ANALYTICAL BIOCHEMISTRY 49, 550–558

Epimerization of *trans*-4-Hydroxy-L-proline to *cis*-4-Hydroxy-D-proline during Acid Hydrolysis of Collagen

DOMINIC D. DZIEWIATKOWSKI, VINCENT C. HASCALL, AND RICK L. RIOLO

Dental Research Institute, The University of Michigan, Ann Arbor, Michigan 48104

Received April 5, 1972; accepted May 1, 1972

1. *trans*-4-Hydroxy-L-proline is epimerized to *cis*-4-hydroxy-D-proline under the conditions generally used for the hydrolysis of proteins for analyses of amino acids.

2. The *cis* epimer elutes from a column of UR-30 resin on a Beckman amino acid analyzer at the same time as threonine and hence would be undetected.

3. About 8% of the *trans*-4-hydroxy-L-proline in collagen can be epimerized to *cis*-4-hydroxy-D-proline in the hydrolysis of collagen with 6 *N* HCl at 110° for 72 hr.

4. A procedure is described whereby the primary amino acids in hydrolyzates are deaminated with nitrous acid and removed so that the amounts of *trans*-4-hydroxyproline, *cis*-4-hydroxyproline, and proline can be determined without changing the temperature of the column of UR-30 resin on the Beckman amino acid analyzer.

In studies on the formation of ester sulfates of hydroxy amino acids (1), we observed that treatment of *trans*-4-hydroxy-L-proline with 6 *N* HCl, 110°, 24 hr, yielded a small amount of a product which eluted from a column of Beckman UR-30 resin later than *trans*-4-hydroxy-L-proline. This product gave a chromophore with ninhydrin with a 440 nm absorption profile typical of imino acid derivatives. These observations suggested that during acid hydrolysis the *trans*-4-hydroxy-L-proline was epimerized, in part, to a different proline derivative. The following experiments were carried out to characterize this reaction in more detail.

MATERIALS AND METHODS

Amino acid analyses were done on a Beckman model 120C amino acid analyzer equipped with an extended range. Typically, 0.05–0.10 μmole of a hydroxyproline sample was applied to the column (0.9 × 62 cm) of

UR-30 resin. The column was maintained at 56° and eluted with citrate buffer, pH 3.25 (2).

Values for the specific rotation of samples, $[\alpha]_D^{26}$, were measured with a Rudolph polarimeter, using an 1 decimeter tube and a sodium lamp. In experiments on the change of the specific rotation of *trans*-4-hydroxy-L-proline with time in 12 *N* HCl at 110°, a Fric polarimeter, using a 2 decimeter tube and a sodium lamp, was employed.

trans-4-Hydroxy-L-proline and *cis*-4-hydroxy-L-proline were purchased from Calbiochem. On analysis with the amino acid analyzer each derivative gave a single peak, with the *trans*-4-hydroxy-L-proline eluting at 45 min and the *cis*-4-hydroxy-L-proline eluting at 54.5 min. Values of the specific rotation of the samples are listed in Table 1. The concentrations of the solutions used for the measurements were 2% (w/v).

cis-4-Hydroxy-D-proline was prepared by the procedure described by Robinson and Greenstein (4). On analysis for amino acids a single peak was seen, which eluted in the same position as the *cis*-4-hydroxy-L-proline described above. Values for $[\alpha]_D^{26}$ of the sample are given in Table 1.

Collagen was extracted from rat skins and was purified as described by Gross and Kirk (5).

All the other reagents were analytical grade.

Approximately 5 mg portions of *trans*-4-hydroxy-L-proline were dissolved in 2 ml of 6 *N* HCl. The solutions were heated in evacuated and sealed tubes for 24 hr or 96 hr at 110°. They were then either dried at 45° in a flash evaporator or dried at room temperature over NaOH and $CaCl_2$ in an evacuated desiccator. The residues were dissolved in citrate buffer, pH 2.2, and analyzed on the amino acid analyzer (2).

