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ISOLATION AND CHARACTERISATION OF GLUTAMATE DEHYDROGENASE FROM *MYCOBACTERIUM SMEGMATIS* CDC 46 *

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Summary

Glutamate dehydrogenase (L-glutamate:$NADP^+$ oxidoreductase (deaminating), EC 1.4.1.4) has been purified from *Mycobacterium smegmatis* CDC 46 using $(NH_4)_2SO_4$ precipitation, negative adsorption on DEAE-cellulose, 2′,5′-ADP-Sepharose affinity chromatography and Sephadex G-200. The enzyme was purified 1041.6-fold and the preparation was found to be homogeneous on column chromatography, polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis. Alanine and threonine were identified as the N- and C-terminal amino acids of glutamate dehydrogenase from *M. smegmatis*. The enzyme kinetics and regulation of glutamate dehydrogenase activity by different nutritional factors has been studied. Initial velocity plots showed that the reaction mechanism of glutamate dehydrogenase from *M. smegmatis* followed an ordered sequential ter-bi mechanism.

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* Supplementary data to this article are deposited with and can be obtained from Elsevier/North Holland Biomedical Press, B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. Reference should be made to No. BBA/DD/155/69115/615 (1980) 299—308. The supplementary information includes: Amino acid composition of the purified enzyme, effect of divalent cations on enzyme activity, effect of high NADPH concentration at high L-α-ketoglutarate concentration in the presence of different concentrations of ammonium chloride, double-reciprocal plots of velocity vs. NADPH concentration at a low concentration of ammonium chloride (20 mM) and various fixed concentrations of L-α-ketoglutarate and the effect of purine nucleotides on the activity of glutamate dehydrogenase.

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Introduction

Intensive studies have been carried out on glutamate dehydrogenase (L-glutamate:NAD^+ oxidoreductase (deaminating), EC 1.4.1.4) from many sources, vertebrates in particular, because of its biological significance and key role in metabolism. Taking into consideration the importance of glutamate dehydrogenase as a link between carbohydrate and protein metabolism, it was thought worthwhile to investigate this particular enzyme in *Mycobacterium smegmatis* in detail. This enzyme also plays a pivotal role in the interconversion of L-amino acids and keto acids.

Materials and Methods

ADP, ATP, AMP, GMP, GTP, GDP, NADH, $NADP^+$, $NADPH_2$, phenazine methosulfate, Nitro-blue Tetrazolium (NET), *p*-chloromercuribenzoate, iodoacetate, fructose 1,6-diphosphate, isocitrate, L-glutamate, Tris (hydroxymethyl) aminomethane, L-asparagine, glutamine, succinate, pyruvate, β-mercaptoethanol, urea, SDS, Blue Dextran, Naphthol Blue-black, Sephadex G-200, riboflavin, cytochrome *c*, xanthine oxidase, bovine glutamate dehydrogenase, catalase, ovalbumin, bovine serum albumin, ribonuclease, pepsinogen and trypsin were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). 2′,5′-ADP-Sepharose 4B was a product of Pharmacia Fine Chemicals (Uppsala, Sweden). Aquacide was from Calbiochem (Switzerland), *N,N′*-methylene bisacrylamide and *N,N,N′,N*-tetramethylethylene diamine were products of Canalco Inc. (Rockville, U.S.A.). All the other chemicals, inorganic and organic, used in the present study were of analytical grade and purchased from British Drug Houses Ltd. (Bombay, India) or E. Merck, (Darmstadt, F.R.G.).

Mycobacterial strains. M. smegmatis CDC 46 was obtained from the Centre for Disease Control (Atlanta, GA, U.S.A.). *M. phlei* ATCC 354 was procured from American Type Culture Collection (Rockville, MD, U.S.A.). *M. tuberculosis* TMC No. 1011) was from Trudeau Mycobacterial Culture Collection (Saranac Lake, NY, U.S.A.). The organism was maintained on Lowenstein-Jensen medium and was usually subcultured fortnightly. Youmans and Karlson [1] medium was used to obtain sufficiently large quantities of cells. Magnesium citrate in the medium was replaced by magnesium carbonate and citric acid to avoid precipitation.

