BBA 66146

PURIFICATION AND PROPERTIES OF A NAD-DEPENDENT GLUTAMATE DEHYDROGENASE FROM CLOSTRIDIUM SB4

E. L. WINNACKER* AND H. A. BARKER
Department of Biochemistry, University of California, Berkeley, Calif. 94720 (U.S.A.)

(Received March 10th, 1970)

SUMMARY

The purification of a NAD-dependent glutamate dehydrogenase from lysine-fermenting Clostridium SB4 is described. The enzyme, purified 250-fold with a 40% yield, was found to be homogeneous by gel electrophoresis and ultracentrifugation criteria. Sedimentation analysis gave a $s^{\circ}_{20,w}$ value of 11.7 and a molecular weight of 275 000. The sedimentation rate is invariant over a concentration range from 50 μg to 5 mg of protein per ml, in contrast to beef liver glutamate dehydrogenase. The enzyme, although isolated from an anaerobic organism is not sensitive to oxygen. It is stable to storage under various conditions, especially in highly dilute solutions.

Amino acid composition, gel filtration behavior, substrate specificity towards various α-keto acids, pH optima and kinetic parameters of the enzymatic reaction were determined and compared with corresponding properties of glutamate dehydrogenases from other sources. Initial velocity measurements result in linear double-reciprocal plots and indicate a sequential order of substrate addition, different from that of other glutamate dehydrogenases.

Low Michaelis constants for ammonia and α-ketoglutarate, high coenzyme and substrate specificity, lack of sensitivity towards purine nucleotides and high stability suggest the use of the enzyme for analytical determinations of ammonia and α-ketoglutarate.

The function of the enzyme in lysine fermentation is discussed.

INTRODUCTION

Glutamate dehydrogenase (L-glutamate–NAD(P) oxidoreductase, EC 1.4.1.2–4) catalyzes the following reaction:

$$\text{Glutamate} + \text{NAD(P)}^+ + \text{H}_2\text{O} \leftrightharpoons \alpha\text{-ketoglutarate} + \text{NAD(P)H} + \text{NH}_4^+$$

The enzyme has been isolated in homogeneous form from various animal sources (beef liver[1], chicken liver[2,3], pig liver[4], frog liver[5], dog fish liver[6]), from fungi

* Present address: Karolinska Institutet, Solnavagen 1, Stockholm 60, Sweden.

(*Neurospora crassa*[7], *Blastocladiella emersonii*[8], baker's yeast[9]) and from *Thiobacillus novellus*[10], a facultative chemo-autotrophic bacterium. Strong glutamate dehydrogenase activities have been demonstrated, but never purified, in cell-free extracts of various strictly anaerobic microorganisms as Clostridium SB4[11], *Clostridium tetanomorphum*[12], *Clostridium sporogenes*[13], *Clostridium kluyverii*[14], *Clostridium propionicum*[15], *Clostridium aminobutyricum*[16], *Clostridium thermoaceticum*[17] and Reiter's treponeme[18]. The physiological significance of glutamate dehydrogenase and its possible link to the energy-yielding process in these anaerobes has generally only been speculated upon. This communication describes the purification and characterization of a NAD-dependent glutamate dehydrogenase from extracts of Clostridium SB4, a lysine fermenting anaerobe. Chemical and physical properties of the enzyme are compared with those of enzymes from other sources and discussed in relation to the pathway of lysine dissimilation in this microorganism.

MATERIALS AND METHODS

Materials

Clostridium SB4 was grown and harvested as described by COSTILOW *et al.*[11]. In large cultures (20 l) sodium thioglycolate was replaced by $Na_2S_2O_4$, (30 mg/ml) as reducing agent. The specific activity of glutamate dehydrogenase in cell-free extracts increases towards the beginning of the stationary phase. Cells were therefore harvested after about 6–8 h of growth, depending on the size of the inoculum, when the apparent $A_{570\ m\mu}$ reached 3.0. Apparent absorbance was tested with a Zeiss-PMQ II spectrophotometer using 3-ml cuvettes and a 1:5 dilution of the culture with distilled water. Cells used for the preparation of the enzyme could be stored at −18° for at least 6 months without any detectable loss of activity.

DEAE-cellulose (Whatman DE 52, preswollen) was washed and equilibrated with the appropriate buffer according to the instructions of the manufacturer. Sephadex G-150 and G-200 (Pharmacia, Uppsala) were soaked in the appropriate buffers and 5 mM EDTA for at least 2 weeks. All column materials were deaerated under reduced pressure before the columns were packed according to the procedure of ANDREWS[19]. Dialysis tubing (Union Carbide) was freed of impurities by heating it at 80° for 30 min in 10 mM EDTA (pH 7.5). After rinsing with water, the tubing was stored in 2 mM EDTA at 4° until needed. Commercially available chemicals were obtained from the following sources: phenazine methosulfate from Aldrich Chem. Co; L-lysine·HCl from Ajinomoto Co.; NAD^+, NADH, $NADP^+$, NADPH and sodium α-ketoglutarate from C. F. Boehringer; sodium α-ketoadipate and amido-black from K and K Laboratories; protamine sulfate from E. Lilly and Co.; L-glutamic acid and nitro blue tetrazolium from Nutritional Biochem. Corp., biological grade $(NH_4)_2SO_4$ from Schwarz Bioresearch Inc.; bovine serum albumin, sodium pyruvate, sodium α-ketobutyrate, sodium α-ketovalerate, sodium α-ketoisovalerate, levulinic acid, AMP, ADP and ATP from Sigma Chem. Co.; lactate dehydrogenase (rabbit muscle) from Worthington Biochem. Corp.

Methods

Glutamate dehydrogenase activity was routinely determined by measuring the decrease of the NADH absorption at 340 mμ in an 1-ml assay solution of the

following composition: 150 mM Tris·HCl buffer (pH 8.1), 180 mM $(NH_4)Cl$, 0.1 mM NADH, 5 mM α-ketoglutarate and 0.01–0.1 unit of glutamate dehydrogenase. The pH of the solution was adjusted to pH 8.1. 1.5 ml silica cells with a 1-cm light path were used in a Model DU Beckman spectrophotometer equipped with a Model 2000 Gilford multiple sample absorbance recorder. The reaction was started by the addition of α-ketoglutarate. This was important in the early stages of the purification since crude extracts contained considerable NADH oxidase activity.

