Ingles, D. W., and Knowles, J. R. (1966), *Biochem. J. 99,* 275.
Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N.Y., McGraw-Hill, pp 500-501.
Johnson, C. H., and Knowles, J. R. (1966), *Biochem. J. 101,* 56.
Kezdy, F. J., and Bender, M. L. (1962), *Biochemistry 1,* 1097.
Kezdy, F. J., and Bender, M. L. (1965), *Biochemistry 4,* 104.
Kezdy, F. J., Clement, G. E., and Bender, M. L. (1964), *J. Am. Chem. Soc. 86,* 3690.
Lucas, E. C., and Caplow, M. (1972), *J. Am. Chem. Soc. 94,* 960.
Lucas, E. C., Caplow, M., and Bush, K. J. (1973), *J. Am. Chem. Soc. 95,* 2670.
McConn, J., Ku, E., Himoe, A., Brandt, K. G., and Hess, G. P. (1971), *J. Biol. Chem. 246,* 2918.
Marini, J. L., and Caplow, M. (1971), *J. Am. Chem. Soc. 93,* 5560.
Martin, R. B., and Hedrick, R. I. (1962), *J. Am. Chem. Soc. 84,* 106.
Morihara, K., Oka, T., and Tsuzuki, H. (1969), *Biochem. Biophys. Res. Commun. 35,* 210.
Oppenheimer, H. L., Labouesse, B., and Hess, G. P. (1966), *J. Biol. Chem. 241,* 2720.
Philipp, M. (1971), Dissertation, Northwestern University.
Philipp, M., and Bender, M. L. (1973), *Nature (London), New Biol. 241,* 44.
Philipp, M., Pollack, R. M., and Bender, M. L. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 517.
Polgar, L. (1972), *Acta Biochim. Biophys. Acad. Sci. Hung. 7,* 319.
Rajender, S., Lumry, R., and Han, M. (1971), *J. Phys. Chem. 75,* 1375.
Renard, M., and Fersht, A. R. (1973), *Biochemistry 12,* 4713.
Robertus, J. D., Kraut, J., Alden, R. A., and Birktoft, J. J. (1972), *Biochemistry 11,* 4293.
Smallcombe, S. H., Ault, B., and Richards, J. H. (1972), *J. Am. Chem. Soc. 94,* 4585.
Tables of Chemical Kinetics (1950), Homogeneous Reactions, National Bureau of Standards Circular 510.
Weiner, H., and Koshland, D. E., Jr. (1965), *J. Biol. Chem. 240,* 2764.
Williams, A. (1970), *Biochemistry 9,* 3383.
Williams, R., and Bender, M. L. (1971), *Can. J. Biochem. 49,* 210.
Zeeberg, B., Caplow, M., and Caswell, M. (1975), *J. Am. Chem. Soc. 97,* 7346.
Zerner, B., Bond, R. P. M., and Bender, M. L. (1964), *J. Am. Chem. Soc. 86,* 3674.

Comparison of the Biosynthetic and Biodegradative Ornithine Decarboxylases of *Escherichia coli*†

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ABSTRACT: Biosynthetic ornithine decarboxylase was purified 4300-fold from *Escherichia coli* to a purity of approximately 85% as judged by polyacrylamide gel electrophoresis. The enzyme showed hyperbolic kinetics with a K_m of 5.6 mM for ornithine and 1.0 μM for pyridoxal phosphate and it was competitively inhibited by putrescine and spermidine. The biosynthetic decarboxylase was compared with the biodegradative ornithine decarboxylase [Applebaum, D., et al. (1975), *Biochemistry 14,* 3675]. Both enzymes were dimers of 80 000-82 000 molecular weight and exhibited similar kinetic properties. However, they differed significantly in other respects. The pH optimum of the biosynthetic enzyme was 8.1, compared with 6.9 for the biodegradative. Both enzymes were activated by nucleotides, but with different specificity. Antibody to the purified biodegradative ornithine decarboxylase did not cross-react with the biosynthetic enzyme. The evolutionary relationship of these two decarboxylases to the other amino acid decarboxylases of *E. coli* is discussed.

