New Substrates of 5-Oxo-L-prolinase*

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Interaction of 5-oxo-L-prolinase (which catalyzes stoichiometric cleavage of 5-oxo-L-proline to L-glutamate coupled to that of cleavage of ATP to ADP and Pᵢ), with a number of new 5-oxo-L-proline analogs was examined in studies in which (a) analogs were substituted for 5-oxo-L-proline and the formation of products was determined, (b) the effects of the analogs on the normal catalytic reaction were observed, and (c) the effects of the analogs on the ITPase activity exhibited by the enzyme were examined. Some of these reactions are partially coupled, i.e. the molar formation of ADP exceeds that of amino acid; others are uncoupled, i.e. cleavage of ATP, but not that of imino acid occurs. Analog in which the 4-methylene moiety of 5-oxo-L-proline is replaced by O, S, and NH (L-2-oxooxazolidine-4-carboxylate, L-2-oxothiazolidine-4-carboxylate, and 2-imidazolidone-4-carboxylate) participate in reactions that are, partially coupled, coupled, and uncoupled respectively. Partial coupling occurs with cis- and trans-2-oxo-5-methyloxazolidine-4-carboxylate, and uncoupled reactions occur with L-2-iminohiazolidine-4-carboxylate, and 2-oxo-5,5-dimethylthiazolidine-4-carboxylate. These and earlier findings indicate that significant binding of the imino acid substrate requires a 5-carbonyl (or =NH), an un-substituted N-1 and a C-2 of the L-configuration; substantial modification of 5-oxo-L-proline in the region of C-3 and C-4 is possible with retention of binding properties. Uncoupling and partial coupling may be associated with significant differences in the orientations of the analogs at the active site that facilitate (to various extents) the access of water to the nucleoside triphosphate.

5-oxo-L-prolinase catalyzes the ATP-dependent cleavage of 5-oxo-L-proline1 to yield stoichiometric amounts of ADP, glutamate, and inorganic phosphate (1). The requirement for ATP as substrate is mandated by the unusual stability of the internal peptide bond of 5-oxoproline at physiological values of pH (2, 3). The enzyme, which has been found in a variety of organisms, has recently been purified to homogeneity from rat kidney (4). Previous studies in this laboratory have shown that the stoichiometry of the enzyme-catalyzed reaction can be affected by replacement of ATP with certain other nucleo-

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1Synonyms: L-pyroglutamic acid, 2-pyrrolidine-5-carboxylate acid.

side triphosphates (5), or by replacement of 5-oxoproline with certain other imino acids (1). The analogs of 5-oxoproline previously examined are of two types: (a) compounds such as L-2-imidazolidone-4-carboxylate and L-dihydrooxorotate, which do not undergo ring cleavage, but which stimulate formation of inorganic phosphate from ATP, and (b) compounds such as 2-piperidine-6-carboxylate and 3-oxo-5-oxoproline, whose rings are opened in reactions which are partially coupled, i.e. the molar ratio of inorganic phosphate to amino acid formed is significantly greater than unity.

A plausible mechanism of the reaction is formation of an enzyme-bound phosphorylated derivative of 5-oxoproline followed by its hydrolysis to yield glutamate and inorganic phosphate. Previous studies are consistent with, but do not prove, such a mechanism (1, 6, 7). The possibility that the binding of ATP at one enzyme site induces conformational changes in the enzyme that favor the decyclization of substrate has also been considered; previous studies have provided evidence that the interaction of the enzyme with its substrates is associated with conformational changes (4, 8).

In the present work, we sought to explore further the uncoupling phenomena associated with replacement of 5-oxo-L-proline by several new analogs of 5-oxoproline in the hope that studies of such modified reactions might yield information useful in deducing the normal reaction mechanism. Such data are important for the mapping of the substrate binding site and are required for the design of inhibitors and active site-directed agents. The present studies indicate that the 5-oxo-L-proline binding site can accept a number of analogs, and that such interactions are accompanied by various degrees of coupling between the cleavage of nucleoside triphosphates and that of the imino acid.

