Ornithine Cyclase (Deaminating)

III. MECHANISM OF THE CONVERSION OF ORNITHINE TO PROLINE*

Ornithine cyclase catalyzes the transformation of ornithine to proline in a unique enzymatic reaction (1-4). The cyclase, which is a single protein, appears to catalyze a balanced, oxidation-reduction reaction in which the intermediates remain enzyme-bound. It is stimulated by catalytic amounts of NAD⁺ and the pure enzyme contains approximately 1 mol of bound NAD⁺ per mol of enzyme (4). Some stimulation of the cyclase was apparent with ADP or ATP, although no sigmoidal kinetics were observed and the apparent Kₘ for ornithine was unchanged by the addition of ADP. No other cofactors have been identified. Two alternative mechanisms are proposed, depending on the amino group deaminated during the reaction. The present study demonstrates that it is the α-amino group which is removed from ornithine during the cyclase reaction. In addition, evidence is presented concerning the role of the enzyme-bound NAD⁺.

MATERIALS AND METHODS

Heavy Nitrogen Experiment

Synthesis of [6-15N]Ornithine—[8-15N]Ornithine was synthesized from α-amino adipic acid and potassium 15N-labeled (99.4 atom % enriched, Mallinckrodt Nuclear, St. Louis, Mo.) in a Schmidt degradation reaction by a procedure adapted from two sources (5, 6).

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The newly synthesized amino acid was purified on Dowex 50 (H⁺-form columns), dried, and dissolved in distilled water. The identity of the amino acid synthesized was confirmed as ornithine from (a) paper isoelectric focusing in 0.125 M sodium acetate (pH 5.2), 0.5 M acetic acid (pH 2.6), and 0.2 M formic acid (pH 2.0); (b) descending chromatography on Whatman No. 3MM paper developed in butanol-acetic acid-water (12:3:5); and (c) by ascending chromatography on thin layer silica gel developed with chloroform-methanol-15% ammonium hydroxide (36:46:20). Assay of the product by the reduced ninhydrin method (7) indicated the yield was 250 pmol or 16.6%. Samples of the synthesized product and authentic ornithine were examined in the combined gas chromatograph-mass spectograph after preparing TMS derivatives of each.

Enzyme Reactions Involving [6-15N]Substrate Labeled [6-15N]ornithine and unlabeled ornithine were converted to proline in reaction mixtures similar to the standard anaerobic, radiochemical assay (3), except that Cu²⁺ was added to these reactions to inhibit proline reductase activity. Each reaction mixture contained 15 μl each of 0.25 M Tris chloride buffer (pH 8.0), 0.1 mM mercaptoethanol, 1 mM NAD⁺, and 66 mM CuCl₂. The source of the cyclase was ammonium sulfate-treated and dialyzed extract from Clostridium sporogenes, which still contained some proline reductase activity. The reaction mixtures contained 75 μl each of either 0.25 M Tris chloride buffer (pH 8.2), 50 mM α-ketoglutarate, 20 mM ADP, and 40 mM NAD⁺. The reaction was started by adding 35 μl of either 0.2 μl labeled or unlabeled substrate. The reaction mixtures were incubated for 2 hours at 57°C to allow for maximum conversion to take place. The protein was heat-inactivated. A radiochemical assay run concurrently indicated that about 3.3 μmol of proline were formed by the enzyme.

Some of the NH₃ liberated by the cyclase reaction was trapped in glutamate by the use of glutamate dehydrogenase. The following reagents for this reaction were added to each of the reaction mixtures after heating to stop the cyclase reaction: 30 μl each of 1.5 M Tris chloride buffer (pH 8.2), 50 mM α-ketoglutarate, 20 mM ADP, and 40 mM NADH. The reaction was started by adding 8.5 units (10 μl) of glutamate dehydrogenase. The reaction mixtures were incubated at 30°C for 1 hour. The protein was precipitated by heating each tube to 80°C for 5 min. The residual ornithine and newly formed proline and glutamate were separated from other reaction components by Dowex 50 (H⁺-form) column chromatography. The amino acids were dried in a rotary evaporator and then dissolved in a minimum volume of distilled water.

