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GLUTAMATE DEHYDROGENASE FROM TUNA LIVER

PURIFICATION, CHARACTERISTICS AND SEQUENCE OF A PEPTIDE CONTAINING AN ESSENTIAL LYSINE RESIDUE

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Summary

Glutamate dehydrogenase (EC 1.4.1.2—4) has been purified and crystallized from the acetone powder of tuna liver.

The enzyme has a molecular weight of 333 000 ± 15 000 as evaluated by sedimentation equilibrium and consists of six identical subunits. Unlike the bovine enzyme the molecular weight does not increase with increasing protein concentration indicating that the tuna enzyme has no tendency to polymerize.

The amino acid composition and peptide maps of the tuna and bovine liver enzyme are similar, suggesting considerable homology between the two enzymes. Furthermore, from the tryptic digest a hexadecapeptide containing a lysine residue reactive to pyridoxal 5′-phosphate exhibits the same composition and sequence as the peptide containing the reactive lysine-126 in the sequence of the bovine enzyme.

The molecular activity is 25 and 510 mol of substrate per mol enzyme per s, respectively, for the glutamate oxidation and the α-ketoglutarate reduction with NAD or NADP as coenzymes. The enzyme is regulated by pyridine nucleotides like other vertebrate enzymes, but it also exhibits some coenzyme specificity, the activity being about fifteen times higher with NAD than with NADP.

Introduction

Structural properties of glutamate dehydrogenase (EC 1.4.1.2—4) from fish have not yet been described, although the enzyme from dogfish, purified to homogeneity and characterized in its kinetics a few years ago [1—2], showed

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some characteristics different from other glutamate dehydrogenases. On the other hand, much information regarding the structure of the enzyme from a variety of other sources, ranging from microorganisms [3—6] to mammals [7], has appeared from various laboratories.

With a view to overcoming the present gap in knowledge concerning fish glutamate dehydrogenase, with particular regard to phylogenesis, we initiated a study on the enzyme from the liver of tuna (*Thunnus thymnus*) which is readily available in this country.

This paper reports the isolation, physical, chemical and kinetic characterisation of the enzyme and the sequence of a peptide containing a lysine residue involved in the catalytic activity.

Experimental procedure

Materials. The sources of all chemicals, enzymes and resins used have been described [5,6,8]. Frozen tuna livers were kindly supplied by Mr. Ferrari of the Maruzella Fish Company, Marano Lagunare, Venice.

Enzyme assay. During the purification procedures the assay mixture (2.5 ml) was 5 mM glutamate/0.1 mM NAD in $I = 0.1$ phosphate buffer (pH 8). The reduction of NAD at 25°C was followed by changes in absorbance at 340 nm with a Gilford Model 2000 recording spectrophotometer. Enzyme activity was defined in Standard Units. In the reverse reaction the assay mixture (2.5 ml) was 5 mM α-ketoglutarate/0.1 mM NADH/0.1 M $(NH_4)_2SO_4$ in 0.01 M Tris/acetate buffer (pH 8).

Protein concentration in the early stages of the enzyme purifications was determined spectrophotometrically [9]. The concentration of the pure enzyme was calculated from the value $A^{0.1\%}_{280\,nm} = 0.97$ [10].

Purification of the enzyme. Glutamate dehydrogenase was prepared from the acetone powder of thawed frozen livers. It was found that approximately 1 kg wet weight of tuna livers (each weighing about 350 g) yielded on average 250 g light tan, fluffy acetone powder. For the extraction the acetone powder was suspended for a few min, in a Waring Blendor in 8 vols. 0.05 M phosphate buffer (pH 7.2)/10^{-3} M EDTA (w/v) followed by 1.5 h stirring at 4°C. The suspension was spun and ethanol (20% v/v) was slowly added to the supernatant solution while the temperature was decreased to —10°C. After 30 min the precipitate, collected by centrifugation, was discarded and the clear supernatant was brought to pH 5.2 by dropwise addition of 4 M ammonium acetate buffer (pH 4). The precipitate containing crude enzyme was collected, dissolved in 300 ml 0.1 M phosphate buffer (pH 7) and fractionated by addition of ammonium sulfate. Solid ammonium sulfate (59 g) was slowly added while stirring at 4°C. After 30 min the precipitate was removed by centrifugation and the supernatant (310 ml) added to 23 g ammonium sulfate. After 30 min standing the suspension was centrifuged and the pellet containing enzyme activity was dissolved in about 100 ml 0.005 M phosphate buffer (pH 7.2)/10^{-3} M EDTA/2-mercaptoethanol and dialyzed overnight against the same buffer. To the dialyzed solution were added 100 ml of settled DE-32 cellulose equilibrated with the same 0.005 M buffer, and the mixture was stirred for 30 min. The

cellulose was filtered, washed with 150 ml of the same buffer and twice with 0.01 M buffer.