The above experiment was repeated, except that 5 mg portions of *trans*-4-hydroxy-L-proline were dissolved in 1 ml of 6 *N* HCl and the heating periods were 24 and 72 hr. At the same time, approximately 5 mg portions of collagen were similarly hydrolyzed. All of the hydroly-

TABLE 1
Values for Specific Rotation, $[\alpha]_D^{26}$, for Isomers of 4-Hydroxyproline

Isomer	$[\alpha]_D^{26}$ measured in		$[\alpha]_D^{25}$ reported in	
	H_2O	12 *N* HCl	H_2O	5 *N* HCl
trans-4-Hydroxy-L-proline	−73.4°	−46.9°	−76.0°[a]	−50.5°[a]
cis-4-Hydroxy-L-proline	−59.9°	−22.7°	−59.5°[a]	−18.8°[a]
cis-4-Hydroxy-D-proline	+60.7°	+23.9°	+59.5°[b]	—

[a] From "Handbook of Biochemistry" (3).
[b] From Robinson and Greenstein (4).

zates were diluted by the addition of 2 ml of 6 *N* HCl. 1 ml of each was then evaporated to dryness in a flash evaporator. The residues were dissolved in citrate buffer, pH 2.2, and analyzed with the Beckman amino acid analyzer. To a second 1 ml of each sample in 6 *N* HCl, 0.5 ml of a 3 *M* solution of sodium nitrite and 1 ml of 8.6 *N* HCl were added in that order. The resultant solutions, 0.6 *M* in nitrous acid, 6 *N* in HCl, were heated at 100° for 3 min and then evaporated to dryness in a flash evaporator. Each residue was dissolved in 1 ml of 6 *N* HCl and heated at 100° in a water bath for 90 min. After flash evaporation, each residue was dissolved in 1 ml of 3 *N* HCl and extracted 3 times with 3 ml portions of ethyl ether. The aqueous phase for each sample was dried in a flash evaporator, and the residues were dissolved in citrate buffer, pH 2.2, for analysis on the amino acid analyzer.

The above procedure for deamination of primary amino acids is a modification of the procedure of Hamilton and Ortiz (6). The amino acids, proline and hydroxyproline, are stable to the procedure and can be recovered quantitatively.

2 gm of *trans*-4-hydroxy-L-proline was dissolved in 100 ml of concentrated HCl, approximately 12 *N* (Baker Analyzed Reagent, sp.gr. 1.185–1.192, 36.5–38.0%). The solution was maintained at 110° for up to 96 hr, except for an aliquot which was set aside at 0° for the same period of time. The optical rotations of aliquots of the solution were measured after various intervals of time. At the same times aliquots were flash evaporated and analyzed on the amino acid analyzer. The experiment was repeated with a solution of 2.5 gm of *trans*-4-hydroxy-L-proline in 100 ml of 12 *N* HCl. However, samples were removed for analysis after intervals of time up to 12 days.

RESULTS

When *trans*-4-hydroxy-L-proline was dissolved in 6 *N* HCl and immediately dried at 45° with a flash evaporator, or more slowly at room temperature over NaOH and $CaCl_2$ in an evacuated desiccator, only one component, with an elution time of 45 min, was observed on the column of UR-30 resin. On the other hand, if such solutions were heated at 110°, the amount of material in the effluent at 45 min decreased and a correspondingly increased amount of ninhydrin-reactive material was found at 54.5 min (Table 2). Similar results were obtained when collagen was hydrolyzed with 6 *N* HCl (Table 3). The fact that the isomer formed from *trans*-4-hydroxy-L-proline eluted from the column at the same position as a standard of *cis*-4-hydroxy-L-proline (Table 4) suggested that the isomer was either the D or L form of *cis*-4-hydroxyproline or a mixture of both.

TABLE 2
Epimerization of *trans*-4-L-Hydroxyproline at 110° in 6 *N* HCl

Time of heating, hr	Treatment	Epimer found, % of total[a]
0	Flash evaporation at 45°	0
0	Desiccation at room temp.	0
24	Flash evaporation at 45°	10.2
24	Desiccation at room temp.	5.8
96	Flash evaporation at 45°	27.8
96	Desiccation at room temp.	31.2

The hydrolyzates were evaporated to dryness either in a flash evaporator at 45° or at room temperature in an evacuated desiccator which contained pellets of NaOH and $CaCl_2$. The residues were dissolved in sodium citrate buffer, pH 2.2, for analysis on an amino acid analyzer.