Protein was estimated by the method of Lowry et al. [2] using crystalline bovine serum albumin as standard.

Enzyme assays. Glutamate dehydrogenase was assayed by measuring spectrophotometrically the oxidation of $NADPH_2$ at 340 nm, 27°C [3]. The reaction system (1 ml) consisted of 0.1 M Tris-HCl buffer (pH 8.0)/0.1 M NH_4Cl/10 mM α-ketoglutarate/0.1 mM NADPH. The reaction was inititated by the addition of enzyme substrate. The assay mixture of oxidative deamination of glutamate consisted of 1 ml containing 0.1 M Tris-glycine buffer, pH 9.0/200 mM glutamate/0.1 mM $NADP^+$.

Molecular weight and subunits. Molecular weight of the enzyme was determined by the method of Whitaker [4] using Sephadex G-200. Polyacrylamide gel electrophoresis of the protein was conducted according to Davis [5] and

SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn [6]. In all instances, protein was localized by staining with Amido Black [6]. GDH was located on the gels by active staining after electrophoresis for 29 min in a mixture consisting of 22 ml 0.01 M Tris-HCl buffer, (pH 8.0), 2 ml 1.0 M sodium glutamate, 0.6 ml $NADP^+$ (30 mg/ml), 1.0 ml phenazine methosulfate (5 mg/ml). The position of the NADPH-dependent GDH coincided with the band of the enzyme located in a separate gel by Amido Black staining [6]. Amino acid composition was determined by the method of Piepy and Morris [7]. The N-terminal residue of the enzyme was determined according to Gray [8] and C-terminal residue according to Ambler [9].

Isolation of the enzyme. M. smegmatis CDC 46 cells (800 g) were suspended in 0.05 M Tris buffer, pH 7.4 (2 l) and 25 ml aliquots of the cell suspension were sonicated for 5 min in a MSE ultrasonic disintegrator at 16 kHZ. Unbroken cells and debris were removed by centrifugation at 2000 × *g* for 30 min in a refrigerated centrifuge (International Equipment Co., Model 20 B). Solid $(NH_4)_2SO_4$ was added to the supernatant solution to 0.25 saturation. After standing for 3 h, the precipitated protein was removed by centrifugation at 20 000 × *g* for 30 min and the supernatant fraction was raised to 0.65 saturation by adding solid $(NH_4)_2SO_4$. The precipitate obtained on centrifugation was dissolved in a small amount of 0.1 M phosphate buffer, pH 7.0. It was then dialyzed against the same buffer of 0.05 M for 24 h with four changes of buffer (2 l each time). The dialyzed extract was passed through a DEAE-cellulose column (1.2 × 10 cm) and the unadsorbed fraction was loaded on to a 2′,5′-ADP-Sepharose 4B column (1 × 8 cm). The column was washed free of unadsorbed proteins with phosphate buffer 0.1 M, pH 7. The adsorbed protein was desorbed by stepwise elution with 25 μM $NADP^+$ (40 ml). The enzyme was eluted with 100 μM $NADP^+$. Fractions of 1 ml were collected and assayed for protein and enzyme activity. The active fractions were pooled and concen-