The enzyme was finally eluted by washing the cellulose with two 150 ml portions of 0.05 M buffer and recovered by precipitation with ammonium sulfate (50% saturation). The precipitate was dissolved in 0.1 M phosphate buffer at pH 6.8 containing EDTA and 10^{-3} M 2-mercaptoethanol to reach a protein concentration of about 25 mg per ml, and saturated ammonium sulfate solution was slowly added to about 30% saturation at 4°C. After a few hours crystallization occurred. Recrystallization was accomplished by dissolving the protein, 10—15 mg per ml in 0.1 M phosphate buffer; saturated solution of ammonium sulfate was slowly added to reach faint turbidity. The sample was then left standing at 4°C for few days. At this protein concentration the crystallization usually occurred in the range 30—33% ammonium sulfate saturation. To assure constancy of pH during the addition of the saturated ammonium sulfate, this solution was made in 0.1 M phosphate buffer (pH 6.8).

Amino acid analysis. Analyses were performed with a Jeol Model GAH automatic amino acid analyzer by the procedure of Hamilton [11] for the single column. In this procedure ϵ-pyridoxyl-lysine is eluted between ammonia and histidine. Samples of protein were hydrolyzed with 6 M HCl containing 0.1% mercaptoacetic acid and 0.05% phenol for 22, 44 and 72 h at 110°C.

Sedimentation equilibrium. Weight average molecular weights were determined at 20°C by the meniscus depletion method of Yphantis [12] in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics and a temperature control unit. A three double-channeled centerpiece, solution column heights of 2.7 mm and sapphire windows were employed.

Three different protein concentrations between 0.2 and 1.4 mg per ml were used in each cell, at speeds of 10 000 or 9000 rev./min for the native enzyme and 32 000 rpm for the samples in guanidinium chloride. The interference patterns were recorded on Kodak metallographic plates with an exposure of 35 s and the plates were read on a Gaertner microcomparator. Plates were analyzed at 24 and 48 h to insure that equilibrium was complete. The x coordinate at each half-fringe was measured and the computations were performed with the aid of a Monroe Epic 3000 programable calculator. Weight average molecular weights ($\overline{M}$) were determined from the plotted slopes of ln c vs. x^2, from Equation 1 where x is the distance from the axis of rotation to the position of the half-fringe of c (concentration).

$$\overline{M} = \frac{2RT}{(1 - \overline{V}p)\omega^2} \cdot \frac{\mathrm{d} \ln c}{\mathrm{d}x^2} \qquad (1)$$

R is the gas constant, T is the absolute temperature, $\overline{V}$ is the partial specific volume, p is the density of the solvent, and ω is the angular velocity of the rotor.

The solutions were dialyzed for 48 h at 4°C. The density of the solutions was determined by pycnometry. Partial specific volumes were calculated from the amino acid composition.

Modification of enzyme. In kinetic studies of inactivation a sample of stock enzyme suspension in $(NH_4)_2SO_4$ was centrifuged and the resulting pellet dissolved in buffer to give a final concentration of 1 mg per ml. The procedures

and calculations for inactivations by pyridoxal 5'-phosphate were similar to those described for the bovine enzyme [13].

Isolation of the ε-pyridoxyl-lysine containing peptide. To the enzyme (200 mg) in 200 ml 0.1 M phosphate buffer at pH 7.2, 20 ml $6.4 \cdot 10^{-4}$ M pyridoxal 5'-phosphate were added. After 20 min the sample was treated with 20 ml 1% $NaBH_4$ to complete reduction of the aldimine while the pH was maintained at 7.0—7.2 by continuous addition of dilute acetic acid. The labelled protein, which retained about 8% of the starting activity was purified from reagents by repeated precipitation with 50% saturated $(NH_4)_2SO_4$. The pellet was dissolved in 8 ml 6 M guanidinum chloride in Tris-acetate buffer (0.2 M, pH 8.5) containing 0.04 M 2-mercaptoethanol. After 5 h under nitrogen 75 mg iodoacetic acid were added and, when no reactivity to the Ellman reagent was detected, 2-mercaptoethanol was added to destroy excess iodoacetic acid. The samples were exhaustively dialyzed against water.

The modified carboxymethylated protein was suspended in 70 ml water at room temperature and at pH 8.5, and 4 mg of trypsin were added. The pH was maintained at 8.0 with an autotitrator by addition of 0.1 M NaOH. After 2 h an additional 2 mg trypsin were added and the temperature was increased to 37°C. The reaction was terminated 1 h later by the addition of 20 ml 30% acetic acid. The mixture was concentrated to approximately 8 ml by rotary evaporation under vacuum.

