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PURIFICATION AND SOME PROPERTIES OF L-GLYCOL DEHYDROGENASE FROM HEN'S MUSCLE

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Summary

1. An enzyme which catalyzes the NAD(P)H-linked reversible reduction of uncharged vicinal dicarbonyls and α-hydroxycarbonyls to L-(+)-glycols has been purified from hen's muscle This enzyme has not been previously described

2. According to the rules of the I U.P.A C.-I U.B. Enzymes Commission, the systematic name of L-(+)-glycol NAD(P) oxidoreductase and the trivial name of L-glycol dehydrogenase are proposed for the enzyme.

3. Three forms of this enzyme differing in p*I* have been isolated; two forms, which together represent about 90% of total recovered activity, are electrophoretically pure.

4. Molecular weight, pH profiles and affinity for substrates are also described.

Introduction

Previous work from this laboratory [1] has demonstrated the presence in hen's muscle of an enzyme which catalyzes acetoin reduction coupled to NADPH oxidation. The present paper describes a purification procedure, which allows us to obtain electrophoretically pure preparations of two forms of this enzyme and to isolate a third form in a less pure state, its characterization as L-glycol.NAD(P) dehydrogenase — as far as we know a new enzyme — and some of its properties.

Materials and Methods

NAD, NADP, NADH and NADPH were supplied by Boehringer; α-NADH by Sigma. Acetoin (BDH) was purified as previously described [2] and diacetyl by fractional distillation through a Hempel column, collecting the material which distils in the range 88—89°C at normal pressure; 2,3-pentanediol and hydroxy-

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butyrate were a gift of Dr Størmer, Oslo University, and diacetylmethylcarbinol was donated by Dr López, then at the University of Bochum (F R G). Acetylethylcarbinol was synthesized from hydroxybutyrate by using the procedure recommended by Larsen et al [3]. Calcium phosphate gel was prepared following the method of Keilin and Hartree as described by Dixon and Webb [4]. Sephadex was supplied by Pharmacia Fine Chemicals, Ampholites by LKB and hydroxyapatite by Bio-Rad All other chemicals and biochemicals used were of the best available grade from Merck, BDH, Fluka, Sigma or Calbiochem The biological material consisted of dissected leg muscles free of surface fat and connective tissue from culled laying hens

Protein concentrations were generally determined by the biuret method, as described by Chance and Redfearn [5], absorbance measurements at 280 and 260 nm were used when the protein concentration was low and the method of Fenner et al. [6] when samples contained Ampholites. Bovine serum albumin was taken as standard.

Enzyme activities were spectrophotometrically measured in the reductase direction at 25°C in 0.1 M phosphate buffer, pH 7, 0.2 mM coenzyme and 4 mM of the appropriate oxidized substrate. For assays in the reverse direction the concentration of the oxidized coenzymes was established at 1 mM and that of the reduced substrate at 64 mM The purification procedure was monitored through the NADPH-linked acetoin reductase reaction, 1 unit of enzyme activity is defined as the amount of enzyme that reduces 1 nmol of acetoin per min under assay conditions.

Electrofocusing was performed during 72 h in a 440 ml LKB Svensson and Vesterberg column at 0—0 5°C. Electrophoresis in 7 5% polyacrylamide gels (bisacrylamide/acrylamide, 1 37 5) was performed at 0—4°C in 0 1 M sodium and potassium phosphate buffer, pH 7 8, 8 mA/tube (6 mm inner diameter) was applied until the Bromophenol blue used as tracer reached the end of the tube (12 cm), the protein bands were stained with Coomassie blue following the ORTEC procedure [7] Enzyme activity in the electrophoresis tubes was revealed as diacetyl reductase by the procedure already described [8] except for the following (a) molarity of the incubation buffer (0.5 M instead of 0 05 M) and NADH (1 mM instead of 3 mM), (b) the introduction of a 30 min period of rest (under dry conditions, at room temperature) between incubation with substrates and staining of the residual coenzyme, the rest period being aimed at exhausting the reduced pyridine nucleotide in the enzyme position, and (c) staining buffer 0.5 M Tris-H_3BO_3, pH 9.