Ornithine decarboxylase is widely distributed in nature and catalyzes the synthesis of putrescine from ornithine (for recent reviews of polyamine biosynthesis, see Tabor and Tabor, 1972; Morris and Fillingame, 1974). In *Escherichia coli,* ornithine decarboxylase is induced to high levels by low pH and the presence of substrate (Applebaum et al., 1975), thus classifying it as one of the biodegradative amino acid decarboxylases (Gale, 1946; Morris and Fillingame, 1974). The biodegradative ornithine decarboxylase of *E. coli* has been purified and characterized (Applebaum et al., 1975). Under noninducing conditions, i.e., at neutral pH and in minimal culture medium, *E. coli* contains low levels of ornithine decarboxylase activity for synthesis of putrescine and spermidine (Morris and Pardee, 1965, 1966). It is not known whether this low activity is due to a distinct ornithine decarboxylase or to simply the uninduced level of the biodegradative enzyme. Both biosynthetic and biodegradative enzymes for the decarboxylation of arginine have been demonstrated in *E. coli* (Blethen et al., 1968; Wu and Morris, 1973a,b). In this paper, we describe the purification of ornithine decarboxylase from uninduced cells and compare its properties with those of the biodegradative enzyme.

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Materials and Methods

Unless otherwise stated, materials and methods were as described previously (Applebaum et al., 1975).

For production of antiserum to biodegradative ornithine decarboxylase, 2–3 mg of the highly purified enzyme (Applebaum et al., 1975) was emulsified with Freund's complete adjuvant and injected interdermally into a New Zealand female rabbit. The animal was reinjected 8 and 10 weeks after the initial injection; for this, the enzyme solution (approximately 2 mg per mL of 0.15 M NaCl) was mixed with an equal volume of 2% aluminum potassium sulfate and 1 mL was administered intravenously. One week after the last injection, the animal was bled by heart puncture. After clotting, the serum was collected and fractionated by precipitation with ammonium sulfate at 50% saturation. The precipitated fraction was exhaustively dialyzed against 1 mM potassium phosphate buffer (pH 7.4), clarified by centrifugation, and brought to 0.15 M with NaCl. The antiserum was stored at −20 °C. This dialyzed antibody fraction was used for Ouchterlony double immunodiffusion studies in 1% agarose (0.15 M NaCl–0.4 mM pyridoxal phosphate).

The activity assay measured the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine at 37 °C as described by Applebaum et al. (1975). Unless otherwise stipulated, the reaction mixture (0.3 mL) contained 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 8.25), 0.04 mM pyridoxal phosphate, 1.67 mM dithiothreitol, and 7.4 mM L-[1-^{14}C]ornithine of appropriate specific activity. For assays at pH 7.0, 100 mM 3-(*N*-morpholino)propanesulfonic acid was substituted as buffer in the above reaction mixture. One unit of ornithine decarboxylase was defined as the amount of enzyme catalyzing the release of 1 μmol/min under the above conditions.

Results

Purification and Properties of Biosynthetic Ornithine Decarboxylase: Growth of E. coli. E. coli strain UW44, which produces both the biosynthetic and biodegradative ornithine decarboxylases (Applebaum et al., 1975), was grown with aeration at 37 °C in medium 63 of Cohen and Rickenberg (1956) supplemented with trace elements (Ames et al., 1960), 0.2% glucose, and yeast extract (0.05%). The cells were harvested in late log phase (A_{540} = 1.2), washed once with 0.9% NaCl, and stored as a paste at −20 °C. For 100 L of culture, the yield of cells ranged from 150 to 200 g wet weight.

