-82 to yield the pyruvoyl group of the α subunit, rather than by hydrolysis. Possible mechanisms for the reaction are discussed briefly.

L-Histidine decarboxylase (histidine carboxy-lyase, EC 4.1.1.22) of *Lactobacillus* 30a is the best-characterized representative of a small group of enzymes whose catalytic action is dependent upon an amide-bound pyruvoyl group at the active site (1). This enzyme arises from a pyruvate-free proenzyme (2) by the overall process shown in Eq. 1.

$$\underset{\text{proenzyme}}{(\pi)_6} + 6H_2O \rightarrow \underset{\text{active enzyme}}{(\alpha\beta)_6} + 6NH_3 \qquad [1]$$

Each π subunit of $M_r \approx 37{,}000$ is converted to an α subunit ($M_r \approx 28{,}000$) and a β subunit ($M_r \approx 9{,}000$) by a posttranslational process depicted in Fig. 1, in which cleavage of the amide bond between serines 81 and 82 of the proenzyme π chain occurs and serine 82 is converted to a pyruvoyl residue (4, 5). The nature of this activation reaction is not understood. It occurs upon incubation of the homogeneous proenzyme around pH 7 in the presence of monovalent cations and is apparently intramolecular because (*i*) the reaction is first order with respect to both proenzyme and total protein; (*ii*) proenzyme from single crystals and proenzyme immobilized on a Sepharose support both activate normally; and (*iii*) no complementation is observed by combining wild-type and mutant cell extracts (6). Activation of the wild-type proenzyme occurs about three times faster than that of the mutant 3 proenzyme, and the derived enzymes differ in some structural, chemical, and catalytic properties (7). Activation requires tertiary structure of the proenzyme because urea-denatured preparations do not activate but do so after renaturation by removal of the urea (6).

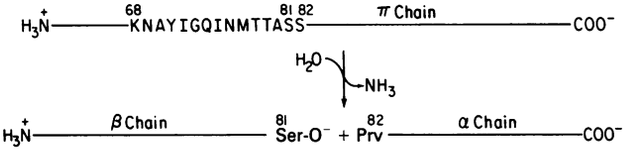

FIG. 1. Conversion of the π chain of prohistidine decarboxylase to the α and β chains of the active enzyme. Prv designates the pyruvoyl group at the NH_2 terminus of the α chain; the standard single-letter notation, in which S is the symbol for serine, is used. The partial sequence of the π chain shown is taken from that determined for the β chain (3) and the sequence of the proenzyme fragment overlapping the β and α chains (4).

In this paper we present evidence that chain cleavage is not a simple hydrolysis but proceeds by a hitherto unobserved route in which the hydroxyl oxygen of serine-82 is incorporated into the carboxyl group of serine-81. The mechanistic implications of these findings are discussed briefly.

MATERIALS AND METHODS

Materials. $H_2{}^{18}O$ (92 atom %) was obtained from Prochem (London), β-chloro-L-alanine from Vega Biochemicals, *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (TPCK-trypsin) from Millipore, DE-52 (DEAE-cellulose) from Whatman, and Dowex-50W cation-exchange resin from Sigma. Carboxypeptidase Y, acetonitrile, and *N,O*-bis(trimethylsilyl)-trifluoroacetamide were from Pierce.

Synthesis of L-[*carboxyl*-^{18}O]Serine. Dry HCl gas was bubbled into a solution of L-serine (100 mg) in 0.2 ml of 82 atom % $H_2{}^{18}O$ as described by Murphy and Clay (8) until the pH reached 0.5. Exchange of the carboxyl oxygens was complete after incubation at 70°C for 3 days. No exchange of the hydroxyl oxygen occurred under these conditions.

