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ENHANCED HEAT, ALKALINE AND TRYPTIC STABILITY OF ACETAMIDINATED PIG HEART LACTATE DEHYDROGENASE *

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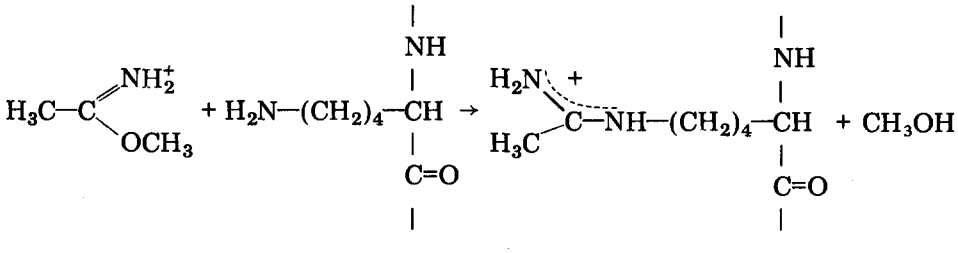
Summary

Modification of 17 from 24 lysine residues in pig heart lactate dehydrogenase (L-lactate : NAD^+ oxidoreductase, EC 1.1.1.27) with methyl acetimidate yields an enzyme derivative with enhanced stability toward heat and alkaline denaturation as well as tryptic digestion. The specific activity of the modified enzyme is only slightly reduced.

Introduction

Acetamidination of proteins has been described in the literature as a selective and mild modification procedure for the ϵ-amino groups of the lysines in proteins without altering the charge [1—3]:



Acetamidinated lysine has an arginine-like structure with a similar pK value (pK = 12.5). The reactions of imidates with lysine have been studied in detail [4], side reactions with other amino acid residues have not been reported.

In general, a high incorporation of acetamidino groups into the proteins was observed. Because the acetamidino group is too small for hydrophobic inter-

* Part of the doctoral thesis of P.T.
Abbreviation: TNBS, trinitrobenzene sulfonate.

actions, it could be expected that the physical and chemical properties of the modified enzymes are very similar to those of the native ones. This has been demonstrated for carbonic anhydrase [5,6], carboxypeptidase A [7], trypsin [8] and lysozyme [9]. Observed alterations in the physical constants of acetamidinated enzymes are due to modification of lysines in the active center, as has been reported for ribonuclease A [10] and cytochrome b_5 reductase [11].

Lactate dehydrogenase (L-lactate : NAD^+ oxidoreductase, EC 1.1.1.27) is a tetrameric enzyme with a molecular weight of 144 000. There are 24 lysines per subunit. No essential lysine could be detected by chemical methods [12,27] or X-ray crystallography [13]. Acetamidination, therefore, does not effect the active site of this enzyme and can be used to study the enzyme surface where most lysines are located [14].

Materials and Methods

Pig heart lactate dehydrogenase, NAD^+ (grade III) and NADH (grade II) were purchased from Boehringer, Mannheim; proteinase K and trypsin (ox) from E. Merck, Darmstadt, and α-chymotrypsin from Worthington. Lactate dehydrogenase was used without further purification since the only impurity visible in disk electrophoresis is a small amount of pig muscle lactate dehydrogenase occurring as isoenzyme II. Upon scanning the gel slabs, the impurity amounts to not more than 1%. Trypsin was purified by the method of Jany et al. [15]. Methyl acetimidate hydrochloride was prepared by the Pinner synthesis from acetonitrile in methanol saturated with HCl. Protein concentrations were determined by the biuret method [16] or by ultraviolet absorption using $E_{280}^{1\%} = 13.8$. Activities were measured in 67 mM phosphate buffer, pH 7.2, containing 0.5 mM pyruvate and 0.3 mM NADH following the decrease of the absorbance at 340 nm. Kinetic constants were calculated according to the method of Eisenthal and Cornish-Bowden [17].

Modifications of the enzyme were performed in 0.1 M pyrophosphate buffer, pH 8.7. For 50 mg enzyme, 140 mg of the reagent was neutralized and added immediately to the protein solution. After stirring for 1 h at room temperature the reactions were stopped by dialysis or gel filtration using 67 mM phosphate buffer, pH 7.2.