Initial velocity measurements for kinetic studies were performed in 3 ml, 1-cm light path silica cells or 18 ml, 5-cm light path silica cells on a Cary Model 14 recording spectrophotometer. Homogeneous enzyme preparations were kept in highly dilute solutions (1 μg/ml) at −15°. Samples from these could be used without further dilutions and were stable for several weeks without measurable losses in activity determined in a standard assay. The nomenclature of CLELAND[20] was used for the description of reaction mechanisms and kinetic constants. NAD^+ and NADH were determined in enzymic spectrophotometric assays with glucose dehydrogenase[21] and lactate dehydrogenase[22], respectively.

One enzyme unit catalyzes the oxidation of 1 μmole of NADH per min at 25° under the assay conditions. Specific activities are given in units per mg protein.

Protein determinations in crude and partially purified extracts were done after the method of LOWRY *et al.*[23] with crystalline bovine serum albumin as standard. After the DEAE-cellulose step, protein was estimated from $A_{280\ m\mu}$. The absorbance $E^{0.1\%}_{280\ m\mu}$ was determined from a synthetic boundary experiment[24] in a Spinco Model E ultracentrifuge equipped with a Rayleigh interference optical system. A solution of the enzyme ($A_{280\ m\mu} = 0.535$), purified through Step 7 and equilibrated with 50 mM potassium phosphate buffer (pH 7.0), was placed in a 11.92-mm synthetic boundary cell with the dialysis buffer as the reference solvent. From the number of fringes across the boundary which had formed after 20 min of rotation at a rotor speed of 5227 rev./min at 23°, from the wavelength used, 5460 Å, the known[25] average refractive increment (dn/dc) for proteins at 23°, $1.9 \cdot 10^{-3}$ dl/g and the average fringe number, 41.5, of a 1% protein solution, an extinction coefficient of 1.07 A units/mg protein per ml was determined. The validity of the method and the experiments was checked by running bovine serum albumin and sucrose ($dn/dc = 1.43 \cdot 10^{-3}$ dl/g)[25] solutions of known concentrations.

Nucleic acid content in the crude extracts was estimated from the $A_{280\ m\mu}/A_{260\ m\mu}$ ratio (ref. 26).

Analytical ultracentrifugation experiments were performed on a Spinco Model E ultracentrifuge equipped with either a Schlieren optical system or a combination monochromator split beam photoelectric scanning absorption system. Sedimentation velocity experiments with the Schlieren system were performed at 8.1° and 59 870 rev./min; experiments with the ultraviolet absorption optical system permitting the observation of the boundary at 280 mμ, were conducted at 10.9° and 52 000 rev./min. Sedimentation coefficients were calculated according to a procedure of SCHACHMAN[24] and corrected to the viscosity and density of water at 20°. In experiments with the Schlieren optical system the migration of the boundary was determined by measurements of photographs, taken at 8-min intervals, on a Nikon Model 6C microcomparator.

Sedimentation equilibrium experiments were performed by the high speed or

meniscus depletion method of YPHANTIS[27] by using the ultraviolet absorption optical system and a 12-mm six-channel Yphantis centerpiece. Partial specific volumes ($\bar{v}$) were calculated from the amino acid composition according to the method of COHN AND EDSALL[28]. The average molecular weight was determined as described by YPHANTIS[27]. Enzyme samples were prepared for analysis by dialyzing a 5 mg/ml solution of the enzyme against two changes of 1000 vol. of 50 mM potassium phosphate buffer (pH 7.0). Samples were diluted, as required, with the dialysis buffer.

Polyacrylamide gel electrophoresis at pH 8.9 and pH 7.9 was conducted according to the methods of DAVIS[29] and HEDRICK AND SMITH[30], respectively. All runs were made at 23–25° and a constant current of 3 mA per tube. Protein samples (10–200 μg) were added to 100 μl of the application buffer, containing 0.003% bromophenol blue, and applied directly on top of the stacking gel. Gels were stained for protein for at least 2 h in a 0.5% solution of amido black in 7% acetic acid. They were destained electrophoretically and stored in 7% acetic acid at 4°. Enzyme activities were located by incubation of the gels at 23–25° for 30 min in a staining solution, described by FINE AND COSTELLO[31].

Molecular weights were determined by gel filtration on a Sephadex G-200 column (2 cm × 100 cm) with 0.1 M sodium citrate (pH 6.0) as running buffer according to the description of LEACH AND O'SHEA[32]. The column was standardized with blue dextran, determined by its absorption at 625 mμ, and three proteins of known molecular weight. Bovine serum albumin (mol. wt. 65 000)[19] was determined by its absorption at 280 mμ. Lactate dehydrogenase (mol. wt. 140 000)[19] was assayed by the method of KORNBERG[33]. Catalase (mol. wt. 250 000)[34] was determined according to CHANCE AND MAEHLY[35]. In a typical run the following quantities of proteins and blue dextran were used, dissolved in 1 ml of citrate buffer: 5 mg blue dextran, 500 μg (50 μl; 250 units) glutamate dehydrogenase, 10 μl of a crystalline suspension of catalase, 1 mg of lactate dehydrogenase (40 μl of a crystalline suspension (25 mg/ml) in 50% $(NH_4)_2SO_4$) and 10 mg of crystalline bovine serum albumin. The solution was centrifuged to remove undissolved crystals of catalase. The proteins were eluted at a rate of 3.3 ml $cm^{-2} \cdot h^{-1}$. The fraction size was 1.67 ml with a deviation of $\pm 2\%$, determined by weighing each fifth fraction. Enzyme activities were determined on 20-μl aliquots of the eluate.

For amino acid analysis 4.7 mg of enzyme (1 ml; specific activity: 480 units/mg) were dialyzed for 24 h against 10 mM potassium phosphate buffer (pH 6.8). In constricted tubes aliquots of 300 μl were diluted with 0.7 ml of water, mixed with 1 ml of conc. HCl and, after evacuation, hydrolyzed at 110° for 24 and 48 h, respectively. The hydrolysates were then taken to dryness in a Buchner evaporator, the amino acids dissolved in 2 ml of 0.2 N citrate buffer (pH 2.2), and analyzed in a Spinco Model 120C amino acid analyzer according to the method of BENSON AND PATTERSON[36]. Tryptophan and tyrosine were determined spectrophotometrically by the methods of BEAVEN AND HOLIDAY[37] and BENCZE AND SCHMID[38]. No attempt has been made to measure either amide groups or cysteine by independent methods.