Initial fractionation was performed on a Sephadex G50 SF column (3.8 × 138 cm) with 20% acetic acid. Fractions of 8 ml were collected at a flow rate of 25 ml $\cdot$ h^{-1} and were monitored for fluorescence at 410 nm (excitation at 300 nm), absorbance at 280 and 340 nm and for peptide content with ninhydrin [14] after alkaline hydrolysis [15]. The fraction containing the chromophore was further fractionated by preparative high voltage paper electrophoresis at pH 1.9 (formic/acetic acid) and preparative descending paper chromatography with 1-butanol/pyridine/glacial acetic acid/water (15 : 10 : 3 : 12, v/v) on Whatman No. 3MM paper. Peptides containing ε-pyridoxyl-lysine residues were detected on paper by their fluorescence when viewed under an ultraviolet lamp while other peptides were located by dipping the paper in ninhydrin/collidine reagent [16]. Final purification was achieved by fractionation on a Sephadex G25 column (1.6 × 110 cm) with 20% acetic acid.

Small scale enzymatic digestions. Digestion by trypsin or by carboxypeptidases A and B was performed in 0.1 M ammonium carbonate buffer at 37°C with a level of enzyme to protein or peptide of 2%. Before peptide mapping the tryptic mixture was repeatedly lyophilized and dissolved in water for complete removal of NH_4HCO_3.

Edman degradation. The method of Peterson et al. [17] with some modifications [6] was used.

Results

Purification of tuna glutamate dehydrogenase

It was observed that the preparation of acetone powder is necessary for a satisfactory extraction of enzyme. Table I presents a summary of the purification procedure of tuna liver glutamate dehydrogenase starting from acetone

TABLE I

PURIFICATION OF TUNA LIVER GLUTAMATE DEHYDROGENASE

Procedure	Volume (ml)	Protein (g)	Units	Specific activity	Yield (%)
Acetone powder extract *	1000	66	1250	0.019	100
20% ethanol, supernatant	1100	50	1250	0.025	100
pH 5.2 precipitation, precepitate	450	15	760	0.056	62
0—32% $(NH_4)_2SO_4$ precipitation, supernatant	500	10	645	0.065	52
20—45% $(NH_4)_2SO_4$ precipitation, precipitate	105	6.3	560	0.089	45
DE-32, batch elution	20	0.5	465	0.93	37
0—50% $(NH_4)_2SO_4$ precipitation, precipitate	6.2	0.29	360	1.24	29
1st crystals	8.0	0.19	320	1.68	26
2nd crystals	8.0	0.15	284	1.90	23
3rd crystals	8.0	0.13	250	1.93	21

* For the extraction 150 g acetone powder were suspended in 1200 ml of phosphate buffer (see Text).

powder. Usually three to four crystallizations are necessary to obtain completely colorless concentrated solutions of enzyme; however, there is no significant increase in specific activity with the last crystallizations. The homogeneity of the enzyme is proved by (a) the constancy of the specific activity in eight different preparations, (2) a single band in analytical sodium dodecyl sulfate polyacrylamide gels, (3) a single symmetrical Schlieren peak in the ultracentrifuge at high concentration of enzyme (6.5 mg/ml) (see Fig. 1) and (4) by the linearity of the plots of log c. vs. x^2 [12] in the sedimentation equilibrium experiments both with the native enzyme and the dissociated subunits.

The enzyme is stable for months when suspended in ammonium sulfate.

Molecular weight

The molecular weight of the native enzyme was determined by sedimentation equilibrium after dialysis against 0.1 M phosphate buffer containing 1 mM EDTA and 1 mM 2-mercaptoethanol at pH 7.2. A partial specific volume of 0.735 was calculated from the amino acid composition. An average molecular weight of 333 000 ± 15 000 was obtained at protein concentrations of 0.2, 0.4,

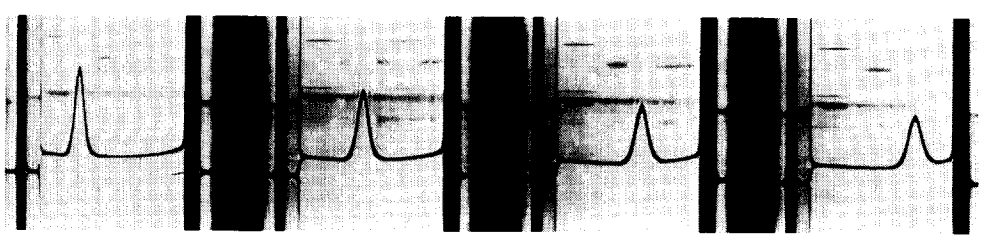

Fig. 1. Sedimentation pattern of purified tuna glutamate dehydrogenase. The protein concentration was 6.5 mg/ml in 0.1 M phosphate buffer, (pH 6.8)/0.1 mM 2-mercaptoethanol. Migration is from left to right. The photographs were taken at various times after reaching a speed of 40 000 rev./min. The temperature was 10° C and the Schlieren plate angle was 60° C.

TABLE II

MOLECULAR WEIGHT EVALUATION BY SEDIMENTATION EQUILIBRIUM

	Protein concentration (mg/ml)			Molecular weight	Average of two runs
Native enzyme in 0.1 M phosphate buffer	0.2	0.4	0.8 [a]	336 575 ± 14 399	333 204 ± 16 465
	0.7	1.05	1.4 [b]	329 855 ± 4 851	
Dissociated enzyme in 6 M guanidine	0.5	0.75	1.0 [c]	54 054 ± 1 774	53 898 ± 2 557
	0.4	0.6	0.8	53 751 ± 2 400	

Rotor speed: [a] 10 000 rev./min, [b] 9000 rev./min, [c] 32 000 rev./min.

