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CHARACTERISATION OF A HIGHLY HYDROPHOBICALLY MODIFIED LACTATE DEHYDROGENASE

WOLFGANG KAPMEYER * and GERHARD PFLEIDERER **

Lehrstuhl für Biochemie, Abteilung Chemie, Ruhr-Universität Bochum, Postfach 2148, 4630 Bochum (G.F.R.)

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Summary

1. Lysine residues of porcine H_4 lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase EC 1.1.1.27) were modified with methyl-ϵ-(*N*-2,4-dinitrophenyl)aminocaproimidate · HCl. With increasing incorporation of the reagent a linear decrease of enzymatic activity was noticed. No essential lysyl group with an extraordinary reactivity was modified.

2. The active forms of the modified enzyme with different incorporation values were separated from denatured material by fractional precipitation and gel chromatography. An ϵ-(*N*-2,4-dinitrophenyl)aminocaproamidinate lactate dehydrogenase was obtained with an average incorporation of 38 groups per tetramer and a residual activity of 42%. This material proved to be homogenous in cellulose electrophoresis.

3. The ϵ-(*N*-2,4-dinitrophenyl)aminocaproamidinate lactate dehydrogenase is soluble only in glycine buffer at pH 8 and can be stabilized as ternary complex with NAD^+ and sodium sulfite. Gel chromatography and ORD measurements show no strong conformational change.

4. ϵ-(*N*-2,4-dinitrophenyl)aminocaproamidinate lactate dehydrogenase has similar K_m values for pyruvate, NADH, lactate and NAD^+ as the native enzyme, and shows a lower thermostability due to a diminished stabilization by the hydrate layer on the surface.

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* Present address: University of California, San Diego, Department of Chemistry, La Jolla, California 92093, USA.

** Present address: Institut für Organische Chemie, Biochemie und Isotopenforschung der Universität Stuttgart, Pfaffenwaldring 55, 7000 Stuttgart 80 (German Federal Republic).

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Abbreviations: NPCA, ϵ-(*N*-2,4-dinitrophenyl)aminocapramidino-,Nbs_2, disodium 5,5′-dithio-bis-(2-nitrobenzoate), NPCI, methyl-ϵ-(*N*-2,4-dinitrophenyl)aminocaproimidate · HCl.

Introduction

Several amino acids proved to be essential in lactate dehydrogenase (L-lactate:NAD^+dehydrogenase, EC 1.1.1.27 including cysteine, tyrosine, histidine, arginine and lysine [1—5]) and a tentative mechanism of action had been proposed for the lactate dehydrogenase by Holbrook and Gutfreund [6]. Contradictionary results had been found according to the function of lysines. Pfleiderer and Zaki [7] acetylated in the presence of sulfite ions 58% of the lysines in porcine heart muscle lactate dehydrogenase without any loss of activity. Chen and Engel [5] modified porcine skeletal muscle lactate dehydrogenase with pyridoxal 5′-phosphate and found after reduction with $NaBH_4$ a residual activity of 9% and an incorporation of 5.3 mol pyridoxal 5′-phosphate per subunit. On the other hand Tatschek and Jeckel [8,17] had found that the modification of heart muscle lactate dehydrogenase with pyridoxal 5′-phosphate yielded a totally unfolded enzyme with no catalytic activity. To determine the function of non essential lysine residues we synthesized a reagent consisting of a dinitroaromatic chromophore connected by a spacer with an imidoester (Fig. 1).

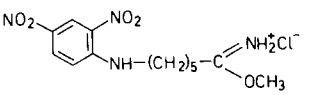

Fig. 1. Structure of the modification reagent methyl-ε-(*N*-2,4-dinitrophenyl)aminocaproimidate · HCl.

Imidoesters are specific modification reagents for lysines. This reagent offers the obvious advantage of an intensive yellow colour and allows a simple determination of incorporation of amidinated groups. Amidination of amino groups maintains the charge of the protein, but the loci of the individual positive charges are displaced by almost 1.5 Å.

Materials and Methods

NAD^+, NADH and pig heart lactate dehydrogenase were purchased from Boehringer, Mannheim; carboxypeptidase A and B, trypsin and chymotrypsin from Worthington Bioch. Corpn. Subtilisin was obtained from Novo Industri A/S, pronase E, proteinase K and aminopeptidase K from Merck, Darmstadt. 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from EGA Chemie Steinheim/Albuch, 3-(4,5-dimethylthiazolyl-2-)2,5-diphenyl tetrazoliumbromid and ninhydrine from Serva, Heidelberg. Phenazine methosulfate was obtained from Aldrich Europe, Belgium. Precoated TLC-plates SIL G 25 and cellulose acetate strips were obtained from Macherey & Nagel, Düren. Acetic acid and hydrochloric acid were Baker Analysed Reagents. All other chemicals were products from Merck, Darmstadt. Dimethyl sulfoxide (reagent grade) was stored over Linde type 4 A molecular sieve and was distilled under reduced pressure before use; b.p.: 75.5—76.0° C, 12 mm.