Absorption spectra were determined on a Cary Model 14 spectrophotometer. pH was measured with a Corning Model 12 pH-meter, relative ionic strengths during chromatographic experiments with a radiometer conductivity meter.

RESULTS

Purification of glutamate dehydrogenase

Step 1. Sonic extraction

75 g of Clostridium SB4 cells, fresh or frozen, were suspended in 200 ml of a 50 mM potassium phosphate buffer (pH 6.8), and homogenized at 0° with a Waring blender at medium speed. The cells were then disrupted in 50-ml portions with 250 mg of Corundum FFF powder per batch in a 10 kcycles Raytheon sonic oscillator for 10 min. The sonicated extracts were centrifuged for 40 min at 14 000 × *g*. The supernatant was decanted, the precipitate washed with 100 ml of the phosphate buffer and recentrifuged under the same conditions. The combined supernatant solutions, which usually had a protein content of 30–35 mg/ml, were either immediately used or frozen and stored at −15°. No change in enzyme activity was observed after 6 months of storage at this temperature.

Step 2. Protamine sulfate treatment

Sonic extract (630 ml) containing 30 mg protein per ml was adjusted to a protein concentration of 12 mg/ml by adding 940 ml of a 100 mM potassium phosphate buffer (pH 7.2). To this solution was added under stirring during 30 min a solution of 3.78 g protamine sulfate in 378 ml 100 mM potassium phosphate buffer (pH 7.2) (0.2 g protamine per g protein). After 30 min at 0° the solution was centrifuged at 14 000 × *g* for 30 min. The precipitate was discarded. The final volume of the clear solution was 1890 ml and the protein concentration 8.5 mg/ml. The increase in the $A_{280\ m\mu}/A_{260\ m\mu}$ ratio from 0.62 in the crude extract to 0.87 corresponds to an estimated decrease in the nucleic acid content from 18 to 5% (ref. 26).

Step 3. First $(NH_4)_2SO_4$ precipitation

576 g of solid $(NH_4)_2SO_4$ were added during 30 min under stirring to the clear solution from Step 2. The pH was kept at 6.8 by adding 45.6 g K_2HPO_4 to the solution after half of the $(NH_4)_2SO_4$ was added. The solution (0.50 saturated in $(NH_4)_2SO_4$) was centrifuged at 14 000 × *g* and the precipitate discarded. To the supernant solution (2190 ml) were added 189 g of $(NH_4)_2SO_4$ during 20 min to make the solution 0.65 satd. After standing for 30 min the solution was centrifuged and the supernatant fraction discarded. The precipitate was dissolved in 50 ml of 0.1 M potassium phosphate buffer (pH 7.2) to give a final volume of 63 ml and a protein concentration of 44 mg/ml.

Step 4. Heat treatment

The enzyme solution from Step 3 (63 ml) was diluted with 16 ml of 0.1 M potassium phosphate buffer (pH 7.2) to a protein concentration of 35 mg/ml. The solution was stirred for 15 min in a 65° waterbath and immediately transferred to an icebath, stirred for 5 min and then centrifuged at 20 000 × *g* for 25 min. The supernatant solution was decanted, the precipitate stirred up with 10 ml of the same phosphate buffer and recentrifuged. The volume of the combined supernatant solutions was 103 ml; the protein concentration 12.6 mg/ml.

Step 5. Second $(NH_4)_2SO_4$ precipitation

The conditions were the same as in the first precipitation. The solution from Step 4 was refractionated with $(NH_4)_2SO_4$, retaining the fraction precipitating between 0.50 and 0.65 satn. The precipitate was dissolved in 5 ml of 0.1 M potassium phosphate (pH 7.2), and this solution was dialyzed against three changes of 1 l of

50 mM potassium phosphate buffer (pH 6.9) for 24 h in preparation for the following step. The final volume was 11 ml and the protein concentration was 79 mg/ml.

Step 6. DEAE-cellulose column fractionation and concentration

The solution from Step 5 was run into a column (3 cm × 50 cm) of DEAE-cellulose equilibrated with 50 mM phosphate buffer (pH 6.9). The column was eluted at the rate of 30 ml/h with a gradient linear from 50 mM to 280 mM potassium phosphate (pH 6.9). Fractions of 5 ml were collected. $A_{280\ m\mu}$ of the effluent solution was continuously monitored with an ISCO dual-beam optical unit attached to a Model AU-2 ISCO UV-analyzer. A major protein peak was eluted between 60 and 135 mM phosphate; the glutamate dehydrogenase activity appeared with a small protein peak eluting between 150 and 170 mM phosphate. Fractions 61–69 (vol. 46.8 ml), containing about 60% of the enzyme of relatively high specific activity (242 units/mg; 1.82 mg protein/ml), were combined. An additional 17% of the enzyme of lower specific activity (106 units/mg) was collected separately in Fractions 70–81 (vol. 59 ml; 0.7 mg protein/ml). The protein solution (Fractions 61–69) was concentrated by ultrafiltration using a UM-1 membrane in a Diaflo Model 50 ultrafiltration cell applying a pressure of 35 p.s.i. A 10–15% loss of activity sometimes occurred during this operation. In some experiments therefore this concentration step was performed by vacuum dialysis which caused virtually no loss in activity.

Step 7. Sephadex G-150 column fractionation and concentration

The high specific activity material from Step 6, concentrated to 5 ml, was divided into two 2.5-ml portions. One portion at a time was applied on a column (2 cm × 100 cm) of Sephadex G-150 which had been equilibrated with either 50 mM potassium phosphate (pH 7.0), or 100 mM Tris acetate (pH 7.5). Both buffer systems gave comparable results. The enzyme was eluted from the column with one of the two buffers; seventy 3.6-ml fractions were collected at a rate of 3.1 ml $cm^{2-} \cdot h^{-1}$. The major protein peak appeared at the void volume of the column, determined in a separate run with 1 ml of a 5 mg/ml solution of blue dextran. A minor protein peak which eluted between Fractions 41–51 was identified as glutamate dehydrogenase. The most active Fractions 43–48 with an $A_{280\ m\mu}/A_{260\ m\mu}$ of 2.05–2.15 and a specific activity of 450 to 500 units/mg were pooled and concentrated by vacuum dialysis. The recovery of activity was better than 80%, the purification from the previous step 2-fold. The gel-filtration step was occasionally repeated when there was too much overlap between the two major protein peaks.