0.7, 0.8, 1.05 and 1.4 mg/ml. These experiments indicate no concentration dependence within the range 0.2—1.4 mg enzyme per ml. A summary of these results is reported in Table II.

A further indication of the low tendency of the enzyme to polymerize is the symmetry of the Schlieren peak obtained at high protein concentration.

Subunit size was investigated by sedimentation equilibrium [12—5] on samples containing 6 M guanidinium · HCl and 0.5% mercaptoethanol at pH 7.3 after dialysis for 48 h at 4°C. An average value from six different enzyme concentrations of 53 900 ± 2500 (see Table II) indicates that the native enzyme is composed of six subunits per molecule.

TABLE III

AMINO ACID COMPOSITION OF TUNA LIVER GLUTAMATE DEHYDROGENASE COMPARED WITH THE BOVINE AND THE DOGFISH ENZYME

Amino acid residues for subunit of mol. wt. 55 400.

Amino acid	Tuna		Bovine [a]	Dogfish [b]
	Residues per subunit	Nearest integer residue		
Lysine	31.22	31	33	30.64
Histidine	14.62	15	14	10.40
Arginine	28.13	28	30	22.16
Aspartic acid	52.04	52	50	47.86
Threonine	27.29	27	28	25.15
Serine	25.28	26	30	23.26
Glutamic acid	47.44	47	45	50.19
Proline	28.13	28	21	23.54
Glycine	47.82	48	47	48.47
Alanine	37.98	38	37	35.95
Valine	30.94	31	34	22.76
Methionine	11.65	12	13	15.62
Isoleucine	33.91	34	37	29.75
Leucine	28.69	29	31	28.47
Tyrosine	16.87	17	18	16.73
Phenylalanine	20.82	21	23	21.49
Tryptophan	3.79	4	3	
Cysteine	5.72	6	6	

[a] Values obtained from sequence [7].

[b] Values recalculated from the data of Corman et al. [1].

Amino acid composition

Table III presents a comparison of the composition of the tuna enzyme with the bovine and the dogfish enzymes. Numbers of residues were calculated for a subunit of molecular weight 55 400 which, obtained from the sequence of

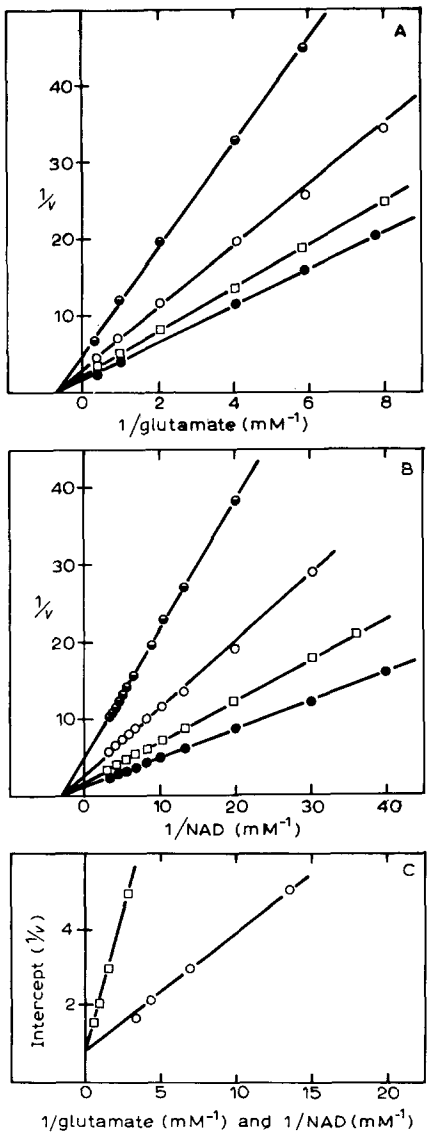

Fig. 2. A. Variation of the reciprocal of enzyme activity with the reciprocal of glutamate concentration, with potassium phosphate buffer (0.2 M, pH 7.6) at 25°C, for several constant values of NAD concentration: ●———●, 0.075 mM; ○———○, 0.15 mM; □———□, 0.225 mM; ●———●, 0.3 mM. B. Variation of the reciprocal of enzyme activity with the reciprocal of NAD concentration, buffer conditions as above, for several constant values of glutamate concentration: ●———●, 0.38 mM; ○———○, 0.75 m: □———□, 1.5 mM; ●———●, 3 mM. C. Secondary plots of the previous experiments: the vertical intercepts of Figs. 2A and B were plotted as a function of the reciprocal of the concentrations of the changing fixed substrates: □———□, glutamate, and ○———○, NAD, respectively.