ε-Aminocapronitrol. This was prepared according to the method of Arbuzov [9]. After fractional distillation a gas-chromatographically pure product was obtained.

ε-(N-2,4-dinitrophenyl)aminocapronitril. This was synthesized as described by Hunter and Ludwig [10].

Methyl-ε-(N-2,4-dinitrophenyl)aminocaproimidate HCl. The previously described procedure [10] was altered to avoid absolutely any contact with moisture because the imidata hydrochloride is hydrolysed in the presence of water. 3.6 g highly dried ε-(*N*-2,4-dinitrophenyl)-aminocapronitril was placed into a two-necked bulb with an inside-fitted sintered glass funnel. 135 ml dry methanol and 145 ml dry ethyl acetate, saturated with 53.6 g HCl was pressed with nitrogen into the reaction vessel. After a 25-h incubation at 4° C, 1.4 l dry ethyl ether was added under nitrogen and the product was precipitated at —20° C. The supernatant was pressed up over the sintered glass funnel and the product was washed three times with dry diethyl ether and dried under reduced pressure. Yield: 3.35 g. The crystallized product was chromatographically pure on silica-gel thin-layer plates (MeOH/ether 1 : 10), m.p. 102—103° C.

Calculated for $C_{13}H_{18}O_5N_4 \cdot HCl$: C, 45.03; H, 5.62; N, 15.91; Cl, 10.06;

Analytically found: C, 45.19; H, 5.52; N, 16.16; Cl, 10.

NPCA-aminocaproic acid HCl. 540 mg NPCI and 360 mg ε-aminocaproic acid were dissolved in a mixture of 4.5 ml methanol and 1.5 ml H_2O. 1 M NaOH was added to give a final pH of 9.5. After 1 h reaction at 22° C the solvents were removed in a rotavapor and 30 ml water were added. The solution was extracted three times with diethyl ether and acetic acid was added to make pH 5. Further separation was performed on a 1 × 30 cm cation exchange column of Bio-Rex 70 (acetate). The product was eluted with a linear gradient of 0.3 l water and 0.3 l 0.1 M ammonia and gave 200 mg NPCA-ε-aminocaproic acid. It was suspended in 5 ml tetrahydrofuran and dissolved by adding 2.5 ml 6 M HCl. The product crystallised at 4° C, was separated from the solvent by suction on a sintered glass funnel and washed with tetrahydrofuran/H_2O 1 : 2. Yield: 152 mg.

Calculated for $C_{18}H_{27}N_5O_6 \cdot HCl$: C, 48.49; H, 6.33; N, 15.71; Cl, 7.95;

Analytically found: C, 48.57; H, 6.46; N, 15.73; Cl, 7.99.

Ultraviolet spectrum: Maxima at 262 and 366 nm, shoulder at 406 nm. Molar extinction coefficients at 366 nm:

$\epsilon = 1.54 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ in 0.1 M glycine buffer, pH 8;

$\epsilon = 1.60 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ in 2% formic acid.

NPCA-lysine. 170 mg methyl-ε-(*N*-2,4-dinitrophenyl)aminocaproimidate HCl was dissolved in 3 ml methanol and 1.5 ml *N*-ethylmorpholine (pH 9). 112 mg α-*N*-carbobenzoxylysine was added. After 4.5-h incubation at 37° C the solvent was removed in vacuum giving NPCA-α,*N*-carbobenzoxylysine. The product was redissolved in 1 ml formic acid and 5 ml M HCl was added. In an evacuated sealed tube the sample was hydrolysed for 3 h at 108° C and the solvent removed. The product was purified on a preparative silica-gel thin-

layer plate with a solvent of butan-1-ol/pyridine/acetic acid/water, 68 : 40 : 14 : 25. The sample was scraped from the plate and redissolved in methanol/20% acetic acid, 5 : 1. The lyophilized product was dissolved in 1 ml methanol/water 10 : 1 and insoluble silica gel was removed by centrifugation. Further silica gel was removed by precipitation with isopropanol. The product was precipitated after an addition of 50 ml of isopropanol. The precipitate was redissolved in 2 ml 6 M HCl forming the hydrochloride. The NPCA-lysine proved to be homogenous on silica gel chromatography. Yield after lyophilisation: 45 mg. The infrared spectrum is very similar to that of NPCA-ϵ-aminocaproic acid · HCl.