Crystallization

The material from the DEAE-cellulose step crystallized readily by adding solid $(NH_4)_2SO_4$ to a 0.5% solution of the enzyme to about 55% satn. The experiments were performed in either 100 mM potassium phosphate (pH 7.0) or 100 mM Tris·HCl (pH 7.5), the pH values being controlled by the addition of either basic potassium phosphate or Tris base. Crystals began to form after a few minutes standing at 25°; after 24 h at 4° 80% of the protein had crystallized as visualized by the Schlieren effect of the stirred solution. Under the phase microscope small crystals with a rhombohedric shape and defined edges were clearly visible. The specific activity of the crystalline material shortly after crystallization reached about a value of 480 units/mg but never exceeded it. In addition, the high specific activity of the crystals decreased sharply with a half-life time of approx. 18 h upon storage

TABLE I

PURIFICATION OF GLUTAMATE DEHYDROGENASE

Step		*Vol. (ml)*	*Total protein (mg)*	*Specific activity (units/mg)*	*Total activity (units × 10^{-3})*	*Yield (%)*
1	Crude extract	1570	18800	2.1	39.6	100
2	Protamine treatment	1890	16100	3.1	49.5	125
3	$(NH_4)_2SO_4$ ppt. (0.5–0.65 satn.)	63	2770	14.3	39.5	99.7
4	Heat treatment	103	1300	30	38.9	98.5
5	$(NH_4)_2SO_4$ ppt. (0.5–0.65 satn.)	11	868	40.4	35.1	86.8
6	DEAE-cellulose chromatography (conc.)	5	85.5	242	20.6	52
7	Sephadex G-150 chromatography (conc.)	6.5	33.5	485	16.25	41

in 0.55 satd $(NH_4)_2SO_4$. The column procedure is therefore preferable for obtaining pure material.

Table I gives typical results of the purification procedure. Glutamate dehydrogenase was purified 220–240-fold, depending on the activity of the crude extract. Yield varied between 35 and 45%; final specific activities between 450 and 490 units/mg. The individual steps and the entire purification procedure are highly reproducible.

Characterisation of glutamate dehydrogenase

Homogeneity

The degree of homogeneity of glutamate dehydrogenase preparations, purified through Step 7, was determined by polyacrylamide gel electrophoresis and sedimentation velocity ultracentrifugation. Only one protein band could be detected after analytical gel electrophoresis (Fig. 1) under various conditions with up to 200 μg of protein/gel. Two buffer systems, pH 8.9 (ref. 29) and 7.9 (ref. 30) were used as well as different gel concentrations, ranging from 5 to 9% acrylamide. The sharp

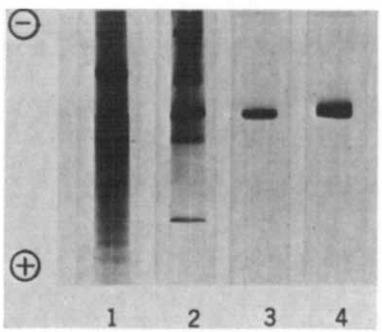

Fig. 1. Polyacrylamide gel electrophoresis of glutamate dehydrogenase. Gels containing 7.5% acrylamide, 0.25% methylene bisacrylamide and 0.075 M Tris·HCl (pH 8.9), were run at 3 mA per tube for 3 h. Protein migration is towards the anode, as indicated. (1) 250 μg of protein, purified through Step 4; (2) 250 μg protein, purified through Step 6; (3) 50 μg of glutamate dehydrogenase, purified through Step 7; and (4) 150 μg of glutamate dehydrogenase, purified through Step 7.

protein band coincided with a single glutamate dehydrogenase activity band visualized by an activity staining technique (see *Methods*). Schlieren patterns obtained from a sedimentation velocity experiment with a 5 mg protein per ml solution taken at 8-min intervals at 8.1° and 59 870 rev./min showed one major component. The origin of a minor (<2%), very slowly moving peak with an estimated sedimentation coefficient of 0.4 S is not understood.

Stability

The enzyme, purified through Step 7, is stable at 4° or −15° in solutions containing five levels of enzyme from 4–500 μg of protein per ml in either 0.1 M sodium citrate buffer (pH 6.0), potassium phosphate buffer (pH 6.8), or Tris·HCl (pH 7.5). No loss of activity was observed under these conditions after 6 weeks; 15% of the activity was lost after 8 months. The presence of 1 mM EDTA did not influence the stability of the enzyme. More concentrated solutions of glutamate dehydrogenase were less stable. A solution containing 5.3 mg of protein per ml in Tris·HCl buffer lost about 15% of its activity while stored at −15° for a month and 25% after 8 months. At 4° this preparation lost 35% of its activity over a period of 2 weeks at either pH 7.5 or pH 6.9.

The stability of the enzyme in crude extracts is remarkable. At −15° in 0.1 M potassium phosphate buffer (pH 6.8), no change in activity could be observed after 6 months. At 30°, between pH 4.0 (0.1 M acetate) and pH 8.5 (0.1 M phosphate or Tris·HCl), no activity was lost after 5 days. The half-life time at 50° and pH 6.8 (0.1 M phosphate) is 5 days, at 70° 20 min.

Optical properties

The purified enzyme shows a typical protein absorption spectrum in either 50 mM potassium phosphate (pH 7.0), or 50 mM Tris·HCl (pH 7.4), with an absorbance maximum at 279 mμ and a minimum at 249 mμ. The extinction coefficient $E^{0.1\%}_{280\,m\mu}$ was determined to be 1.07 absorbance units. The protein concentration was determined by ultracentrifugation in a synthetic boundary experiment (see Methods). The 280 : 260 absorbance ratio of 2.05 to 2.12 indicates that the preparation is free of nucleic acid or enzyme bound NAD. A 5.3 mg protein per ml solution showed no absorption above 325 mμ.

TABLE II

DEPENDENCY OF THE SEDIMENTATION COEFFICIENT $s_{20,w}$ ON PROTEIN CONCENTRATION

The rotor speed was 59 780 rev./min in the experiment with 5 mg protein per ml (Schlieren optical system) and 52 000 rev./min in the other experiments (ultraviolet optical system).

