PRODUCTS OF PROLINE PEROXIDATION

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1. Introduction

Proline can be oxidized to hydroxyproline *in vitro* by a system containing Fe^{2+}, L-ascorbate and EDTA, and employing O_2 or H_2O_2 as oxidant [1,2]. The system has been suggested as a model for collagen-proline hydroxylation [3–5]. We report here on a study of products from H_2O_2 hydroxylation of proline and prolylpeptides. It is shown that to be hydroxylated by H_2O_2, proline must have its ring nitrogen unsubstituted. Peroxidation of free proline yields a variety of products, including hydroxyprolines, whereas N-substituted proline yields mainly a non-hydroxylated end product. A pathway of *in vivo* hydroxylation mechanistically similar to Fe^{2+}/H_2O_2 hydroxylation of proline is not consistent with our results.

2. Experimental

The standard reaction mixture and conditions have been described [6]. Hydroxylated products were assayed according to Woessner [7]. Prior to assay, free proline and dipeptides were desalted on Dowex 50 (H^+) and dipeptides were hydrolyzed in 6 N HCl in a closed tube at 135° for 3 hr; polypeptides were dialyzed before hydrolysis. For amino acid separation on paper, an aqueous solvent was used [8]; spots were developed with ninhydrin [9]. Amino acid analyzer runs were performed on an Aminco analyzer. Chemical were obtained commercially unless otherwise noted.

3. Results

Seventeen replicates of peroxidation of 1 mg free proline gave a mean (± S.D.) of 33 μg (± 7) hydroxyproline. Omission of ascorbic acid from the reaction mixture in 15 replicates gave a mean yield of hydroxylated products of 32 μg (± 5). Yield is insensitive to pH changes between 2 and 6.5, averaging 34 μg hydroxyproline/mg proline over this range.

Yields of hydroxylated product from the peroxidation of prolylpeptides are shown in table 1 (columns 2 and 3). Since high though narrowly reproducible proline losses accompanied desalting of untreated dipeptides on Dowex (column 4), the μg hydroxyproline found (column 2) have been normalized to 100% proline recovery (column 3). Only N-terminal proline appears to be hydroxylated. Particularly striking is the absence of hydroxylation in crystalline β-casein *A* which contains 15% proline residues.

Fig. 1 compares amino acid analyzer effluent traces

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Table 1

Peroxidation of prolylpeptides. The reaction mixture contained (in μmole/3.6 ml final vol.): $FeSO_4$, 44; L-ascorbate, 55; EDTA, 15, citric acid, 300; Na_2HPO_4, 150; H_2O_2, 880; proline 8.7. Incubation time; 15 min at 40°; pH 4.

Substrate (mg)	Hydroxyproline Yield		Proline
	Found (μg)	Normalized to pro = 100% (μg)	% found after passage over Dowex
pro.pro (1)	5	16	32
pro.gly (1)	9	17	54
gly.pro (1)	1	2	59
leu.pro (1)	0.4	2	17
gelatin (4)	minus 130[a]	–	–
β-casein *A* (10)	2[a,b]	–	–

[a]Final value minus initial value.
[b]Reaction mixture at pH 6.5.

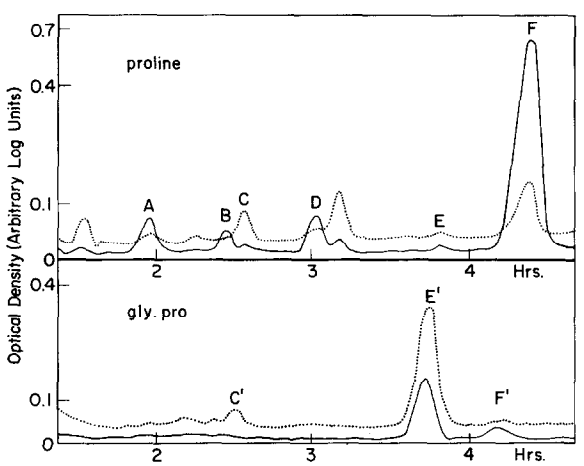

Fig. 1. Peroxidation of proline and gly.pro: amino acid analyzer effluent traces. . . . . 570 mμ absorption of ninhydrin adduct; —— 440 mμ absorption of ninhydrin adduct. Peaks: *A*, 3-*trans*-hydroxyproline; *B*, 4-*trans*-hydroxyproline; *C*, *C'*, unknown peak appearing in reagent blank; *D*, 4-*allo*-hydroxyproline; *E*, *E'*, unknown peak, chief product of gly.pro peroxidation; *F*, *F'*, proline.

