Structure of the Dimeric \(\alpha\)-Keto Acid Analogue of Asparagine*

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SUMMARY

Studies on the dimer of \(\alpha\)-ketosuccinamic acid indicate that its structure is 4,6-dihydroxy-2-oxo-piperidine-5-carboxamide-4,6-dicarboxylic acid. The dimer was converted in acid solution to 2-hydroxy-pyridine-4,6-dicarboxylic acid; ultraviolet spectral evidence was obtained for intermediates in this transformation and studies with \(L\)-\([4\text{-}C]\)asparagine confirmed the pathway. On hydrogenation the dimer gave 4-hydroxy-2-oxo-piperidine-5-carboxamide-4,6-dicarboxylate. The proposed structure of the dimer is in accord with these transformations and is consistent with earlier data on the chemical and enzymatic properties of this compound.

\(\alpha\)-Keto acids are well established intermediates in the metabolism of amino acids; they are formed from amino acids by transamination and oxidative deamination, and in several instances the formation of an \(\alpha\)-keto acid is the penultimate step in the biosynthesis of the corresponding amino acid. Transamination or oxidation of most of the naturally occurring amino acids yields \(\alpha\)-keto acids which exhibit typical carbonyl group reactivity and which undergo decarboxylation in the presence of hydrogen peroxide (1). However, in several instances (notable examples are glutamine and asparagine), the newly formed \(\alpha\)-keto acid is spontaneously converted to a product that does not possess a reactive carbonyl group (2). Thus, \(\alpha\)-glutamylglutamic acid, the \(\alpha\)-keto acid analogue of glutamine, undergoes spontaneous cyclization to 5-hydroxy-2-pyrollidone-5-carboxylate (3).

In earlier work on the oxidative deamination of \(L\)-asparagine to \(\alpha\)-ketosuccinamic acid by \(L\)-amino acid oxidase it was observed that the isolated \(\alpha\)-keto acid product does not possess a reactive carbonyl group, but can be converted to a compound with the expected properties of \(\alpha\)-ketosuccinamic acid by treatment with mild alkali (2). Later it was found that the unreactive form of \(\alpha\)-ketosuccinamic acid is a dimer (3); to our knowledge, the structure of this compound has not been elucidated (Fig. 1, Structure III).

In previous studies (2) \(L\)-asparagine was oxidized by \(L\)-amino acid oxidase in the presence of catalase; after removal of the proteins, the solution containing the reaction products was passed through a column of Dowex 50 (Na+) which was eluted with water. A crystalline sodium salt was obtained from the concentrated effluent. This compound (as well as the corresponding free acid) did not give an immediate precipitate when treated with 2,4-dinitrophenylhydrazine. A very small precipitate formed gradually on standing for several days. On hydrolysis in 1 N HCl at 100\(^\circ\), only about 40\% of the total nitrogen was liberated as ammonia. This compound is a very poor substrate for both lactate dehydrogenase and \(\alpha\)-keto acid-\(\omega\)-amidase, and it is not decarboxylated by hydrogen peroxide under conditions in which a large number of other \(\alpha\)-keto acids are rapidly decarboxylated. When the unreactive form of \(\alpha\)-ketosuccinamic acid was dissolved in dilute alkaline solution, the reactive form of the \(\alpha\)-keto acid was produced as judged by the following properties. (a) A crystalline 2,4-dinitrophenylhydrazone precipitated soon after addition of the reagent, and it analyzed for the expected derivative of \(\alpha\)-ketosuccinamic acid. (b) Quantitative deamidation was observed in 1 N HCl at 100\(^\circ\), and pyruvic acid formation was demonstrated. (c) It was an excellent substrate of both lactate dehydrogenase and \(\alpha\)-keto acid-\(\omega\)-amidase, and it was rapidly and quantitatively decarboxylated by hydrogen peroxide.

The initial product of the oxidative deamination of asparagine is undoubtedly \(\alpha\)-ketosuccinamic acid (Structure II); since oxidation in the absence of catalase is accompanied by utilization of 1 mole of oxygen and formation of 1 mole of carbon dioxide per mole of asparagine oxidized (2). Furthermore, studies of \(\alpha\)-keto acid formation indicated that an \(\alpha\)-keto acid was formed initially as judged by formation of an alkali-soluble hydrazone; however, this property disappears on further incubation. Analytical ultracentrifugation studies by the method of Klainer and Kegeles (4) established that the unreactive form of \(\alpha\)-ketosuccinamic acid is a dimer (3).

