SIMULTANEOUS INCORPORATION OF ^{18}O INTO SUCCINATE AND HYDROXYPROLINE CATALYZED BY COLLAGEN PROLINE HYDROXYLASE

George J. Cardinale, Robert E. Rhoads* and Sidney Udenfriend

Roche Institute of Molecular Biology
Department of Physiological Chemistry
Nutley, New Jersey 07110

Received March 16, 1971

SUMMARY: The sequential copolymer $(Pro\text{-}Gly\text{-}Pro)_{\sim 12}$ was hydroxylated in an $^{18}O_2$ atmosphere with a partially purified preparation of collagen proline hydroxylase. Mass spectral analysis demonstrated the incorporation of one atom of ^{18}O into hydroxyproline and one atom into succinic acid. Since the enzyme produces equal amounts of peptidyl hydroxyproline and succinate these findings strongly suggest that one molecule of O_2 takes part in the simultaneous oxygenation of peptidyl proline and α-ketoglutarate. A possible mechanism for this reaction is presented.

During the biosynthesis of collagen, conversion of proline to hydroxyproline is carried out after incorporation of the proline into peptide linkage. The finding that the hydroxyl group of hydroxyproline is derived from molecular oxygen (1) (2) established collagen proline hydroxylase as a mixed function oxygenase. It was subsequently found that this enzyme has an absolute and specific requirement for α-ketoglutarate (3). Following the original report from this laboratory, several other oxygenase systems which require α-ketoglutarate were described (4-7). Recently it has been demonstrated that one mole of α-ketoglutarate is converted to succinate and CO_2 for each mole of hydroxylated product formed by collagen proline hydroxylase (8) and by γ-butyrobetaine hydroxylase (9). More recently, Lindblad et al. (10) reported that not only is ^{18}O from molecular oxygen incorporated into carnitine during the γ-butyrobetaine hydroxylase

---

*Present address:

Department of Pharmacology
Stanford University School of Medicine
Palo Alto, California 94304

reaction, but also into the succinate derived from α-ketoglutarate. We have now been able to carry out a similar experiment with collagen proline hydroxylase and have found that atmospheric ^{18}O is incorporated in equivalent amounts into the two products, peptidyl hydroxyproline and succinate.

MATERIALS AND METHODS

The sequential copolymer $(Pro\text{-}Gly\text{-}Pro)_{\sim 12}$ average mol. wt. 3400 was a gift from Dr. J. Kurtz, Weizmann Institute of Science, Rehovoth, Israel. DL-hydroxyproline-2-^{14}C was purchased from Amersham/Searle, Regisil (bis (trimethylsilyl)trifluoroacetamide) from the Regis Chemical Company, and $^{18}O_2$ (99.5 atom %) from the Isomet Corporation. Analyses for hydroxyproline were performed on a Beckman Model 120C amino acid analyzer equipped with a range card for high sensitivity.

Each reaction mixture contained in a total volume of 15 ml; Tris-HCl, pH 7.2, 750 μmoles; α-ketoglutarate, 1.5 μmoles; ascorbate, 7.5 μmoles; ferrous ammonium sulfate, 1.5 μmoles; $(Pro\text{-}Gly\text{-}Pro)_{\sim 12}$, 2.25 mg; bovine serum albumin, 30 mg; bovine liver catalase (Boehringer), 6.0 mg; and collagen proline hydroxylase, purified from newborn rat skin through the DEAE-Sephadex chromatography step, 2.5 arbitrary units (11). When these components were incubated under the conditions described below, 1.0 - 1.3 μmoles of succinic acid and hydroxyproline were formed.