Modification of porcine heart muscle lactate dehydrogenase. 409 mg suspension in 3 M ammonium sulfate of porcine heart muscle lactate dehydrogenase were sedimented by centrifugation and the precipitate redissolved in 20 ml water. The sample was dialysed twice against 4 l 67 mM phosphate buffer to remove completely the ammonium sulfate. The sample was then dialysed twice against 4 l of the modification buffer, pH 9.5, containing 0.2 M triethanolamine · HCl, 0.025 M KCl, $3 \cdot 10^{-4}$ M mercaptoethanol and 10^{-4} M EDTA. 22 ml of a mixture containing modification buffer/dimethyl sulfoxide 1 : 2 were added to the dialysed lactate dehydrogenase (18.2 mg/ml protein, specific activity 400 units/mg) *. 94 mg NPCl dissolved in 6.67 ml dimethyl sulfoxide and 3.24 ml 0.1 M NaOH were added to give a 24-fold excess of reagent per subunit and a dimethyl sulfoxide concentration of 40%. At the beginning of the modification the enzymatic activity was 324 units/mg. After 60 h, once again 22.5 mg reagent in 0.6 ml dimethyl sulfoxide and 0.97 ml 0.1 M NaOH were added making overall a 30-fold excess of the reagent per subunit of lactate dehydrogenase. After 90-h incubation the sample had an average specific activity of 173 units/mg. During the modification the NPCA-lactate dehydrogenase precipitated and formed a fine suspension. The excess of imidoester was removed by reaction with 25 ml 0.1 M glycine buffer, pH 8. 40 mg NAD^+ and 20 mg sodium sulfite dissolved in 25 ml 0.1 M glycine buffer were added in order to stabilize the enzyme. The main part of the modified enzyme was dissolved as a ternary complex with NAD^+ and sodium sulfite. A precipitate of hydrolyzed reagent and denatured enzyme was formed by centrifugation (20 min; 48 000 × *g*). The precipitate was suspended twice in 12 ml 0.2 M glycine buffer, pH 8, and centrifuged again. The combined supernatants containing the modified enzyme were dialysed twice against 5 l 67 mM phosphate buffer, pH 7.2 and centrifuged (the modified lactate dehydrogenase is insoluble in phosphate buffer). The precipitate was dissolved in 28 ml 0.2 M glycine buffer, pH 8 ($5 \cdot 10^{-4}$ M NAD^+, 10^{-4} M sulfite and 0.02% sodium azide). The denatured protein was removed from the active NPCA-lactate dehydrogenase on Sephadex G-200 f (3 × 180 cm), elution was effected with 0.1 M glycine buffer, pH 8, containing $5 \cdot 10^{-4}$ M NAD^+ and 10^{-4} M sodium sulfite. An NPCA-lactate dehydrogenase with a mean incorporation value of 9.4 NPCA groups per subunit and a specific activity of 170 units/mg (42% residual activity) was obtained.

Enzyme assay. Lactate dehydrogenase activity was estimated by determining

* 1 unit = 1 μmol NADH oxidized per min.

the initial velocity of oxidation of NADH by pyruvate in the standard test [11]. Highly modified samples were diluted in 0.1 M glycine buffer, pH 8, before the test, to avoid aggregation. Protein concentrations of 1—10 mg/ml were determined by the biuret method adapted to small volumes (0.25 ml biuret solution, 0.01 ml sample, 0.24 ml water). The absorbance at 578 nm was measured in microcuvettes (volume 0.5 ml, light path 2 cm) using a molecular weight for lactate dehydrogenase of 4 × 36 000. Protein was also determined by measuring the extinction at 280 nm using a factor of 1.32 for a solution containing 1 mg/ml.

Electrophoresis on cellulose acetate strips. Electrophoresis was performed on 25.5 × 145 mm cellulose acetate strips in a Boskamp-Mikrophor-Apparat (30 V/cm, 0.05 M glycine buffer, pH 8.7, 80 min. To suppress the adsorption of the NPCA-lactate dehydrogenase the strip was dipped in a mixture of 0.05 glycine buffer, pH 8.7 (containing 10^{-3} M NAD^+ and $2 \cdot 10^{-4}$ M sodium sulfite) with 40% dimethyl sulfoxide. Protein staining was performed in 0.02% nigrosine, 2% acetic acid; enzyme-specific staining was carried out on a agarose gel with Tetrazolium Blue and phenazine methosulfate [12].