the bovine enzyme, is also in good agreement with that of the tuna enzyme obtained by sedimentation equilibrium experiments. For an easier comparison with the composition of the tuna enzyme, numbers of residues for the dogfish glutamate dehydrogenase were recalculated for a molecular weight of 55 400 from the data of Corman et al. [1] where the composition was reported in μmol per mg of protein. The results for the tuna enzyme are given as the averages of analysis performed on separate hydrolysates of different preparations. Linear extrapolation to zero time was used to obtain values for serine, threonine and tyrosine. The values obtained on the 72 h sample were used for valine and isoleucine. Tryptophan was estimated spectrophotometrically by the method of Goodwin and Morton [19] as modified by Beaven and Holliday [20]. The half-cystine content was determined by spectrophotometric-titration with dithionitrobenzoate in 6 M guanidinium chloride. *S*-Carboxymethylation of the enzyme in 6 M guanidinium chloride in the presence of 2-mercaptoethanol followed by acid hydrolysis and amino acid analysis yielded 6—6.3 *S*-carboxymethylcysteine residues per subunit. These results indicate that all half-cysteine residues are present as cysteine.

Kinetic studies

Initial-rate data measurements were made in 0.2 M potassium phosphate buffer at pH 7.6 at 25°C with the concentration of NAD in the range 0.075—0.3 mM and glutamic acid in the range of 0.38—3 mM. Lineweaver-Burk plots with each reactant as variable substrate (Figs. 2A and B) are linear within the experimental error, and secondary plots of the slopes and intercepts (Fig. 2C) are also linear.

A family of straight lines intersecting at the horizontal axis was obtained with both substrates. As the plots did not show any parallel lines, it is evident that a reversible connection between the binding of glutamate and NAD exists. This indicates the formation of a ternary complex between the enzyme and the substrate; both glutamate and the coenzyme must bind to the enzyme before the release of any of the product, but no clue is afforded by these experiments as to the sequence of addition.

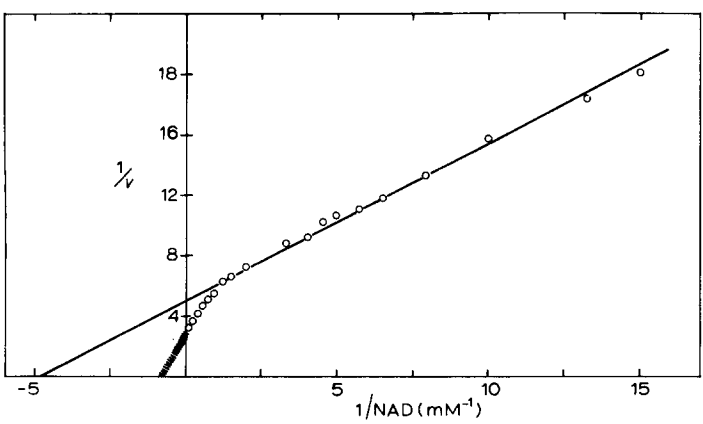

Fig. 3. Variation of the reciprocal of the enzyme activity with reciprocal of NAD concentration for glutamate concentration 50 mM, with potassium phosphate buffer (0.11 M, pH 7) and 10^{-5} M EDTA at 25°C.

From the points of intersection of Fig. 2 the K_m for glutamate and NAD were calculated to be $1.4 \cdot 10^{-3}$ and $3.3 \cdot 10^{-4}$ M, respectively. The molecular activity measured as mol substrate per mol enzyme per s could also be estimated as 25. In the reverse reaction, with NADH with coenzyme, a molecular activity of 510 was calculated.

The enzyme was found to be about 15 times less active with NADP as coenzyme; its lower specificity is also revealed by its higher K_m which is $0.7 \cdot 10^{-3}$ M.

Engel et al. [21] observed that for the bovine enzyme NAD at high concentration is an activator. Fig. 3 shows that this holds also for the tuna enzyme: an activation is observed at high NAD concentration. In these activation conditions higher values of K_m for NAD may be calculated which are in the range of 10^{-3} M.

Tuna enzyme is strongly inhibited by isophthalic acid: the inhibition is competitive with glutamic acid, and from the slopes a K_i value of $2.8 \cdot 10^{-4}$ M was calculated. This value is of the same order as that reported by Canghey et al. [22] for the bovine enzyme ($5.6 \cdot 10^{-4}$). Furthermore, the enzyme activity is enhanced by ADP and inhibited by GTP to the same extent as is the bovine enzyme under identical conditions.

