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THE SEPARATION OF PARTIALLY MODIFIED LACTATE DEHYDROGENASE BY AFFINITY CHROMATOGRAPHY *

THE SPECIFIC ACTIVITY OF PROTOMERS?

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Summary

Pig heart lactate dehydrogenase (L-lactate : NAD^+ oxidoreductase, EC 1.1.1.27) was partially inactivated by reaction with phenylmaleimide. The resulting protein mixture was separated by affinity chromatography on a Sepharose-linked oxamate column yielding three distinct enzyme fractions with one, two and three out of four subunits being modified. The specific activities of these samples were lower than expected from the degree of modification, while the maximum binding capacity of NADH-oxamate related well to the number of catalytic centers modified. A possible cooperative effect in the native enzyme is discussed.

Introduction

A valuable tool for the study of subunit interactions in oligomeric enzymes is the inactivation of some of the subunits by chemical modification. This can be done by total modification and subsequent hybridization with unmodified enzyme. However, even when hybridization itself is possible, this method often fails due to the instability and rapid denaturation which may occur in highly modified proteins. A different approach is the partial chemical modification under limiting conditions, i.e. reaction time, concentration of the reagent. Unfortunately, this yields a mixture of all possible intermediates from the unmodified oligomer to a totally modified species. We describe a method for the separation of such a mixture in the case of pig heart lactate dehydrogenase.

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Methods

Preparation of N-phenyl[2,3-$^{14}C_2$]maleimide

N-Phenyl[2,3-$^{14}C_2$]maleimide was prepared in the usual way [1] from [2,3-$^{14}C_2$]maleic anhydride, a product of Amersham-Buchler, Wenden, G.F.R. The specific radioactivity was 0.04 Ci/mol.

Protein determination

The concentration of unmodified lactate dehydrogenase was determined by its ultraviolet absorption at 280 nm using a factor of 1.32 for a solution containing 1 mg/ml. For the modified samples the Lowry method [2] was used. The same factor as for unmodified enzyme based on the 280 nm absorption was employed. NADH from the elution buffer of the affinity chromatography had to be largely removed by repeated dialysis prior to the protein estimation.

Enzyme assays

Standard conditions: The initial velocity of the decrease of absorbance at 366 nm was measured at 25°C upon enzyme addition to a cuvette containing 2 ml of a solution of 0.35 mM NADH and 0.5 mM pyruvate in 67 mM phosphate buffer, pH 7.2.

The substrate optimum was determined by varying the pyruvate concentration from 0.1 to 2 mM.

Modification experiments

Pig heart lactate dehydrogenase (EC 1.1.1.27), purchased from Boehringer, Mannheim, G.F.R., as an ammonium sulfate suspension, was dialyzed against 67 mM phosphate buffer, pH 7.2 and subsequently centrifuged at 44 000 × *g*. The preparation was pure H_4 isoenzyme with a maximum contamination of H_3M of about 3% as shown by low voltage electrophoresis on cellulose acetate strips. To 10 ml of a $3.5 \cdot 10^{-5}$ M solution (calculated on the basis of 144 000 as molecular weight) kept at 25°C were added 2.8 mmol of solid *N*-phenyl[2,3-$^{14}C_2$]maleimide. Depending on the desired degree of incorporation, the reaction was stopped after 20—50 min. For this the solution was decanted from unreacted reagent, the temperature was brought to 0°C and mercaptoethanol was added to a final concentration of 0.1 M. After 30 min this mixture was dialyzed against 20 mM pyrophosphate buffer, pH 8.0 containing 50 mM sodium chloride and 3 mM sodium azide. It was then chromatographed on Sephadex G-200 (1.5 × 100 cm) equilibrated with the same buffer system. Flow rate 10—12 ml/h. The combined fractions containing protein in the native conformation (elution volume 145 ml) were concentrated by pressure dialysis in Ultratubes UH 100 from Schleicher and Schüll, G.F.R. The incorporation of phenylmaleimide was estimated from the radioactivity of aliquots counted in Pinaud's solution [3] in a Berthold-Frieseke liquid scintillation counter Betaszint BF 5000 operating in the external standard channel ratio manner. The various fractions from the affinity column had first to be dialyzed to remove the sodium chloride thus avoiding precipitation in the scintillation cocktail.

Affinity chromatography

Sepharose-linked immobilized oxamate was prepared according to O'Carra et al. [4] with the modifications as suggested by Spielmann et al. [5]. The protein binding capacity was 1 mg of lactate dehydrogenase per ml of packed gel. About 30 mg of modified enzyme in 3—4 ml of phosphate buffer, pH 7.2 containing 0.5 M sodium chloride and 0.2 mM NADH were applied to an oxamate column (1.2 × 35 cm) equilibrated with the same buffer system. When the third protein band appeared in the eluate as monitored by its 290 nm absorption NADH was omitted from the irrigant. After complete removal of the NADH from the column as followed by its 340 nm absorption, elution was discontinued for 12—15 h before the unmodified sample was collected. The column was regenerated by irrigation with 1 M sodium chloride and subsequently with the appropriate buffer.