**EXPERIMENTAL PROCEDURES**

### Material

| Substrate analogs | Calculated for C₆H₉O₅ | Found | Percent | Literature
|-------------------|------------------------|-------|---------|------------------------|
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
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| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |
| L-pyroglutamic acid | C 32.65 H 3.42 | 32.65 | 100% | L-2-imidazolidone-4-carboxylate acid from L-proline |

### Notes

2 Portions of this paper (including "Experimental Procedures" and Tables I-III and V) are presented in Miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, MD 20814. Request Document No. 52M-1071, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
New Substrates of 5-Oxo-L-prolinease

Table I gives data on the ability of several 5-oxoproline analogs to serve as substrates of the enzyme. The inhibitory effects of the analogs on the normal coupled reaction catalyzed by the enzyme are given in Table II, and their effects on ITPase activity are described in Table III. Table IV gives the structures of the analogs together with relevant kinetic constants.

Structural modification of 5-oxo-L-proline by replacement of its 4-methylene moiety with O, S, or N leads to compounds that stimulate cleavage of ATP and inhibit ITPase activity. Replacement of the 4-methylene moiety of 5-oxo-L-proline with oxygen (L-2-oxooxazolidine-4-carboxylate) leads to a partially coupled reaction (coupling ratio, 1.7; Table IV). In contrast, replacement of the 4-methylene moiety of 5-oxo-L-proline with nitrogen increases ATP cleavage substantially, but there is no ring cleavage. Substitution of sulfur in place of the 4-methylene moiety of 5-oxo-L-proline (L-2-oxothiazolidine-4-carboxylate) leads to the only compound now known (other than 5-oxo-L-proline) that reacts in a fully coupled manner. Thus, we observed equivalent formation of cysteine and ADP when this compound was incubated with the enzyme (Table I).

L-2-Oxothiazolidine-4-carboxylate exhibits a very high affinity for the enzyme and, as expected, is an excellent inhibitor of the normal reaction (Table II), and inhibits ITPase (Table III). L-Thioproline, which lacks the carbonyl at the 5 position of 5-oxoproline, is neither a substrate nor a significant inhibitor. 2-Thiazolinone-4-carboxylate (Table IV) and 2-imidazolinone-4-carboxylate (which differs from 2-imidazolidone-4-carboxylate in that it has an \(\alpha,\beta\)-double bond) stimulate ATP cleavage, but notably stimulate rather than inhibit ITPase (Table III). Introduction of a methyl group into 2-oxooxazolidine-4-carboxylate at C-5 increases the coupling ratio from 1.7 to about 20; similar results were obtained with the cis- and trans-5-methyl derivatives (Table IV). Introduction of two methyl groups into this position of the corresponding sulfur-containing analog (2-oxo-5,5-dimethylthiazolidine-4-carboxylate) leads to a compound that does not stimulate hydrolysis of the normal reaction (Table II), and inhibits ITPase (Table III). L-Thioproline, which lacks the carbonyl at the 5 position of 5-oxoproline, is neither a substrate nor a significant inhibitor.

Table I

| Substrate | Product formed | \(K_i\) (mM) |
|-----------|----------------|-----------|
| L-2-oxooxazolidine-4-carboxylate | L-thioproline | 0.55 |
| L-2-oxothiazolidine-4-carboxylate | L-thioproline | 0.01 |
| L-2-imidazolinone-4-carboxylate | L-thioproline | 0.005 |

Table II

| Substrate | Inhibition of normal reaction | Inhibition of ITPase |
|----------|------------------------------|---------------------|
| L-2-oxooxazolidine-4-carboxylate | Complete | Complete |
| L-2-oxothiazolidine-4-carboxylate | Near complete | Complete |
| L-2-imidazolinone-4-carboxylate | Partial | Complete |

Table III

| Substrate | Activity (pmol/min/mg) | Inhibition |
|----------|-----------------------|-----------|
| None | 1.35 | 1.35 |
| L-2-oxooxazolidine-4-carboxylate | 0.49 | 0.49 |
| L-2-oxothiazolidine-4-carboxylate | 0.48 | 0.48 |
| L-2-imidazolinone-4-carboxylate | 0.31 | 0.31 |

Table IV

| Substrate | Structure | Reactions |
|----------|-----------|-----------|
| L-2-oxooxazolidine-4-carboxylate | O-alkylation, O-dealkylation | Complete |
| L-2-oxothiazolidine-4-carboxylate | O-alkylation, O-dealkylation | Complete |
| L-2-imidazolinone-4-carboxylate | O-alkylation, O-dealkylation | Complete |