The incorporation of acetamidino groups into the protein was estimated by determination of the number of free ε-amino groups with trinitrobenzene sulfonate (TNBS) according to Habeeb [18]. This method was modified in the way that the reaction buffer was 0.1 M borate buffer, pH 9.5, and the reaction time 3 h as otherwise native lactate dehydrogenase did not react completely. For acetamidinated lactate dehydrogenase a heat denaturation step before the addition of TNBS has to be inserted as otherwise there is no reaction with TNBS. The extinction coefficient $\epsilon_{342} = 14\,400\ mol^{-1} \cdot cm^{-1}$ was determined from fully trinitrophenylated lactate dehydrogenase of which no free lysines were found in amino acid analysis after extrapolating different hydrolysis times to zero. Amino acid analyses were performed on a Biocal BC 200 amino acid analyser according to Spackman [19].

Alkaline stability was measured in 0.05 M glycine/NaOH buffer containing

0.1 M NaCl and 25 μg/ml enzyme. The glass electrode for pH measurements was adjusted with standard buffers up to pH 13.0. Heat stability was tested in 67 mM phosphate buffer, pH 7.2, containing 0.1 mg/ml enzyme. ORD spectra were recorded on a Cary 60 spectrophotometer in 0.1 M phosphate buffer. Calculations of the helix content were performed according to Simmons et al. [20].

Tryptic digestions were carried out in 0.1 M NaCl solution with 5 mg/ml lactate dehydrogenase at pH 8.7 and 25°C in a Metrohm autotitrator by addition of 0.01 M NaOH.

The NAD^+/sulfite binding capacity was determined according to Pfleiderer et al. [21] by measuring the change of absorption at 320 nm of the enzyme solution (2 mg/ml) in 67 mM phosphate buffer containing 0.1 mM NAD^+ after the addition of 1.5 mM sulfite ($\epsilon = 4800\ mol^{-1} \cdot cm^{-1}$).

Results

The incorporation of acetamidino groups into the enzyme is based on the pH of the reaction. The modification procedure described above was found to give the highest incorporation with minimal enzyme inactivation. Thus modified lactate dehydrogenase contained 6.5 ± 0.5 free lysines, that means 17.5 ± 0.5 acetamidino groups per subunit. Without a heat denaturation step the content of free lysines was even smaller. The incorporation was also studied by amino acid analysis. The ε-amidinated lysine is rather stable upon hydrolysis with 6 M HCl at 104°C, yet normally elutes together with ammonia in amino acid analysis. With special techniques, however, it was possible to elute ε-acetamidinated lysine prior to ammonia and to determine it quantitatively (Kiltz, H.H., personal communication). By extrapolation the values from different hydrolysis times to zero a content of 17.3 ± 1.0 acetamidinated lysines per subunit lactate dehydrogenase was found, which fits exceedingly well with the values from the TNBS test.

After modification a small fraction of denatured enzyme could be separated by chromatography on Sephadex G-200. The activity of the acetamidinated lactate dehydrogenase was reduced by 20% as compared to the native enzyme, whereas the NAD^+/sulfite binding capacity and the K_m value for pyruvate is not altered (Table I). In disk electrophoresis the acetamidinated enzyme migrates like the native one.

The stability of both native and acetamidinated lactate dehydrogenase in alkaline buffers is given in Fig. 1. It was found that the modified enzyme is much more stable in media between pH 10 and 12 than the native one. This

TABLE I

	Native lactate dehydrogenase	Acetamidinated lactate dehydrogenase
K_m (pyruvate) (μM)	39.5	43.0
υ (pyruvate) (units/mg)	436	348
NAD^+/sulfite binding capacity (mol/mol subunit)	0.99	1.04

TABLE II

VALUES OF THE HELIX CONTENT OF NATIVE AND ACETAMIDINATED LACTATE DEHYDROGENASE

	Native lactate dehydrogenase		Acetamidinated lactate dehydrogenase	
	pH 7.5	pH 12.0	pH 7.5	pH 12.0
$(\alpha)'_{232}$	5790	4250	5880	5970
$(m)'_{232}$	4880	3580	4952	5033
% Helix	28	16	29	29
Activity	389 I.U./mg	0 I.U./mg	291 I.U./mg	259 I.U./mg

observation was confirmed by measurement of the ORD spectra of both enzymes in buffers of pH 7.5 and 12.0 (Table II). Under the latter conditions the native enzyme is inactive and highly unfolded, whereas the acetamidinated one is still active and has, within accuracy of the measurement, the same helix content as at pH 7.5.

Thermal stability profiles are given in Fig. 2. It was found that acetamidinated lactate dehydrogenase is more resistant to heat denaturation. The low protein content of 0.1 mg/ml was chosen to avoid precipitation although the results are similar for higher protein contents. Half-life times for the different temperatures, estimated by plotting the logarithm of the residual activity against time, are given in Table III.

