Study of the 5-Oxoprolinase Reaction by $^{13}$C NMR*

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5-Oxoprolinase catalyzes the ATP-dependent decyclization of 5-oxo-L-proline to L-glutamate. Previous studies provided evidence for the intermediate formation of a phosphorylated form of 5-oxoprolinase (Seddon, A. P., and Meister, A. (1986) J. Biol. Chem. 261, 11538–11541) and of a tetrahedral intermediate (Li, L., Seddon, A. P., and Meister, A. (1987) J. Biol. Chem. 262, 11020–11025). A new approach to the study of the reaction mechanism using the $^{18}$O isotope effect on the $^{13}$C NMR signals for 5-oxoprolinase and glutamate is reported here. The $^{13}$C chemical shifts induced by $^{18}$O substitution for the carboxyl group of 5-oxoprolinase and the $\gamma$-carboxyl group of glutamate are about 0.03 ppm with respect to the corresponding $^{16}$O-compounds. Using 5-[13C]oxo-[5-18O]proline (97 and 79.5 atom % excess, $^{13}$C and $^{18}$O, respectively), the disappearance of the $^{18}$O-labeled and unlabeled 5-oxoproline and formation of the corresponding glutamate species were followed in the reactions catalyzed by purified preparations of 5-oxoprolinase isolated from Pseudomonas putida and from rat kidney. This procedure permits simultaneous determinations of the rates of $^{18}$O exchange and of the overall decyclization reaction. The ratios of $^{18}$O exchange rates to the overall reaction rates for the bacterial and kidney enzyme catalyzed reactions were 0.28 and 0.14, respectively. The findings support the view that the coupling of ATP hydrolysis to 5-oxoproline decyclization involves formation of a phosphorylated tetrahedral intermediate. Although the exchange phenomena are consistent with the mechanistic interpretations, they seem not to be required for catalysis.

5-Oxoprolinase catalyzes the conversion of 5-oxo-L-proline to L-glutamate according to Reaction 1. In the

\[ \text{5-Oxoprolinase + ATP + 2H}_2\text{O} \rightarrow \text{5-oxo-L-proline} \rightarrow \text{L-glutamate} + \text{ADP + P}_i \]

**Reactions**

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‡ Reversible transfer of oxygen from the $\gamma$-carboxyl group of glutamate to inorganic phosphate occurs in the reaction catalyzed by glutamine synthetase (6, 7), which involves intermediate formation of $\gamma$-glutamyl phosphate (8). Although $\gamma$-glutamyl phosphate is postulated as an intermediate in the reaction catalyzed by 5-oxoprolinase, this reaction also involves formation of a phosphorylated tetrahedral intermediate (see Fig. 4 in Ref. 4).
EXPERIMENTAL PROCEDURES

Materials—L-[5-13C]Glutamic acid (97-99 atom % excess) and H218O (97-99 atom % excess) were purchased from MSD Isotopes. Sources of all other chemicals and materials were as described (2).

P. putida (strain ALA) was that previously obtained by soil enrichment culture (1). 5-Oxoprolinase (320 units/mg) was isolated and purified as described (1, 2). Rat kidney 5-oxoprolinase (22 units/mg) was purified essentially as described (5), except that chromatography on phenyl-Sepharose was omitted. One unit of enzyme activity is defined as the amount of enzyme required for the conversion of 1 µmol of substrate to product in 1 h at 37 °C under the assay conditions.

5-[18O]Oxo-L-[5-13C]proline was synthesized, as described (4), from L-[5-13C]glutamate. 18O enrichment in the amide carbonyl group of the synthesized 5-oxoproline was determined to be 79.5 atom % excess by 13C NMR spectrometry.