Table V

| Substrate | Reaction | \(k_{cat}\) (s\(^{-1}\)) | \(K_m\) (mM) |
|----------|---------|----------------|---------|
| L-2-oxooxazolidine-4-carboxylate | Hydrolysis | 0.51 | 0.01 |
| L-2-oxothiazolidine-4-carboxylate | Hydrolysis | 0.25 | 0.005 |
| L-2-imidazolinone-4-carboxylate | Hydrolysis | 0.15 | 0.002 |

**Legend to Tables**

The reaction mixtures contained (in a total volume of 0.5 ml) 200 mM sodium buffer (pH 8.0), 100 mM KCl, 8 mM ATP, 10 mM of pyruvate, 0.5 mg/ml of protein, and 0.1 mg/ml of 5-oxoproline. The reactions were incubated at 37°C for 15 min. The reactions were terminated by the addition of 0.2 ml of 0.4 M perchloric acid, and the acid extracts were centrifuged at 10,000 g for 15 min. The supernatant solutions were removed and assayed for cysteine and ADP.

The inhibition of thioesterase activity by L-2-oxothiazolidine-4-carboxylate was determined by following the formation of thioamidic acid as a function of time at 37°C.

For the determination of the inhibition constant, a plot of \(V/V_0\) vs. [I] was constructed, where \(V_0\) is the initial rate of the uninhibited reaction and \(V\) is the observed velocity at different inhibitor concentrations.
of 5-oxoproline analogs are neither substrates nor inhibitors. If the amide moieties of the D-compounds bind to the enzyme in a manner similar to that for the L-substrates, then the carboxyl groups of the D-isomers must project toward the enzyme at a position normally occupied by the α-hydrogen atom of the substrate. The findings suggest that such interaction is sterically prevented. This interpretation is consistent with the inability of 2-methyl-5-oxoproline to bind to the enzyme. It seems notable that 2-thiazololone-4-carboxylate and 2-imidazolone-4-carboxylate (which have αβ-unsaturation) evidently bind to the enzyme without such hindrance. That these compounds stimulate substantial ATPase suggests that they are oriented on the enzyme in such a way as to permit access of water to the nucleoside triphosphate. These are the only compounds thus far studied that stimulate rather than inhibit ATPase activity (Table III).

It is of interest that analogs in which the 4-methylene moiety of 5-oxo-L-proline is replaced with S, O, or NH stimulate cleavage of ATP and inhibit that of ITP. Whereas the NH analog is not cleaved, the S analog is an excellent coupled substrate, suggesting that sulfur serves as an isomorphous replacement in this system for the 4-methylene moiety of 5-oxo-L-proline. It was previously suggested (1, 6) that the uncoupling found with L-2-imidazolone-4-carboxylate might be a consequence of the ability of the 4-hetero atom to facilitate hydrolysis of the corresponding phosphoryl compound without producing decyclization. If such hydrolysis is related to the ability of the hetero atom to donate electrons to the ring, then the partial uncoupling observed here with the 4-position analogs of 5-oxo-L-proline would be in accord with the expected order of reactivity, i.e. NH > O > S (12). It is evident from the data, however, that other types of analogs (e.g. 4-hydroxy-5-oxoproline, 2-piperidone-6-carboxylate, 3-methyl-5-oxoproline) also yield partially coupled reactions. Such reactions are most probably associated with an alignment of analog on the enzyme which differs from that obtained with 5-oxo-L-proline, and the factors involved may be both steric and electronic. An alternative to the possibility discussed above, i.e. that the analog is phosphorylated, and then dephosphorylated without decyclization, is that the binding of the amino acid produces a conformational change in the enzyme that facilitates (to a greater or lesser degree, depending on the analog) entrance of water to the site at which the nucleoside triphosphate binds, thus promoting its hydrolysis in some or all of the enzyme turnovers.