The observation that the dimeric form of \(\alpha\)-ketosuccinamic acid does not readily form a hydrazone derivative indicates the absence of a free carbonyl group; that it does give a hydrazone on prolonged treatment suggests the presence of a carbonyl group which is masked or sterically prevented from reacting. It may be noted that \(\alpha\)-ketosuccinamic acid possesses an active methylene group between the amide and \(\alpha\)-keto groups, and is thus favored to undergo an aldol condensation with itself to give a...
dimeric carbinol. However, such a dimer would possess a reactive carbonyl group, and this structure would therefore not be consistent with the observed properties of the dimer of α-ketosuccinamic acid. On the other hand, such a dimeric carbinol could readily form the ketolactam (4,6-dihydroxy-2-oxo-piperidine-5-carboxamide-4,6-dicarboxylic acid; Fig. 1, III) in a manner analogous to the cyclization of α-ketoglutarate to 5-hydroxy-2-pyrrolidine-3-carboxylic acid (3). Structure III is consistent with the observation that acid hydrolysis releases less than 2 moles of the nitrogen as ammonia. Hydrolysis of an equivalent amount (2 moles) of Structure II liberates 2 moles of ammonia.

To examine further the possibility that Structure III is the dimeric form of α-ketosuccinamic acid, the dimer was allowed to stand in an acid solution. Under these conditions, one would expect III to undergo dehydration and aromatization. These transformations were followed spectrophotometrically (Fig. 2).

A freshly prepared solution of III in 1 M HCl exhibits an ultraviolet absorption curve with a shoulder at 250 to 270 nm (Fig. 2, Curve 2). After allowing the solution to stand for 1 hour at 37°, a maximum absorbance appears at 280 nm (Curve 3), which increases with time (Curve 4). This maximum is ascribed to the unsaturated imino chromophore in Structure IV. Before this band reaches its maximum, a new band appears at about 330 nm. On further incubation, the band at 260 nm decreases and the band at about 330 nm increases (Curve 4). This change appears to be associated with the dehydration of IV. After 48 hours at 37°, the spectrum shown in Fig. 2, Curve 5, appears. α-Ketosuccinamic acid (II and III) exhibits an absorbance maximum at 292 nm in alkaline solution (2). A similar absorbance maximum was observed when alkali was added during the reaction when the maximum at 270 nm was present. This is in accord with the reversibility of the initial dehydration to yield IV; presumably, once the aromatic system (Structure V) is formed, the reaction is not reversible. Alkaline solutions of Structure VII gave absorption curves that were virtually the same as those obtained in acid solution (Fig. 2; Curve 5). The observed spectral changes thus appear to be correlated with dehydration and aromatization (III → V). Structure V could not be isolated under the conditions employed; crystals of VII began to form as the band at 230 nm began to appear.

Structure VII (2-hydroxy-pyridine-4,6-dicarboxylic acid) was suggested by nuclear magnetic resonance and analytical data.
The product obtained had a melting point of 325-328°, a melting point of 318-319° has been reported for this compound (5). Assuming that VI is formed by deamination of V prior to decarboxylation, there are three possible structures for the pyridinol dicarboxylic acid depending on which carboxyl group is lost, i.e., 4,5-, 5,6-, or 4,6-pyridinol carboxylic acids. The 4,5- and 5,6-isomers have been described and reported to have melting points of 287-289° (6) and 253-254° (7), respectively. Confirmation of VII as the product obtained here was achieved by studies with 14C-labeled compounds. Thus, L-[4-14C]asparagine was converted via III to VII according to the scheme given in Fig. 1; (the labeled carbon atoms are marked with a closed circle). The specific radioactivity of VII was close to 50% of that of the L-[4-14C]asparagine from which it was derived. The data are therefore in accord with transformation of III to VII involving (a) removal of 2 molecules of water to yield IV and V, successively, (b) deamination of V to VI, and (c) decarboxylation of VI to VII.

Additional evidence for the proposed structure of the dimeric form of α-ketosuccinamic acid (III) and the intermediate Structure IV was obtained by catalytic hydrogenation of α-ketosuccinamic acid dimer in the presence of hydrochloric acid. The product VIII was obtained and identified as described below. Aqueous solutions of VIII exhibited maximum absorbance in the ultraviolet at 262 nm; this maximum did not change on acidification, but in alkaline solution the maximum shifted to 282 nm. This bathochromic shift is ascribed to the base-catalyzed opening of the ring to yield the enolic form of IX. Structure VIII was therefore in accord with transformation of III to VII involving (a) removal of 2 molecules of water to yield IV and V, successively, (b) deamination of V to VI, and (c) decarboxylation of VI to VII.

EXPERIMENTAL PROCEDURE

2-Hydroxy-pyridine-4,6-dicarboxylic Acid (VII) — The sodium salt of the dimer of α-ketosuccinamic acid (150 mg; prepared as previously described (2)) was dissolved in 15 ml of 1 N HCl and incubated at 37°. After 12 hours dark brown crystals began to form, and after 48 hours the crystals were collected and dried (30 mg; m.p. 320-325°). The product was recrystallized from 1

\[
\text{C}_4\text{H}_6\text{N}_2\text{O}_5
\]

Calculated: C 45.9, H 2.75, N 7.64

Found: C 45.8, H 2.89, N 7.49

UV\text{\textsubscript{max}} (H_2O), 325 nm (ε 1220); NMR (dimethylsulfoxide), δ 2.60 (d, 1, J = 1 Hz), δ 2.85 (d, 1, J = 1 Hz).