Protein concn. (mg/ml)	*Temperature*	$s_{20,w}$
5.0	8.1°	11.78
0.9	11.0°	11.45
0.52	10.9°	11.79
0.125	9.8°	11.73
0.075	9.8°	11.74
0.058	10.9°	11.25

TABLE III

WEIGHT AVERAGE MOLECULAR WEIGHT ($\bar{M}_w$) OF GLUTAMATE DEHYDROGENASE FROM SEDIMENTATION EQUILIBRIUM EXPERIMENTS

Protein concn. (mg/ml)	*Temperature*	$\bar{M}_w$
0.1	10°	283 000
0.2	10°	268 000
0.3	10°	276 000
0.2	15°	270 000
0.4	15°	278 000

Ultracentrifugal analysis

The results of a series of sedimentation coefficient determinations designed to determine the sedimentation rate of the clostridial glutamate dehydrogenase as a function of enzyme concentration are summarized in Table II. The sedimentation coefficient $s_{20,w}$ appears to be constant over a 100-fold concentration range. The observed deviations are within the experimental error. The $s_{20,w}$ value, extrapolated to zero protein concentration is 11.7. There is no indication therefore, that the enzyme undergoes the concentration dependent dissociation which is characteristic of the mammalian[1] and frog enzymes[5].

The results of five sedimentation equilibrium experiments on the same enzyme preparation at four different protein concentrations are summarized in Table III. In all experiments the plot of log protein concentration, which is proportional to the measured absorbance, against the square of the distance from the center of rotation was strictly linear from the bottom of the cell to the meniscus, indicating the homogeneity of the enzyme preparation. The mean value of the weight-average molecular weight from these five determinations was 275 000±7000. From the known values of both the molecular weight and the corrected $s^\circ_{20,w}$ values, the frictional coefficient f/f_0 can be determined as outlined by ONCLEY[39]. Table IV summarizes published $s^\circ_{20,w}$ values and molecular weights of glutamate dehydrogenases from various sources. The axial ratios have been obtained from frictional coefficients as described by SCHERAGA AND MANDELKERN[40]. The data show that high frictional coefficients and axial ratios are common to all these enzymes with the exception of the beef liver enzyme subunit. The results are reflected in the gel-filtration behavior, in particular of the clostridial enzyme.

TABLE IV

FRICTIONAL COEFFICIENT f/f_0 AND AXIAL RATIOS OF GLUTAMATE DEHYDROGENASES FROM VARIOUS SOURCES

Source	$s^\circ_{20,w}$	$\bar{M}_x \times 10^{-3}$	f/f_0	*Axial ratio*
Clostridium SB_4	11.7	275	1.30	5.8
Dogfish liver[6]	13.0	330	1.24	5.0
Neurospora crassa[7]	10.0	267	1.35	6.0
Beef liver subunit[41]	13.0	270	1.004	2

Molecular weight estimation by gel-filtration

A molecular weight estimation of the Clostridium SB4 glutamate dehydrogenase was obtained by gel filtration on a Sephadex G-200 column, calibrated with a high molecular weight dextran and three proteins of known molecular weight. With bovine serum albumin, lactate dehydrogenase and catalase as standards a linear relationship was obtained between the ratios (V/V_0) of the elution volumes of the proteins to the exclusion volume (V_0) of the column and the logarithm of their molecular weights (Fig. 2). Assuming that the enzyme follows this relationship, an

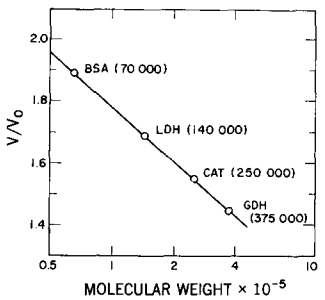

Fig. 2. Molecular weight of glutamate dehydrogenase by gel filtration. The enzyme (GDH, 250 units) was applied on a Sephadex G-200 column (2 cm × 100 cm) along with 5 mg of blue dextran, 1 mg of lactate dehydrogenase (LDH), 100 μg of catalase (CAT), and 10 mg of bovine serum alumbin (BSA). The void volume (V_0) of the column is 165 ml. Other details are given under *Methods*. The data are plotted in a semilogarithmic plot.

apparent molecular weight of 350 000–390 000 is found for the bacterial glutamate dehydrogenase. The deviation of this value from 275 000, determined by equilibrium centrifugation, is significant. It probably can be attributed to the high axial ratio of 5.8 of this enzyme, since according to ANDREWS[19], the deviation of a protein molecule from standard behavior in a gel-filtration experiment increases with the axial ratio. The beef liver enzyme subunit with an axial ratio of 2 shows little, if any, deviation from the usual molecular weight–elution volume relationship[19].

Amino acid analysis

The amino acid composition of Clostridium SB4 glutamate dehydrogenase, obtained from two 24-h and one 48-h hydrolysates, is reported in Table V. No attempt has been made to measure either amide groups directly or cysteine by an independent method. Serine and threonine are corrected for destruction during hydrolysis, assuming first order kinetics, as outlined by STEIN AND MOORE[44]. The total amino acids account for only 90.5% of the mass of the hydrolyzed protein. Part of the remaining 9.5% represents amide ammonia, part may have been lost during the preparation of the hydrolysates. Data for the beef liver enzyme from two different sources are presented for comparison. Based on 90.5% of the composition of the native enzyme, the partial specific volume was calculated from the weight percentage of the amino acids and their specific volumes according to COHN AND EDSALL[28]; a value of 0.737 (66.74/90.55) ml/g was obtained. This value was used for analysis of data from ultracentrifugal experiments.

TABLE V

AMINO ACID COMPOSITION OF GLUTAMATE DEHYDROGENASES FROM BEEF LIVER AND CLOSTRIDIUM SB4

$\bar{v}_{\text{amino acid}}$ denotes the partial specific volume of the free amino acids.