Peptide mapping. Peptide maps were produced by the standard 2-dimensional analytical method with electrophoresis in pyridine/acetic acid/water, 10 : 90 : 900 at pH 3.2 (50 V/cm; 2 h) followed by chromatography in a solvent containing butan-1-ol/pyridine/acetic acid/water 68 : 40 : 14 : 25, pH 5. Electrophoresis was performed in a Pherograph-Original-Frankfurt.

Determination of sulfhydryl groups. The total number of sulfhydryl groups was determined according to the method of Ellmann [13] with 5-5′-dithiobis-(2-nitrobenzoic acid) in 67 mM phosphate buffer, pH 7.2, and 8 M urea. Reactive sulfhydryl groups were determined in the following procedure: 0.02 ml of a NPCA-lactate dehydrogenase solution (7.3 mg/ml in 0.1 M glycine buffer, pH 8) was mixed with 0.45 ml 0.1 M glycine buffer, pH 8. 0.02 ml of a saturated 5-5′-dithiobis(2-nitrobenzoic acid) solution in 0.1 M glycine buffer, pH 8 was added and the increasing extinction at 405 nm was measured for 5 h at 25° C (the reference contained the same solutions except the NPCA-lactate dehydrogenase).

ORD measurements. The samples were dissolved in 0.1 M glycine buffer, pH 8 and centrifuged to remove insoluble material. The ORD spectrum was measured in a spectral polarimeter Carry 60 (Applied Physic Corporation Monrovia, California). The helix content was calculated from the mean effective residual rotation at the minimum of the spectrum at 232 nm by the method of Simmons et al. [14] using the equation:

$$\% \text{ helix} = \frac{m(232-1800)}{10\,900} \cdot 100,$$

as described by Jeckel and Pfleiderer [15].

Thermostability measurements. The thermostability was determined by incubation of the samples in 0.2 M glycine buffer, pH 8 at 55° C and measuring the residual activity (protein concentration: 7.2 mg/ml).

Gel chromatography for determination of Stokes radius. 1.5 mg NPCA-lactate dehydrogenase (9.4 NPCA-groups/subunit) and 3.0 mg unmodified lactate dehydrogenase were chromatographed on Sephadex G-200 (3 × 180 cm), equi-

librated with 0.1 M glycine buffer, pH 8 containing $5 \cdot 10^{-4}$ M NAD^+, 10^{-4} M sodium sulfite and 0.02% sodium azide and eluted with the equilibration buffer; flow rate 20 ml/h. Detection of both proteins by the absorption at 280 nm. The concentration of NPCA-lactate dehydrogenase was measured at 366 nm, that of pure unmodified lactate dehydrogenase at 280 nm after precipitation of NPCA-lactate dehydrogenase with 67 mM phosphate buffer, pH 7.2.

Preparation of small samples of NAD^+ and sulfite free NPCA-lactate dehydrogenase. For kinetic measurements the NAD^+/sulfite stabilized NPCA-lactate dehydrogenase was separated from NAD^+ and sulfite by gel chromatography. Samples of 20 µl NPCA-lactate dehydrogenase solution (7—10 mg/ml) were desalted by gel filtration on Sephadex G-25 (0.6 × 7 cm superfine) with 0.2 M glycine buffer, pH 8. The column was cooled with ice/water. The protein was collected according to the yellow colour and diluted 1 : 5 with 1 M glycine buffer, pH 8, to obtain a better stabilization of the enzyme.

Kinetics. The NPCA-lactate dehydrogenase was diluted in 0.8 M glycine buffer, pH 8. K_m and V for the pyruvate/NADH reaction were determined in 67 mM phosphate buffer, pH 7.2; those for the lactate/NAD^+ reaction were measured in 0.1 M glycine buffer pH 9.5; 25°C. Pyruvate kinetics: 0.35 mM NADH; 0.03—0.45 mM pyruvate. NADH kinetics: 0.5 mM pyruvate; 0.015—0.1 mM NADH. Lactate kinetics: 0.8 mM NAD^+; 0.468—7.5 mM lactate. NAD^+ kinetics: 7.5 mM lactate; 0.012—0.093 mM NAD^+. The initial velocity was determined and the values for K_m and V calculated according to the procedure of Lineweaver and Burk [16].