Inactivation by pyridoxal 5'-phosphate

The inactivation of glutamate dehydrogenase by pyridoxal 5'-phosphate was extensively studied for the bovine enzyme [23] since it offered a convenient method of specifically labelling the enzyme. The tuna enzyme is inactivated by

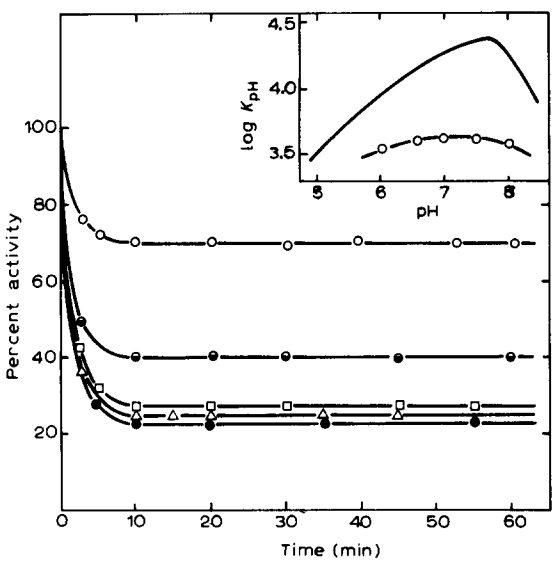

Fig. 4. Inactivation of tuna glutamate dehydrogenase by pyridoxal 5'-phosphate. Reactions were performed at 25°C in I = 0.1 phosphate buffer. Enzyme concentration was 0.7 mg/ml. At pH 6.0, pyridoxal 5'-phosphate concentration was $7.8 \cdot 10^{-4}$ M (□———□), at pH 7.2, pyridoxal 5'-phosphate concentrations were $1.1 \cdot 10^{-4}$ M (○———○), $3.8 \cdot 10^{-4}$ M (◒———◒), $7.8 \cdot 10^{-4}$ M (●———●). At pH 8, pyridoxal 5'-phosphate concentration was $7.8 \cdot 10^{-4}$ M (△———△). Insert: Effect of pH on the logarithm of the equilibrium constant, K_{pH}, for the formation of the pyridoxal 5'-phosphate inactivated enzyme complex (○———○), in comparison with that observed in the bovine enzyme (———) [12].

pyridoxal 5′-phosphate and Fig. 4 shows the time course of the reaction with $1.9 \cdot 10^{-5}$ M enzyme and excess pyridoxal 5′-phosphate in molar ratio ranging from 10 to 50 at pH 6.5, 7.2 and 8.0. The rate of inactivation was generally faster than that of the bovine enzyme under similar conditions [13] and was almost independent of pyridoxal phosphate concentration. Similar behaviour had already been observed with the NAD-specific enzyme of *Neurospora* and it was ascribed to a rate-limiting interaction between enzyme and pyridoxal 5′-phosphate due to the phosphate group [24]. The extent of inhibition was related to reagent concentration, and equilibrium constants for formation of the enzyme-pyridoxal 5′-phosphate adduct obtained at pH 6—8 ranged from $3.5—4.1 \cdot 10^{-3}$ (Fig. 4, insert). As expected the inactivation was completely reversed by dialysis while stabilized by $NaBH_4$ reduction.

To characterize the inhibition product, 10 mg of enzyme was dissolved in 15 ml 0.1 M phosphate buffer (pH 7.2), pyridoxal 5′-phosphate (3.2×10^{-4} M) was added and, after equilibrium was reached (8% activity remaining), $NaBH_4$ was added to complete reduction of the chromophore. The spectrum of the protein after purification from excess reagents showed a maximum at 325 nm, characteristic of ϵ-*N*-pyridoxyl-lysine [25]. From the extinction coefficient of the reduced aldimine at 325 nm the presence of about 1.2 residues of ϵ-*N*-pyridoxyl-lysine was calculated. A similar value (1.2—1.5 residues per mol) was obtained by analysis of the acid hydrolysate of the modified protein.

Peptide mapping performed on a tryptic digest of the *S*-carboxymethylated modified enzyme showed, in addition to 30—40 ninhydrin positive spots, one single fluorescent spot under ultraviolet irradiation. A similar picture was obtained with the bovine enzyme treated in the same way.

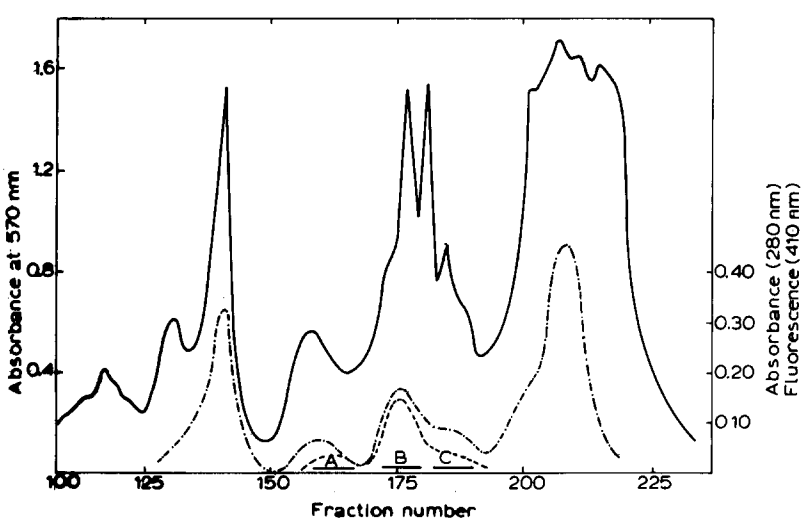

Fig. 5. Fractionation of the tryptic digest of tuna glutamate dehydrogenase, previously inactivated with pyridoxal 5′-phosphate and carboxymethylated, on a column (3.8 × 140 cm) of Sephadex G-50 in 20% acetic acid. Fractions of 8 ml were collected at a flow rate of 50 ml/h. Tubes were monitored by ninhydrin assay after alkaline hydrolysis (———), for absorption at 280 nm (— · — · —), and fluorescence at 410 nm by illumination at 300 nm (- - - - -). Pooled fractions are indicated by solid bars.