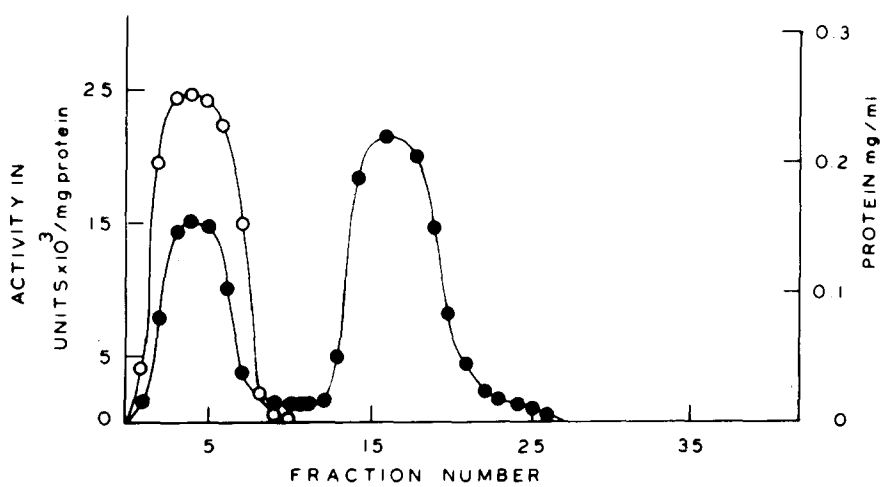

Fig. 1. Elution profile on Sephadex G-200. Protein obtained from the affinity column was loaded on Sephadex G-200 (2.5 × 50 cm) eluted with 0.05 M phosphate buffer, pH 7. Fractions of 2 ml each were collected. ○———○, enzyme; ●———●, protein.

trated to a small volume (1.0 ml) using Aquacide III. This procedure resulted in a 350-fold increase in specific activity over the previous step. The enzyme after affinity chromatography was passed through Sephadex G-200 column (2.5 × 50 cm) and the protein was eluted with phosphate buffer, 0.05 M, pH 7, and 3 ml fractions were collected (Fig. 1). Fractions containing maximum specific activity (80207 units) were pooled and concentrated using Aquacide III. The above purification procedure resulted in a 100-fold increase in specific activity with about 40% recovery.

Results

Criteria of purity. The constant specific activity of the final preparation suggested that the enzyme was homogeneous. This was further confirmed by the appearance of a single and compact band on the polyacrylamide gel electrophoresis at pH 8.6. The fractions gave two bands after affinity chromatography (Fig. 2c). The identity of the protein band as the enzyme was established by simple staining of the gel (Fig. 2d) for activity. The enzyme protein on SDS-polyacrylamide gel electrophoresis under denaturing conditions gave a single band.

Molecular weight. The enzyme had a molecular weight of 245 500 ± 5500 as determined by Sephadex G-200 gel filtration using bovine serum albumin dimer (138 000), catalase (232 000), xanthine oxidase (275 000) and bovine glutamate dehydrogenase (336 000) as marker proteins. The molecular weight of the subunit was determined by SDS-polyacrylamide gel electrophoresis under denaturing conditions, using cytochrome *c* (11 700), trypsin (232 800), pepsinogen (40 000), ovalbumin (43 000) and bovine serum albumin (69 000) as marker proteins and was found to be 40 000. The single band obtained sug-

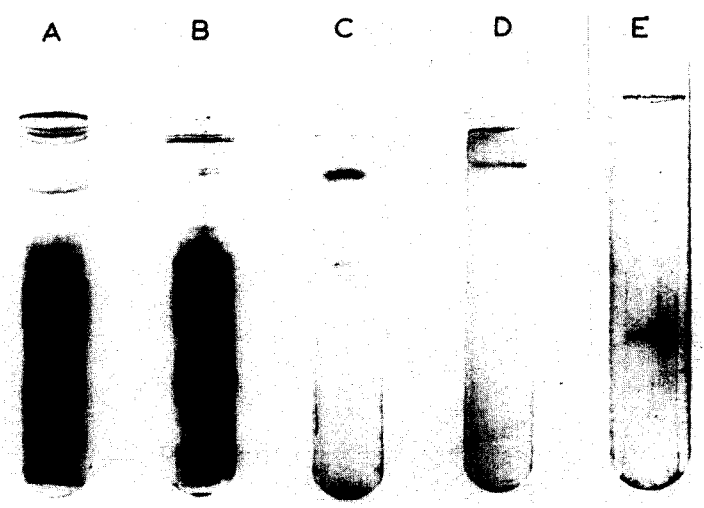

Fig. 2. Electrophoretic pattern of protein at various stages of purification. A, Crude extract; B, 25—65% NH_4Cl fraction; C, 2′,5′-ADP-Sepharose 4B; D, Sephadex G-200; E, SDS-polyacrylamide gel electrophoresis.

gested that the protein was made up of identical subunits and also supported the view that the enzyme preparation was homogeneous. The theoretical amino acid composition calculated for a subunit molecular weight of 40 000 is deposited in BBA data bank. The N- and C-terminal residues were found to be alanine and threonine.