Diacetyl was determined by using the procedure of Owades and Jakovac [9] and acetoin and butylene glycol by a method developed in our laboratory [10], which is based on their oxidation into diacetyl after extensive protein-depletion. Optical rotation was measured in a Perkin Elmer 241 electropolarimeter using the D-line of the sodium spectrum as the source of light

Purification

The first stages of the purification were carried out using the procedure already described [1] Muscles were homogenized with 5 vol of cold distilled water using a blade homogenizer. The homogenate was centrifuged for 10 min at 1500 × *g* Calcium phosphate gel was added to the crude extract until

10—15% of the enzyme activity was bound (usually about 0 5 vol. of gel with 2.8 g dried wt./100 ml) which resulted in the adsorption of about 80% of the protein. The gel was removed by centrifugation for 10 min at 1500 × *g* and the supernatant was freeze-dried. 8 g of powder were dissolved in 20 ml of the elution buffer (3 mM sodium and potassium phosphate, pH 7), cleared by centrifugation and chromatographed on a column (5 × 50 cm) of Sephadex G-100. These first stages of purification allowed us to obtain enrichment factors of about 80—90 with a yield of 65—75% of the original activity.

Molecular sieving through Sephadex G-75 superfine allowed the estimation of the molecular weight of the enzyme, the result being 28 000, cytochrome *c* (M_r = 12 500), myoglobin (M_r 17 000), chymotrypsin (M_r = 24 500), β-lactoglobulin (M_r = 35 000), egg albumin (M_r = 45 000) and serum albumin (M_r = 65 000) were used as standard proteins. These experiments also revealed that the enzyme was essentially contaminated with proteins of low molecular weight. Consequently, after freeze-drying, the enriched material from Sephadex G-100 gel filtration was redissolved in water (about 150 mg of protein/2.5 ml) and sieved through a bed of 2 5 × 40 cm of Sephadex G-75 superfine using as eluant 3 mM sodium and potassium phosphate buffer, pH 7, which allowed us to obtain enrichment factors of 170—190 with 50—55% recovery of the activity of the crude extracts The fractions selected from the former purification stage were adsorbed in hydroxyapatite beds and eluted with a discontinuous molarity gradient of sodium and potassium phosphate buffer, pH 7, consisting of 3 mM (three bed volumes), 10 mM (four bed volumes) and 30 mM (six bed volumes). 44—48% of the activity present in the crude extracts, with a specific activity of 500—750 U/mg protein, was recovered in the 30 mM fraction; no more enzyme is eluted by increasing the concentration of the buffer up to 250 mM

Results

The result of electrofocusing these preparations is shown in Fig. 1. Several enzyme forms which catalyze acetoin reduction coupled to the oxidation of NADPH were detected, those of p*I* 4 8, 6 2 and 7 2 being the major ones. Their relative proportions varied widely in different experiments, but the total recovered activity, about 35—38% of that present in the crude muscle extracts, is usually distributed as follows p*I* 7 2/p*I* 6.2/p*I* 4 8 = 10 2 : 1 3 The material of each of these three main bands, obtained by performing the electrofocusing in the pH range 4—8, was bulked and freed of sucrose and Ampholites by two consecutive filtrations (with a freeze-drying concentration stage in between) through short and wide columns (2 6 × 24.5 cm) of Sephadex G-25 coarse. These preparations can be frozen and stored at —18°C for several months without noticeable loss of activity.

