BBA 66010

STUDIES ON AMINO ACID RACEMASES

II. PURIFICATION AND PROPERTIES OF THE GLUTAMATE RACEMASE FROM *LACTOBACILLUS FERMENTI**

WARREN F. DIVEN

Department of Biochemistry, School of Medicine, University of Pittsburgh, Pittsburgh, Pa. 15213 (U.S.A.)

(Received July 14th, 1969)

SUMMARY

A purification procedure has been developed for the glutamate racemase (EC 5.1.1.3) from *Lactobacillus fermenti* (ATCC 9338) which results in preparations purified 2000-fold with respect to a cell extract. The enzyme is stabilized by the presence of mercaptoethanol and other sulfhydryl containing compounds. The molecular weight of the enzyme has been estimated to be 23 000 by chromatography on Sephadex G-150. Kinetic studies indicate a K_m for D-glutamic acid of 2.2 mM and that hydroxylamine, riboflavin, FMN, FAD, and certain structural analogues of FAD are inhibitors of the racemase catalyzed reaction. The enzyme appears to be extremely specific for glutamic acid since aspartic acid, alanine, α-aminobutyric acid, or α-methylglutamic acid are completely inactive either as substrates or inhibitors.

INTRODUCTION

The generally accepted mechanism of amino acid racemization[1,2] involves the formation of a Schiff base by reaction of the amino acid with an enzyme-bound pyridoxal phosphate. Studies of partially purified enzymes from several different microorganisms[3–8], however, have suggested that very different mechanisms exist. The previous paper in this series has presented a study of the purification and properties of the alanine racemase from *Lactobacillus fermenti*[9] and here the further purification and some studies on the glutamate racemase (EC 5.1.1.3) are presented.

EXPERIMENTAL PROCEDURE

Organisms were grown at 37° for 18–20 h as described previously[9]. Enzyme activity was determined by incubating 17 μmoles potassium phosphate, 0.5 μmole

* A preliminary report of part of this material has been presented (*Federation Proc.*, 26 (1967) 387). The previous paper in this series is ref. 9.

EDTA, 8 μmoles of D-glutamic acid, 1 mg bovine serum albumin, 10 μmoles mercaptoethanol and enzyme in a final volume of 1.0 ml at pH 7.5. After a 30-min incubation at 37°, the reaction was stopped by immersing the tubes in boiling water for 5 min. Aliquots were then assayed for L-glutamate formation using glutamate dehydrogenase by the method of GLASER[4]. For kinetic studies the racemase reaction was stopped at appropriate time intervals. Protein determinations were made by the method of LOWRY *et al.*[10] using bovine serum albumin as reference.

All operations were carried out at 3° unless otherwise indicated. From 100 to 150 g of cells were suspended in 500 ml of 0.05 M phosphate buffer (pH 7.5) containing 0.01 M mercaptoethanol. The cell suspension was incubated with constant stirring for 2 h with 300 mg egg-white lysozyme. At the end of the incubation the suspension was cooled in an ice bath and the solids removed by centrifugation for 20 min at 13 000 $\times g$. All subsequent centrifugations were performed using these conditions. To the supernatant, 10 g of streptomycin sulfate were added and after 15 min 100 ml of 2% protamine sulfate. After standing for 15 min, the precipitate was removed by centrifugation.

$(NH_4)_2SO_4$ fractionation was carried out by slowly adding the solid salt with constant stirring. The fraction precipitating between 35 and 80% of saturation was retained. The $(NH_4)_2SO_4$ precipitate was dissolved in sufficient 0.025 M phosphate buffer (pH 6.0) containing 0.01 M mercaptoethanol and 1 mM DL-glutamic acid to make 100 ml of solution. This fraction was applied to a Sephadex G-100 column (80 mm $\times$ 420 mm) and eluted with the same buffer. Fractions of about 60 ml were collected and assayed for enzymatic activity. The active fractions were combined and $(NH_4)_2SO_4$ to 75% of saturation was added. The resulting precipitate was separated by centrifugation and dissolved in 0.025 M phosphate buffer (pH 6.4) containing 0.01 M mercaptoethanol and 1 mM DL-glutamic acid. The solution was vacuum dialyzed against 4 l of this buffer overnight until the final volume was between 5–10 ml. The dialyzed enzyme solution was applied to a 15 mm $\times$ 820 mm DEAE-cellulose column and eluted with the same buffer. Fractions of approx. 5 ml were collected and assayed for enzymatic activity. The active fractions were pooled and vacuum dialyzed *vs.* phosphate buffer (pH 5.0) containing 0.01 M mercaptoethanol and 1 mM DL-glutamic acid until the volume was from 1 to 2 ml. At this stage of preparation approx. 2000-fold purification had been obtained and this is the material used in all the studies reported here.