Synthesis of L-[*hydroxyl*-^{18}O]Serine. β-Chloro-L-alanine hydrochloride (40 mg) was dissolved in 0.5 ml of 92 atom % $H_2{}^{18}O$

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Abbreviation: Me_3Si, trimethylsilyl. Prv, pyruvoyl residue.

and the pH was adjusted to 7 by careful addition of powdered KOH. The solution was incubated at 80°C and the pH was readjusted to 7 with KOH every 4 hr. The reaction was followed by amino acid analysis and was complete after 52 hr, with a yield of 35%. Analysis of the trimethylsilyl (Me_3Si) derivative (see below) showed that the hydroxyl group contained 70 atom % ^{18}O and that the carboxyl group contained less than 2 atom % ^{18}O.

Trimethylsilylation of Amino Acids and Enzymatic Digests. Dried samples were incubated with *N,O*-bis(trimethylsilyl)-trifluoroacetamide/acetonitrile, 2:1 (vol/vol), at 125°C for 40 min in 1-ml vials sealed with Teflon-lined caps and analyzed after standing at room temperature for 24 hr. The concentration of individual amino acids was approximately 1 μmol/ml.

Gas Chromatography/Mass Spectrometry. Trimethylsilyl derivatives were analyzed by gas chromatography/mass spectrometry with a Finnigan 9610 gas chromatograph interfaced to a Finnigan automated 4023 mass spectrometer with an INCOS data system. Samples were chromatographed on a 40-m SE-30 (J & W Scientific, Rancho Cordova, CA) fused silica capillary column. The injector and transfer lines were maintained at 250°C, and the linear velocity of helium carrier gas was 25 cm/sec. The column temperature was maintained at 100°C for 2 min after injection and then increased to 270°C at 8°C/min. The mass spectrometer was operated with an ionizing potential of 70 eV with an ion source temperature of 250°C. Mass spectra were recorded at 1-sec intervals. The elution and fragmentation patterns obtained permitted identification of all amino acids except for arginine, cysteine, histidine, lysine, and tryptophan. The ^{18}O content of the carboxyl oxygens of Me_3Si-serine was determined from the relationship:

$$\text{atom \% } ^{18}O = 100(I_{220} + 2I_{222})/2(I_{218} + I_{220} + I_{222}),$$

in which I is the ion abundance (corrected for contributions by naturally occurring isotopic peaks) at the m/z (mass-to-charge ratio) values denoted by the subscript. The ^{18}O content of the hydroxyl group of Me_3Si-serine was determined from the relationship:

$$\text{atom \% } ^{18}O = 100I_{206}/(I_{204} + I_{206}).$$

The ^{18}O content of the Me_3Si derivatives of other amino acids was determined from intensities of the molecular ion (M) and the ions at m/z M − 15, M − 117, and 218.

Purification of Histidine Decarboxylase and Prohistidine Decarboxylase. Mutant and wild-type cells of *Lactobacillus* 30a were grown to stationary phase on the defined medium of Guirard and Snell (9) modified to contain yeast extract (150 mg/liter), L-histidine (5 g/liter), and unlabeled or ^{18}O-labeled L-serine (16 mg/liter). Correction was made for the dilution of labeled serine by the serine present in yeast extract. Wild-type enzyme and proenzyme from mutant 3 were purified from acetone-dried cells as described (2, 5).

Activation of Prohistidine Decarboxylase. For activation in $H_2{}^{18}O$, unlabeled mutant proenzyme (5 mg) was dissolved in 0.1 ml of 82 atom % $H_2{}^{18}O$ containing 1 M KCl and 0.1 M potassium phosphate, pH 7.6. After 18 hr at 37°C, 1 ml of 0.2 M ammonium acetate, pH 4.8, was added and the protein was precipitated by addition of ammonium sulfate to give 80% saturation. ^{18}O-Labeled mutant proenzyme was activated in unlabeled water by incubation in 1 M potassium phosphate, pH 7.6, for 18 hr at 37°C.