after peroxidation of free proline and of the peptide gly.pro. It is significant that peroxidation of the peptide leads essentially to a single product (*E*) visible in the analyzer trace, while a variety of products is obtained from peroxidation of free proline. These products include three peaks absorbing at 440 mμ; on the basis of comparison of effluent times of authentic standards, two of these were identified as 4-*trans*-hydroxyproline (*B*) and 4-*allo*-hydroxyproline (*D*). Peak *A*, for which no authentic standard was available, has on the basis of published relative effluent times [10] tentatively been identified as 3-*trans*-hydroxyproline. These compounds, as well as several whose ninhydrin adducts absorb at 570 mμ, are absent from the trace of gly.pro peroxidation. Peak *E* on the latter trace emerges within a few minutes of glutamic acid and its ninhydrin-adduct absorbs at 570 mμ. However, compound *E* is unreactive with glutamic decarboxylase and on paper chromatograms its pink ninhydrin color (R_f 0.28) develops slowly in contrast to the deep purple of glutamic acid (R_f 0.27). The same compound is obtained in peroxidation of all N-substituted prolines investigated. The tentative structure Δ^1-pyrroline-2-carboxylic acid has been assigned for the following reasons: (1) the infrared spectrum shows a peak in the region of C=N stretching frequencies; (2) compound *E* easily decarboxylates but is slow to react with ninhydrin; (3) peroxidation of analogous substituted tetrahydrofurans leads chiefly to products of 3° hydrogen abstraction [11]. The synthesis of Δ^1-pyrroline 2-carboxylic acid for added confirmation of structure assignment is in progress.

4. Discussion

A free-radical mechanism for proline hydroxylation by hydrogen peroxide has been assumed [3,12], and a summary of evidence supporting such a mechanism has been published [6]. The free radical produced by homolytic splitting of H_2O_2, presumably $HO\cdot$ [13], can abstract hydrogen from proline, leaving a prolyl-free radical which reacts further to give, e.g., substitution products such as alcohols [14].

When the ring nitrogen of proline is substituted by a group R, where R≠H, its basicity will be lowered. Free or N-terminal proline is therefore protonated under our reaction conditions, while N-linked proline is not. The hydroxylation observed with free or N-terminal proline can be understood as an effect of N-protonation on the site of free-radical attack. The full positive charge conferred on the basic ring nitrogen by protonation discourages attack at ∝ carbons 2 and 5; from the consequent "hunt" for hydrogen abstraction sites by the attacking radical, a variety of prolyl radicals may result leading to the variety of products seen on the analyzer. When N-protonation is prevented by substitution, e.g. when the ring N is involved in peptide linkage, the electrophilic free radical will tend to attack at carbons α to the nitrogen. The 3° hydrogen on carbon 2 of such a prolyl moiety should be favored for abstraction because of the relative stability of the resultant radical [15]. A reaction sequence is plausible that leads *via* production of 2-hydroxyproline by dehydration to Δ^2-pyrroline 2-carboxylic acid. With hydrolysis, this eneamine will rearrange to Δ^1-pyrroline 2-carboxylic acid [16].

Shibata et al. [17] have reported that the proline of pro.leu.gly is hydroxylated by Fe^{2+}/H_2O_2, while hydroxylation of proline in gly.pro.leu is much diminished. While our work confirms and explains this observation, it conflicts with results of Hurych [12] who reported hydroxylation of proline in gly.pro.leu.gly.pro to an extent approximating that of proline in pro.gly. It is conceivable that out postulated intermediate, 2-hydroxyproline, might have survived his milder reaction conditions to react like 4-hydroxyproline in the colorimetric assay.

Proline appears to be in peptide linkage when hydroxylated *in vivo* [3,18,19]. Although a free-radical mechanism has been suggested for the hydroxylase reaction [3–5], such a mechanism is made unlikely by our results. An alternate mechanism for hydroxylase activity has been put forth by Hayaishi and Nozaki [20]

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