Amino acid	*Beef*[6] *(μmole/mg)*	*Beef*[41] *(μmole/mg)*	*Clostridium SB4*			
			μmole/mg	*Wt. % (g/100 g)*	$\bar{v}_{\text{amino acid}}$[28]	$\bar{v} \cdot wt.$ %
Lys	0.551	0.594	0.587	7.524	0.82	6.17
His	0.225	0.242	0.211	2.89	0.67	1.94
Arg	0.484	0.515	0.347	5.428	0.70	3.80
Asp	0.860	0.927	0.903	10.38	0.61	6.33
Thr	0.433	0.497	0.265	2.70	0.70	1.89
Ser	0.476	0.558	0.291	2.53	0.63	1.59
Glu	0.811	0.832	0.890	11.48	0.67	7.69
Pro	0.364	0.418	0.362	3.52	0.76	2.68
Gly	0.875	0.858	1.000	5.71	0.64	3.65
Ala	0.659	0.683	0.634	3.52	0.76	2.68
Val	0.595	0.555	0.599	5.94	0.86	5.11
Met	0.222	0.233	0.222	2.91	0.75	2.18
Ile	0.606	0.616	0.524	5.94	0.90	5.34
Leu	0.570	0.550	0.527	5.97	0.90	5.37
Tyr	0.294	0.308	0.340	5.55	0.71	3.94
Phe	0.403	0.401	0.433	6.38	0.77	4.91
Cys		0.229	0.060	0.62	0.61	0.38
Trp		0.080	0.085	1.57	0.74	1.16
			Total	90.55		66.74

Buffer and pH effects

The effect of pH on the rates of glutamate oxidation and α-ketoglutarate amination was studied in several buffer systems. Initial velocities of glutamate oxidation were determined with 100 mM Tris–HCl or ethanolamine–HCl buffer between pH 7 and 10.75 in the presence of 50 mM glutamate, 0.15 mM NAD and 0.2 μg of enzyme. Rates of amination of α-ketoglutarate were obtained between

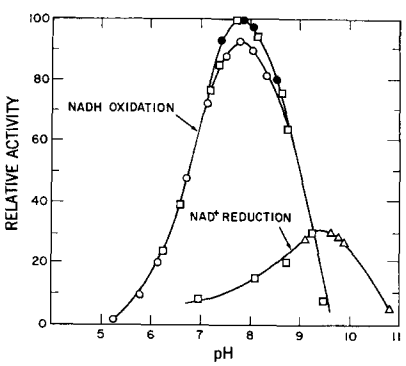

Fig. 3. Effect of pH on activity of glutamate dehydrogenase. NADH oxidation was measured in 100 mM Tris–HCl (□—□), potassium phosphate (○—○) or glycylglycine chloride (●—●) under standard conditions with 0.05 μg of enzyme. NAD^+ reduction was measured in 100 mM Tris–HCl (□—□) or ethanolamine–HCl (△—△) in the presence of 50 mM sodium glutamate, 0.15 mM NAD^+ and 1 μg of enzyme.

pH 5.5 and 9.5 under standard conditions with 100 mM potassium phosphate, Tris–HCl or glycylglycine chloride buffer. Fig. 3 shows that the pH optimum of NADH oxidation is about pH 7.8; activity is slightly lower (93%) in phosphate than in Tris·HCl buffer. The pH optimum of coenzyme reduction is about 9.4. Different rate dependencies of the forward and backward reaction in an enzyme catalyzed reaction are a commonly encountered phenomenon. With glutamate dehydrogenases, however, identical pH optima for both directions have been reported for all pure enzymes so far studied with the exception of a NAD-dependent enzyme from *Thiobacillus novellus*[10]. Since a NAD-dependent glutamate dehydrogenase in extracts of a treponeme, a strict anaerobe, has been observed[18] to have identical optima (at pH 9.7), no general rule can be formulated as to a general occurence of different pH optima in enzymes from bacteria.

Kinetic studies

Initial velocity measurements of the oxidation of glutamate were performed in 18 ml, 5-cm ligh path silica cells with varying concentrations of glutamate (1.25–40 mM) or NAD (0.02–0.25 mM). With NAD or glutamate as variable substrates the double reciprocal plots were linear and intersecting. The kinetic constants, as outlined by CLELAND[20] could therefore be determined from Eqn. 1

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \qquad (1)$$

(where A and B are substrate concentrations, K_{ia} is the dissociation constant for A and K_a and K_b are Michaelis constants of A and B, respectively). The results are summarized in Table VI together with data from enzymes of other sources. Eqn. 1 represents a sequential mechanism, involving either a rapid equilibrium random or an ordered addition of substrates. Without additional evidence from binding, product inhibition or isotope exchange studies a decision between these alternatives cannot be made.

TABLE VI

KINETIC CONSTANTS FOR SUBSTRATES OF GLUTAMATE DEHYDROGENASES FROM VARIOUS SOURCES

	Clostridium SB4[a]	*Thiobacillus*[b,10]	*Beef liver*[c,5]	*Frog liver*[c,5]	*Dogfish liver*[c,6]
K_{NADH} (mM)	0.01	0.004	0.026	0.2	
K_{NADPH} (mM)			0.026	0.2	0.4
$K_{\alpha\text{-ketoglutarate}}$ (mM)	0.65	0.66	0.70	5.0	4.5
$K_{ammonia}$ (mM)	0.32	0.5	3.2	5.0	4.5
$K_{glutamate}$ (mM)	1.8	13.3	1.8	1.8	84
K_{NAD^+} (mM)	0.01	0.19			
K_{NADP^+} (mM)			0.047	0.5	0.08
V_f (sec^{-1})[d]	2,200[f]		250[g]	100[g]	175[h]
V_r (sec^{-1})[e]	270[f]		8[g]	4[g]	2.5[h]

[a] pH 8.0, 0.1 M Tris–acetate, 25°.
[b] pH 7.5, 0.2 M Tris·HCl, 25°.
[c] pH 8.0, 0.01 M Tris–acetate, 23°.
[d] Measured as moles of NAD(P)H per mole of enzyme per sec.
[e] Measured as moles of NAD(P) per mole of enzyme per sec.
[f] Based on a mol. wt. of 275 000.
[g] Based on a mol. wt. of 250 000.
[h] Based on a mol. wt. of 330 000.