A 200-ml round-bottomed flask, slightly modified from the design of Hayaishi (12), was used for carrying out the reaction. Prior to adding the reactants the flask was filled with four parts of nitrogen and one part of $^{18}O_2$ at atmospheric pressure. Nitrogen was bubbled through the non-protein components of the reaction mixture for 10 minutes. The three protein components were then added and the total mixture (15 ml) gently swirled under a stream of nitrogen. This solution was injected through the rubber vaccine stopper into the reaction flask using a syringe with a 5 inch #20 needle. The flask was kept at 0° for 20 minutes with occasional swirling to allow equilibration with the atmosphere and then

incubated at 30° for 60 minutes. At the end of the incubation period, the reaction mixture was removed with a syringe and needle and a small portion of the gas phase was removed with a Hamilton gas-tight syringe. The solution was quickly cooled, acidified to pH 2 with HCl and extracted five times with three volumes of diethyl ether. The ether phase was evaporated under a stream of nitrogen and the residue, which contained the succinic acid (100-150 µg), was set aside for mass spectral analysis. To the acidified aqueous layer, which had been extracted with ether, 1.5 ml of 50% trichloroacetic acid was added to precipitate proteins. After 30 minutes at 0°, the precipitate was removed by centrifugation at 12,000 x g for 15 minutes and the supernatant solution was taken to dryness at 90° under a stream of nitrogen. The residue was dissolved in 5.0 ml of constant boiling HCl and hydrolyzed under nitrogen at 110° for 16 hours. The HCl was removed under a stream of nitrogen at 90°, the residue was dissolved in 0.005N HCl, a trace amount of DL-hydroxyproline-2-^{14}C was added as a marker, and the solution was applied to a 0.9 cm x 54 cm UR-30 column of the Beckman Amino Acid Analyzer equipped with a bypass which led to a fraction collector. Elution was carried out with the standard sodium citrate buffer, 0.2 M, pH 3.5. The fractions containing hydroxyproline were pooled and desalted on a 1.5 ml Dowex 50-X 4 (H^+) column, using 2N ammonia as the eluant. After removal of ammonia under a stream of nitrogen at 90°, a small amount of methanol was added and the insoluble residue was removed by filtration through glass wool. The methanol was then evaporated and the residue (70-90 µg hydroxyproline) set aside for mass spectral analysis.

The succinic acid residue was dissolved in 100 µl of pyridine and the hydroxyproline residue in 100 µl of acetonitrile. An equal volume of Regisil was added and the solutions were heated at 130° for 15 minutes. The trimethylsilyl derivatives of the compounds isolated from the reaction mixtures were shown to have the same retention times on gas

chromatography as the trimethylsilyl derivatives of authentic succinic acid and hydroxyproline. These comparisons were made with a Barber Colman Model 5000 gas chromatograph, equipped with a 1.8 m x 2 mm column of 3.6% OV101 on Gas Chrom Q 100/120 mesh. The ^{18}O content of the isolated reaction products was measured by feeding the output from a gas chromatograph into a Finnigan Model 1015 Quadrupole Mass Spectrometer with direct feed via splitter (i.e., no concentrating device). Atmospheric $^{18}O_2$ abundance was measured with a Jeolco 01SG mass spectrometer.

RESULTS AND DISCUSSION

The ^{18}O contents of succinic acid and hydroxyproline isolated from several experiments carried out in an atmosphere of $^{18}O_2$ are shown in Table I. Typical mass spectra on which these data are based are shown

Table I

^{18}O CONTENTS OF ENZYMATICALLY FORMED SUCCINIC ACID AND HYDROXYPROLINE

Experiment[a]	Atmospheric $^{18}O_2$ Abundance[b]	% of Molecules Containing 1 Atom of ^{18}O [c]	
		Hydroxyproline	Succinic Acid
1	89	--	85
2	88	--	82
3	> 98	92	90
4	98	92	88

[a] Experiments 1 and 2 were consecutive incubations in the same flask. Reaction mixture 2 was injected into the flask as soon as reaction 1 had been removed. Experiments 3 and 4 were run on another day in a similar manner.

[b] $\frac{^{18}O_2}{^{18}O_2 + ^{16}O_2} \times 100$

[c] These values were calculated by determining the ratio of the 232/230 mass/charge peaks for hydroxyproline and the 249/247 mass/charge peaks for succinic acid.

in Figs. 1 and 2. It is apparent that succinic acid and peptidyl hydroxyproline are both formed by a direct addition of oxygen. Since equimolar quantities of succinate and hydroxyproline are produced in this reaction (8) these results strongly suggest that one molecule of O_2 takes part in the simultaneous oxygenation of both substrates. This is in contrast to other mixed function oxygenases which catalyze the reduction of one atom of oxygen to water.