Cellulose acetate electrophoresis

Electrophoresis was carried out on 2.5 × 14.5 cm cellulose acetate strips from Macherey, Nagel and Co., Düren, G.F.R., in 0.03 M 5,5-diethylbarbiturate buffer, pH 8.6, at 17 V/cm.

Coenzyme binding studies

To remove bound NADH, 5 mg samples of lactate dehydrogenase in phosphate buffer, pH 7.2 were passed through a Sephadex G-25 column (1 × 20 cm) containing 150 mg of finely powdered charcoal (Norit A from Serva, Heidelberg, G.F.R., washed with HCl). The fluorescence was measured with a Perkin Elmer spectrofluorimeter model MPF-2A. A protein concentration of 0.3 mg/ml was used. The NADH concentration varied from 0—30 μM. Excitation and emission settings were 340 and 430 nm, respectively. For the estimation of the maximum binding capacity of NADH/oxamate the cuvette contained 10 mM oxamate. 5-μl samples of a 0.54 mM NADH solution were added to a final concentration of 15 μM. All solvents were passed through Millipore filters, 0.65 μm (Millipore Filter Corp., Bedford, Mass., U.S.A.) before the titrations to remove any fines.

Results

Pig heart lactate dehydrogenase was inactivated by reaction with *N*-phenyl[2,3-$^{14}C_2$]maleimide [6]. The modification was stopped when a residual specific activity between 30 and 50% was reached. Soluble denatured protein and excess reagent were separated by gel filtration on Sephadex G-200. The denatured fraction elutes in the void volume under these conditions*. The incorporation of the maleimide into the enzyme with native conformation corresponded to 1.5—2.5 mol per tetramer.

Unmodified native pig heart lactate dehydrogenase has been separated from other dehydrogenases by affinity chromatography on Sepharose-linked

* Lactate dehydrogenase always contains some unfolded material which may be separated by this method [7].

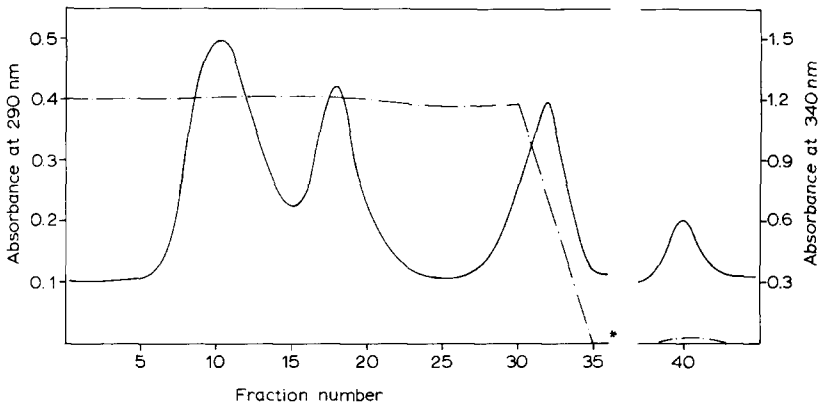

Fig. 1. Elution pattern of partially modified lactate dehydrogenase on a Sepharose-linked oxamate column. ———, protein absorbance at 290 nm; - - - - - -, NADH absorbance at 340 nm. * Elution was discontinued for 15 h.

immobilized oxamate. When NADH is added to the irrigant the enzyme is retained by ternary complex formation. Elution may be accomplished by omitting NADH from the elution buffer [4].

We applied the native portion of our partially modified enzyme to such a column and obtained four well resolved bands as shown in Fig. 1. The last band contains pure unmodified enzyme exhibiting full activity. It elutes as a sharp peak only when complete dissociation of the ternary complex is achieved by discontinuing irrigation for at least 12 h. The three preceeding bands are modified enzyme with an incorporation of phenylmaleimide corresponding to three, two and one mol per tetramer of the protein in the order of their elution from the column. These ratios have been determined for every fraction and are uniform within the bands. Only the first two or three fractions of the first band contain the fourfold modified species. However, low-voltage cellulose-acetate electrophoresis at pH 8.6 reveals that this is denatured although it remains in solution. It does not migrate. Only the native isozymes of lactate dehydrogenase migrate under these conditions as shown for unmodified species by Jeckel [7] in analogy to results obtained for horse liver alcohol dehydrogenase [8]. Enzyme retained at the origin is inactive and completely unfolded as demonstrated by optical rotatory dispersion.