For the amination of α-ketoglutarate, three initial velocity patterns were obtained by varying the concentrations of a given pair of substrates while the concentration of the third substrate was held constant and saturating. Three subsets of data were obtained (1) at a saturating concentration of NADH (0.1 mM), ammonium acetate concentration being varied between 0.25 and 5 mM at several fixed levels of α-ketoglutarate (0.10–1.0 mM); (2) at constant ammonium acetate concentration (75 mM), NADH being varied between 12.5 and 100 mM at several fixed levels of α-ketoglutarate (0.25–2 mM); and (3) at constant α-ketoglutarate concentration (3 mM), NADH being varied between 6.6 and 80 μM at constant levels of ammonium acetate (5–50 mM). The double reciprocal plot for the subset with constant and saturating NADH concentration was linear with an intersecting pattern whereas the double reciprocal plots of the two other subsets were linear but showed a parallel pattern of lines. Data from the first, intersecting subset are represented by Eqn.1, data from the subsets with the parallel pattern by Eqn. 2 which lacks the term $K_{ia}K_b$

$$v = \frac{VAB}{K_bA + K_aB + AB} \tag{2}$$

in the denominator[20]. Michaelis constants, determined according to these equations from replots of slopes and/or intercepts versus reciprocal substrate concentrations are summarized in Table VI.

Initial velocity measurements of the amination reaction were performed at low concentrations of α-ketoglutarate and NADH because of a pronounced inhibition by higher levels of these substrates. Plots of initial velocity *versus* α-ketoglutarate concentration (Fig. 4) at two different NADH concentrations (0.1 and 0.3 mM) show an activity maximum at about 3 mM α-ketoglutarate and a 40% decrease in the maximum velocity at the higher NADH concentration. The dotted line indicates the expected curve in the absence of substrate inhibition.

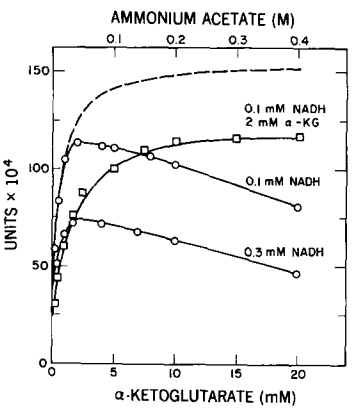

Fig. 4. Demonstration of substrate inhibition. ○, initial velocity against α-ketoglutarate concentration in the presence of 200 mM ammonium acetate, 100 mM Tris–acetate (pH 8.0), 0.1 μg of enzyme, and 0.1 or 0.3 mM NADH, as indicated. The dotted line shows the expected curve in the absence of substrate inhibition. □, initial velocity against ammonium acetate concentration in the presence of 100 mM Tris–acetate (pH 8.0), 2 mM α-ketoglutarate (α-KG), 0.1 μg of enzyme and 0.1 mM NADH.

No change in the inhibition pattern or in absolute velocities was observed in the presence of 0.1 or 1 mM AMP, ADP or ATP. A plot of initial velocity versus ammonium acetate concentration revealed no inhibition up to 400 mM ammonium acetate with 0.1 mM NADH and 2 mM α-ketoglutarate.

Substrate specificity

Initial velocity experiments with NADPH as coenzyme in the amination reaction of α-ketoglutarate gave the following results: In 100 mM Tris–acetate buffer (pH 8.0), in the presence of 150 mM ammonium acetate, 3 mM α-ketoglutarate and NADPH, varying between 0.04 and 0.1 mM, the maximum velocity was found to be 1.2 units/per mg of protein, or 0.0031 that of the NADH-catalyzed reaction under otherwise identical conditions. Under standard conditions the specific activity was found to be 0.0017 that of the NADH-dependent reaction. The maximum velocity and specific activity ratios were not influenced by the presence of 0.1 or 1 mM AMP, ADP or ATP. No α-ketoglutarate inhibition was observed in the NADPH-catalyzed reaction. The possibility of a contamination of NADPH by NADH could be eliminated in a spectrophotometric assay. In the presence of 0.1 mM NADPH and large amounts of enzyme (200 μg) a linear decrease of $A_{340\ m\mu}$ could be observed over 80% of the absorbance range.

It was shown by polyacrylamide gel electrophoresis that the observed NADP activity of the enzyme preparations is not caused by a contaminating enzyme of different specificity. Gels of different acrylamide concentrations, ranging from 5 to 10%, were run in pH 8.9 (ref. 29) and pH 7.9 (ref. 30) buffer systems and enzyme activity was determined by coupling the enzyme-catalyzed reduction of NAD^+ or $NADP^+$ with a redox dye system (*Methods*). Gels were first stained in the presence of $NADP^+$ with the appearance of only a very weak band and subsequently brought into a NAD^+-containing staining solution. Since under all conditions of electrophoresis, both bands appeared at the same position it was concluded that the activity towards NADP is not due to a second enzyme. Similar activity staining experiments on polyacrylamide gels of the crude extract led to similar results; only one glutamate dehydrogenase could be detected.

Substrate specificity towards various α-keto acids was determined in 100 mM Tris–HCl buffer, 0.1 mM NADH, 180 and 450 mM NH_4Cl and varying concentrations of the α-keto acids, mostly between 5 and 50 mM. All reactions were shown to have an absolute requirement for NH_4^+. The results are summarized in Table VII, which shows K_m, v_{max} values and initial velocities. The ratio of the maximum velocities of α-ketoglutarate and pyruvate was not influenced by the presence of 0.1 or 1 mM AMP, ADP or ATP. In addition, no alanine dehydrogenase activity could be observed in the presence of NADPH as coenzyme. The results (Table VII) indicate that the γ-carboxyl group of α-ketoglutarate is not essential for activity although it enhances binding considerably. The binding of α-keto acids other than α-ketoglutarate is at least one order of magnitude lower than with the beef liver enzyme. In view of the high K_m for α-ketobutyrate and α-ketovalerate the relatively high maximum velocities for these compounds probably have no physiological significance.

The high specificity of the clostridial enzyme could be used for analytical determinations of α-ketoglutarate and ammonia. Low Michaelis constants for both substrates would allow determinations of submicromole amounts of the two sub-

TABLE VII

SUBSTRATE SPECIFICITY OF GLUTAMATE DEHYDROGENASE

The reaction mixtures contained 100 mM Tris–HCl (pH 8.0), 180 and 450 mM NH_4Cl, 0.1 mM NADH, 5–50 mM of the indicated substrates, and 0.1–20 μg of enzyme in a total volume of 1 ml. Michaelis constants (K_m) and maximum velocities (v_{max}) are obtained from double-reciprocal plots. Values in parentheses are for the bovine liver enzyme[46]. Column v denotes initial velocities at 2 mM concentrations of the indicated substrates. Levulinate and α-ketoadipate did not react at a detectable rate.