Crude Extract. One kilogram of thawed cells was suspended in 3 L of buffer A (0.05 M potassium phosphate, pH 6.0, containing 1 mM dithiothreitol, 5 mM $MgSO_4$, and 0.04 mM pyridoxal phosphate). Aliquots (250 mL) of the cell suspension were cooled in a bath at −15 °C during sonication for 20 min with a Bronson S-75 sonifier at 5.5 A. Particulate matter was removed by centrifugation at 16 000*g* for 50 min. The pooled supernatant fractions were then diluted with buffer A to A_{280} = 116.

Streptomycin Precipitation. For every 100 mL of the diluted crude extract, 7.5 mL of a freshly prepared 10% (w/v) streptomycin sulfate solution was added dropwise with stirring. After 30 min of stirring in an ice bath, the preparation was centrifuged at 12 500*g* for 40 min and the supernatant solutions were pooled.

Ammonium Sulfate Fractionation. Buffer B (0.05 M potassium phosphate, pH 6.0, containing 15 mM β-mercaptoethanol, 5 mM $MgSO_4$, and 0.04 mM pyridoxal phosphate) was saturated with ammonium sulfate at room temperature and stored at 5 °C. This solution was added dropwise with stirring to the streptomycin supernatant fluid at 0 °C. After stirring for 30 min, the suspension was centrifuged at 12 500*g* for 30 min. The precipitate formed between 0 and 39% saturation was discarded. The fraction between 39 and 50.5% saturation, containing biosynthetic ornithine decarboxylase, was resuspended in 2 L of buffer A.

Heat Treatment. The ammonium sulfate fraction was dialyzed for 24 h against three 20-L changes of 0.2 M potassium phosphate buffer, pH 6.3, supplemented as for buffer B. After dialysis, the pyridoxal phosphate concentration was increased to 0.44 mM and the solution was stirred for 2 h. Aliquots (30 mL) of the solution were heated at 62 °C for 10 min in 25 × 150 mm Pyrex culture tubes with periodic shaking. The samples were cooled rapidly to 0 °C, combined, and centrifuged at 16 000*g* for 25 min. The supernatant solutions were pooled and dialyzed against three 20-L changes of buffer B.

Hydroxylapatite Chromatography. A hydroxylapatite (Bio-Rad Laboratories) column (5 × 20 cm) was washed with 5 column volumes of 0.05 M potassium phosphate buffer, pH 6.0, followed by 3 volumes of buffer B. The dialyzed, heat-treated preparation was applied to the column, followed by 800 mL of the final washing buffer. A 2-L linear gradient from 0.05 to 0.25 M potassium phosphate buffer, pH 6.0, supplemented as for buffer A, was applied. The fractions containing ornithine decarboxylase activity were pooled, concentrated to 20 mL in an Amicon ultrafiltration cell with a PM10 membrane, and dialyzed twice against 1 L of 0.4 M sodium acetate, pH 5.6 (containing 0.04 mM pyridoxal phosphate and 15 mM mercaptoethanol).

Gel Filtration on G-200 Sephadex. The dialyzed enzyme was made 10% in glycerol and layered onto the top of the gel bed (5 × 51 cm). Ornithine decarboxylase activity eluted after 400 mL had passed through the column. The active fractions were pooled, concentrated by Amicon ultrafiltration, and dialyzed twice for 6 h against 1 L of 0.05 M sodium succinate buffer, pH 5.9, containing 0.04 mM pyridoxal phosphate and 15 mM β-mercaptoethanol.

DEAE[1]-A25 Sephadex. The dialyzed enzyme was applied to a DEAE-A25 Sephadex column equilibrated with the same buffer used for dialysis. The column was further washed with 20 mL of buffer and the enzyme was eluted by a 2-L linear gradient from 0.05 to 0.2 M sodium succinate buffer, pH 5.9, supplemented as for dialysis. The active fractions were pooled and concentrated with the Amicon ultrafiltration cell.