[a] The amount of epimer formed was calculated assuming that it yielded the same amount of chromophore per mole as did *trans*-4-hydroxy-L-proline.

TABLE 3
Epimerization of *trans*-4-L-Hydroxyproline of Collagen at 110° in 6 *N* HCl

Material	Time of heating, hr	Treatment[a]	Epimer found, % of total
trans-4-L-hydroxyproline	24	Evap. only	2.4
	24	D & E	2.8
	72	Evap. only	7.4
	72	D & E	8.7
Collagen	24	D & E	2.7
	72	D & E	8.1

trans-4-Hydroxy-L-proline and collagen were each heated in 6 *N* HCl for the specified periods of time. Subsequently a portion was analyzed directly and a second equal portion was treated with nitrous acid as detailed in the text.

[a] D & E, deamination and evaporation.

TABLE 4
Time of Appearance of Some Amino Acids in Effluent from Column of UR-30 Resin

Amino acid	Elution time, min
trans-4-Hydroxyproline	45.0
Aspartic acid	45.5
cis-4-Hydroxyproline	54.5
Threonine	55.0
Proline	73.0

A Beckman amino acid analyzer was used as specified (2). The temperature of the long column was 56°.

The experiments on the change in the optical rotation of a solution of *trans*-4-hydroxy-L-proline in 12 *N* HCl at 110° as correlated with the appearance of an isomer lead to the conclusion that the isomer is *cis*-4-hydroxy-D-proline. The plot of observed values for $[\alpha]_D^{25}$ against per cent of *trans*-4-hydroxyproline present in the solution after various incubation times (Fig. 1) yields a straight line which extrapolates to a

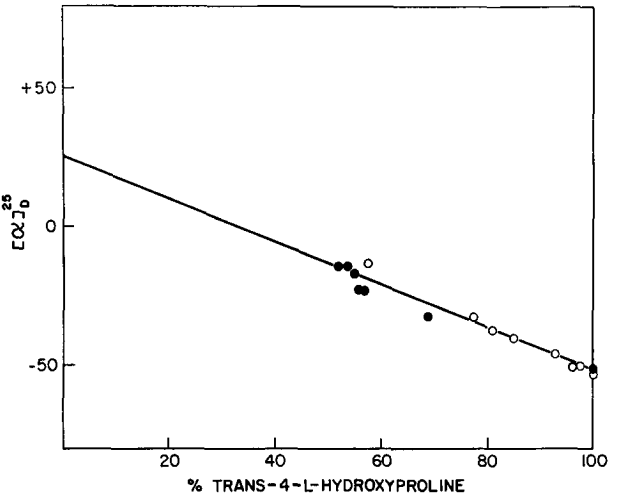

FIG. 1. The curve indicates the optical rotation observed for a solution of *trans*-4-hydroxy-L-proline as it was epimerized on heating at 110°C in 12 *N* HCl as a function of the relative amount of the *trans* epimer present: (○) from experiment 1, (●) from experiment 2. The extrapolated value for $[\alpha]_D^{25}$ at 0% *trans* epimer is positive, suggesting that the *cis* epimer formed is of the D series. The graph shows the best first-order least-squares line through the data.

value of $[\alpha]_D^{25}$ of +25° for the isomer (0 per cent *trans*-4-hydroxy-L-proline). This agrees well with a value of $[\alpha]_D^{26}$ of +23.9° determined in 12 *N* HCl for a sample of *cis*-4-hydroxy-D-proline prepared according to Robinson and Greenstein (4) (Table 1). Indeed, the acid conditions used in the experiment are similar to those these authors used to epimerize the α-carbon of 4-hydroxyprolines, namely, boiling in a mixture of acetic anhydride and glacial acetic acid.