Isolation and sequence of the pyridoxal 5'-phosphate-labelled peptide

The above reported results prompted us to isolate and characterize the pyridoxal 5'-phosphate-labelled peptide, therefore sufficient enzyme (200 mg) was labelled, carboxymethylated and digested with trypsin.

The elution of the tryptic peptides from a column of Sephadex G-50 is shown in Fig. 5: one major peak with both absorption at 280 and fluorescence at 410 nm following excitation at 300 nm was revealed. Fraction B was further purified by paper chromatography followed by high voltage paper electrophoresis. At this step, while the peptide appeared pure by chromatographic tests, analysis revealed contaminant material that was eliminated by chromatography on Sephadex G 100. Analysis indicated the following composition for peptide TS6B: *S*-carboxymethyl cysteine 0.8 (1); aspartic acid 1.1 (1); proline 1.2 (1); glycine 3.3 (3); alanine 3.2 (3); valine 3.2. (this value increased to 3.7 (4) after 48 h hydrolysis); phenylalanine 1.0 (1); lysine 1.1 (1) and ϵ-pyridoxyl-lysine 0.6 (1). This peptide, the only one labelled by pyridoxal 5'-phosphate as revealed by fluorescence, was recovered in 20% yield without taking into account the losses during the various steps of purification.

Carboxypeptidases A and B released after 5 min lysine, valine and a trace amount of glycine, and after 20 min lysine, valine, glycine and alanine. No further hydrolysis was observed even after 20 h, indicating the proximity of a non-hydrolyzable peptide bond. Edman degradation allowed identification of the NH_2-terminal five residues in the sequence CmCys-Ala-Val-Val-Asp.

From these data and the analysis of the whole peptide the following partial sequence could be deduced:

CMCys—Ala—Val—Val—Asp—(Val, Pro, Phe, Gly, Gly, Ala, Lys)—Ala—Gly—Val—Lys.
PLP

Discussion

Crystalline tuna liver glutamate dehydrogenase can be obtained in good yield from acetone powder with only a few steps of purification. The crystalline enzyme can be stored in ammonium sulfate for months without appreciable loss of activity. Also, the acetone powder retains activity for long periods, this being important for an unusual source of enzyme.

The molecular weight of tuna glutamate dehydrogenase is very close to that already found for other vertebrate glutamate dehydrogenases. Furthermore the tuna enzyme does not appear to polymerize at increasing protein concentrations. The polymerisation of glutamate dehydrogenase is a characteristic commonly observed among the vertebrates and found absent only in the rat and dogfish enzymes [26,1] although recently, with the rat liver enzyme, the difficulty to safely assess this property was demonstrated [26].

In any case the low tendency of the tuna enzyme to polymerise is a very welcome property since it may offer a new opportunity to obtain crystals satisfactory for X-ray analysis. In fact Josephs recently demonstrated that the difficulty in obtaining good crystals for X-ray analysis from bovine and chicken enzymes is due to their polymerisation properties, and suggested that enzymes which polymerize weakly or not at all may be more suitable for X-ray studies

[28]. This observation is now prompting us to make an extensive study of the properties of the crystals of the tuna enzyme.

In comparison with the well known bovine enzyme (see Table III), the amino acid composition of the tuna shows significant differences (over 10%) only in serine and proline content. The composition of the parent dogfish enzyme, in addition to the difference of these amino acids, differs markedly also in methionine, valine, isoleucine, histidine and arginine. The closer similarity in amino acid composition between mammals and tuna (Teleosts) than between mammals and dogfish (Chondricthyes) is not surprising from the evolutionary point of view since the latter is an offshoot derived earlier from the main stock of vertebrates than the bony fishes, the amphibians, reptiles and mammals [29].

Kinetic studies of glutamate oxidation revealed similar constants, a sequential order of mechanism, activation by NAD, inhibition by isophthalic acid, already described for the bovine enzyme. For this reason, further kinetic investigations of the reductive amination of α-ketoglutamate were deemed unwarranted. What appeared to be of interest in the kinetic analysis is the large difference in activity observed with NAD and NADP as coenzymes, the enzyme being about 15 times less active with the latter.