A typical purification from 1 kg of thawed cells is summarized in Table I. Biosynthetic ornithine decarboxylase was routinely purified to a specific activity of 100 units per mg with an average recovery of 10%. Supplementation of the chromatography buffers with additional pyridoxal phosphate, $MgSO_4$, or β-mercaptoethanol did not increase the yield.

Purity of Biosynthetic Ornithine Decarboxylase Preparations. The purified material showed less than 0.1% decarboxylase activity toward arginine (at pH 5.2 and 8.3) and lysine (at pH 5.6, 7.3, and 8.3), indicating no significant contamination by these decarboxylases. The results from analysis of the DEAE-A25 Sephadex fraction by polyacrylamide disc gel electrophoresis appear in Figure 1. One distinct band was seen at pH 8.9 and 7.6 with some minor contaminants. In the presence of 0.1% sodium dodecyl sulfate a single band was also seen (Figure 1C). On the basis of these gels, we estimated the

[1] Abbreviations used: DEAE, diethylaminoethyl; Mes, 2-(*N*-monopholino)ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; SD, standard deviation.

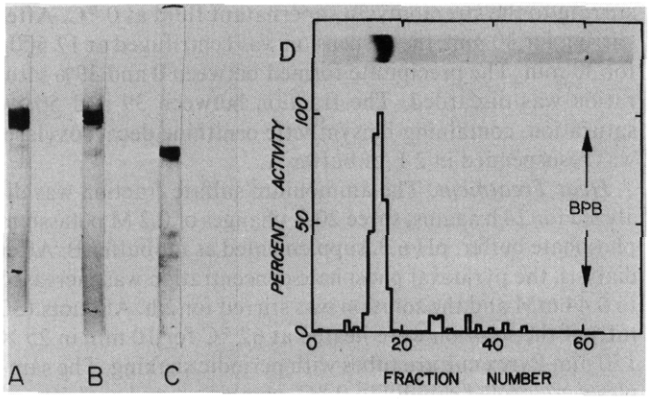

FIGURE 1: Polyacrylamide gel electrophoresis of biosynthetic ornithine decarboxylase at (A) pH 8.9 and (B) pH 7.6. Gel C was run in the presence of sodium dodecyl sulfate. Gels A through C contained respectively 14.2, 14.2, and 2.1 μg of material from DEAE-A25 Sephadex chromatography. Part D demonstrates the coincidence of biosynthetic ornithine decarboxylase activity and material staining as protein after gel electrophoresis. Ornithine decarboxylase was assayed in gel slides (see Applebaum et al., 1975) after electrophoresis of 9.7 μg of purified enzyme at pH 7.6. An identical gel was stained with Coomassie blue. The position of the bromophenol blue marker (BPB) is indicated by the arrows.

TABLE I: Summary of Purification of Biosynthetic Ornithine Decarboxylase.

Purification Step	Total Act. (units)	Total Protein (mg)	Spec Act. (units/mg)	Recovery (%)
Crude extract	1820	79 000	0.023	100
Streptomycin sulfate (supernatant solution)	1520	70 900	0.021	83
Ammonium sulfate	1740	20 400	0.085	96
Heat treatment	1170	6 020	0.19	64
Hydroxylapatite	738	345	2.1	41
G-200 Sephadex	367	62.8	5.8	20
DEAE-A25 Sephadex	178	1.8	99.0	10

purity to be at least 85%. The ornithine decarboxylase activity in a gel at pH 7.6 migrated as a single peak corresponding to the major band staining as protein (Figure 1D).

Enzyme Kinetics. The dependence of activity on ornithine concentration showed classical Michaelis–Menton kinetics. When these data were plotted as V vs. V/S, and the resulting points fitted by the least-squares method, a K_m of 5.6 mM was obtained (Table II). Both putrescine and its biosynthetic product, spermidine, inhibit ornithine decarboxylase. In the presence of either polyamine, the apparent K_m was increased while the V_m did not significantly change (Table II), suggesting simple competition with the substrate.