Methods—Assays for 5-oxoprolinase activity were carried out as described (1). 13C NMR spectra were obtained at 24 °C with a 500-MHz Bruker spectrometer (AM series) using a 10-mm broad-band probe tuned at 125.78 MHz. Samples for analysis were dissolved in 0.6 ml of deuterium oxide (99 atom % excess 'H). A 500-Hz spectral width and a 90° pulse angle (20 µs) were applied. Exponential multiplication was used for Fourier transform spectra using a line-broadening factor of 0.2. Relative intensities of the corresponding 13C NMR signals were calculated from integrated areas of the peaks. To ensure the accuracy of the measurements, [5-13C,-18O]glutamate was isolated by AG 50W-X8 H' chromatography, and 18O enrichment was determined by mass spectrometry as described (4). The results obtained by NMR and by mass spectrometry were in close agreement (±6%).

RESULTS

5-Oxoproline highly enriched with 18O and 13C in the amide carbonyl group (79.5 and 97 atom % excess, respectively) gave two well-resolved 13C NMR signals, with an upfield shift of 0.03 ppm for the 18O-containing 5-oxoproline relative to the 16O-labeled species (Fig. 1). Similarly, the signals for [5-13C,18O]glutamate showed an upfield shift of 0.027 ppm relative to that for the 16O-compound (Fig. 2). Chemically syn-

Fig. 1. 13C resonance signals for 5-[18O]oxo5-[13C]proline and 5-[18O]oxo5-[12C]proline. The NMR signal for the 13C-enriched carbonyl carbon of 5-oxoproline is shifted upfield (0.03 ppm) by 18O substitution. 18O and 13C enrichments were 79.5 and 97 atom % excess, respectively. The spectrum (eight acquisitions) was obtained from a 10 mM solution of the sample in D2O.

Fig. 2. 13C resonance signals for [5-13C,18O]glutamate and [5-13C,18O]glutamate. The NMR signal for the 13C-enriched 5-carboxyl carbon of glutamate is shifted upfield (0.027 ppm) by 18O substitution. The spectrum (256 acquisitions) was obtained from a sample of glutamate (10 mM in D2O) prepared in a reaction catalyzed by bacterial 5-oxoproline using 5-[18O]oxo5-[13C]proline. 18O and 13C enrichments in glutamate were 59 and 97 atom % excess, respectively.

Fig. 3. Bacterial 5-oxoprolinase-catalyzed intermediate-water exchange of 18O in 5-oxoproline and glutamate, followed by 13C NMR spectrometry. Reaction mixtures were incubated at 37 °C and contained, in a final volume of 6.0 ml, 10 mM 5-[18O]oxo5-[13C]proline (79.5 atom % excess 18O), 10 mM ATP, 20 mM MgCl2, 12 mM phosphoenolpyruvate, 15 units of pyruvate kinase, 150 mM KCl, 2 mM dithiothreitol, and 100 mM Tris-HCl (pH 8.2). The reaction was initiated by addition of 60 units of bacterial 5-oxoprolinase. At the indicated intervals, 0.6-ml portions were removed, adjusted to pH 6.0 by addition of HCl, and rapidly frozen. After sample collection, the solutions were thawed, centrifuged, neutralized by addition of NaOH, and lyophilized. Each sample was then dissolved in 0.6 ml of D2O and analyzed by NMR spectrometry (48 acquisitions/spectrum).

thesized [5-13C,18O]glutamate gave a signal that was 0.06 ppm upfield relative to that for [5-13C]glutamate; thus, the isotope effect is additive, as previously noted (11). The 13C resonance...
signal for the γ-carboxyl carbon of glutamate is 0.475 ppm upfield of that for the amide carbonyl carbon of 5-oxoproline (Fig. 3). Thus, the signals for 13C-enriched glutamate and 5-oxoproline and the corresponding 16O-labeled compounds can be simultaneously observed directly without isolation and purification of the samples prior to analysis. Since the intensities of the NMR signals are directly proportional to the peak areas of the isotopic species (10), the concentrations of the reactant, 5-oxoproline, and of the product, glutamate, can be quantitated.