Isolation of the α and β Chains of Histidine Decarboxylase. Unlabeled or labeled homogeneous active enzyme (5 mg) was denatured by incubation in 2 ml of 5 M guanidinium chloride in buffer A (0.03 M sodium phosphate, pH 7.6) for 2 hr at 45°C. The solution was then dialyzed against buffer A for 5 hr at 25°C and applied to a column of DE-52 DEAE-cellulose (3 ml) equilibrated with buffer A. The β chain was eluted first with buffer A containing 0.1 M NaCl and the α chain was then eluted with buffer A containing 0.5 M NaCl (10). Protein-containing fractions were dialyzed against distilled water and lyophilized. Purity of preparations was confirmed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (11).

Enzymatic Digestion of α and β Chains. The β chain (0.5 mg) was incubated in 0.2 ml of 0.2 M pyridine/acetic acid, pH 6.2, for 1 hr at 37°C and then digested with carboxypeptidase Y (10 μg) for 1 hr at 37°C. The solution was heated at 100°C for 4 min to stop the reaction and lyophilized. The α chain (0.5 mg) was digested with TPCK-trypsin (5 μg) in 0.2 ml of 0.2 M *N*-ethylmorpholine/acetic acid, pH 8.0, for 5 hr at 37°C. The solution was heated at 100°C for 4 min and lyophilized. The residue was dissolved in 0.2 ml of 0.2 M pyridine/acetic acid at pH 5.5, digested with carboxypeptidase Y (10 μg) for 12 hr at 37°C, and lyophilized. The lyophilized digests were then trimethylsilylated and analyzed by gas chromatography/mass spectrometry as described earlier.

Analyses for Exchange Reactions. Determination of the ^{18}O content of serine-81 required prior denaturation of active enzyme and trimethylsilylation of the carboxypeptidase digest of the isolated β chain. The following samples were therefore analyzed for ^{18}O content to test for possible interfering exchange reactions during these procedures: (*i*) L-[*carboxyl*-^{18}O]Serine (0.2 μmol) was trimethylsilylated in the presence of the β chain (1 mg). (*ii*) L-[*carboxyl*-^{18}O]Serine (1.0 μmol) was incubated in 0.1 ml of 5 M guanidinium chloride in buffer A for 2 hr at 45°C. The sample was applied to a column of Dowex 50W X-8 (1 ml) equilibrated and eluted with 0.1 M pyridine/acetic acid, pH 3.2. Fractions containing serine were pooled, lyophilized, and trimethylsilylated. (*iii*) The β chain (0.5 mg) dissolved in 0.1 ml of 0.2 M pyridine/acetic acid, pH 6.5, containing 82 atom % $H_2{}^{18}O$ was digested with carboxypeptidase Y (10 μg) for 1 hr at 37°C and the lyophilized digest was trimethylsilylated. None of these samples showed detectable exchange of the carboxyl oxygens of the serine standards or of serine-81. Other amino acids released by carboxypeptidase digestion in $H_2{}^{18}O$ contained the theoretical amount of ^{18}O in their carboxyl groups.

RESULTS

Mass Spectrum of Me_3Si-Serine. The most abundant ions above m/z 100 in the electron-impact mass spectrum of

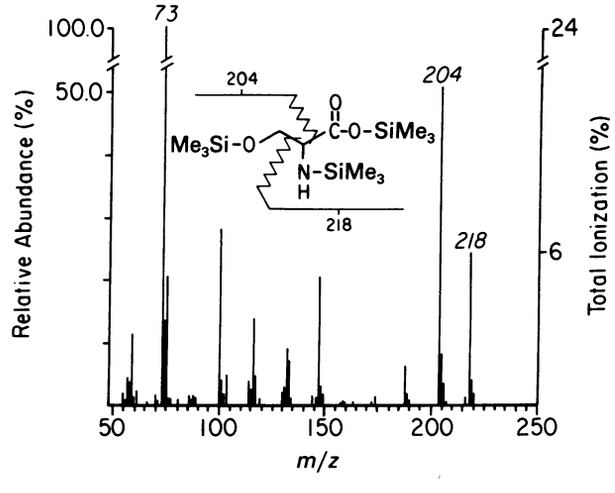

FIG. 2. Electron-impact mass spectrum from m/z 50 to 250 of tris(trimethylsilyl)serine. The fragment at m/z 73 is the trimethylsilyl cation (12). The fragmentation pattern that gives rise to signals at m/z 204 and 218 is also shown.