The present studies show that both cis- and trans-2-oxo-5-methylthiazolidine-4-carboxylate are substrates. It seems notable that trans-2-oxo-5-methylthiazolidine-4-carboxylate is a much better inhibitor of the normal reaction than is the corresponding cis compound, suggesting that a methyl group located on the same side of the ring as the carboxyl group may hinder binding. This is supported by the observation that DL-trans-3-methyl-5-oxoproline (formed by cyclization of erythro-β-methylglutamate) is a good inhibitor, whereas the cis isomer (formed by cyclization of threeo-β-methylglutamate) is a poor inhibitor. DL-cis-3-Hydroxy-5-oxoproline (formed by cyclization of threeo-β-hydroxyglutamate) is also a poor inhibitor (17). The present findings indicate that 2-oxo-5,5-di- methylthiazolidine-4-carboxylate interacts poorly with the enzyme.

### TABLE IV

| COMPOUND                  | REACTION TYPE | K2 (µM) | K1 (µM) | V1/0 (µmol/min/mg) |
|--------------------------|---------------|---------|---------|-------------------|
| 5-oxo-L-proline          | COUPLED (I 0) | 1.34    |         |                   |
| L-2-thiazolone-4-carboxyl| COUPLED (I 0) | 2.75    |         |                   |
| L-2-imidazolone-4-carboxyl| COUPLED (I 0) | 3.54    |         |                   |
| L-2-thiophosphonic acid  | UNCOUPLED (w) | 14      | 3       | 1.45              |
| L-thiophosphate          |              |         |         |                   |
| 2-thiazolone-4-carboxyl   | UNCOUPLED (w) | 3.55    | 270     | 2.2               |
| DL-cis-2-oxo-5-methylthiazolidine-4-carboxyl | PARITALLY COUPLED (1 2) | 8.3 | 0.83 |
| L-trans-3-oxo-5-methylthiazolidine-4-carboxyl | PARITALLY COUPLED (1 2) | 5 | 0.46 |
| DL-2-oxo-5,5-di-methylthiazolidine-4-carboxyl | UNCOUPLED (w) | 650 | 0.10 |

* Data are given in Table I.
* Determined by following ADP formation continuously with the pyruvate kinase-lactate dehydrogenase-coupled assay (see "Experimental Procedures").
* Determined by measuring inhibition of conversion of 5-oxo[3H]proline to [3H]glutamate (see "Experimental Procedures").
* Determined by measuring the rate of cleavage of ATP observed in the absence of a second substrate was 0.07 µmol/min/mg.
* Determined at a concentration of 5 mM.

### DISCUSSION

The data permit an approach to the mapping of the enzyme site that binds 5-oxo-L-proline, 5-Oxo-D-proline and D-isomers

**ATP.** The findings with the 2-oxo-5-methylthiazolidine-4-carboxylates led us to re-examine the earlier finding (17) that the enzyme did not cleave the ring of 3-methyl-5-oxoproline. We have now found that this compound is a substrate, although a poor one; it is converted to 3-methylglutamate at about 1% of the rate found with 5-oxoproline and the coupling ratio is about 21–23 (Table V).

### TABLE V

| Substrate                  | Products (mmol) | Coupling Ratio |
|----------------------------|-----------------|----------------|
| 2-oxo-5-methylthiazolidine | ATP | ADP | 34 |
| 2-oxo-5-methylthiazolidine | B | B | 2 |

**1** The reaction mixture contained initially 300 mM Na borate buffer (pH 10.0), 120 mM KC1, 5 mM MgCl2, 5 mM phosphopyruvate, 0.1 unit of pyruvate kinase, 1 mM ATP, 0.1 mM 3-oxo-4-carboxylic acid (0.1 mg of 3-oxo-4-carboxylic acid was used). The reaction mixture was incubated at 37°C for 30 min. The observed rate of ADP formation in about 12–16 cycles and was corrected for the 0.1 mg of 3-oxo-4-carboxylic acid. ATP was determined by the coupled pyruvate kinase-lactate dehydrogenase procedure.

In the earlier work (17), a relatively high concentration (20 mM) of this imino acid was used. This may have obscured the low activity of this substrate, which is also an effective inhibitor (Table V).

The data permit an approach to the mapping of the enzyme site that binds 5-oxo-L-proline, 5-Oxo-D-proline and D-isomers.
enzyme suggesting that even though the presence of a substituent on carbon atom 3 of 5-oxo-L-proline other than hydrogen does not prevent binding, the position of the amide bond is probably perturbed in such a manner as to interfere with proper alignment on the enzyme for substrate cleavage.