5-[16O]Oxo-1-[5-13C]proline was used as substrate for the reaction catalyzed by bacterial 5-oxoprolinase (Fig. 3). Fig. 3 shows a stacked plot composed of a series of NMR spectra obtained on samples of the reaction mixture removed at the time intervals indicated. The disappearance of 13C, 16O, and 13C, 16O-labeled 5-oxoproline and the formation of corresponding glutamate species are inversely related. It is apparent that the ratio of 16O to 18O in the substrate is greater than that in the product glutamate. Similar results were obtained in reactions catalyzed by rat kidney 5-oxoprolinase (Fig. 4).

Conversion of the integrated area for each peak in Fig. 3 to the amount of each 13C-labeled species, plotted as a function of time, is shown in Fig. 5. At saturating levels of substrates and in the presence of an ATP-regenerating system (phosphoenolpyruvate and pyruvate kinase), the decrease of 5-oxoproline and the formation of glutamate were linear with time for about 90% of the reaction time course. Initial 16O enrichment of 5-oxoproline was 79.5 atom % excess in the amide carbonyl group. 16O-Labeled and unlabeled 5-oxoproline decreased at rates of 0.093 and 0.024 μmol/min, respectively. The rate of formation of 16O-labeled glutamate was 0.077 μmol/min, whereas that of unlabeled glutamate was 0.049 μmol/min, giving rise to an enrichment in the γ-carboxyl group of glutamate of 59.4 atom % excess. If no exchange between medium water and the amide carbonyl oxygen had occurred, the rate of disappearance of the 16O-labeled substrate would be equal to the rate of appearance of the 16O-labeled glutamate species. Similarly, the rate of disappearance of unlabeled 5-oxoproline would be equal to that of the appearance of unlabeled glutamate. It is apparent that this is not the case. In addition to labeled glutamate, 16O-labeled 5-oxoproline was also converted to unlabeled glutamate during the course of the reaction.

Analyses similar to those described above were performed under various conditions (Table I). Addition of phosphoenolpyruvate and pyruvate kinase to the reaction mixture markedly accelerated the rate of glutamate formation for the reaction catalyzed by bacterial 5-oxoprolinase. The loss of 16O label from glutamate, however, was not significantly influenced by an increase in reaction rate (Table I, Systems 1 and 2). The effect of decreasing pH on isotope exchange was also investigated. At pH 7.6, the rate of glutamate formation was approximately half that at pH 8.2; however, 16O enrichment in glutamate was not significantly different at these two pH values. Thus, a decrease in the pH of the reaction or removal

![FIG. 4. Rat kidney 5-oxoprolinase-catalyzed intermediate-water exchange of 16O in 5-oxoproline and glutamate, followed by 13C NMR spectrometry. The reaction conditions were identical to those described for Fig. 3, except that the reaction was catalyzed by rat kidney 5-oxoprolinase (90 units). The pH value of the reaction mixture was 8.0, and the molar ratio of Mg2+ to ATP was 1:1.](image-url)

![FIG. 5. Time course for reaction catalyzed by bacterial 5-oxoprolinase using 5-[16O]oxo[5-13C]proline. From the data shown in Fig. 3, the integrated peak areas for the corresponding 13C NMR signals were converted to micromoles of 5-[16O]oxo proline (●), 5-[16O]oxo proline (○), [5-13C]glutamate (△), and [5-13C]glutamate (△). The rates of disappearance and formation of each 13C-labeled species were obtained from the linear portions of the corresponding curves.](image-url)
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FIG. 6. Ratio of $^{18}$O to $^{16}$O in 5-oxoproline and glutamate in reaction catalyzed by bacterial 5-oxoprolinase as function of reaction time. Experimental conditions were identical to those described for Fig. 3, except that the total reaction time was 210 min. Aliquots of the reaction mixture were removed at the indicated intervals and the ratios of $^{18}$O to $^{16}$O in 5-oxoproline (5-OP) and glutamate were determined by $^{13}$C NMR spectroscopy. The progress of the reaction (as percent of completion) was derived from the sum of the integrated areas for both glutamate species.

FIG. 7. Simplified scheme to illustrate oxygen exchange between enzyme-bound intermediates and water.