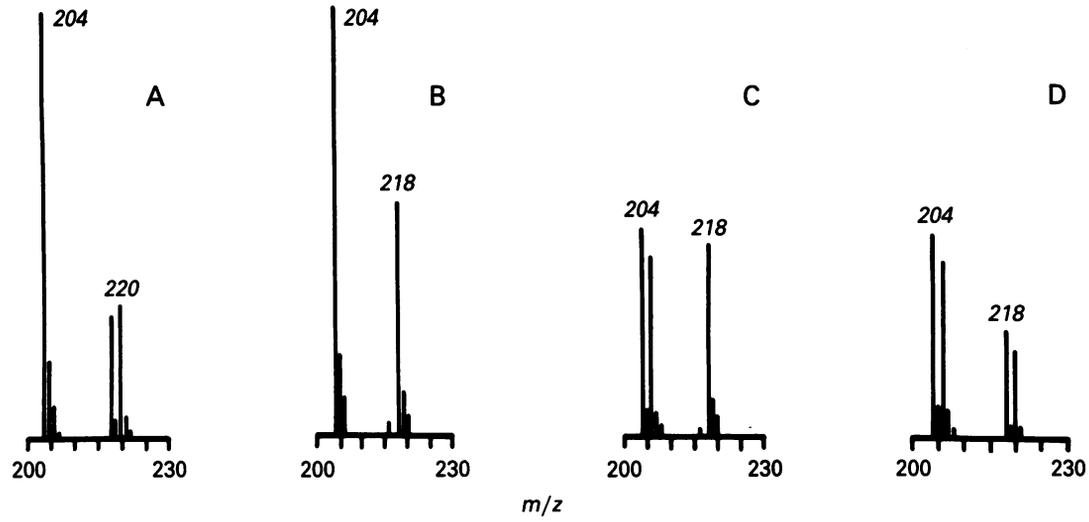

FIG. 3. Mass spectra from m/z 200 to 230 of Me_3Si-serine from mutant 3 histidine decarboxylase. (*A*) Serine-81 from the β chain derived from proenzyme labeled with L-[*carboxyl*-^{18}O]serine. Serine residues from the α chain showed the same spectrum. (*B*) Serine-81 from unlabeled proenzyme activated in $H_2{}^{18}O$. Serine residues from the α chain showed the same spectrum. (*C* and *D*) Serine residues from the α chain (*C*) or serine-81 from the β chain (*D*), both derived from proenzyme labeled with L-[*hydroxyl*-^{18}O]serine.

tris(trimethylsilyl) serine appear at m/z 218 and 204 (Fig. 2). The ion at m/z 218 results from loss of the amino acid side chain from the parent ion and therefore contains the carboxyl but not the hydroxyl oxygen atoms (12). The ion at m/z 204 results from loss of the trimethylsilylcarboxyl group from the parent ion and therefore contains only the hydroxyl oxygen atom of serine (13).