RESULTS AND DISCUSSION

The results of a typical enzyme fractionation are shown in Table I. All steps in the procedure are reproducible and excellent yields are obtained. A purification of 1800-fold was obtained in the experiment shown in Table I; however, purifications of 1500–2000-fold have been obtained by this procedure with 40–60% recovery.

Correlations have been found between the log of the molecular weight and the elution volume for chromatography of globular proteins on columns of Sephadex[11–13]. Fig. 1 shows such a plot for γ-globulin, bovine serum albumin, ovalbumin, myoglobin and glutamate racemase on a Sephadex G-150 column.

The molecular weight of the protein associated with glutamate racemase activity, calculated from these data, is 23 000 $\pm$ 2000. The sedimentation coefficient calculated

TABLE I

PURIFICATION OF GLUTAMATE RACEMASE

Fraction	*Protein (mg)*	*Activity* (units)*	*Specific activity (units/mg)*	*Recovery (%)*
Cell extract	9000	38.7	0.0043	—
35–80% $(NH_4)_2SO_4$ fraction	5250	42	0.0080	100
Combined Sephadex G-100	210	40	0.19	95
0–75% $(NH_4)_2SO_4$ fraction	98.8	38	0.38	90
Combined DEAE-cellulose	3.0	23.1	7.7	55

* The unit of activity is defined as that amount of enzyme necessary to catalyze the formation of 1 μmole D-glutamic acid per min.

from preliminary sedimentation-velocity experiments is compatible with this molecular weight.

Several other amino acids were examined for their effect on glutamate racemase activity. L-aspartic acid, α-L-aminobutyric acid, L-alanine and α-methylglutamic acid were all completely inactive either as substrates for the enzyme or as inhibitors of D-glutamic acid racemization. These results are in agreement with the suggestions of others that each amino acid racemase is specific for a single amino acid[5,14].

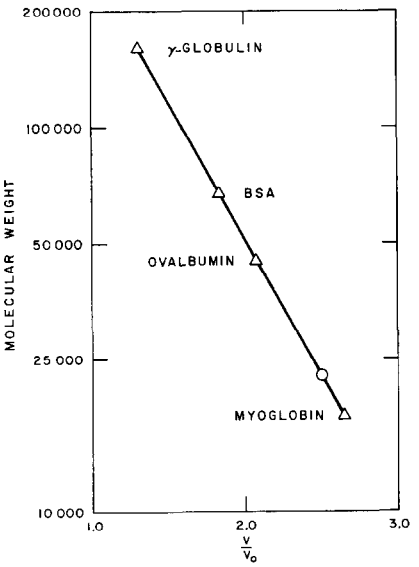


Fig. 1. Molecular weight estimation for glutamate racemase. A 25 mm × 450 mm chromatographic column was packed with Sephadex G-150 which had been prepared according to the manufacturers instructions. The void volume (V_0) of the column was determined by the elution of a 1.0-ml sample of dextran blue. A 2.0-ml sample containing from 10 to 20 mg each of the indicated reference proteins was allowed to run into the column and was eluted with 0.025 M phosphate buffer. A sample of glutamate racemase of specific activity 7.7 was eluted from the column in a similar manner. Collected fractions were assayed for both protein and enzymic activity and a single symmetrical peak was obtained. V/V_0 for the glutamate racemase activity is represented by the open circle. BSA = bovine serum albumin.

TABLE II

INHIBITION OF GLUTAMATE RACEMASE

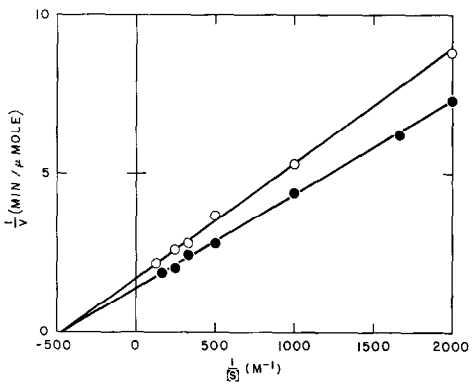
A glutamate racemase sample containing 0.5 μg protein of specific activity 7.7 units/mg was incubated with the indicated compounds under the standard assay conditions as described in the text.