Properties of the enzyme. The enzyme functioned optimally at pH 8.0 for the reductive amination of α-ketoglutarate and between 8.8—9.8 for the oxidative deamination of glutamate. The enzyme was stable to heat inactivation at 60°C for 10 min. Mercuric ions and *p*-chloromercuribenzoate inhibited the enzyme to 90% and 70%, respectively at 1 mM concentration while complete inhibition was observed at 10 mM. Iodoacetate had no effect. Cobalt and nickel ions inhibited the activity, whereas Zn^{2+} activated at low concentrations (approx. 0.1 mM) and inhibited at higher concentrations (greater than 10 mM). Monovalent cations such as Na^+, K^+, Li^+ and Cs^+ had no effect on the enzyme activity.

Kinetics of the enzyme reaction. The enzyme catalyses the following reaction:

$$\text{L} - \alpha - \text{ketoglutarate} + \text{NADPH} + \text{NH}_4^+ \rightleftharpoons \text{L-glutamate} + \text{NADP}^+ + \text{H}_2\text{O}$$

The reaction can be classified as a ter-bi reaction. The K_m of the substrates L-α-ketoglutarate, $NADPH_2$ and NH_4Cl was determined to be 5, 0.045 and 33 mM. The K_m for glutamate and $NADP^+$ were found to be 62.5 and 0.029 mM. The order of binding of the substrates in each direction was determined by varying one substrate at fixed concentration of the other and at saturating and unsaturating concentrations of the third substrate.

Fig. 3 depicts the Lineweaver-Burk plot at a varying concentration of $NADP^+$ at different fixed concentrations of glutamate. These intersecting lines suggest that the mechanism is probably sequential rather than ping-pong. The nature of

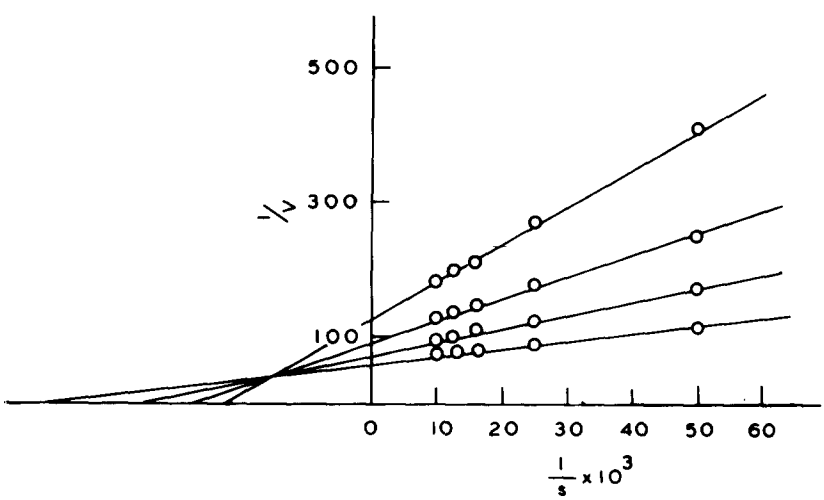

Fig. 3. Double-reciprocal plots of velocity vs. $NADP^+$ concentration in the presence of different fixed concentrations of glutamate. Concentrations of glutamate used were: 40, 70, 90 and 120 mM. Experiment was performed at 27°C in 0.1 M Tris-HCl buffer, pH 8.0.