Preparation and Analysis of Histidine Decarboxylase Labeled with L-[*carboxyl*-^{18}O]Serine. Prohistidine decarboxylase was purified from mutant 3 of *Lactobacillus* 30a grown on defined medium containing L-[*carboxyl*-^{18}O]serine (70 atom % ^{18}O). The proenzyme was activated by incubation in potassium phosphate, pH 7.6, and the α and β chains were purified by ion-exchange chromatography after protein denaturation. Comparison of the amino acids released by carboxypeptidase digestion of the β chain with the COOH-terminal sequence of the β chain (3) showed that digestion terminated at lysine-68 and that therefore the COOH-terminal serine 81 was the only serine residue released (Fig. 1). Digestion of the α chain with trypsin followed by carboxypeptidase resulted in the release of approximately 7 mol of serine per mol of α chain and similar amounts of most other amino acids. Analysis of the Me_3Si derivatives by gas chromatography/mass spectrometry showed that 50% of the serine-81 residues contained one atom of ^{18}O in the carboxyl group with no ^{18}O in the hydroxyl group (Fig. 3*A*). Serine residues from the α chain showed similar labeling. No ^{18}O was present in the other amino acids in the digests, demonstrating that carboxyl oxygen was not transferred from serine to other residues. The decrease in the ^{18}O content of enzyme-bound serine compared to that of serine added to the growth medium is attributed to some synthesis of L-serine (about 60 μmol/liter of culture) by mutant 3 because (*i*) control analyses showed no detectable exchange of the carboxyl oxygens during protein denaturation, carboxypeptidase Y digestion, or trimethylsilylation; (*ii*) the same dilution was observed when cells were grown in the presence of L-[*hydroxyl*-^{18}O]serine; and (*iii*) mutant 3 shows some growth on defined medium lacking serine.

Activation of Prohistidine Decarboxylase in $H_2{}^{18}O$. Purified unlabeled prohistidine decarboxylase from mutant 3 was activated in $H_2{}^{18}O$ containing 1 M KCl and 0.1 M potassium phosphate, pH 7.6. Analysis of serine-81 showed no incorporation of ^{18}O into the carboxyl group (Fig. 3*B*). Because results described in the preceding section showed no exchange of oxygen from the carboxyl group during procedures used to isolate serine-81, the result demonstrates that carboxyl formation at the terminus of the newly formed β chain does not involve acyl transfer to a water molecule or hydroxide ion derived from the solvent. There also was no ^{18}O in any of the other amino acids derived from the α or β chains.

Analysis of Histidine Decarboxylase from Cells Grown in the Presence of L-[*hydroxyl*-^{18}O]Serine. Prohistidine decarboxylase was purified from mutant 3 grown on defined medium containing 60 atom % L-[*hydroxyl*-^{18}O]serine. Serine residues from the α chain of the activated proenzyme contained 44 atom % ^{18}O in the hydroxyl group and essentially no ^{18}O in the carboxyl group (Fig. 3*C*). The dilution of label is similar to that observed with cells grown on L-[*carboxyl*-^{18}O]serine and is attributed to limited synthesis of L-serine by the cells. The COOH-terminal serine-81 of the β chain also contained 45 atom % ^{18}O

Table 1. ^{18}O content of serine residues from histidine decarboxylase

Proenzyme	Activation in	Source of serine	^{18}O content, % Hydroxyl group	^{18}O content, % Carboxyl group*
Labeled with [*carboxyl*-^{18}O]-serine	$H_2{}^{16}O$	α chain†	0	50
		Ser-81 of β chain‡	0	50
Unlabeled	$H_2{}^{18}O$	α chain†	0	0
		Ser-81 of β chain‡	0	0
Labeled with [*hydroxyl*-^{18}O]-serine	$H_2{}^{16}O$	α chain†	44	≈1
		Ser-81 of β chain‡	45	42

Prohistidine decarboxylase (proenzyme) was isolated from mutant 3 of *Lactobacillus* 30a grown in the presence of serine labeled as indicated in column 1, then converted to the active enzyme in $H_2{}^{16}O$ or $H_2{}^{18}O$ as indicated in column 2. α and β subunits were then isolated and separately examined for the ^{18}O content of their serine residues.

* Percentage of residues containing one atom of ^{18}O per carboxyl group.

† Determined after digestion of the α chain in $H_2{}^{16}O$ with trypsin and then carboxypeptidase. No ^{18}O was present in tyrosine, threonine, aspartate, glutamate, or other residues from the α chain.

‡ Determined after digestion of the β chain in $H_2{}^{16}O$ with carboxypeptidase.