In conclusion, the present and previous findings indicate that binding of the imino acid substrate requires a carbonyl (or =NH) group at position 5 (neither L-proline nor L-thioproline is significantly bound). Although the ring nitrogen can be replaced by oxygen (with low activity), the enzyme cannot accommodate an N-methyl moiety (7). The substrate interacts with the enzyme in a manner such that its α-hydrogen atom is directed toward the enzyme, but the presence of an α-hydrogen atom is not an absolute requirement for binding. That 2-pyrrolidone is not a substrate (1) suggests that the 2-carboxyl group may be required. Other data indicating that DL-2-pyrrolidone-4-carboxylate and DL-5-carboxymethyl-2-pyrrolidone are poor inhibitors (8) and that they are not substrates also suggest that the 2-carboxyl group is required, but other structural possibilities remain to be explored. The present and previous studies indicate that substantial modification of the 5-oxo-L-proline molecule in the region of C-3 and C-4 is possible with retention of binding properties. For example, 2-piperidone-6-carboxylate, 3- and 4-oxy-5-oxoproline, 3- and 4-methyl-5-oxoproline, as well as a number of the compounds reported here which contain sulfur, oxygen, or nitrogen in the ring exhibit significant interaction with the active site. The markedly varied results observed with respect to coupling ratio and degree of inhibition reflect significant differences in the orientations of the substrates at the active site. It is remarkable that thus far only two compounds have been found (5-oxo-L-proline and L-2-oxothiazolidine-4-carboxylate) which exhibit very high affinity for the enzyme and coupling ratios of unity.

The activity of the enzyme toward L-2-oxothiazolidine-4-carboxylate has proven to be of practical value as a cysteine delivery system; thus, its administration to mice leads to a marked increase in the glutathione content of the liver (18). Presumably, the corresponding 4-oxygen analog, L-2-oxo-oxazolidine-4-carboxylate, would serve similarly for intracellular delivery of L-serine, just as 5-oxo-L-proline is itself an efficient precursor of intracellular L-glutamate (19).

REFERENCES
1. Van Der Werf, P., Griffith, O. W., and Meister, A. (1975) J. Biol. Chem. 250, 6686–6692
2. Wilson, H., and Cannan, P. K. (1937) J. Biol. Chem. 119, 309–331
3. Meister, A., Bukenberger, M. W., and Strassburger, M. (1963) Biochem. Z. 328, 217–229
4. Williamson, J. M., and Meister, A. (1982) J. Biol. Chem. 257, 9161–9172
5. Griffith, O. W., and Meister, A. (1976) Biochem. Biophys. Res. Commun. 70, 756–765
6. Griffith, O. W., Van Der Werf, P., and Meister, A. (1976) in Glutathione: Metabolism and Function (Arias, I. M., and Jakoby, W. B., eds) pp. 63–69, Raven Press, New York
7. Griffith, O. W., and Meister, A. (1981) J. Biol. Chem. 256, 9981–9985
8. Griffith, O. W., and Meister, A. (1982) J. Biol. Chem. 257, 4392–4397
9. Kaneko, T., Shimokobe, T., Otta, Y., Toyokawa, E., Unui, T., and Shibata, T. (1964) Bull. Chem. Soc. Jpn. 37, 242–244
10. Shah, H., Hartman, S. P., and Weinhouse, S. (1979) Cancer Res. 39, 3942–3947
11. Kaneko, T., and Inui, T. (1961) Nippon Kagaku Zasshi 82, 1075–1078
12. Cooper, A. J. L., and Meister, A. (1973) J. Biol. Chem. 248, 8409–8505
13. Ravel, J. M., McCord, T. J., Skinner, C. G., and Shive, W. (1958) J. Biol. Chem. 232, 159–168
14. Carter, H. E., and Zirkle, C. L. (1949) J. Biol. Chem. 178, 709–714
15. Penefsky, H. S. (1977) J. Biol. Chem. 252, 2891–2899
16. Segel, I. H. (1975) in Enzyme Kinetics, pp. 57 John Wiley and Sons, New York
17. Griffith, O. W., and Meister, A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3330–3334
18. Williamson J. M., and Meister, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 936–939
19. Sekura, R., and Meister, A. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2896–2899