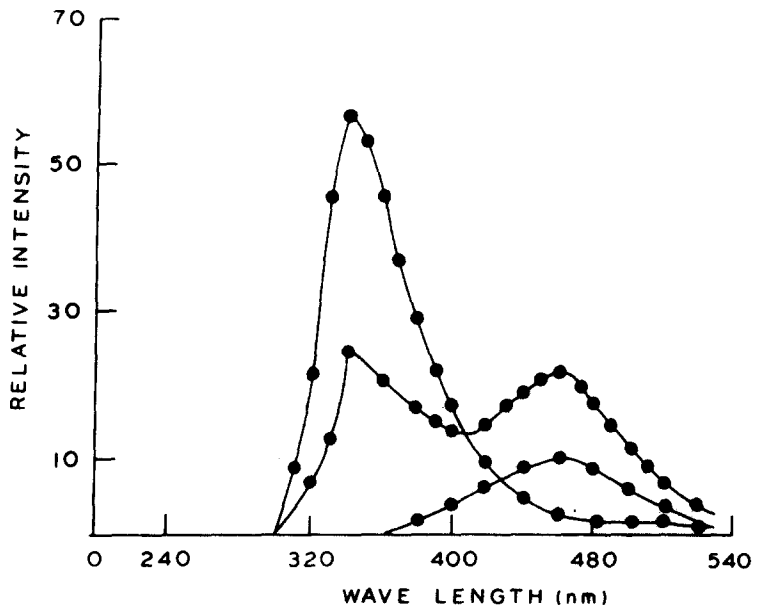

Fig. 4. Effect of binary complex formation with NADPH on glutamate dehydrogenase emission spectrum. NADPH was added to a cuvette containing 2.0 ml (0.1 mg/ml glutamate dehydrogenase in 0.05 M phosphate buffer, pH 7), so that the final NADPH concentration was 0.1 mM. Excitation was constant at 280 nm. The uppermost line shows the emission spectrum of glutamate dehydrogenase alone. The middle line shows the partially quenched curve with two maxima and represents the mixture of glutamate dehydrogenase with NADPH. The lowest line shows the emission spectrum of an equal amount of NADPH in the absence of enzyme.

the reverse reaction was examined by varying the $NADPH_2$ concentration at different fixed concentrations of NH_4Cl at a saturating concentration of L-ketoglutarate. An intersecting pattern of lines was obtained. In another set of experiments, at various concentrations of L-ketoglutarate and at a subsaturating concentration of NH_4Cl, the velocity of the reaction was determined. An intersecting pattern of lines was obtained in the double-reciprocal plots. A parallel set of lines was obtained when the above experiment was repeated at a saturating concentration of NH_4Cl.

Fluorescence studies. The glutamate dehydrogenase exhibited a typical protein fluorescence spectrum with an excitation maximum at 280 nm and an emission maximum at 340 nm. The binding of $NADPH_2$ was monitored by quenching of the fluorescence at 340 nm and an enhancement of fluorescence at 460 nm (Fig. 4). NH_4Cl and L-α-ketoglutarate had no effect on the protein fluoresence spectrum.

Substrate specificity. The enzyme was specific for $NADP^+$ and $NADPH_2$. No activity could be detected in the presence of NAD and $NADH_2$. The activity in the presence of several L-amino acids is shown in Table I. A marginal activity of 10% of the activity with glutamate was shown with aspartate and leucine and about 5% with isoleucine, valine and lysine. Very little activity was observed with norvaline, glycine, serine and phenyl alanine. L-Ketoglutarate, L-ketovalerate, L-ketoisovalerate and L-ketobutyrate were poor substrates while pyruvate, oxaloacetate and glyoxylate were not acted upon.

TABLE I

ACTIVITY OF GLUTAMATE DEHYDROGENASE ON OTHER AMINO ACIDS AND KETO ACIDS

The specificity of glutamate dehydrogenase from *M. smegmatis* CDC 46 was checked for different amino acids and keto acids. The assay conditions for the different amino acids remained the same as for the oxidative deamination of glutamate and the conditions for different alpha-keto acid remained the same as for the reductive amination of alpha-ketoglutarate, respectively. The concentration used for the amino acids as substrate was 50 μmol and for all the keto acids was 10 μmol in their assays, respectively.