Pyridoxal Phosphate Requirement. Apoenzyme, assayed in the absence of added pyridoxal phosphate, showed less than 2% of the holoenzyme activity. The apoenzyme could be reactivated with coenzyme. Hyperbolic saturation kinetics were observed with pyridoxal phosphate yielding a K_m of 1 μM.

Molecular Weight and Subunit Structure. When biosynthetic ornithine decarboxylase was subjected to polyacrylamide electrophoresis in 0.1% sodium dodecyl sulfate, it displayed a relative migration of 0.455 ± 0.004 in four determinations corresponding to a subunit molecular weight of 82 000 ± 1% when compared with marker proteins. In sucrose gradients, the enzyme behaved similarly at pH 5.9 and 7.0 with an estimated sedimentation coefficient of 7.9–8.0 S and a calculated molecular weight of 146 000. Thus, the enzyme appears to be a dimer of 82 000 subunit molecular weight.

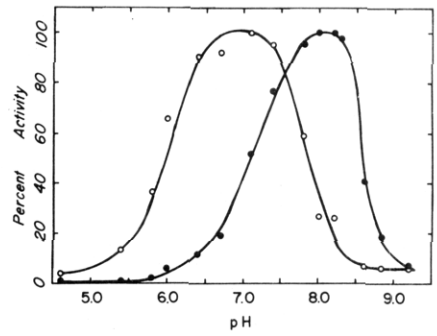

FIGURE 2: Dependence of the activities of biosynthetic and biodegradative ornithine decarboxylase on pH. Biosynthetic (●—●) and biodegradative (○—○) ornithine decarboxylases were measured as described in the text. The following buffers were used: 0.1 M sodium acetate (pH 4.6, 5.4), 0.05 M Mes (pH 6.0), 0.1 M Pipes (pH 6.4, 6.7), 0.1 M Hepes (pH 7.1, 7.4, 7.8, 8.0), 0.1 M Bicine (pH 8.6), and 0.1 M glycine–NaOH (pH 8.2, 8.8, 9.2).

TABLE II: Polyamine Inhibition of Biosynthetic Ornithine Decarboxylase.

Polyamine Added	Concn (mM)	K_m or K_m^{app} [a] (mM)	V_m [a] (nmol min^{-1})
None		5.6 ± 0.4	73 ± 3
Putrescine	6	7.1 ± 0.6	73 ± 4
	12	8.2 ± 0.7	67 ± 4
Spermidine	2	7.8 ± 0.7	65 ± 4
	5	10.3 ± 1.6	61 ± 6

[a] Values for K_m and V_m were obtained from plots of V vs. V/S which were fitted by the least-squares method. The error is expressed as ±1 SD. The enzyme used was from the DEAE fractionation step.

Comparison of Biosynthetic and Biodegradative Ornithine Decarboxylase: pH Dependence. The activities of the purified biosynthetic and biodegradative ornithine decarboxylases showed different dependences on pH (Figure 2). At pH 8.1, where biosynthetic ornithine decarboxylase was maximally active, the activity of the biodegradative enzyme fell to 30%. Optimal activity of the biodegradative decarboxylase was observed at pH 6.9, a difference from the biosynthetic enzyme of 1.2 pH units. This difference in optimal pH was observed throughout the purification of both enzymes. It should be noted that this value of pH 6.9 for the biodegradative decarboxylase (Applebaum et al., 1975) differs considerably from the optimum of pH 5.3 originally reported (Morris and Pardee, 1965). This original biodegradative ornithine decarboxylase activity found in *E. coli* K-12 was actually a low level activity of biodegradative arginine decarboxylase against ornithine (Blethen et al., 1968). We have not been able to detect an authentic biodegradative ornithine decarboxylase in *E. coli* K-12 (see Discussion).