Table I summarizes the purification procedure

Electrophoresis of the final preparations As Fig. 2 shows, the forms of p*I* 7.2 and 6.2 are electrophoretically pure under the experimental conditions used and that of p*I* 4.8 gives wide and diffuse bands of both protein and activity The mobility of the three enzyme forms is very similar

pH profiles and enzyme stability pH profiles of the three forms of the

TABLE I

PURIFICATION OF L-GLYCOL DEHYDROGENASE

L-Glycol dehydrogenase was purified from 1 kg of hen's leg muscle (about 10 fowls)

Step	Total protein (mg)	Specific activity (u/mg protein)	Total activity (U)	Purification (-fold)	Yield (%)
(1) Aqueous extract (homogenization with 5 vol of water)	40 000—45 000	0 5—1 2	20 000—45 000	—	—
(2) Calcium phosphate gel (unadsorbed)	13 000—13 500	2 —3	18 000—40 000	3—4	90
(3) Sephadex G-100 eluate	250—400	60 —80	14 000—30 000	80—90	65—70
(4) Sephadex G-75 superfine eluate	100—140	130 —150	13 000—21 000	170—190	50—55
(5) Hydroxyapatite eluate with 30 mM sodium potassium phosphate buffer, pH 7	30—45	400 —450	12 000—20 000	500—600	44—48
(6) Electrofocusing pI 7 2 form	2—4	2900	6 000—11 000	4000	26—28
pI 6 2 form	0 5—1	2200	1 200— 2 200	3000	5—6 35—38
pI 4 8 form	2—3 5	400	800— 1 500	500	3—4

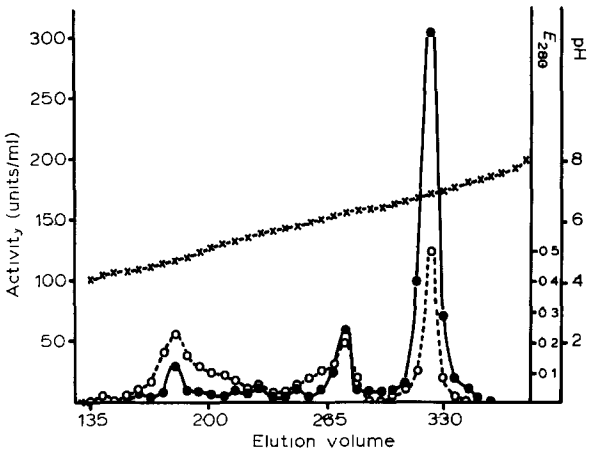

Fig 1 Electrofocusing in the pH range 4—8 of a preparation purified to stage 5 of Table I (X———X) pH gradient, (●———●) enzyme activity, (o- - - - -o) E_{280nm} after discounting the absorbance of Ampholites measured in a blank running

enzyme in 0.1 M sodium and potassium phosphate buffer show a plateau extending from pH 5 to about pH 6.6 and a descending branch at higher pH values At every purification stage, the enzyme is very stable in both water and low molarity buffers of pH 7, provided that the solutions are maintained at low

Fig 2 Electrophoresis on polyacrylamide gel of the three forms of the enzyme obtained by electrofocusing Anode at the bottom (a) Form of p*I* 4 8, (b) form of p*I* 6 2, (c) form of p*I* 7 2 Dark tubes, stained for L-glycol dehydrogenase activity, clear gels, stained for protein

TABLE II

REDUCTION OF DIACETYL TO BUTYLENE GLYCOL AND OXIDATION OF NADPH

Reaction mixture diacetyl, 11 98 μmol, NADPH, 29 71 μmol, enzyme preparation, form of p*I* 7 2, total activity about 1000 U, water, enough to reach a total volume of 25 ml (the reaction mixture was not buffered to avoid difficulties in butylene glycol determinations) Similar results were obtained with the enzyme forms of p*I* 6 2 and 4 8 n d , not determined

Time (min)	Consumed diacetyl (μmol)	Acetoin (μmol)	Butylene glycol (μmol)	Oxidized NADPH (μmol)
5	7 19	n d	n d	7 26
10	10 88	9 71	n d	11 22
90	11 78	8 07	2 64	13 29
390	11 78	4 47	6 75	19 11

temperature, but it is easily inactivated in high molarity buffers

Stoichiometry The stoichiometry of the reaction was studied by incubating at 25°C samples of the enzyme forms of p*I* 7.2, 6.2 and 4 8 with diacetyl and NADPH, periodically removing aliquots and measuring diacetyl, acetoin, butylene glycol and NADPH As shown in Table II for the form of p*I* 7.2, 1 mol of NADPH is consumed per mol of acetoin measured, and 2 mol for each mol of butylene glycol formed This demonstrates that the enzyme catalyzes the reduction of keto groups to secondary alcohols; in the case studied (1) diacetyl + NADPH → acetoin + NADP, (2) acetoin + NADPH → butylene glycol + NADP