The effect of the acetamidination of lactate dehydrogenase on the tryptic digestion of the enzyme is given in Fig. 3, where the base consumption profiles for native and acetamidinated lactate dehydrogenase are shown. Activity profiles are related with the base consumption (Fig. 3). It is further shown that other protease like α-chymotrypsin and proteinase K do digest the acetamidinated enzyme like the native one indicating the significance of the lysines for tryptic stability.

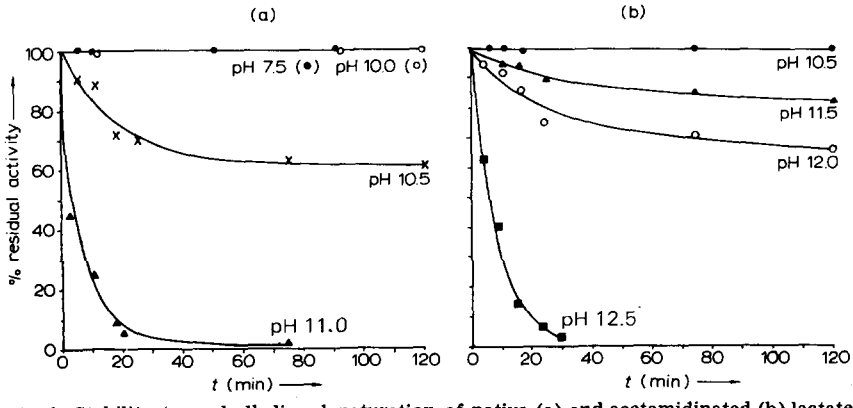


Fig. 1. Stability toward alkaline denaturation of native (a) and acetamidinated (b) lactate dehydrogenase at various pH values each solution containing 25 μg/ml enzyme.

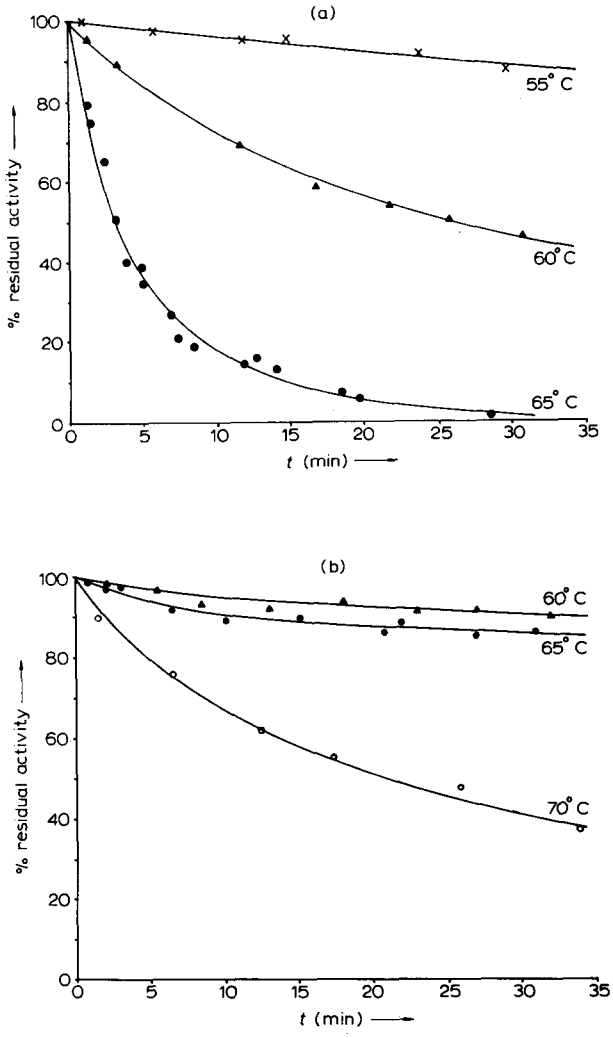


Fig. 2. Temperature stability of native (a) and acetamidinated (b) lactate dehydrogenase at various temperatures. 0.1 mg/ml enzyme in 67 mM phosphate buffer each.