Results

Modification

NPCI is insoluble in water. To obtain a high modification rate an organic solvent must be added. In thorough experiments dimethyl sulfoxide proved to be best suited to solubilize the reagent as it is compatible with lactate dehydrogenase in high concentrations. Enzymatic activity decreased 10—15% after addition of 40% dimethyl sulfoxide but was constant during continuous incubation in the dimethyl sulfoxide buffer.

During modification of porcine heart muscle lactate dehydrogenase a loss of activity is observed depending on the incorporation of the NPCA groups. A modification of the lactate dehydrogenase · NAD^+ · sodium sulfite complex shows also a desactivation of the enzyme, resulting in a 10% higher residual activity compared with the modified apoenzyme. An 18% lower incorporation of NPCA groups was achieved compared with the apoenzyme modification.

To obtain an incorporation of more than 5 NPCA groups per subunit it was necessary to add the reagent in two portions to the modification mixture and to increase the modification time to 90 h (see Materials and Methods.) With increasing incorporation of reagent a precipitate consisting of hydrolysed reagent and of the main part of modified enzyme is formed. To achieve a further modification in this heterogeneous mixture vigorous shaking was necessary.

In order to redissolve the highly modified NPCA-lactate dehydrogenase after an extensive modification procedure 0.1 M glycine buffer, pH 8, NAD^+

and sodium sulfite were added and insoluble products were removed by centrifugation. Since the NPCA-lactate dehydrogenase with an incorporation of more than 7 NPCA groups per subunit is insoluble in 67 mM phosphate buffer, pH 7, a separation of lower and higher modified material was achieved by a simple precipitation of the material with the highest incorporation of NPCA groups during dialysis against 67 mM phosphate buffer, pH 7.2. Denatured modified enzyme was removed by gel chromatography on Sephadex G-200 according to the method of Jeckel [17] (Fig. 2). The modification of lactate dehydrogenase and the separation of the most highly modified fraction of active enzyme yielded a porcine H_4 NPCA-lactate dehydrogenase with a mean incorporation value of 9.4 NPCA groups per subunit and a residual activity of 42%. The incorporation of NPCA groups per subunit increases with modification time. Fig. 3 shows that no lysine with a greater reactivity and an essential character was modified. During the statistical modification of all available lysines the enzyme is inactivated, although we cannot refer the inactivation to the modification of a special lysine.

Identification of amidinated residues

The identification of NPCA-lysine in the modified protein sample was obtained either by enzymatic digestion with a mixture of trypsin, chymotrypsin, pronase, subtilisin, proteinase K, aminopeptidase M, carboxypeptidase A and carboxypeptidase B or by hydrolysis with 6 M HCl for 20 h at 108°C in a sealed evacuated tube. The proteolytically digested NPCA-lactate dehydrogenase was submitted to a fingerprint on a thin-layer silica-gel plate and only one yellow spot was detected which had identical mobility and § F values identical to those of synthetic ϵ-NPCA-lysine. A fingerprint of the HCl-hydrolysed NPCA-lactate dehydrogenase gave the same result.

Electrophoresis

Electrophoresis of the NPCA-lactate dehydrogenase was performed on cellu-

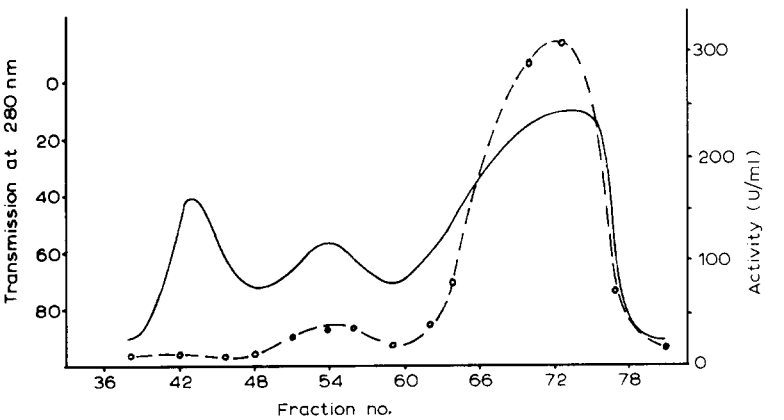

Fig. 2. Gel chromatography of the modified lactate dehydrogenase. The separation of denatured and active forms of NPCA-lactate dehydrogenase was performed on a 3 × 180 cm Sephadex column G-200 f in 0.1 M glycine buffer, pH 8 ($5 \cdot 10^{-4}$ M NAD^+, 10^{-4} M sodium sulfite, 0.02% sodium azide). ———, transmission at 280 nm; ○— —○, enzymatic activity.