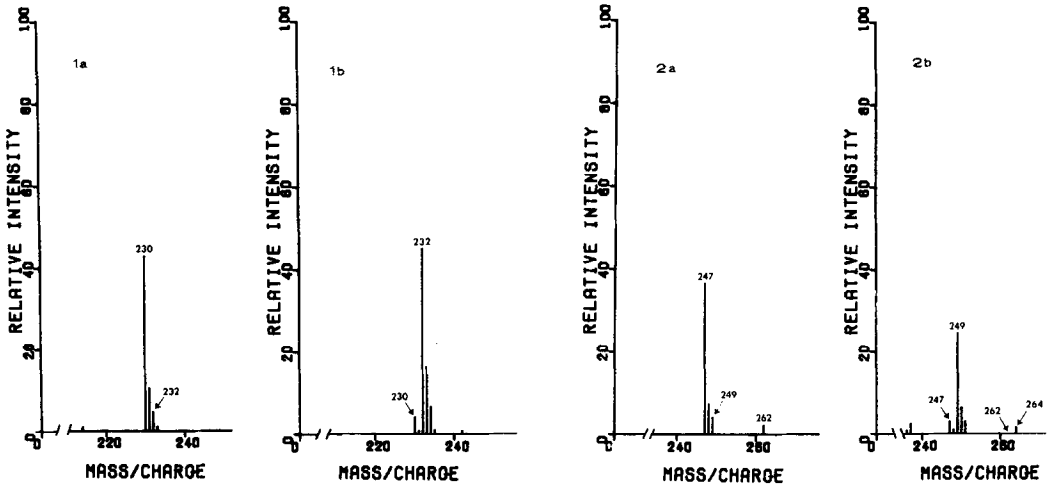

Fig. 1 - Segments of the mass spectra of the trimethylsilyl derivatives of authentic (1a) and enzymatically formed (1b) hydroxyproline. The peak at 230 is the positive ion resulting from the loss of the trimethylsilylated carboxyl group. The incorporation of one atom of ^{18}O in over 90% of the enzymatically formed hydroxyproline is evident from the magnitude of the corresponding ion plus two mass units in 1b.

Fig. 2 - Segments of the mass spectra of the trimethylsilyl derivatives of authentic (2a) and enzymatically formed (2b) succinic acid. The peak at 262 is the molecular ion and at 247 is the positive ion after the loss of one methyl radical from a trimethylsilyl group of the ester. The incorporation of one atom of ^{18}O in 80-90% of the enzymatically formed succinic acid is evident from the magnitude of the corresponding ions plus two mass units in 2b.

Recently Lindblad *et al.* (10) have shown that γ-butyrobetaine hydroxylase, another α-ketoglutarate-requiring enzyme, catalyzes the incorporation of atmospheric ^{18}O into succinic acid. These authors proposed that γ-butyrobetaine, α-ketoglutarate and O_2 are associated as a ternary complex on the enzyme, peroxide bridging the carbonyl carbon of

α-ketoglutarate to the β-carbon of γ-butyrobetaine. A comparable peroxide intermediate, which was suggested many years ago by Goodwin and Witkop (13)(14) for the conversion of p-hydroxyphenylpyruvic acid to homogentisic acid, was recently established by Lindblad et al. (15) with the use of $^{18}O_2$. By analogy with these two reactions the hydroxylation of prolyl residues by collagen proline hydroxylase may involve intermediates of the type shown in Fig. 3. A hydroperoxide at the 4 (trans) position of the proline would be the initial product. The anionic form of the hydroperoxide would then be capable of attacking α-ketoglutarate to yield an intermediate complex which would decompose to peptidyl hydroxyproline, succinic acid and CO_2. It should be noted that ascorbic acid, which was once considered a specific factor in hydroxyproline formation, plays no role in this mechanism. In vitro ascorbic acid serves as a reductant which can be replaced by dithiothreitol (11) ascorbic acid analogues (16), and tetrahydropteridines (16).