These results were not due to the presence in our preparations of two enzymes independently catalyzing the reduction of diacetyl and acetoin, as proved by. (a) The purity of the enzyme preparations used. (b) Diacetyl reductase and acetoin reductase activities show entirely parallel elution profiles in Sephadex G-100 and G-75 superfine chromatographic analyses and in electrofocusing fractionations. (c) Enzyme assays with mixtures of acetoin and diacetyl (4 mM each) gave activities clearly lower than the addition of those obtained with 4 mM acetoin or 4 mM diacetyl, very similar to the rates reached with diacetyl alone, as expected since diacetyl is a much better substrate than acetoin (see Table III).

Similar results were obtained with the other two enzymatic forms.

Coenzyme specificity Assays with equimolar mixtures of NADH and NADPH yielded activities lying between those obtained in tests with either coenzyme, enzyme activities with NADH or NADPH show parallel elution profiles in gel filtration and electrofocusing experiments This proves that the enzyme accepts both pyridine nucleotides.

None of the three forms of the enzyme shows measurable activity with α-NADH

Substrate specificity To investigate the substrate structural requirements for carbonyl reduction, a number of aldehydes, monoketones, ketoacids, diketones, ketoacid esters and carbinols were tested (Table III) The highest activities were found with vicinal diketones and glyceraldehyde, but the enzyme also utilizes α-ketoacid esters, α-ketocarbinols, glyoxal and methylglyoxal quite

TABLE III

SUBSTRATE AND COENZYME SPECIFICITY OF HEN'S MUSCLE L-GLYCOL DEHYDROGENASE

Activity nmol of substrate reduced per min under standard assay conditions by 100 U enzyme tr, trace activities, less than 5 nmol of substrate reduced per min The following compounds were also tested, resulting in either zero or trace activities acetaldehyde, acetone, 3-butanone, 3-pentanone, pyruvate, oxaloacetate, α-ketoglutarate, 2,4-pentanedione and 2,5-hexanedione

Substrate	pI 4 8		pI 6 2		pI 7 2	
	NADPH	NADH	NADPH	NADH	NADPH	NADH
Vicinal dicarbonyls						
Glyoxal	241	81	305	6	59	9
Methylglyoxal	64	9	373	16	194	34
Diacetyl	454	125	537	33	203	101
2,3-pentanedione	528	143	666	36	217	113
Ketoacid esters						
Ethyl pyruvate	390	104	374	29	182	115
Methyl pyruvate	259	74	157	0	145	66
α-Hydroxycarbonyls						
Glycolaldehyde	99	46	51	tr	35	7
Glyceraldehyde	621	187	549	40	191	110
Acetoin	100	29	100	12	100	74
Acetylethylcarbinol	225	66	198	13	103	80
Diacetylmethylcarbinol	241	56	200	13	101	63

well, it does not accept acetaldehyde, monoketones, non-vicinal diketones or free ketoacids Therefore, the structural requirement for the substrate is an uncharged carbonyl vicinal to a second carbonyl or hydroxycarbonyl group.

As reported for other enzymes able to reduce diacetyl and/or acetoin [8,11—17], all three forms of this enzyme operate much better in the reductase direction. Reversibility assays were performed with the more characteristic of its products 2,3-Pentanediol was the best accepted of them, but the ratio of forward to backward activities was only about 4 1, even when increasing the coenzyme and substrate concentrations by a factor of 5 and 16, respectively, with respect to the usual value in experiments in the forward direction. At the same concentrations, assays with acetoin of butylene glycol as substrate revealed only traces of acetoin dehydrogenase and about 10-times lower butylene glycol dehydrogenase than acetoin reductase activity. Only trace activities were equally observed with glycol, glycolaldehyde, glycerol, acetylethylcarbinol and ethyl lactate.