Substrates	Relative rate
L-Glutamate	100.0
L-Aspartic acid	10.0
L-Leucine	9.0
L-Isoleucine	7.0
L-Methionine	6.0
L-Valine	5.0
L-Lysine	3.0
L-Norvaline	2.0
L-Glycine	0.8
L-Serine	0.8
L-Phenyl alanine	0.5
L-Ketoglutarate	100.0
L-Ketovalerate	12.0
L-Ketoisovalerate	8.0
L-Ketobutyric acid	2.5
Pyruvic acid	Not acted upon
Oxaloacetic acid	Not acted upon
Glyoxylate	Not acted upon

Effect of nutritional factors, some glycolytic and tricarboxylic acid cycle intermediates on the activity of glutamate dehydrogenase. When both glycerol and asparagine were substituted by glucose and NH_4Cl, respectively, or glucose and glutamic, respectively, in Youmans and Karlson medium [1] on equimolar basis, a 5.5-fold and 4.3-fold increase in activity, respectively, was observed. The effect of some glycolytic and tricarboxylic acid cycle intermediates on the enzyme activity is listed in Table II.

TABLE II

EFFECT OF VARIOUS INTERMEDIATES ON THE ACTIVITY OF PURIFIED GLUTAMATE DEHYDROGENASE

The glycolytic and TCA cycle intermediates were tried on the activity of purified glutamate dehydrogenase from *M. smegmatis* CDC 46 at various concentrations as given in the table. The enzyme was assayed spectrophotometrically as described under Materials and Methods. Values represent the average of three separate experiments. (a) represents percentage activity of the enzyme during oxidative deamination and (b) represents percentage activity of the enzyme during reductive amination.

Concentration (mM)	Glu-6-*P*		Fructose-1,6-P_2		Citrate		Isocitrate		Pyruvate	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
None	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	100.0	100.56	99.0	102.7	110.0	100.0	100.0	100.0	100.0	86.6
25	100.0	167.56	87.5	175.6	145.4	100.0	125.0	100.0	100.0	82.2
50	56.25	205.4	75.0	216.21	181.8	98.0	125.0	86.6	100.0	75.4
100	37.5	62.4	25.0	100.0	181.8	77.7	93.5	75.5	99.0	44.4

Comparison of levels of various enzymes related to glutamate metabolism. The levels of several enzymes of glutamate metabolism when *M. smegmatis* was grown on different carbon and nitrogen sources have been investigated. A reciprocal relationship between the levels of glutamate dehydrogenase and isocitric dehydrogenase was observed. L-α-ketoglutarate dehydrogenase and glutamate dehydrogenase activity increased when glutamate was present in the medium. NH_4Cl and glucose increased the activity of glutamate dehydrogenase while the activity of L-α-ketoglutarate dehydrogenase was only marginally affected.

Discussion

Glutamate dehydrogenase has been intensively studied in several microorganisms [10,11], animal [12] and plant sources [13,14]. In view of the importance of this enzyme in the nitrogen metabolism and as a link between protein and carbohydrate metabolism, this enzyme from *M. smegmatis* CDC 46 was purified. The uniqueness of the purification procedure was the use of 2′,5′-ADP-Sepharose 4B. Earlier methods of purification involved the use of a glutamate- or an AMP-hooked affinity column [12]. A 300-fold increase in specific activity and removal of large number of contaminating proteins was observed (Table III). In view of the fact that a group specific affinity reagent was used, the enzyme preparation at this step was not homogeneous (Fig. 5) and passage through a sieving column yielded an enzyme preparation which was homogeneous (Fig. 5). The molecular weight, subunit molecular weight, pH optima and