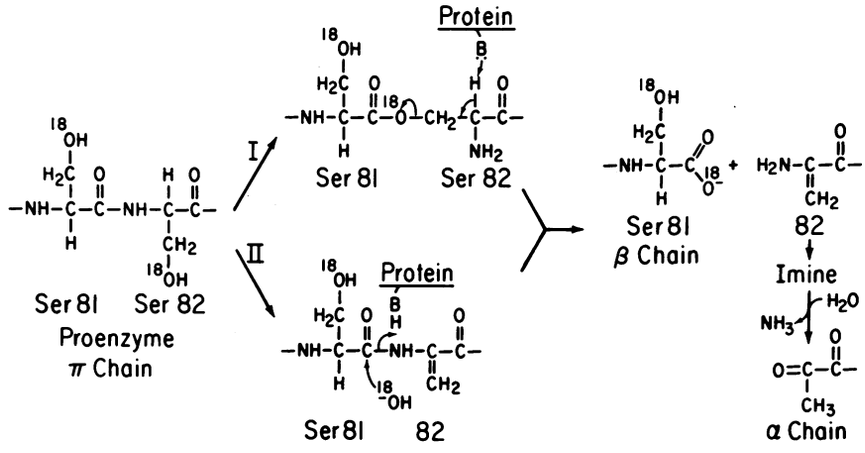

FIG. 4. Possible routes for transfer of the hydroxyl oxygen of serine-82 to the carbonyl group of serine-81. Route I, formation of an ester intermediate between the two residues followed by α,β-elimination. Route II, α,β-elimination at serine-82 followed by addition of a sequestered hydroxide ion (or water molecule) to the scissile peptide bond (or to an acyl enzyme intermediate). The diagram is not meant to reflect the stereochemistry of the amino acid residues concerned.

in the hydroxyl group, but in addition, 42% of the carboxyl groups of this residue contained one atom of ^{18}O (Fig. 3*D*). No ^{18}O was present in the other amino acids of the α and β chains, including those residues with potentially nucleophilic oxygen atoms in their side chains (threonine, aspartate, glutamate, and tyrosine). These same results were obtained with histidine decarboxylase isolated from wild-type cells grown in the presence of L-[*hydroxyl*-^{18}O]serine. These results are summarized in Table 1 and demonstrate that the hydroxyl oxygen of a serine residue of the proenzyme is specifically incorporated into the carboxyl group of serine-81 during proenzyme activation.