Tuna enzyme is inhibited by pyridoxal 5′-phosphate a modifier that was extensively studied with the bovine enzyme [13,23]. For inactivation a high ratio of pyridoxal 5′-phosphate to protein is necessary since the equilibrium constants for the formation of the adduct are smaller than for the bovine enzyme. The rate of inactivation was faster than that of the bovine enzyme under similar conditions and almost independent of pyridoxal 5′-phosphate concentration as already observed for the NAD-specific glutamate dehydrogenase from *Neurospora* [24]. Despite these differences a single lysine residue is involved in the inactivation since samples inactivated to about 10% remaining activity revealed 1—1.5 residues of ϵ-pyridoxyl-lysine by amino acid analysis after borohydride reduction of the Schiff base. The specificity of the labelling was further demonstrated by peptide mapping of the tryptic digest of the pyridoxal 5′-phosphate modified protein; only a single fluorescent spot was revealed by examination under ultraviolet light and only one labelled tryptic peptide was isolated from the pyridoxal 5′-phosphate inactivated, carboxymethylated protein.

The amino acid composition and partial sequence of this peptide revealed its identicality with the hexadecapeptide (containing lysine-126) already isolated following pyridoxal 5′-phosphate treatment and tryptic digestion from the bovine and chicken enzymes.

This identity in sequence demonstrates the invariance of this part of the molecule of glutamate dehydrogenase in the evolution of the vertebrates. Furthermore, this finding emphasizes the importance of this lysine residue (126 in bovine enzyme) in the catalytic activity of glutamate dehydrogenase [13], although some authors suggest that this residue plays only a minor role in the conformation of enzyme [30].

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References

1 Corman, L., Prescott, L. and Kaplan, N.O. (1967) J. Biol. Chem. 242, 1383—1390
2 Corman, L. and Kaplan, N.O. (1967) J. Biol. Chem. 242, 2840—2846
3 Blumenthal, K.M. and Smith, E.L. (1973) J. Biol. Chem. 248, 6002—6008
4 Wootton, J.C., Chambers, G.K., Holder, A.A., Baron, A.J., Taylor, J.G., Fincham, J.R.S., Blumenthal, K.M., Moon, K.E. and Smith, E.L. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4361—4365
5 Veronese, F.M., Nyc, J.F., Degani, Y., Brown, D.M. and Smith, E.L. (1974) J. Biol. Chem. 249, 7922—7928
6 Veronese, F.M., Degani, Y., Nyc, J.F. and Smith, E.L. (1974) J. Biol. Chem. 249, 7936—7941
7 Moon, K.E., Piszkiewicz, D. and Smith, E.L. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1380—1383
8 Veronese, F.M., Boccù, E. and Conventi, L. (1975) Biochim. Biophys. Acta 377, 217—228
9 Layene, E. in Methods in Enzymology (1975) Vol. 3, pp. 447—454
10 Olson, J.A. and Anfinsen, C.B. (1952) J. Biol. Chem. 197, 67—79
11 Hamilton, B.P. (1963) Anal. Chem. 35, 2055—2063
12 Yphantis, D.A. (1964) Biochemistry 3, 297—317
13 Piszkiewicz, D. and Smith, E.L. (1971) Biochemistry 10, 4544—4552
14 Hirs, C.H.W., Moore, S. and Stein, W.H. (1956) J. Biol. Chem. 219, 623—642
15 Doscher, M.S. and Hirs, C.H.W. (1967) Biochemistry 6, 304—312
16 Bennet, J.C. in Methods in Enzymology (1967) Vol. 3, pp. 330—339
17 Peterson, J.D., Nehrlich, S., Oyer, P.E. and Steiner, D.F. (1972) J. Biol. Chem. 247, 4866—4871
18 McMeekin, T.L. and Marshall, K. (1952) Science 116, 142—143
19 Goodwin, T.W. and Morton, R.A. (1946) Biochem. J. 40, 628—632
20 Beaven, G.H. and Holiday, E.R. (1952) Adv. Prot. Chem. 7, 319—385
21 Engel, P.C. and Dalziel, K. (1969) Biochem. J. 115, 621—631
22 Caughey, W.S., Smiley, J.D. and Hellerman, L. (1957) J. Biol. Chem. 224, 591—607
23 Piszkiewicz, D., Landon, M. and Smith, E.L. (1970) J. Biol. Chem. 245, 2622—2626
24 Degani, Y., Veronese, F.M. and Smith, E.L. (1974) J. Biol. Chem. 249, 7929—7935
25 Johnson, R.J. and Metzler, D.E. (1970) in Methods in Enzymology, Vol. 18, pp. 433—475
26 King, K.S. and Frieden, C. (1970) J. Biol. Chem. 245, 4391—4396
27 Ifflaender, U. and Sund, H. (1972) FEBS Lett. 20, 287—290
28 Josephs, R. (1975) J. Mol. Biol. 97, 127—130
29 Romer, A.S. in Vertebrate Paleontology (1966) University Chicago Press
30 Goldin, B.R. and Frieden, C. (1972) J. Biol. Chem. 247, 2139—2144