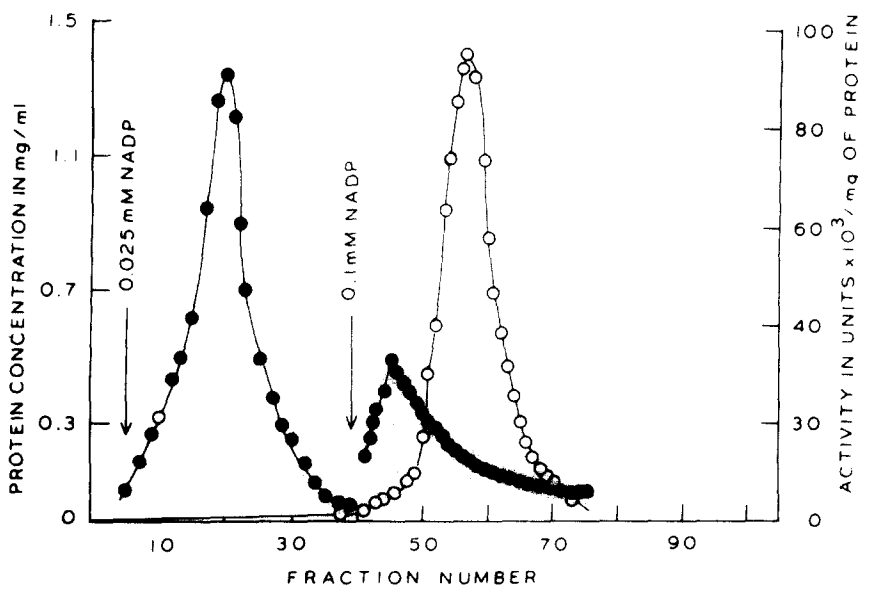

Fig. 5. 2′,5′-ADP-Sepharose affinity chromatography. Protein obtained from negative adsorption on DEAE-cellulose column (1 × 8 cm) was loaded on a 2′,5′-ADP-Sepharose 4B column. The column was washed well to remove the unbound proteins with 0.1 M phosphate buffer, pH 7. The step wise elution with 0.025 mM (40 ml) and 0.1 mM $NADP^+$ was carried out in the same buffer, at the rate of 10 ml/h, in fractions of 1 ml each. ○———○ enzyme; ●———● protein.

TABLE III

PURIFICATION OF GLUTAMATE DEHYDROGENASE FROM *M. SMEGMATIS* CDC 46

Cells grown on Youmans and Karlson synthetic medium were used. Preparation of cell-free extract and purification of the enzyme were described in the section on Materials and Methods. The purification obtained in a typical experiment is given in this table.

Step	Total protein (mg)	Total activity (units) *	Specific activity **	Fold purification
Crude	3125	240 625	77	—
25—65% NH_4Cl fraction	1548	217 960	141	1.8
Negative adsorption on DEAE-cellulose	1250	192 500	154	2.0
2',5'-ADP-Sepharose 4B	2.5	132 314	53 584	696.0
Sephadex G-200	1.2	96 248	80 207	1042.0

* Unit of enzyme activity is the amount of enzyme that converted 1 nmol NADPH to $NADP^+$ per min.
** Specific activity has been expressed as nmol NADPH converted to $NADP^+$ per min/mg protein.

enzyme specificity were comparable to the enzymes isolated from other microorganisms [10].

Preliminary kinetic analysis suggested that the reaction in both the directions was sequential with NADPH being the first substrate to add followed by NH_4Cl and L-α-ketoglutarate. Some support for this proposal is the observation that saturation with NH_4Cl results in a parallel set of lines in the reciprocal plot, whereas both the slope and the intersect effect was observed at subsaturated concentration. The fluoresence quenching observed on $NADPH_2$ addition suggested that this may be binding to the free enzyme. The enzyme was specific for $NADP^+$. An interesting observation was differences in specificity between the fast growing saprophytes of mycobacteria which required $NADP^+$ as the coenzyme, and the NAD^+ specificity for the enzyme from pathogenic and slow-growing mycobacteria. The increased enzyme levels when the organism was grown on glucose or NH_4Cl suggested that these precursors or their metabolites were probably enhancing either synthesis or the activity of the enzyme. Our results now permit a critical examination of the structure, regulation and function of glutamate dehydrogenase in mycobacteria.

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