Subunit Molecular Weight. When purified biosynthetic and biodegradative ornithine decarboxylases were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the calculated molecular weight of each was 80 000–82 000 (also see Applebaum et al., 1975). When the

TABLE III: Influence of Nucleotides on the Activity of Ornithine Decarboxylases.[a]

Nucleotide	Biosynthetic (%)	Biodegradative (%)
GTP	+405	+409
GMP	+167	−8
ATP	+260	+65
UTP	+74	+235
CTP	+190	+35

[a] Assays were performed in the presence of 0.1 mM nucleotide and were buffered with 0.1 M Mops (pH 7.0) for the biodegradative enzyme and with 0.1 M Hepes (pH 8.4) for the biosynthetic decarboxylase. Each assay contained 2.1×10^{-3} unit of the biodegradative and 3.2×10^{-3} unit of the biosynthetic enzymes. Both enzymes were purified through their respective ammonium sulfate fractionation steps (see the text for the biosynthetic and Applebaum et al., 1975, for the biodegradative). The results are presented as % stimulation compared with a control lacking nucleotide.

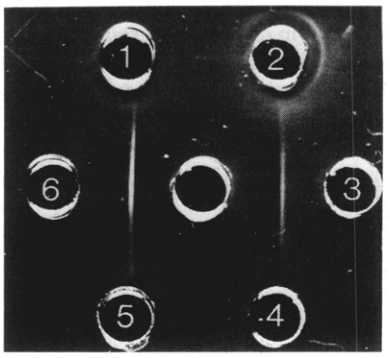

FIGURE 3: Comparison of biosynthetic and biodegradative ornithine decarboxylases by immunodiffusion. Fractionated antiserum to biodegradative ornithine decarboxylase was in the center well. Purified biodegradative ornithine decarboxylase (0.9 μg) was in wells 3 and 6. The other wells contained purified biosynthetic ornithine decarboxylase from the activity peak of the DEAE-A25 Sephadex column (1, 1.6 μg; 2, 16.1 μg) and from the trailing edge of this peak (4, 1.1 μg; 5, 11.0 μg).

two purified proteins were mixed and treated with sodium dodecyl sulfate, only one band of protein could be observed after electrophoresis, indicating no detectable difference in subunit molecular weight.

Nucleotide Activation. The biosynthetic and biodegradative ornithine decarboxylases were both activated by GTP and other nucleoside phosphates (Table III). While activation by GTP was similar, the two enzymes responded quite differently to the other nucleotides. GMP strongly activated the biosynthetic decarboxylase, whereas the biodegradative enzyme was slightly inhibited. The effects of UTP and CTP were also opposite on the two enzymes. CTP more strongly activated the biosynthetic enzyme, whereas UTP was more effective with the biodegradative. Both K_m and V_{max} were influenced by nucleotides (data not shown) as was previously found by Hölttä et al. (1972) with the biosynthetic enzyme. The data in Table III were collected at the respective pH optima of the two enzymes. Similar specificity for activation was obtained with the biosynthetic enzyme when assayed at pH 7.0, the optimum of the biodegradative decarboxylase. Therefore, these differences in nucleoside activation patterns were not due to differences in assay conditions.

Immunodiffusion. Antiserum against the purified biodegradative ornithine decarboxylase was used to investigate its immunological relatedness to the biosynthetic enzyme. The results of Ouchterlony double diffusion analysis are presented in Figure 3. The biodegradative enzyme (0.9 μg) formed a single well-defined precipitin band. Varying the amount of purified biosynthetic ornithine decarboxylase from 1 to 16 μg gave no evidence for cross-reactivity with the antiserum to the biodegradative enzyme. In addition, no cross-reactivity was found with crude extracts or at intermediate stages of purification of the biosynthetic enzyme.