Analytical Methods—Samples containing 100 μg each of 6-15N-labeled or unlabeled ornithine and samples of the suspended enzymatic products estimated to contain approximately 100 μg of proline and 35 μg of glutamate were dried at 50°C in small (6 x 50 mm) tubes inside screw-capped tubes (13 x 100 mm). After drying, the sample tubes were maintained under dry nitrogen or argon. The dried samples were then solubilized in 50 μl of aceeto-
nitrile and 50 μl of bis-(trimethylsilyl)trifluoroacetamide containing 1% (v/v) trimethylchlororosilane (8, 9). Each sample was heated to 77°C for at least 30 min to facilitate the trimethylsilylation reaction.

Samples (2 to 4 μl) of the trimethylsilyl derivatives were analyzed in an LKB-9000 combined gas chromatograph-mass spectrometer equipped with a silanized glass column (3.0 mm × 1.83 m) packed with 1% (w/v) DE-50 on 100 to 200 mesh, Supelco, Inc.). The column temperature was maintained at 100°C until proline was eluted. The temperature was increased at a rate of 7.3°C per min to 140°C, maintained until glutamate was eluted. The temperature was increased to 150°C and at 160°C. The helium carrier gas flow rate was 25 cc per min. The ion source temperature of the mass spectrometer was 290°C, and the ionizing voltage was 70 e.v. All determinations of intensities of the various ions were made from normalized bar graphs (10). Isotope incorporation was measured by determining the ratio I_proline/I_ornithine, where I_proline is the intensity of the ion at m/e = p and I_ornithine is the intensity of the ion at m/e = p + 1.

Reduction of Bound NAD+<sup>+</sup>

Pure cyclase (0.20 ml containing 570 μg of protein, specific activity 0.84), 20 μl of 0.1 mM NAD<sup>+</sup>, and 0.3 ml of H<sub>2</sub>O were placed in each of two 0.5-ml cuvettes (10 mm light path), flushed with argon, sealed, and preincubated for 15 min at 30°C. Then 30 μl of 0.2 M L-ornithine were added to one cuvette while an equal volume of water was added to the other. The absorbance at 340 nm of both cuvettes was measured with a Gilford model 2000 spectrophotometer. The recorder was set for 0 → 0.1 full scale absorbance.

Tritium Exchange from NADT

Chemical Reduction of NADT in T<sub>3</sub>O—The synthesis of NADT labeled with tritium in position 4 of the pyridine ring was similar to the procedures used by Ohlmeyer (11) and Fisher et al. (12). NAD<sup>+</sup> (98% pure), 100 mg, was dissolved in 2 ml of tritiated water (total activity 200 mCi) contained in an acid-cleaned stoppered tube (16 × 150 mm) flushed with argon. Then 50 mg of sodium hydrosulfite (dithionite) and 104 mg of NaHCO<sub>3</sub> were added and dissolved as a mixture. The tube was flushed with argon again and incubated at 30°C for 2 to 5 hours. The reduced NAD<sup>+</sup> was crystallized by addition of 25 ml of absolute ethanol at −20°C. A second crop of crystals was obtained by adding 11 ml of absolute ethanol to the supernatant solution. The crystals of NADT were dissolved in a small volume of water and recrystallized to remove excess tritium from the compound. To remove any residual oxidized tritium, the recrystallized NADT was dissolved in 8 ml of H<sub>2</sub>O and concentrated by ultrafiltration through a Diaflo UM-2 membrane (Amicon Corporation, Cambridge, Mass.). The concentrated compound was diluted to 10 ml and concentrated again to a volume of 2.0 ml. A small sample was counted for tritium content, and an additional sample was assayed for reduced NAD<sup>+</sup> content by measuring the absorbance of a diluted sample at 340 nm. The yield was 69% or 70 μmol of NADT with 15,000 cpm per μmol. This procedure should result in approximately 30% of the reduced compound carrying the tritium label in the A form and 70% in the B form (13).