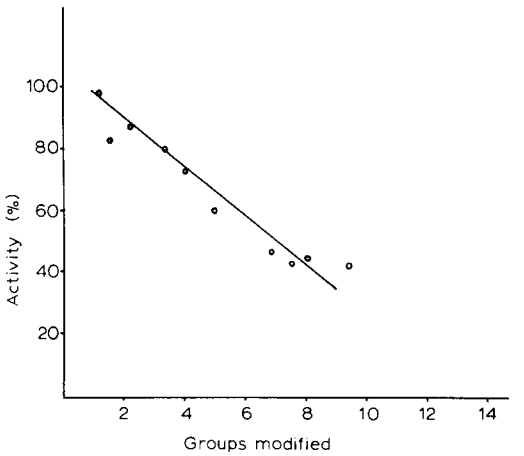

Fig. 3. Lactate dehydrogenase activities as a function of lysine residues modified by NPCI. The activity was determined in the standard test (for NPCA-lactate dehydrogenase with more than five groups per subunit after dilution in glycine buffer). The incorporation of NPCA groups was determined after desalting in a 15 cm Sephadex column G-200 f (for NPCA-lactate dehydrogenase with more than five groups per subunit after removing denatured protein on a 3 × 180 cm column Sephadex G-200).

lose acetate strips dipped in a 40% dimethyl sulfoxide glycine buffer mixture to avoid adsorption of the modified enzyme on the carrier. The electrophoresis showed a homogenous material with the same mobility as unmodified lactate dehydrogenase. After electrophoresis no protein was found at the starting point, indicating that the sample contained no denatured enzyme.

Solubility and stability of NPCA-lactate dehydrogenase

Less modified lactate dehydrogenase (4—5 NPCA groups per subunit) is still soluble in 0.02 M pyrophosphate buffer, pH 8, but in gel chromatography on Sephadex G-200 half of the sample was eluted in the exlusion volume of the column, showing an aggregation of the protein. Gel chromatography on the same column, but in 0.1 M glycine buffer gave an elution volume corresponding to the molecular weight of the lactate dehydrogenase tetramer. Modified lactate dehydrogenase with 7—9 NPCA groups per subunit is insoluble in practically all buffer systems (0.1—0.01 M). In highly diluted buffers for example 1—10 mM Tris, *N*-ethylmorpholine or citrate it is soluble, but instable ($t_{½}$ = 2 h). The NPCA-lactate dehydrogenase is soluble and stable only in glycine buffer. The stability of the enzyme depends on the pH and the ionic strength. Optimal conditions are pH 8 and a high ionic strength of the glycine buffer (1 M). However, the best stabilization was achieved in 0.1 M glycine buffer, pH 8 in the presence of NAD^+ and sodium sulfite, forming a ternary complex with the enzyme. Although highly modified NPCA-lactate dehydrogenase is soluble in 1 M glycine buffer, thin-layer gel chromatography on G-200 sf demonstrated an aggregation as well. This aggregation was suppressed in 0.1 M glycine buffer, pH 8, containing $5 \cdot 10^{-4}$ M NAD^+ and 10^{-4} M sodium sulfite, which could be proved by Sephadex gel chromatography.

Structural determinations

Stokes radius. Gel chromatography on Sephadex G-200 (3 × 180 cm) shows

a similar elution volume for NPCA-lactate dehydrogenase compared with unmodified lactate dehydrogenase. We found an elution volume of 410 ml for NPCA-lactate and of 405 ml for unmodified lactate dehydrogenase, suggesting an unchanged Stokes radius of the NPCA-lactate dehydrogenase tetramer.

Helix content. NPCA-lactate dehydrogenase gave the same ORD spectrum as unmodified lactate dehydrogenase. The helix content was determined according to the method of Simmons and Blout [14] and gave for NPCA-lactate dehydrogenase 31 ± 3%, for unmodified lactate dehydrogenase 31%. No greater deformation or denaturation of the NPCA-lactate dehydrogenase was stated.

Total number of sulfhydryl-groups of NPCA-lactate dehydrogenase. Sulfhydryl groups were estimated with Nbs_2 after denaturation of the protein in 8 M urea at pH 7.2 in 67 mM phosphate buffer. We found 4.7 sulfhydryl groups per subunit for NPCA-lactate dehydrogenase; this is the same value as for unmodified lactate dehydrogenase.