DISCUSSION

The present report describes procedures for labeling prohistidine decarboxylase with ^{18}O-containing amino acids and for recovery of amino acids from active enzyme without exchange of label. These procedures have been used to determine the origin of the oxygen atom incorporated into the carboxyl terminus (serine-81) of the β chains of histidine decarboxylase during activation of prohistidine decarboxylase. Because activation in $H_2{}^{18}O$ does not label this carboxyl group, proenzyme chain cleavage, unlike other known enzymatically catalyzed proteolytic reactions, is not a hydrolytic reaction; i.e., it does not involve acyl transfer to a hydroxide ion or water molecule derived from the solvent. Activation of proenzyme labeled with L-[*hydroxyl*-^{18}O]serine showed that whereas serine residues from the α chain contained ^{18}O only in the hydroxyl group, serine-81 contained similar amounts of label in both hydroxyl and carboxyl groups. No ^{18}O was present in other amino acids of the protein, including those with oxygen atoms in their side chains. The result shows that the hydroxyl oxygen of a proenzyme-bound serine residue is incorporated into the carboxyl group of serine-81 during activation. Transfer of a serine hydroxyl oxygen to the carboxyl group could occur (*i*) by ester formation between the carbonyl group of serine-81 and the hydroxyl group of another serine residue followed by α,β-elimination or (*ii*) by transfer of a sequestered hydroxide ion or water molecule derived from a serine residue to the carbonyl group. Oxygen transfer from any serine residue other than serine-82, the precursor of the pyruvoyl group, would result in formation of an internal dehydroalanine residue (via α,β-elimination) or a crosslink between the β carbon atom of the hydroxyl donor and the side chain of some other amino acid residue (via nucleophilic substitution at the β carbon). Because analysis by the method of Weiner *et al.* (14) and sequence studies (unpublished) show the absence of such modified residues, we conclude that the oxygen atom incorporated into serine-81 is derived from serine-82. Possible routes for such transfer are depicted in Fig. 4. Ester formation between serines 81 and 82 (route I, Fig. 4) could result directly by attack of the hydroxyl oxygen of serine-82 at the scissile peptide bond, with displacement of the amide nitrogen (N → O shift) in a reaction similar to the first phase of proteolysis by serine proteases. It could also result indirectly by attack of this hydroxyl group on an acyl-enzyme intermediate (e.g., a thioester, ester, or anhydride) formed between the serine-81 carbonyl group and a nucleophilic amino acid side chain of the proenzyme. Formation of such an ester would facilitate subsequent α,β-elimination because the carboxylate anion (pK ≈ 4) is a good leaving group. Formation of a sequestered hydroxide ion or water molecule (route II, Fig. 4), on the other hand, is considered less likely because the hydroxide ion (pK ≈ 15) is a poor leaving group. In either case, α,β-elimination at serine-82 of the proenzyme and chain cleavage yields the β chain with its COOH-terminal serine-81 containing one oxygen atom derived from serine-82, and an α chain initially containing an NH_2-terminal dehydroalanine residue (Fig. 4). Conversion of the latter to the pyruvoyl group and ammonia is expected to proceed via imine and carbinolamine intermediates to yield the mature α chain. Mechanisms that involve catalysis of these intermediate steps by appropriately placed proton-accepting or donating groups from the protein are easy to write, but they have not yet been studied.

Further work is required to provide a more detailed understanding of this interesting and apparently unprecedented reaction and to determine whether it represents an isolated instance of an unusual processing reaction or has a more general significance in metabolism.

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1. Snell, E. E. & Recsei, P. A. (1981) in *Structural and Functional Aspects of Enzyme Catalysis*, eds. Eggerer, H. & Huber, R. (Springer, New York), pp. 177–191.
2. Recsei, P. A., & Snell, E. E. (1973) *Biochemistry* **12**, 365–371.
3. Vaaler, G. L., Recsei, P. A., Fox, J. L. & Snell, E. E. (1982) *J. Biol. Chem.* **257**, 12770–12774.
4. Snell, E. E., Recsei, P. A. & Misono, H. (1976) in *Metabolic Interconversion of Enzymes 1975*, ed. Shaltiel, S. (Springer, New York), pp. 213–219.
5. Riley, W. D. & Snell, E. E. (1968) *Biochemistry* **7**, 3520–3528.

6. Recsei, P. A. & Snell, E. E. (1981) in *Metabolic Interconversion of Enzymes 1980*, ed. Holzer, H. (Springer, New York), pp. 335–344.
7. Recsei, P. A. & Snell, E. E. (1982) *J. Biol. Chem.* **257**, 7196–7202.
8. Murphy, R. C. & Clay, K. L. (1979) *Biomed. Mass Spectrom.* **6**, 309–314.
9. Guirard, B. M. & Snell, E. E. (1964) *J. Bacteriol.* **87**, 370–376.
10. Riley, W. D. & Snell, E. E. (1970) *Biochemistry* **9**, 1485–1491.
11. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
12. Vandenheuvel, W. J. & Cohen, J. S. (1970) *Biochim. Biophys. Acta* **208**, 251–259.
13. Bergstrom, K. & Gurtler, J. (1971) *Acta Chem. Scand.* **25**, 175–188.
14. Weiner, H., White, W. N., Hoare, D. G. & Koshland, D. E. (1966) *J. Am. Chem. Soc.* **88**, 3851–3859.