When the transformation of *trans*-4-hydroxy-L-proline to *cis*-4-hydroxy-D-proline was related to time of heating in 12 *N* HCl at 110°, it was found that after 6 days there was nearly an equal amount of the two forms in solution and that the relative concentrations of the epimers do not change thereafter (Fig. 2). The equilibrium constant for the reaction under these conditions, then, is about 1. It is noteworthy that the optical rotation of the solution was the same at the end of 12 days as at the

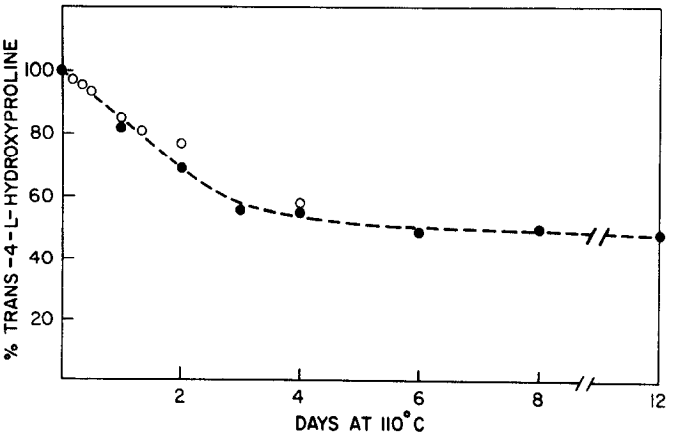

FIG. 2. Transformation of *trans*-4-hydroxy-L-proline to *cis*-4-hydroxy-D-proline with time in 12 *N* HCl at 110°. The data have been plotted against the time the solution was incubated at 110°.

beginning of the experiment if the solution was kept at 0°, and the *cis* epimer was not detected when the solution was analyzed with the amino acid analyzer.

Table 4 indicates the elution times of the 4-hydroxyproline derivatives and of the amino acids which interfere with their detection, aspartic acid and threonine, when standard conditions are used for the analyses. For adequate quantitation of *trans*-4-hydroxyproline and aspartic acid it was suggested by Spackman, Stein, and Moore (7) that an analysis of the hydrolyzates be run at 30°. However, no conditions for resolving *cis*-4-hydroxyproline in amino acid analyses have been reported, and the probable presence of this derivative in acid hydrolyzates of collagen has not been detected. Both 4-hydroxyproline derivatives can be measured using standard conditions on the amino acid analyzer if the primary amino acids are first deaminated and removed by the procedures described in "Materials and Methods." Figures 3 and 4 show analyses for a hydrolyzate of collagen, before and after such treatment. In this example, about 8% of the *trans*-4-hydroxyproline has been epimerized to the *cis* derivative, during the hydrolysis for 72 hr, which is readily apparent in Fig. 4 (see also Table 3).

DISCUSSION

trans-4-Hydroxy-L-proline was first isolated from hydrolyzates of gelatin in 1902 by Fischer (8). It is regarded as a characteristic of the collagen and its concentration in tissues is frequently the basis for the calculation of the approximate concentration of collagen. However, *cis*-4-

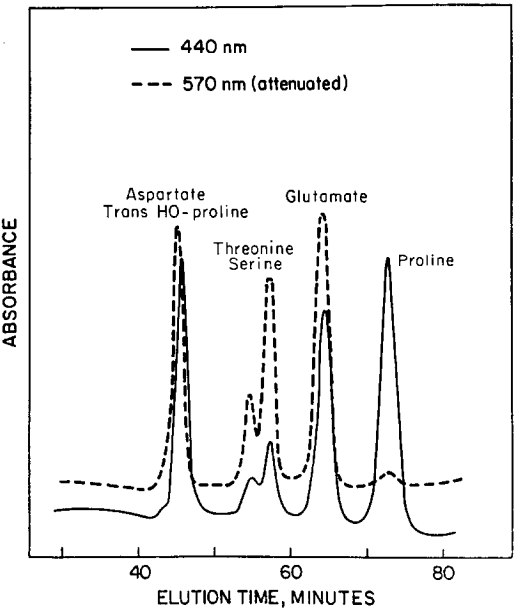

FIG. 3. Elution pattern, 40 to 80 min, of hydrolyzate of collagen from an amino acid analyzer run at 56°C according to the directions supplied by the manufacturer (2). Note that *trans*-4-hydroxyproline is not resolved from aspartic acid and the "peak" for threonine does not indicate the presence of the *cis*-4-hydroxyproline.

hydroxy-L-proline (*allo*-hydroxy-L-proline) also occurs in nature. The latter has been isolated from the toxic peptide of *Amanita phalloides* by Wieland and Witkop (9) and from *Santalum album* L. by Radhakrishman and Giri (10).