Number and reactivity of active site cysteines. The decrease of enzymatic activity was plotted against the number of modified sulfhydryl groups per subunit. It showed that only one thiol group has been attached per monomer. The modification with 5,5′-dithiobis(2-nitrobenzoic acid) followed in the case of NPCA-lactate dehydrogenase a biphasic process indicating a first fast reaction of 0.3 mol sulfhydryl groups per subunit due to a small content of denatured enzyme which was formed during the preparation of NAD^+ and sodium sulfite-free enzyme. Further modification showed a pattern parallel to that of the unmodified lactate dehydrogenase. No additional reactive sulfhydryl groups are present in the native part of the NPCA-lactate dehydrogenase. However, the reaction velocity of 5,5′-dithiobis(2-nitrobenzoic acid) with NPCA-lactate dehydrogenase was much slower compared with unmodified lactate dehydrogenase. The half-life of residual activity of NPCA-lactate dehydrogenase in the reaction with the reagent was 300 min, but that of unmodified lactate dehydrogenase only 15 min, indicating a shielding effect of the NPCA groups on the surface of the modified enzyme against a fast reaction with 5,5′-dithiobis(2-nitrobenzoic acid).

Kinetics. The kinetic parameters of the highly modified NPCA-lactate dehydrogenase (9.4 NPCA groups per subunit) were determined and compared with the kinetic parameters of the unmodified enzyme [18]. The assays for the reaction of pyruvate and NADH were performed at pH 7.2 in phosphate buffer. (The enzyme sample must be pre-diluted in 1 M glycine buffer, pH 8 to prevent aggregation in the test). For the reaction of lactate and NAD^+ the assays were performed at pH 9.5 in glycine buffer.

Table I shows that the K_m value for pyruvate and NAD^+ is identical in native and modified lactate dehydrogenase. The K_m value for NADH is doubled in NPCA-lactate dehydrogenase and divided in half for lactate. The ratio of V for the forward and back reaction of the unmodified and modified enzyme is 0.4 and 0.3.

Thermostability. To prove the lability of the modified enzyme the thermal stability was determined at 55°C. While measuring the kinetics of residual activity a biphasic pattern of denaturation was found for NPCA-lactate dehydrogenase, indicating at least two species of NPCA-lactate dehydrogenase with different thermal stabilities (Fig. 4). The half-life of NPCA-lactate dehydrogenase

TABLE I

KINETIC CONSTANTS FOR UNMODIFIED LACTATE DEHYDROGENASE AND NPCA-LACTATE DEHYDROGENASE

The letters a, b, p and q represent NADH, pyruvate, NAD and lactate, respectively. K is a Michaelis constant; V_1 is the maximum velocity in the reaction of NADH and pyruvate (67 mM sodium phosphate buffer, pH 7.2) and V_2 in the reverse reaction (0.1 M glycine buffer, pH 9.5).

	Unmodified lactate dehydrogenase	NPCA-lactate dehydrogenase	Ratio
K_a	21 μM	40 μM	1.9
K_b	59 μM	57 μM	1.0
K_p	10 μM	11 μM	1.1
K_q	48 μM	18 μM	0.4
V_1	400 units/mg	150 units/mg	0.4
V_2	100 units/mg	30 units/mg	0.3

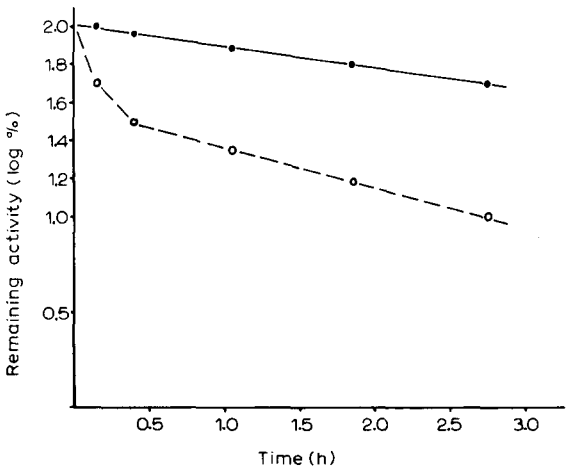

Fig. 4. Thermostability experiment. Lactate dehydrogenase activities as a function of incubation time in 0.2 M glycine buffer, pH 8, at 55°C. ●———●, unmodified lactate dehydrogenase; ○— —○, NPCA-lactate dehydrogenase.

was 15 min during the initial phase of the thermodenaturation experiment and 75 min during the last phase. On the other hand unmodified had a half-life of 160 min in the same experiment.