TABLE III

VALUES FOR $\tau_{1/2}$ OF THE ACTIVITY OF NATIVE AND ACETAMIDINATED LACTATE DEHYDROGENASE AT DIFFERENT TEMPERATURES

T (°C)	$\tau_{1/2}$ (min)	
	Native lactate dehydrogenase	Modified lactate dehydrogenase
55	150	∞
60	23.7	200
65	3.4	82
70	<0.5	26

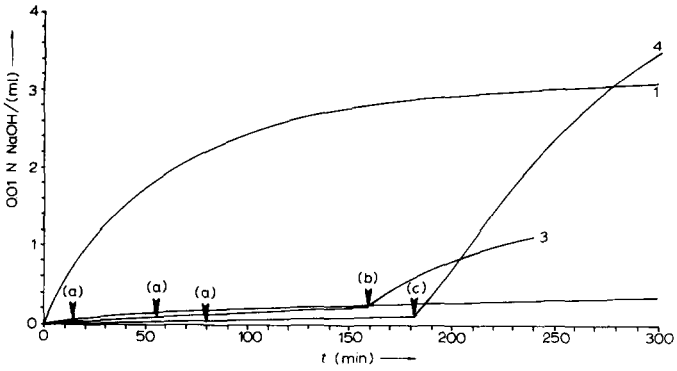


Fig. 3. Base consumption for the proteolysis of native and acetamidinated lactate dehydrogenase. (1) native enzyme, 30 mg; trypsin, 0.6 mg; (2) acetamidinated enzyme, 30 mg; trypsin, 0.6 mg: (a) addition of new trypsin, 0.6 mg; (3) as (2): (b) chymotrypsin, 6 mg; (4) as (2): (c) proteinase, K, 1 mg.

Discussion

From the results of the TNBS test it was concluded that all available lysines on the surface of the lactate dehydrogenase are affected by acetamidination. Normally denaturation in the TNBS test is carried out by hydrophobic interactions of the incorporated trinitrophenyl groups, as denaturing agents interfere with the test [18]. For acetamidination, however, there is only a small colour reaction with TNBS under these conditions deceiving a complete modification, as all available lysines on the surface are already blocked by acetamidino groups. After a heat denaturing step the free lysines which are not on the surface are now accessible to trinitrophenylation and the real incorporation of acetamidino groups can be determined.

The fact that there are 17—18 lysines on the surface of the enzyme per subunit is in exceedingly good agreement with the results from acetylated lactate dehydrogenase (Tuengler, P. and Pfleiderer, G., unpublished) and methyl-ε-(*n*-2,4-dinitrophenyl)-aminocaproimidate-lactate dehydrogenase [27] where only 10 groups are incorporated per subunit, yet 16—18 lysine residues affected by modification. The extent of modification is restricted here by ionic or hydrophobic interactions which play no role for acetamidination.

The reduced activity of acetamidinated lactate dehydrogenase is directly connected with the extent of modification. The loss of activity is smaller upon modification at a lower pH than 8.7, when fewer acetamidino groups are incorporated. Further, the NAD^+/sulfite binding capacity is not reduced as compared to the native enzyme and the activity was not raised after chromatography on Sephadex G-200. The attempt to regenerate the lost activity by cleavage of the acetamidino groups with 0.6 M hydrazine at pH 9.0 [22,10] failed because the native enzyme is inactivated considerably under these conditions.

The higher stability of acetamidinated lactate dehydrogenase in alkaline media is based on the shift of the pK values of the lysine residues from 10.5 to 12.5 after acetamidination [4]. Native lactate dehydrogenase is irreversibly

inactivated at pH values greater than 10.5, probably by a high increase of the net negative charge on the surface due to the deprotonation of lysines. A partial unfolding is the consequence as demonstrated by ORD measurements. This effect is raised to pH 12.5 after acetamidination of the enzyme.

The reason for the stabilization towards heat denaturation by acetamidination is not yet understood. Possible explanations could be an increase of the water layer around the enzyme or ionic effects due to the change of the ϵ-amino groups of lysines to arginine-like groups. Supporting evidence is given by the fact that native lactate dehydrogenase is stabilized to heat denaturation by high ion concentrations [23,24], on the other hand, acetamidinated pig muscle lactate dehydrogenase is stabilized in ion-free water, too (Tuengler, P. and Pfleiderer, G., unpublished). Nevertheless the effect is very interesting with respect to analogous results found for thermostable lactate dehydrogenase from thermophilic bacteria [25]. The thermostable enzyme differs from the thermolabile one of the same bacteria cultivated at different temperatures only by a high exchange of lysines to arginines.

In spite of the similarity of ϵ-acetamidinated lysine with arginine the former is not cleaved in proteins by trypsin. The effect is explained by the additional methylene group of ϵ-acetamidinolysine as compared to arginine. Similarly, homoarginine is not cleaved in proteins by trypsin either [26]. Since the arginines are not very accessible in lactate dehydrogenase the acetamidinated enzyme is highly stable toward tryptic digestions. The normal digestion found with other proteases confirm this hypothesis.

The results from this work indicate that acetamidination generally should increase heat and alkaline stability of enzymes with a high content of lysines and further limit tryptic hydrolysis to the arginines. The effects might be of interest for the technical application of the enzymes.

Acknowledgement

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