Compound	*Concn. (mM)*	*Inhibition (%)*
FAD	1.0	45
FMN	0.1	50
Riboflavin	0.1	43
3-Hydroxypropyl FAD	0.5	19
4-Hydroxybutyl FAD	0.5	21
Hydroxylamine	0.4	33

During an investigation of the cofactor requirements of glutamate racemase an inhibition of the enzyme was observed by FAD and hydroxylamine. It was decided to examine other riboflavin-containing compounds as inhibitors and the results are presented in Table II. Drs. Donald B. McCormick and Bruce Chassy kindly provided the FAD analogues, 3-hydroxypropyl FAD and 4-hydroxybutyl FAD.

The effect of substrate concentration on the initial velocity of the reaction catalyzed by glutamate racemase is presented in Fig. 2 as a double reciprocal plot. The K_m calculated from these data is 22 mM. The results of a similar series of experiments in the presence of 1.0 mM hydroxylamine are also presented.

The effects of 0.1 mM FMN on the initial velocity of the racemase reaction are shown in Fig. 3. It can be seen that the curve in the presence of inhibitor is parallel to



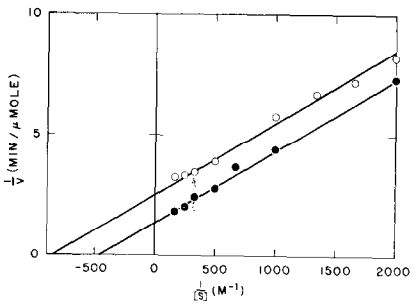


Fig. 2. Inhibition of the racemization of D-glutamic acid by hydroxylamine. Glutamate racemase (0.5 μg protein, specific activity 8.0) was incubated with 17 μmoles potassium phosphate, 0.5 μmole EDTA, 1 mg bovine serum albumin, 10 μmoles mercaptoethanol and D-glutamic acid as indicated in a final volume of 1.0 ml and at pH 6.6. ●, experiments performed in the absence of any inhibitor; ○, experiments in the presence of 1.0 mM hydroxylamine. Each point is the average of at least four determinations and all values were corrected for any variation in enzymic activity.

Fig. 3. Inhibition of the racemization of D-glutamic acid by FMN. The incubation mixtures contained 17 μmoles potassium phosphate, 0.5 μmole EDTA, 1 mg bovine serum albumin, 10 μmoles mercaptoethanol, enzyme, and D-glutamic acid as indicated in a final volume of 1.0 ml at pH 6.6. ○, experiments in the presence of 0.1 mM FMN; ●, experiments in the absence of inhibitor.

that obtained for the uninhibited reaction. Similar results were obtained with the structural analogues of FAD, 3-hydroxypropyl FAD and 4-hydroxybutyl FAD as well as riboflavin.

TANAKA *et al.*[5] have reported that hydroxylamine is not an inhibitor of glutamate racemase and those results have been confirmed at pH 7.5. However, at pH 6.6, as shown in Fig. 2, inhibition occurs. The inhibition by flavin-containing compounds observed here can be explained in terms of the reversible oxidation of an enzyme–substrate complex to an inactive form. Further detailed kinetic studies of the reaction catalyzed by glutamate racemase are necessary to allow a more complete description of the mechanism of action of this enzyme.

ACKNOWLEDGMENTS

This investigation has been supported in part by Grant GM 11984 from the National Institutes of Health, United States Public Health Service and Grant K 9 from the Health Research Services Foundation.

REFERENCES

1 A. E. BRAUNSTEIN AND M. M. SHEMYAKIN, *Biokhimiya*, 18 (1953) 393.
2 D. E. METZLER, M. IKAWA AND E. E. SNELL, *J. Am. Chem. Soc.*, 76 (1954) 648.
3 W. F. DIVEN, J. J. SCHOLZ AND R. B. JOHNSTON, *Biochim. Biophys. Acta*, 85 (1964) 322.
4 L. GLASER, *J. Biol. Chem.*, 235 (1960) 2095.
5 M. TANAKA, Y. KATO AND S. KINASHITA, *Biochem. Biophys. Res. Commun.*, 4 (1961) 114.
6 T. C. STADTMAN AND P. ELLIOTT, *J. Biol. Chem.*, 228 (1957) 983.
7 G. J. CARDINALE AND R. H. ABELES, *Biochemistry*, 7 (1968) 3570.
8 E. ADAMS AND J. L. NORTON, *J. Biol. Chem.*, 239 (1964) 1525.
9 M. M. JOHNSTON AND W. F. DIVEN, *J. Biol. Chem.*, in the press.
10 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
11 P. ANDREWS, *Nature*, 196 (1962) 36.
12 J. R. WHITAKER, *Anal. Chem.*, 35 (1963) 1950.
13 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
14 W. A. WOOD AND I. C. GUNSALUS, *J. Biol. Chem.*, 190 (1951) 403.