Discussion

After the removal of denatured enzyme the modified lactate dehydrogenase proved to be free of denatured enzyme in the electrophoresis. The unchanged Stokes radius and the same number of reactive sulfhydryl groups as well as ORD measurements indicated that the structure of the enzyme has not undergone a drastic conformational change. The stabilization of the NPCA-lactate dehydrogenase as ternary complex with NAD^+ and sulfite shows that the active site of the enzyme is fully intact. A fluorescence titration to determine the active sites was not possible as the incorporated chromophore quenched the fluorescence. The K_m values of NPCA-lactate dehydrogenase for pyruvate and NAD^+

are identical with those of the unmodified enzyme. The K_m value for NADH is doubled and the K_m value for lactate is divided in half in the NPCA-lactate dehydrogenase. These results state that no lysine is involved in the binding of substrate and coenzyme. An unchanged K_m value for pyruvate had been found for pyridoxal 5′-phosphate modified lactate dehydrogenase as well [5]. Moreover, the modification of the lactate dehydrogenase-NAD^+-sodium sulfite complex obtains a modified enzyme with an incorporation of 1—2 lysine NPCA-groups lower and an enzymatic activity 10% higher than that of the modified apoenzyme, indicating that the loss of activity cannot be due to an essential lysine.

The fact that 14 lysines per subunit of lactate dehydrogenase apoenzymes were acetylated without loss of activity [7] is another argument for our hypothesis that no essential lysine is present in lactate dehydrogenase. The incorporation of acetyl groups on the surface may have no effect on the structure of the peptide backbone surrounding the active center. But the incorporation of a voluminous NPCA group may have a steric effect, although the dinitroaromatic group is connected with a spacer on the imidoester residue. The deactivation of lactate dehydrogenase of pyridoxal-5′-phosphate observed by Engel [5] does not discredit our hypothesis because the reagent is directly bound on the surface of the protein and can denature the enzyme due to the incorporation of the highly charged group [8].

We cannot refer the loss of activity of the NPCA-lactate dehydrogenase to the modification of a single lysine with a greater reactivity and a special influence on the active center. We rather presume that the loss of activity is due to the hydrophobically modified surface of the protein. The enzyme is no longer completely surrounded by an aqueous environment. The altered hydration of lysines may have effects on the structure of the enzyme which are too small to be proved by the employed methods.

The poor solubility of the modified enzyme is due to the hydrophobic groups attached to the surface. The NPCA-lactate dehydrogenase shows a precipitation with increasing concentration of buffer ions from 0.01 to 0.1 M. It is soluble in most buffer systems with low ionic strength, but is quickly denatured. A similar loss of solubility has been noted for polytyrosylated trypsin and polyvalylated ribonuclease. Katchalski [19] polytyrosylated trypsin and gained a medium incorporation of 2.5 tyrosines on 57% of all lysines. Polytyrosyltrypsin showed an unchanged enzymatic activity, the same sedimentation constant and a poor solubility due to its hydrophobic character. Nishikowa [20] studied the aggregation of polyvalylated ribonuclease and found that the aggregation was due to hydrophobic interactions of the covalently attached valine peptides.

Only glycine buffer has the favourable effect of dissolving and stabilizing the NPCA-lactate dehydrogenase, as glycine ions are small and highly charged dipoles. Adsorbed on the surface of the protein glycine may disturb the hydrophobic interactions between NPCA-lactate dehydrogenase molecules and prevent aggregation. The function of lysines on the surface of the lactate dehydrogenase is to promote a binding of a hydrate layer and buffer ions to stabilize the native structure of the enzyme. Moreover no dissociation of the tetramer in buffer of low ionic strength is observed on thin-layer gel chromatog-

raphy. Jaenicke too has found that under optimal dissociation conditions at neutral pH the equilibrium still favours the tetrameteric state [21]. The denaturation of the dissolved NPCA-lactate dehydrogenase must be due to the low stability of the modified apoenzyme, demonstrated by the thermodenaturation experiment. Forster and Thomson [22] obtained a pentandial cross-linked porcine heart muscle lactate dehydrogenase with a lightly hydrophobically modified surface too. It had only 50% initial activity but a higher thermal stability. Since we do not yield any cross-linking on the surface of the NPCA-lactate dehydrogenase this modified lactate dehydrogenase shows a very low thermostability. A significant reduced thermal stability and 70% activity was also found when lactate dehydrogenase was coupled directly to activated dextrans [22]. In this case the hydrophobic surrounding of the dextran decreased the enzymatic activity and the thermal stability.

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