Some of the products of the forward reaction have stereoisomers The steric form of the reduced compounds obtained was investigated by incubating NADPH with a large excess of diacetyl to displace the equilibrium of the reaction toward acetoin accumulation, thus avoiding further reduction to butylene glycol, the α_D^{20} value of which is much lower. NADPH oxidation was spectrophotometrically monitored and the optical rotation periodically measured (Fig. 3). At the end of the experiments, acetoin and butylene glycol concentrations were determined in the reaction mixture, no butylene glycol was detected and the acetoin measured was in agreement with the spectrophotometric data of NADPH oxidation. The acetoin produced was shown to be the L-(+) form, with an α_D^{20} value of 226.4° (S.D ± 20 5), this value is about twice as high as the largest reported for this compound, but it is probably the purest L-(+)-ace-

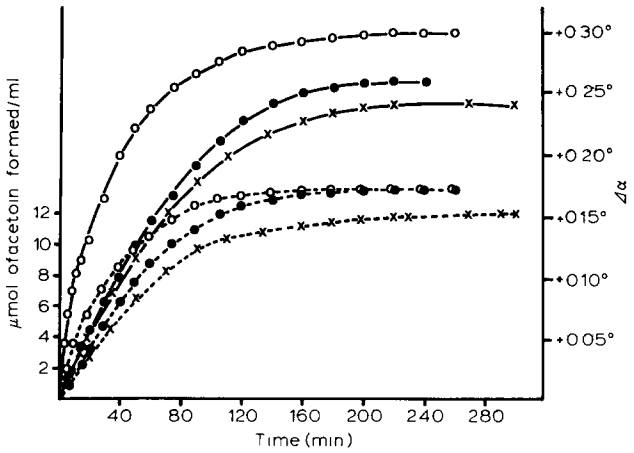

Fig 3 Optical rotation of the acetoin produced in the reduction of diacetyl by L-glycol dehydrogenase Incubation mixture NADPH, 0 11 mmol, diacetyl, 18 2 mmol, enzyme preparation, about 500 U of the p*I* 4 8 (•———•), 6 2 (○———○) or 7 2 (X———X) enzyme forms, volume 8 ml in 0 2 M sodium and potassium phosphate buffer, pH 7 Continuous lines, optical rotation, dashed lines, acetoin formed

TABLE IV

AFFINITY FOR SUBSTRATES K_m^{app} VALUES CALCULATED AT pH 7 AND 25°C WITH SATURATING CONCENTRATION OF NADPH (0 2 mM)

Values of K_m are expressed in μM and are means ± S D

Substrate	p*I* 7 2	p*I* 6 2	p*I* 4 8
Glyoxal	522 ± 49	53 ± 3 8	411 ± 19
Methylglyoxal	102 ± 5 4	98 ± 3 4	—
Diacetyl	109 ± 2 5	38 ± 2 6	—
2,3-Pentanedione	60 ± 1 2	97 ± 2 9	675 ± 53
Glyceraldehyde	26 ± 1 6	29 ± 4 3	566 ± 39
Acetoin	2590 ± 186	2013 ± 77	2164 ± 167

toin ever obtained, since our preparations should lack acetoin racemases (see Ref. 18).