The irreversibility of the 5-oxoprolinase reaction, previously demonstrated by the finding that the enzyme does not catalyze the formation of 5-oxoproline from glutamate in the presence of ATP or ADP and $P_i$, or of ATP from $P_i$ and ADP in the presence of glutamate (4), was also examined by $^{13}$C NMR. The time course of $^{18}$O exchange for an experiment in which bacterial 5-oxoprolinase was incubated with 5-$^{18}$O oxo[5-$^{13}$C]proline in the presence of ATP and an ATP-regenerating system is shown in Fig. 6. The ratio of $^{18}$O to $^{16}$O in 5-oxoproline and in glutamate was essentially complete in 30 min. Incubation was extended to 210 min to detect possible reversibility of the reaction which would be reflected by a further decrease in the ratio of $^{18}$O to $^{16}$O in glutamate after the reaction had gone to completion. It is apparent from Fig. 6 that the reaction is irreversible since the decrease in the ratio of $^{18}$O to $^{16}$O in glutamate was parallel to the reaction course and was not significantly affected by the extended incubation period. A decrease in the ratio of $^{18}$O to $^{16}$O in the amide carbonyl oxygen of 5-oxoproline was also observed. This is consistent with exchange of oxygen between the amide carbonyl group and medium water during the enzymatic reaction, an observation made earlier in the experiments using mass spectrometry (4). The results demonstrate that the exchange between water and reaction intermediates cannot be accounted for by nonenzymatic oxygen exchange or by partial reversibility of the reaction.

Oxygen exchange between enzyme-bound intermediates and medium water may be described by the scheme presented in Fig. 7. $S_i$, $T_1$, and $P_i$ are 5-oxoproline, transitional intermediates, and glutamate, respectively, that are labeled with $^{18}$O. $S_2$, $T_2$, and $P_2$ are the corresponding unlabeled species; $k_{tx}$ and $k_{glu}$ are the apparent rate constants for isotope exchange and glutamate formation, respectively. Under steady-state conditions, the decrease in $S_i$ and $S_2$ and the formation of $P_1$ and $P_2$ are linear functions of reaction time. Loss of $^{18}$O label via conversion of $T_1$ to $T_2$ is an irreversible step since medium water is not enriched with the isotope. This branch of the reaction pathway contributes to the rate of formation of unlabeled glutamate ($P_2$) by a factor of $k_{tx}[T_2]$. The rate of disappearance of labeled 5-oxoproline ($S_i$) for the linear portion of the reaction is therefore equal to the rate of labeled glutamate ($P_i$) formation plus $k_{tx}[T_2]$, which results in the loss of label and gives rise to an additional amount of unlabelled glutamate in $P_2$. Similarly, the rate of formation of unlabeled glutamate ($P_2$) is equal to the rate of disappearance of labeled 5-oxoproline ($S_i$) plus the rate of conversion of $T_1$ to $T_2$ ($k_{tx}[T_1]$). A ratio of the rate of glutamate formation to the rate of exchange, equivalent to $k_{tx}/k_{glu}$, can be obtained from either of the following two relations: $k_{tx}/k_{glu} = (v_{glu} - v_{tx})/v_{tx}$ and $k_{tx}/k_{glu} = (v_{glu} - v_{tx})/v_{tx}$. Values of $k_{tx}/k_{glu}$ obtained by calculation using the equations given above are presented in Table I. The ratio of the rate of oxygen exchange between water and $^{13}$C-labeled intermediates to the rate of glutamate formation for the bacterial enzyme-catalyzed reaction (Systems 1 and 2) was 0.25-0.3 and was greater than found for the rat kidney enzyme-catalyzed reaction.