Discussion

It is clear from the present studies that the biosynthetic decarboxylase bears quite striking resemblance to the biodegradative enzyme (Applebaum et al., 1975). Both enzymes appear to be dimers with subunits of 80 000–82 000 molecular weight. The kinetic characteristics of both enzymes also appear to be similar. If one assumes the biosynthetic enzyme to be 85% pure based on gel electrophoresis, a turnover number of 9.6 mmol min^{-1} (82 000 $\mu g)^{-1}$ can be calculated. The K_m for ornithine is 5.6 mM. These values are to be compared with 10.7 mmol min^{-1} (82 000 $\mu g)^{-1}$ and 3.6 mM for the biodegradative enzyme. In other respects, the biosynthetic and biodegradative decarboxylases differ significantly. Hölttä et al. (1972) initially demonstrated activation of biosynthetic ornithine decarboxylase by GTP and other nucleoside phosphates. Although the biodegradative decarboxylase is also activated by GTP, the two enzymes are quite different in their specificity for activation by other nucleotides. They also differ by more than 1 pH unit in their pH optima. Although the catalytic differences between the biosynthetic and biodegradative enzymes are significant and reproducible, these observations do not settle the question of whether the two enzymes are separate gene products. The differences in catalytic activity could conceivably be due to covalent modification of the product of a single gene. Unfortunately, detailed structural analysis of the biosynthetic enzyme is severely limited by the small amount of material available after a 4300-fold purification. Important information in this regard has come from tests for immunological cross-reactivity. It is hard to imagine a covalent modification leading to such a drastic structural change that the two enzymes would have no detectable common antigenic determinants. Therefore, the information available to date strongly argues that the biosynthetic and biodegradative ornithine decarboxylases are distinct but very similar proteins. This is analogous to the situation with the two α-ketoglutarate semialdehyde dehydrogenases of *Pseudomonas putida* (Koo and Adams, 1974).

We recently concluded that the three biodegradative decarboxylases for ornithine, lysine, and arginine are structurally quite similar and we suggested that they probably share a common evolutionary ancestor (Applebaum et al., 1975). The biosynthetic ornithine decarboxylase clearly should be included in this group also. However, the strong activation of the two ornithine decarboxylases by nucleotides distinctly sets them apart since this has never been reported for the other decarboxylases. Both arginine decarboxylases of *E. coli* have been tested (unpublished experiments of D. R. Morris and W. H. Wu) and were not activated by nucleotides. This argues that the two ornithine decarboxylases may be more closely related to one another than to the others and consequently of more recent divergence. Two other observations support this hypothesis and suggest that perhaps the biodegradative ornithine decarboxylase derived from an ancestral biosynthetic enzyme. First, the biodegradative decarboxylases appear to be a defense mechanism against low environmental pH and, hence, have uniformly low pH optima (reviewed by Morris and Fillingame,

1974). The biodegradative ornithine decarboxylase has the highest pH optimum of the biodegradative enzymes, consistent with an evolutionary trend downward from the high value characteristic of the biosynthetic decarboxylases. Second, on routine screening of many strains of *E. coli* for amino acid decarboxylases, we found that all contained the biosynthetic ornithine decarboxylase and nearly all possessed the biodegradative lysine and arginine decarboxylases. On the other hand, only about one out of ten produced the biodegradative ornithine decarboxylase (D. Applebaum, unpublished results). Therefore, we would suggest that the two ornithine decarboxylases have only recently diverged in selected strains of *E. coli*, and that the newly derived biodegradative enzyme has been left with a vestigial regulatory mechanism in the nucleotide activation.

References

Ames, B. N., Garry, B., and Herzenberg, K. (1960), *J. Gen. Microbiol. 22,* 369-378.

Applebaum, D., Sabo, D. L., Fischer, E. H., and Morris, D. R. (1975), *Biochemistry 14,* 3675-3681.

Blethen, S. L., Boeker, E. A., and Snell, E. E. (1968), *J. Biol. Chem. 243,* 1671-1677.

Cohen, G. N., and Rickenberg, H. V. (1956), *Ann. Inst. Pasteur, Paris 91,* 693-720.

Gale, E. F. (1946), *Adv. Enzymol. 6,* 1-32.