Production of Δ<sup>1</sup>-Pyrrrole-2-carboxylic Acid—Δ<sup>4</sup>-Pyrrrole-2-carboxylic acid was produced by the oxidation of d-proline by δ-amino acid oxidase in the presence of excess catalase in a Warburg respirometer (2, 14). The protein was removed by addition of trichloroacetic acid (5%, w/v) followed by extraction of the trichloroacetic acid with ether. Ether solubilized in the water was removed by bubbling argon through the solution. Oxygen consumption data indicated that approximately 300 μmol of Δ<sup>1</sup>-pyrrrole-2-carboxylic acid were formed from 50 μmol of NADT at 25°C.

Incorporation of NADT into Proline—Enzyme-catalyzed reactions to determine the extent of incorporation or tritium from NADT into proline by the cyclase in the presence of ornithine alone or with Δ<sup>1</sup>-pyrrrole-2-carboxylic acid were similar to the standard radiochemical assay for the cyclase. Each reaction mixture contained: 19.2 mM Tris chloride (pH 8.0), 7.7 mM mercaptoethanol, 1.5 mM ADP, and 0.025 ml of pure cyclase (specific activity, 0.86) prepared as described previously (4). Where indicated, 2 μmol of ornithine, 1.2 μmol of Δ<sup>1</sup>-pyrrrole-2-carboxylic acid, 5 nmol of NAD<sup>+</sup>, and 0.35 μmol of NADT were added alone or in various combinations. The total volume of each reaction was adjusted to 65 μl with distilled water. Each reaction was incubated for 2 hours at 37°C. Reactions were stopped and protein precipitated by addition of 25 μl of 0.5 M formic acid. An aliquot (40 μl) of each reaction mixture was spotted on Whatman No. 3MM paper and developed in descending fashion with butanol-acetic acid-water (12:3:5). The chromatogram was dried and sprayed with 0.01% ninhydrin. The proline spots were cut out and the radioactivity determined in a liquid scintillation spectrometer as outlined previously.

RESULTS

Heavy Nitrogen Experiment—TMS derivatives prepared from either δ-N-labeled or unlabeled ornithine each yielded gas chromatographic peaks. Mass scans taken as each peak eluted from the column were consistent with a tri-TMS and a tetra-TMS derivative of ornithine corresponding to peaks B and D of Fig. 1, respectively. Analysis of the parent ion and four other prominent peaks obtained from the tetra-TMS-ornithine peak indicated the δ-amino group was 28.0 atom % enriched for <sup>15</sup>N. The method of synthesis ensured that all label was directed to the δ-position.

TMS derivatives of the proline and glutamate from reaction mixtures were readily separated from each other and from residual ornithine and several other compounds present in the derivatized sample by gas chromatography (Fig. 1). Four ion peaks from the mass spectrum of proline were examined (Table 1). The parent ion, (TMS)<sub>3</sub>-proline (M), was not included since it was either of very low intensity or absent in every mass spectrum taken. The largest molecular ion consistently present was that at m/e = 244 (Fig. 2) which corresponds to M − 15 (the loss of one methyl group). Other prominent ions used were those at m/e = 216, 142, and 70. Each of these contain the nitrogen from the proline ring. The ion at m/e = 216 may be regarded as TMS—N = CH—COO—TMS. The base peak at m/e = 142 (M − 117) represents one of the expected products of a fission

Fig. 1 Gas chromatography of the trimethylsilylated amino acids separated from a reaction mixture containing unlabeled ornithine, proline, and glutamate. The tracing is the total ion current detector response and the dotted line is the column temperature. For other components of the reaction mixture and isolation and preparation of samples consult "Materials and Methods." Mass spectral analysis of each peak indicated that Peak A was (TMS)<sub>3</sub>-proline, Peak B was (TMS)<sub>3</sub>-ornithine, Peak C was (TMS)<sub>3</sub>-glutamate, and Peak D was (TMS)<sub>4</sub>-ornithine.
TABLE I

Incorporation of N into proline

For conditions of each reaction and purification scheme see "Materials and Methods."