DISCUSSION

Application of the $^{18}$O isotope effect on the $^{13}$C NMR signals for 5-oxoproline and glutamate permitted simultaneous determination of the rate of conversion of 5-oxoproline to glutamate and of the rate of oxygen exchange between medium water and intermediates on the reaction pathway. Consistent with previous observations (4), the amide carbonyl oxygen of 5-oxoproline and the y-carboxyl oxygen atoms of glutamate were shown to undergo replacement by oxygen atoms derived from water. These results provide insight into the kinetics of oxygen partitioning in the 5-oxoprolinase reaction. The rate of exchange was about one-fourth of that for glutamate formation in reactions catalyzed by bacterial 5-oxoprolinase, whereas the rate of exchange for the kidney enzyme-catalyzed reaction was about one-eighth of that for glutamate formation.
The reactions catalyzed by kidney and bacterial 5-oxoprolinase show essentially the same labeling patterns, as analyzed here by 13C NMR spectrometry and previously by mass spectrometry (4). The difference between the two enzyme systems is in the extent of 18O loss or incorporation. This may probably be ascribed to the structural differences between the two enzymes. Unlike the two tightly associated subunits of the kidney enzyme (5), bacterial 5-oxoprolinase is a reconstituted enzyme. The substrate-dependent interactions between Components A and B in the enzyme complex and the relative strength of these interactions compared to the kidney enzyme may be expected to lead to differences in the microscopic rate constants when ATP hydrolysis is coupled to the decyclization of 5-oxoproline. Some variation in the extent of 18O exchange has been observed with different batches of bacterial enzyme preparations. For example, in previous studies using 5-[18O] o xo-L-proline with an 18O enrichment of 45 atom % excess, mass spectral analysis of the glutamate formed showed a final 18O enrichment of only 8% in the γ-carboxyl group (4). In this study with 5-[18O]oxo[5,13C]proline, in which 18O enrichment was about 80 atom % excess, 13C NMR analysis of the glutamate formed showed that 18O enrichment of the γ-carboxyl group was about 60%. No significant 13C isotope effect to account for these observations was detected when 13C-enriched 5-oxoproline was incubated with the bacterial enzyme in H218O and compared to an experiment carried out using un-enriched 5-oxoproline. Moreover, 18O enrichment of samples determined by 13C NMR was in close agreement with that determined by mass spectrometry (±6%). Previous studies (13) on the reaction catalyzed by rat kidney 5-oxoprolinase using 18O-labeled 5-oxoproline showed complete retention of the isotope in glutamate. Considering that the coupling of ATP hydrolysis to 5-oxoproline decyclization is realized through the formation of the tetrahedral intermediate and that the observed oxygen exchange patterns can be ascribed to the randomization of the phosphoryl group in this intermediate, it is possible that 18O isotope partitioning can be influenced by the extent of randomization or the rate of collapse of the tetrahedral intermediate. If a change in the microenvironment at the active center occurs and imposes a restriction on the phosphoryl transfer reaction, then the extent of the isotope loss would be reduced. Changes in the microenvironment at the active center are most likely to occur in the bacterial enzyme since it is a reconstituted enzyme complex composed of two protein components. It is as yet uncertain as to exactly how Component B participates in the overall reaction. If Component B serves as a catalyst that utilizes phosphorylated 5-oxoproline bound to Component A, it is plausible that the active center of the enzyme complex shares catalytic surfaces on both Components A and B; and thus, these surfaces may be more sensitive to modification or damage during purification.

Exchange between the amide carbonyl oxygen of 5-oxoproline and that of water proceeds without additional hydrolysis of ATP since the ratio of P2 to glutamate is within experimental error (±5%) of unity. An overall or partial reversibility of the reaction was previously excluded by the absence of 5-oxoproline formation from glutamate or of ATP from ADP and P2; this is confirmed by the 13C NMR experiments presented here.

This study provides further support for formation of a tetrahedral intermediate in the 5-oxoprolinase-catalyzed reaction. Mechanistically, oxygen exchange is apparently not required. Thus, it occurs at different rates, relative to the overall catalytic rate. Nevertheless, exchange is a consequence of the coupled reaction and is thus a property that is useful in defining the structure of the tetrahedral intermediate, which is a central complex on the reaction pathway.

**Fig. 8.** Tentative scheme for 18O exchange between water and reaction pathway intermediates in 5-oxoprolinase reaction.
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