Coenzyme binding capacity

Binding of NADH to the three species of modified enzyme was studied by following the increase of the NADH fluorescence at 430 nm upon binding when excited at 340 nm. It was found that in spite of excessive dialysis the enzyme was nearly saturated with coenzyme arising from the elution buffer of the affinity chromatography. Charcoal treatment was necessary for its complete removal. This treatment, however, does in fact alter the coenzyme binding behaviour of the enzyme, at least temporarily. Several control experiments with unmodified samples showed deviations to a varying degree from a normal hyperbolic binding curve. An iterative curve fitting procedure to a theoretical binding hyperbola as described by Engel [9] failed. The maximum binding

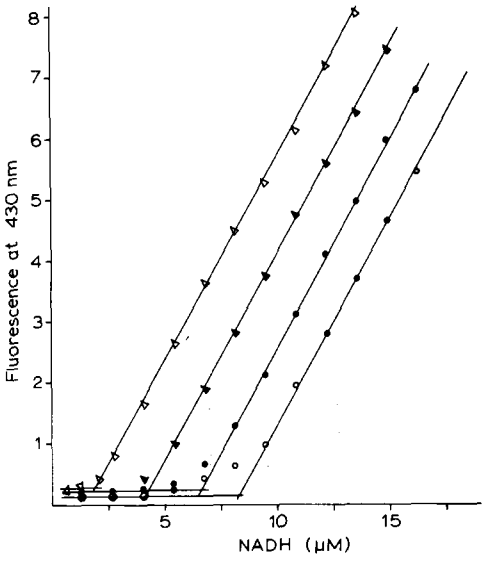

Fig. 2. NADH-oxamate binding capacity of lactate dehydrogenase modified with phenylmaleimide. Titration of 2.08 μM protein and 10 mM oxamate with NADH. Mol phenylmaleimide incorporated per mol of tetramer: ○ = 0, ● = 1, ▲ = 2, △ = 3.

capacity in presence of oxamate was not affected by the charcoal treatment (Fig. 2). The ternary enzyme-NADH-oxamate complex does not fluoresce. Under the conditions applied (10 mM oxamate) the apparent dissociation constant of NADH is in the order of 10^{-9}. Therefore the enzyme is practically completely saturated before the fluorescence of free NADH can be detected [10].

Enzymic activities

The residual specific activities for every fraction of the affinity separation were determined. They were found to be uniform within the three distinct bands. Average values from several modifications are given in Table I. All values

TABLE I

COENZYME BINDING CAPACITY AND SPECIFIC ACTIVITIES OF PARTIALLY MODIFIED LACTATE DEHYDROGENASE FRACTIONS SEPARATED BY AFFINITY CHROMATOGRAPHY ON SEPHAROSE-LINKED IMMOBILIZED OXAMATE.

Mol phenylmaleimide incorporated per tetramer [a]	No. subunits modified	Maximum NADH-oxamate binding capacity [a]	Residual specific activity in % [b]	Theoretical residual specific activity in %	Relative additional loss of specific activity in %
0	0	3.92	100	100	0
1.04	1	3.12	65	75	13
2.06	2	1.91	36	50	27
2.90	3	0.92	15	25	39

[a] ± 5%

[b] average values from 6 separate runs

turned out to be smaller than expected from the degree of modification. The assays were performed under standard conditions [11]. However, these were found to be optimum conditions as well with respect to pyruvate. The modified enzyme showed the same substrate inhibition at high pyruvate concentrations as the native enzyme.

Discussion

The heart isoenzyme of lactate dehydrogenase is known to react rapidly with maleimides by modification of the so called essential cysteine and concomitant loss of enzymic activity [6]. Although this cysteine residue is not involved in substrate binding or catalysis, it is located near the active center [12]. Modification does not occur in the presence of coenzyme. Only one labeled peptide has been found after tryptic digestion [13]. This implies that the three fractions obtained represent enzymes with one, two and three out of four subunits inactivated by modification with one mole of phenylmaleimide each. The residual specific activities of the three types of modified lactate dehydrogenase are reduced as compared to the values to be theoretically expected from the degree of modification provided if there was no interaction between the subunits.

The NADH-oxamate binding capacity agrees well with the number of subunits modified. These modified subunits alone do not form a ternary complex, as demonstrated by the fluorescence titration. The relative additional loss of specific activity shows a nearly linear relationship to the number of active sites inactivated (Table I). A single protomer is still enzymatically active when associated with nonfunctional subunits but to a lesser extent. This could be explained by a cooperative effect between the subunits in the native enzyme. Every protomer makes a constant additional contribution to the total maximum activity.

However, no cooperativity in substrate binding has been found for the heart isozyme yet. Furthermore coenzyme binding studies in our laboratory (Trommer, W.E., Wenzel, H. and Pfleiderer, G., unpublished) using a spin-labeled enzymatically active NAD^+-derivative [14] showed a strict linearity when plotted by the method of Scatchard [15]. Thus an alternative explanation could be given by assuming structural changes in a neighboring subunit upon modification of the "essential" SH-group. The instability of the four-fold modified sample further supports this hypothesis. However, no immunological differences and no changes in the optical rotary dispersion were found in lactate dehydrogenase after reaction of this SH-group with *p*-chloromercuri benzoate [16].

In earlier work it was found that after excessive modification of lactate dehydrogenase by maleimides and removal of denatured enzyme by gel filtration a residual specific activity of 10 to 15% always remained [6]. In view of our results this may be easily explained. When all four subunits are modified the enzyme denatures so rapidly that the fraction remaining in the native conformation is only threefold modified. From Table I it can be seen that such a sample has a residual activity of about 15%.

Acknowledgements

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