| m/e | 1N³ | 1N⁴ | 1N⁴ - 1N³ |
|-----|-----|-----|-----------|
| 70/71 | 12.0 ± 6.8 | 43.5 ± 3.1 | 31.5 |
| 142/143 | 10.9⁴ | 40.2⁴ | 29.3 |
| 216/217 | 18.0 ± 0.63 | 46.5 ± 2.9 | 28.5 |
| 244/245 | 17.1 ± 0.6 | 42.2 ± 6.8 | 25.1 |

Ave. = 28.6 ± 2.6

a See Table I.

b Proline produced from unlabeled ornithine by partially purified cyclase.

c Proline produced from [6N]ornithine by partially purified cyclase.

d The error quoted is the standard deviation of three determinations.

Fig. 2. Comparison of part of the mass spectrum of (TMS)_3-proline derived catalytically from unlabeled ornithine (a) with that of proline from [6N]ornithine (b).

For conditions used in each reaction and purification scheme see "Materials and Methods."

| m/e | 1N³ | 1N⁴ | 1N⁴ - 1N³ |
|-----|-----|-----|-----------|
| 218/219 | 19.9 ± 3.5⁴ | 26.0 ± 2.7 | 6.1 |
| 246/247 | 21.2 ± 8.2 | 20.0 ± 7.0 | -1.1 |
| 348/349 | 21.0 ± 0.9 | 21.7 ± 1.1 | 0.7 |
| 363/364 | 20.1 ± 9.5 | 18.7 ± 3.4 | -1.4 |

Ave. = 11.1 ± 3.9

a See Table I.

b Glutamate generated with the NH₃ released from unlabeled ornithine by partially purified cyclase.

c Glutamate generated with the NH₃ released from [6N]ornithine by partially purified cyclase.

d The error quoted is the standard deviation of three determinations.

Fig. 3. Comparison of part of the mass spectrum of (TMS)_3-glutamate derived catalytically from α-ketoglutarate and the ammonia generated from either unlabeled (a) or [6N]ornithine (b).

The NH₃ group of glutamate must be derived from the α-NH₃ group released from ornithine by the cyclase.

Role of Bound NAD⁺—Following addition of substrate to highly purified cyclase a transient absorption peak at 340 nm appeared (Fig. 4). The data were corrected for absorbance due to both enzyme and NAD⁺. Thus, the net absorbance shown was most likely due to a transient buildup of reduced NAD⁺ (NADH). Addition of a second aliquot of substrate caused the reappearance of the absorbance peak at 340 nm. Calculations based on the cyclase containing 1 mol of NAD⁺ per mol of enzyme, the protein concentration (25.2 mM), the molar absorbance for NADH (6.2 x 10⁶ M⁻¹ cm⁻¹), and the peak absorbance value (0.039) indicate that under these conditions 24.8% of the bound NAD⁺ may be present in the reduced state.

Incorporation of NADT into Proline and Oxidation of NADH—There was no apparent incorporation of tritium from NADT...
0.24 nmol of proline was formed when 2.0 nmol of [U-15N]ornithine and 2.5 nmol of NAD+. In a control reaction, 8.0), 0.1

\[ \text{NAD}^+ \text{specific activity, 0.86} \] added to each reaction tube contained 0.5

\[ \text{mM dithiothreitol, 0.2 mM NADH, approximately 40 mM} \]

[Tris-HCl (pH 8.0), 0.1 mM mercaptoethanol, 0.02 mM ADP, and 0.35 mmol of NADT (specific activity, 15,000 cpm per mmol). Cyclase (29.5 µg, specific activity, 6.84) See "Materials and Methods" for details.