Subtrate	K_m *(mM)*	v_{max} *(units/mg)*	v *(units/mg)*	v *(%)*
α-Ketoglutarate	0.66 (0.70)	480	480	100
Pyruvate	35 (3.3)	0.63	0.029	0.0060
α-Ketobutyrate	153	66.4	0.86	0.179
α-Ketovalerate	50 (6.6)	46.5	1.79	0.372
α-Ketoisovalerate	290	5.82	0.039	0.008
α-Ketoisocaproate	60	0.73	0.021	0.0045

strates. The stability of the enzyme and the lack of sensitivity towards purine nucleotides make the enzyme preferable to the commonly used enzyme from beef liver[42] for this purpose.

DISCUSSION

A stable and highly active NAD-dependent glutamate dehydrogenase constitutes about 0.5% of the soluble protein fraction of the lysine fermenting Clostridium SB4. Although the enzyme is isolated from a strict anaerobe, it is not sensitive to oxygen and, in contrast to other enzymes from this source[43] does not require anaerobic activation to display its activity. The organism produces only one glutamate dehydrogenase. A weak NADP-dependent activity can be attributed to a slight coenzyme unspecificity rather than to the presence of a second NADP-dependent enzyme. The limited amount of information available from other clostridia indicates that they also produce only one glutamate dehydrogenase[12,13], either NAD- or NADP-dependent, whereas the aerobe, *T. novellus*, has been shown to possess two distinct enzymes[10].

The enzyme from Clostridium SB4, with a mol. wt. of 275 000, is large enough to consist of several subunits but it does not undergo the facile association–dissociation reaction on dilution characteristic for the higher molecular weight forms of the beef and frog liver enzymes[45,5]. In this respect it resembles the enzymes from dogfish liver[6] and various fungi and bacteria[7,10]. Unlike several glutamate dehydrogenases from animal[46] and microbial[8,10] sources, the clostridial enzyme appears not to be a regulatory protein. The presence of the purine nucleotides AMP, ADP or ATP does not influence the initial velocity pattern, the substrate inhibition by α-ketoglutarate and NADH or the specificity characteristics.

The enzyme does not display sigmoid kinetics in the investigated concentration ranges of substrates. Initial velocity studies for glutamate oxidation yielded linear, intersecting double-reciprocal plots, consistent with a sequential mechanism of substrate addition. Similar data have been obtained for other glutamate dehydro-

genases although in some cases only in the presence of allosteric effectors[10]. Analysis of the initial velocity pattern of the amination of α-ketoglutarate by regarding the terreactant system as an interrelated set of bireactant subsystems at fixed levels of the third substrate yielded three linear double-reciprocal plots. At saturating coenzyme concentration the double-reciprocal plot was intersecting, whereas at constant ammonia or α-ketoglutarate concentration the plots were parallel. According to CLELAND[20] this distribution of pattern of reciprocal plots indicates a sequential mechanism in which the second and third substrates combine in random order with the complex of the enzyme with the first substrate. Since the parallel patterns were observed with NADH and α-ketoglutarate at saturing concentrations of ammonia, and with NADH and ammonia at high levels of α-ketoglutarate, the data are consistent with a sequential mechanism in which NADH first binds to the enzyme and is followed by α-ketoglutarate and ammonia in random order.

In an extensive analysis by FRIEDEN[47] of the initial velocity pattern of NADP-dependent beef liver glutamate dehydrogenase, a set of two intersecting and one parallel pattern of double-reciprocal plots was obtained. From these results it was concluded that the quarternary complex in this case is formed by an ordered addition of the substrates in the sequence NADPH, ammonia and α-ketoglutarate. NADPH was established as a leading substrate by binding studies, since the kinetic approach does not specify the identity of the first and third substrates.

Similar analyses have been performed for glutamate dehydrogenases from several other sources. The NADP-dependent enzymes from dogfish liver[6] and frog liver[5] showed initial velocity patterns similar to those from the beef liver enzyme. The enzymes from *T. novellus* also appear to follow an ordered ternary mechanism, although the NAD-specific enzyme is ordered only in the presence of AMP (ref. 10). None of the enzymes described so far in the literature shows the initial velocity pattern observed with the clostridial enzyme.

The turnover numbers of the clostridial enzyme at pH 8.0 are 2200 sec^{-1} for NADH oxidation and 250 sec^{-1} for NAD^+ reduction and are at least one order of magnitude higher than corresponding values for other glutamate dehydrogenases and lead to bimolecular rate constants for the association reactions of the enzyme with reduced and oxidized coenzyme of $2.2 \cdot 10^8$ $M^{-1} \cdot sec^{-1}$ and $2.75 \cdot 10^7$, respectively. These high rates suggest that this enzyme participates in the energy yielding processes of this organism. Such a role of glutamate dehydrogenase has been proposed for the degradation of γ-aminobutyrate by *C. aminobutyricum*[16] and for the degradation of β-alanine by *C. propionicum*[15]. The fermentation of lysine by cell-free extracts of Clostridium SB4 is known to be stimulated by α-ketoglutarate[11], suggesting the occurrence of a transamination step in the lysine fermentation pathway. No evidence has been obtained, however, for a transamination reaction with α-lysine, β-lysine or 3,5-diaminohexanoic acid, early intermediates in the α-lysine fermentation[11,48]. It has been proposed[48] that the coenzyme A thioester of β-aminobutyrate may be a cleavage product of 3-keto-5-aminohexanoic acid, known to be formed by a NAD-linked dehydrogenase reaction from 3,5-diaminohexanoic acid[48]. In the further degradation of β-amino butyric acid thioester, leading finally to butyrate, α-ketoglutarate may serve as an amino group acceptor in a transamination reaction. It could be regenerated subsequently through a glutamate dehydrogenase catalyzed reaction, the driving force for this thermodynamically unfavorable step being

provided by the reoxidation of the reduced coenzyme in reactions leading to the formation of butyrate.

ACKNOWLEDGEMENTS

The help of Michael Herbst in the growth of Clostridium SB4 is gratefully acknowledged. This work was supported in part by Research Grant AI-00563 from the National Institutes of Health, U.S. Public Health Services, and by funds from the California Agricultural Experiment Station.

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