Coenzyme and substrate affinity K_m^{app} values for NADPH and NADH were estimated in. the enzyme form of p*I* 7.2, $K_m^{NADPH} = 3.5$ μM (S.D ± 0 12), $K_m^{NADH} = 167$ μM (S D ± 17), p*I* 6 2 form, $K_m^{NADPH} = 5\ 4$ μM (S.D ± 0.16), $K_m^{NADH} = 168$ μM (S D ± 5 1), p*I* 4.8 form, $K_m^{NADPH} = 3\ 8$ μM (S D ± 0 13) Affinities for substrates with possible physiological interest are shown in Table IV

Discussion

Enzyme characterization and nomenclature

The experiments described demonstrate that (a) this enzyme is an L-(+)-dehydrogenase, the reversibility of which can be detected, although it is very much more efficient in the reductase direction, (b) it uses both NADH and NADPH as coenzyme; (c) its substrates are α-dicarbonyls, which are trans-

formed in two independent stages in L-(+)-glycols, the reduction of the first carbonyl group leading to α-hydroxycarbonyls, also accepted as substrates for glycol production In our opinion, according to the rules for enzyme classification and nomenclature of the I.U.P.A C -I U.B. [19], this enzyme must receive the systematic name of L-(+)-glycol NAD(P) oxidoreductase and the trivial name of L-glycol dehydrogenase.

As far as we know this enzyme has not been described until now It has been reported the purification and properties of a rather similar form from *Aerobacter aerogenes* which converts diacetyl and 2,3-pentanedione to acetoin and acetylethylcarbinol and these to 2,3-butanediol and 2,3-pentanediol, respectively, but it fails in reducing other α-dicarbonyls and α-hydroxycarbonyls as well as in oxidizing some glycols and, on the other hand, does not accept NADPH or NADP either as substrates or as inhibitors [3,20]

Purification

A number of commonly used techniques of protein purification were tried and discarded, such as precipitations by salts, heat, acids or solvents, ionic exchange on CM- and DEAE-cellulose and affinity chromatography on NAD-Sepharose 4B The purification procedure which was finally adopted, although rather time consuming, has some advantages it allows us to obtain electrophoretically pure enzyme preparations of at least the two major enzyme forms, it uses only very mild treatments not likely to alter the natural properties of the enzyme, and it results in a very high final yield The latter seems partly due to the behaviour of impure preparations during the Sephadex filtrations, in which greater than 100% recoveries are systematically obtained; this suggests the presence in the aqueous muscle extract of some inhibitor accompanying the enzyme in the first stages of purification On the other hand, the enrichment factors in Table I for each of the three isolated forms of the enzyme are probably underestimated, since they have been calculated with respect to the whole original activity (which is due to all the enzymatic forms) and not to that of each individual enzymatic species. Were the distribution of the three purified forms in the original tissue the same as that observed at the end of the purification process, the enrichment factors would be 5300 (form of p*I* 7 2), 20 000 (form of p*I* 6.2) and 5000 (form of p*I* 4.8).

Microheterogeneity

The existence of multiple molecular forms of an enzyme is a frequent and well known phenomenon detected in no less that 15% of the enzymes characterized to date [21]. To explain the heterogeneity of the enzyme described here the hypothesis to be formulated has to account for its behaviour as a single species during all the purification stages previous to electrofocusing and for the fact that all forms of the enzyme show equal electrophoretic mobility at pH 7 8 (electrophoresis at higher pH values than this leads to great difficulties in enzyme detection, at lower pH values enzyme migration is extremely slow) Since the molecular weights of all the enzymatic forms seem to be equal, in view of the narrow elution bands observed in Sephadex G-75 superfine filtrations, it is likely that the three molecular species have only minor differences in amino acid composition. These differences should imply groups

which remain essentially uncharged at the electrophoresis pH, but are ionized in the pH range of their isoelectric points, i e , cationic groups with pK values of about 6.5; histidine imidazole fulfil these conditions

Physiological substrates

Apparent Michaelis constants for NADPH and NADH indicate that the enzyme must operate in vivo mainly with NADPH, since the physiological concentration of this is about 10—30 K_m^{NADPH} while that of the latter is only about 0 5 K_m^{NADH}. The lack of reports on the concentrations in avian muscle or similar systems of most of the compounds the enzyme uses as substrates makes it very difficult to speculate about which of them can be considered as physiological Nevertheless, acetoin, the compound used for detecting enzyme activity during the purification procedure, must be a poor substrate, since its K_m^{app} value is about 300-times higher than the likely physiological concentration [22,23]

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