Hölttä, E., Jänne, J., and Pispa, J. (1972), *Biochem. Biophys. Res. Commun. 47,* 1165-1171.

Koo, P. H., and Adams, E. (1974), *J. Biol. Chem. 249,* 1704-1716.

Morris, D. R., and Fillingame, R. H. (1974), *Annu. Rev. Biochem. 43,* 303-325.

Morris, D. R., and Pardee, A. B. (1965), *Biochem. Biophys. Res. Commun. 20,* 697-702.

Morris, D. R., and Pardee, A. B. (1966), *J. Biol. Chem. 241,* 3129-3135.

Tabor, H., and Tabor, C. W. (1972), *Adv. Enzymol. 36,* 203-268.

Wu, W. H., and Morris, D. R. (1973a), *J. Biol. Chem. 248,* 1687-1695.

Wu, W. H., and Morris, D. R. (1973b), *J. Biol. Chem. 248,* 1696-1699.

Active Site Specific Inactivation of Chymotrypsin by Cyclohexyl Isocyanate Formed during Degradation of the Carcinostatic 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea†

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ABSTRACT: Prolonged incubation of 1-(2-chloroethyl)-3-([1-^{14}C]cyclohexyl)-1-nitrosourea with chymotrypsin resulted in covalent modification and concomitant inactivation of chymotrypsin via degradation of the nitrosourea to form cyclohexyl isocyanate. Cyclohexyl isocyanate was shown to be an active-site-specific inactivator of chymotrypsin. A cyclohexyl isocyanate to enzyme molar ratio of 0.63 was required to produce 50% enzyme inactivation, thus demonstrating the high specificity of inactivation. At 2.38×10^{-4} M chymotrypsin this near stoichiometric inactivation was not significantly affected by the presence of 1, 5, and 10 mM L-lysine. Degradation of an excess of 1-(2-chloroethyl)-3-([1-^{14}C]-cyclohexyl)-1-nitrosourea in the presence of enzyme yielded 1.11 ± 0.07 mol of covalently bound [^{14}C]cyclohexyl moiety per mol of enzyme inactivated. Short-term incubation demonstrated that the nitrosourea neither inhibited nor protected the enzyme from cyclohexyl isocyanate inactivation. Treatment of chymotrypsin with less than stoichiometric amounts of cyclohexyl isocyanate or titration of the active-site serine with phenylmethanesulfonyl fluoride followed by in situ degradation of excess 1-(2-chloroethyl)-3-([1-^{14}C]cyclohexyl)-1-nitrosourea resulted in a decreased amount of covalently bound ^{14}C proportional to the extent of inactivation by these reagents prior to ^{14}C labeling. These results strongly suggest that cyclohexyl isocyanate, whether added directly or generated by CCNU degradation, reacted almost exclusively with the active site of the enzyme. The extent of this inactivation indicates that 70% of the CCNU degraded in such a manner as to form cyclohexyl isocyanate.

Interest in the antitumor activity of certain nitrosoureas is being directed towards the alkylating and carbamylating intermediates formed during chemical degradation of the nitrosoureas (Montgomery et al., 1967, 1975; Reed et al., 1975; Colvin et al., 1974). Reed et al. (1975) have reported that alkylation by CCNU[1] may occur via a 2-chloroethyl carbonium ion. In vivo, alkylation of thiols occurs producing thiodiacetic acid as a major urinary product after administration of CCNU (Reed and May, 1975).

Wheeler et al. (1975) have emphasized the carbamylation of both the ϵ-amino group of lysine and the α-amino groups of amino acids, peptides, and proteins by nitrosoureas in vitro.

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[1] Abbreviations used are: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; [*chx*-1-^{14}C]CCNU, [^{14}C]CCNU labeled in its cyclohexyl moiety; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; DNBS, 2,4-dinitrobenzenesulfonic acid; Dnp, 2,4-dinitrophenyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; HPLC, high-pressure liquid chromatography.