The observation that *trans*-4-hydroxy-L-proline is epimerized when heated in 6 *N* HCl (1) raised the question as to the nature of the epimer and whether it is formed when collagens are hydrolyzed. The results as given in this paper support the conclusion that the epimer formed is *cis*-4-hydroxy-D-proline. Moreover, on hydrolysis of collagens it is also formed in proportion to the time taken for the hydrolysis. Since a system is frequently employed for amino acid analysis (2) in which the *cis*-4-hydroxyproline is eluted simultaneously with threonine, the presence of the *cis* epimer will remain undetected and the concentration of the *trans* epimer may be underestimated. The data in this paper indicate that the per cent of *cis*-4-hydroxy-D-proline formed during the usual hydrolysis of proteins which contain the *trans*-4-hydroxy-L-proline may be as much as 10%, depending upon time for hydrolysis. The amount of

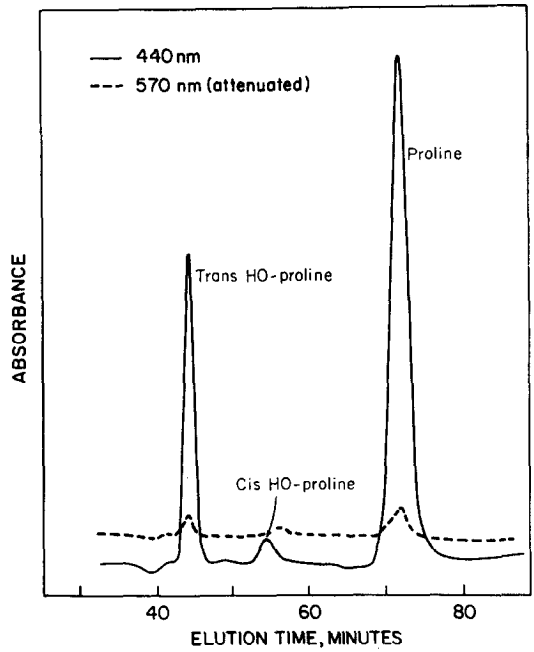

FIG. 4. Elution pattern, 40 to 80 min, of hydrolyzates of collagen after deamination of primary amino acids. The column conditions are the same as for Fig. 3 except that twice as much material was chromatographed. The *trans*-4-hydroxyproline and its *cis* epimer are now readily apparent.

the *cis* epimer in a hydrolyzate can be readily quantitated if the primary amino acids are deaminated by the use of nitrous acid and removed from the hydrolyzate. Such a procedure also allows for good quantitative analyses of all the proline and hydroxyproline in the sample using standard conditions for amino acid analysis.

ACKNOWLEDGMENTS

We are grateful to Dr. J. Distler of the Rackham Research Unit, The University of Michigan, for checking the optical rotations of the amino acids.

This work was supported by U. S. Public Health Service Grant DE-02731.

REFERENCES

1. DZIEWIATKOWSKI, D. D., HASCALL, V. C., AND RIOLO, R. L., *Anal. Biochem.*, to appear.
2. "Procedures Manual" (Amino Acid Analyzer), Beckman, 1966.
3. "Handbook of Biochemistry," p. B20. The Chemical Rubber Co., Cleveland, Ohio, 1968.
4. ROBINSON, D. S., AND GREENSTEIN, J. P., *J. Biol. Chem.* **195**, 383 (1952).

5. GROSS, J., AND KIRK, D., *J. Biol. Chem.* **233**, 355 (1958).
6. HAMILTON, P. B., AND ORTIZ, P. J., *J. Biol. Chem.* **187**, 733 (1950).
7. SPACKMAN, D. H., STEIN, W. H., AND MOORE, S., *Anal. Chem.* **30**, 1190 (1958)
8. FISCHER, E., *Ber.* **35**, 2260 (1902).
9. WIELAND, H., AND WITKOP, B., *Ann. Chem.* **543**, 171 (1940).
10. RADHAKRISHMAN, A. N., AND GIRI, K. V., *Biochem. J.* **58**, 57 (1954).