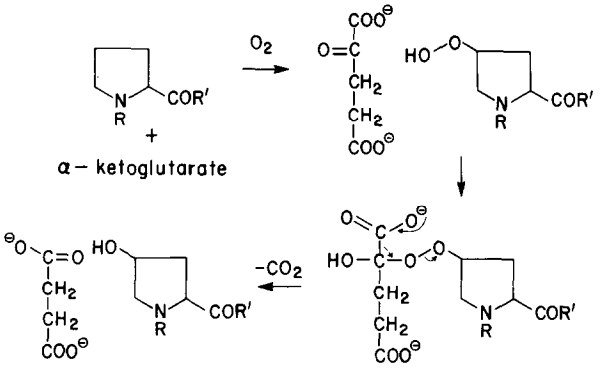

Fig. 3 - Proposed mechanism for the coupled oxygenation of hydroxyproline and α-ketoglutarate by collagen proline hydroxylase. (The prolyl protons were intentially omitted to simplify the drawing).

The α-ketoglutarate requiring hydroxylases and p-hydroxyphenylpyruvate hydroxylase represent a unique class of oxygenases since mixed function oxygenases have generally been considered to transfer one atom of oxygen to substrate and the second atom to water. As noted by Lindblad et al. (15) the present nomenclature of oxygenases must be reconsidered as well

as their classifications into mono and dioxygenases (12).

ACKNOWLEDGEMENT: We are greatly indebted to Dr. C. G. Scott, Mr. Patrick Riggio and Mr. Armen Galustian for performing the gas chromatographic and mass spectral analyses on the isolated products and to Dr. Wolfgang Benz for determining atmospheric $^{18}O_2$ abundance. Mr. James Stone's assistance with the amino acid analyzer is also greatly appreciated.

REFERENCES

1. Fujimoto, D. and Tamiya, N., Biochem. J. 84, 333 (1962).

2. Prockop, D., Kaplan, A. and Udenfriend, S., Biochem. Biophys. Res. Commun. 9, 162 (1962).

3. Hutton, J. J., Tappel, A. L. and Udenfriend, S., Biochem. Biophys. Res. Commun. 24, 179 (1966).

4. Hausman, E., Biochim. Biophys. Acta 133, 591 (1967).

5. Linstedt, G., Linstedt, S., Midtwedt, T. and Tofft, M., Biochem. J. 103, 19P (1967).

6. Abbott, M. T., Schandl, E. K., Lee, R. F., Parker, T. S. and Midgett, R. J., Biochim. Biophys. Acta 132, 525 (1967).

7. Abbott, M. T., Dragila, T. A. and McCrosky, R. P., Biochim. Biophys. Acta 169, 1 (1968).

8. Rhoads, R. E. and Udenfriend, S., Proc. Natl. Acad. Sci., U.S. 60, 1473 (1968).

9. Lindstedt, G., Lindstedt, S., Olander, B. and Tofft, M., Biochim. Biophys. Acta 158, 503 (1968).

10. Lindblad, B., Lindsted, G., Tofft, M. and Lindstedt, S., J. Amer. Chem. Soc. 91, 4604 (1969).

11. Rhoads, R. E. and Udenfriend, S., Arch. Biochem. Biophys. 139, 329 (1970).

12. Hayaishi, O., "Oxygenases", Chapter 1, Academic Press, New York 1962.

13. Goodwin, S. and Witkop, B., J. Amer. Chem. Soc. 79, 179 (1956).

14. Daly, J. W. and Witkop, B., Angewandte Chemie 2, 421 (1963).

15. Lindblad, B., Lindstedt, G. and Lindstedt, S., J. Amer. Chem. Soc. 92, 7446 (1970).

16. Hutton, J. J., Tappel, A. L. and Udenfriend, S., Arch. Biochem. Biophys. 118, 231 (1967).