**Table III**

Incorporation of tritium from NADT into proline with ornithine or with \( \Delta^1 \)-pyrroline-2-carboxylic acid as substrates

| Addition          | Proline  |
|-------------------|----------|
| Ornithine         | 2-PCA\*  | NAD\* | Cyclase | cpm |
| 1                 | 2.0      | -     | -       | -    | 320 |
| 2                 | 2.0      | -     | -       | +    | 315 |
| 3                 | -        | 1.2    | -       | +    | 316 |
| 4                 | -        | -      | 0.005   | -    | 327 |
| 5                 | -        | 1.2    | -       | +    | 301 |
| 6                 | 1.0      | 1.2    | -       | -    | 293 |

* 2-PCA, \( \Delta^1 \)-pyrroline-2-carboxylic acid.

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Transient 340 nm absorbing peak observed upon addition of 30 µl of 0.2 M L-ornithine to 200 µl of cyclase (570 µg of protein, specific activity, 6.84). See “Materials and Methods” for details.

**DISCUSSION**

Previous data (2, 3) indicated that the conversion of ornithine to proline by ornithine cyclase might proceed in one of two possible directions. The direction taken would obviously depend on which amino group was involved in the deamination. Loss of the \( \delta \)-amino group would lead to the formation of glutamic \( \gamma \)-semialdehyde which would undergo ring closure to form \( \Delta^1 \)-pyrroline-5-carboxylic acid. Deamination in the \( \alpha \) position would result in 2-oxo-5-aminopentanoic acid and \( \Delta^1 \)-pyrroline-2-carboxylic acid.

The conservation of 15N from [\( \delta^1 \)-15N]ornithine in proline during the conversion of ornithine to proline by the cyclase enzyme established with certainty that it is the \( \alpha \)-amino group of ornithine which is removed. Thus, it is highly probable that 2-oxo-5-aminopentanoic acid and \( \Delta^1 \)-pyrroline-2-carboxylate are intermediates in the reaction.

The appearance of a transient peak at 340 nm after addition of substrate and the presence of 1 mol of tightly bound NAD\(^+\) per mol of cyclase (4) strongly suggest that the absorbance is due to enzyme-bound NADH. Thus, the enzyme-bound NAD\(^+\) appears to function as a catalytic cofactor in an enzyme-bound oxidative deamination and reduction as postulated. The failure of NADH to exchange onto the cyclase is not unreasonable since the affinity for NAD\(^+\) is very high (apparent \( K_m \) = 6.1 µM, see Reference 3) and NADH generally binds more strongly to a dehydrogenase than NAD\(^+\) (16). Of course, it is possible that isotope selection might hinder the exchange, since Bleville et al. (17) noted a large negative isotope effect with UDP-\( \Delta^1 \)-pyruvate 4\'-epimerase using UDP-\( \Delta^1 \)-pyruvate-4-T as substrate.

In an earlier paper (3), Costlow and Laycock showed that no tritium was incorporated into nonexchangeable positions in proline when the cyclase reaction was run in an assay mixture containing Tracer. Again, barring complete isotope selection, the lack of tritium incorporation into proline is consistent with an enzyme-bound oxidative deamination-reduction mechanism involving NAD\(^+\), since all known dehydrogenases add and remove hydrogens from nonexchangeable positions, while the other involved hydrogen is in a freely exchangeable position on the substrate molecule (18). Also the UDP-\( \Delta^1 \)-pyruvate 4\'-epimerase has a postulated internal oxidation-reduction mechanism and fails to incorporate tritium from Tracer into the product (19).

Thus, we propose that the conversion of L-ornithine to L-proline by ornithine cyclase (deaminating) proceeds as follows: (a) enzyme-bound ornithine is oxidized to 2-oxo-5-aminopentanoic acid with the reduction of enzyme-bound NAD\(^+\) and the release of \( \text{NH}_3 \), (b) the bound 2-oxo-5-aminopentanoic acid undergoes ring closure to form \( \Delta^1 \)-pyrroline-2-carboxylic acid, and (c) the pyrrole ring is then reduced to form L-proline using the bound NADH as the reductant. The proline is then released from the enzyme. However, attempts to demonstrate the half reaction between NADH and \( \Delta^1 \)-pyrroline-5-carboxylic acid were unsuccessful. Therefore, if these two compounds are true
intermediates in the conversion of ornithine to proline, they are apparently irreversibly bound to the enzyme during the reaction.

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