Conversion of L-[4-14C]Asparagine into 2-Hydroxy-4,6-dicarboxylic Acid (VII) — L-[4-14C]Asparagine (Calbiochem) was converted into sodium α-[14C]ketosuccinamic dimer (2), and 40 mg of the 14C keto acid (specific radioactivity, 14.06 mCi/mole) was dissolved in 5 ml of 1 N HCl. After 48 hours at 37°, 16 mg of crystalline pyridinol were obtained as described above (m.p. 320-325°; specific radioactivity, 7.30 mCi/mole).

Hydrogenation of α-Ketosuccinamic Acid — α-Ketosuccinamic acid dimer (500 mg) was dissolved in 10 ml of water; 0.05 ml of concentrated HCl and 100 mg of platinum oxide catalyst were added. Hydrogenation was carried out in a Paar apparatus for 18 hours at 25° and 45 p.s.i. The catalyst was filtered off and the filtrate was evaporated to a colorless oil which crystallized. The product was recrystallized from water-methanol containing a drop of concentrated HCl; yield, 30 mg; m.p. 186-188°. The product gave a positive Nessler's test.

\[
\text{C}_6\text{H}_6\text{N}_2\text{O}_5
\]

Calculated: C 36.5, H 4.97, N 15.9

Found: C 36.5, H 4.72, N 15.8

UV\text{\textsubscript{max}} (H_2O), 262 nm (ε 2100), (1 N NaOH), 286 nm (ε 22400).

DISCUSSION

The findings indicate that α-ketosuccinamic acid undergoes dimerization by an aldol condensation and reaction of its amide nitrogen to form a ketohydrate. The latter reaction is analogous to the cyclization of α-ketoglutaramic acid (8), the condensation of cinnamoylacetaldehyde with cyanacetamide (5), and to the transannular addition of amides to ketones in medium-sized rings (8). Other conceivable structures of the dimer involving rings that contain oxygen atoms or 2 nitrogen atoms are excluded by the present work which demonstrates conversion of the dimer of α-ketosuccinamic acid to pyridine derivatives. The proposed structure for the dimer (III) is in accord with the chemical transformations described and is also consistent with its lack of reactivity with 2,4-dinitrophenylhydrazone and hydrogen peroxide, the limited extent of its deamination on acid hydrolysis, and its lack of susceptibility to lactate dehydrogenase and α-keto acid-ω-amidase.

Transamination or enzymatic oxidation of asparagine in cells that lack α-keto acid-ω-amidase might lead to the in vivo formation of α-ketosuccinamic acid dimer. The formation of dimer
would be expected to drive the transamination reaction in the direction of asparagine utilization. However, the possible physiological significance of the dimerization of \( \alpha \)-ketosuccinamic acid requires additional study. The spontaneous cyclization of \( \alpha \)-ketoglutaramic acid represents a somewhat analogous situation (9, 10). The possibility that the dimerization of \( \alpha \)-ketosuccinamic acid may be enzymatically catalyzed must be considered. The ability of \( \alpha \)-keto acids to undergo dimerization has long been known (11). A number of enzymes that catalyze aldol condensations exist, and an enzyme has been found that catalyzes the aldol cleavage of \( \gamma \)-methyl-\( \gamma \)-hydroxy-\( \alpha \)-ketoglutarate to 2 moles of pyruvate and also the rapid incorporation of pyruvate into this compound (12, 13). The formation of the ketolactam from the dimer would be expected to occur rapidly and nonenzymatically in analogy with \( \alpha \)-ketoglutaramic acid.

REFERENCES

Corrections

Vol. 246 (1971), 7115–7118

In STEPHANI, RALPH A., AND ALTON MEISTER. Structure of the Dimeric α-Keto Acid Analogue of Asparagine.

Page 7117, left-hand column, sixteen lines from the bottom of the page (ε 1220) should be (ε 6800) so that the line reads:

UVₘₐₓ (H₂O), 325 nm, (ε 6800); NMR (dimethylsulfoxide),

Vol. 246 (1971), 7821–7823

In EMURA, JUNJI, TOKUJI IKENAKA, JOHN H. COLLINS, AND KARL SCHMID. The Constant and Variable Regions of the Carboxyl-terminal CNBr Fragment of α-Acid Glycoprotein.

The following note was inadvertently omitted from the final publication:

Editorial Note:

At the request of the Editors the authors furnished a summary of the detailed evidence upon which is based the reported amino acid sequence of the carboxyl-terminal CNBr-fragment. The similarity of this sequence to the sequences in fragments designated III and IV in the present Communication led the Editors to waive a similar request for supporting data concerning these fragments. The authors are preparing fully documented reports of these researches.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
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