THE REACTIVITIES OF THE LYSINE, CYSTEINE AND TYROSINE RESIDUES OF PIG HEART LACTATE DEHYDROGENASE IN THE PRESENCE OF SULPHITE

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1. Introduction

It has been known for some time that many NAD-linked dehydrogenases contain essential cysteine residues [1] but we have little information about the involvement of other amino acid side chains in the binding and the activation of substrates by these enzymes. There is some evidence that amino groups of glutamate dehydrogenase are involved in subunit interaction [2] and in pyridine nucleotide binding [3]. An intramolecular transfer of an acetyl group from the essential cysteine to a single ϵ-amino group has been observed in glyceraldehyde 3-phosphate dehydrogenase [4]. For these reasons we attempted to acetylate the ϵ-amino groups of lactate dehydrogenase with N-acetylimidazole. In the presence of sodium sulphite it was possible to protect all SH-groups, retain the full enzymic activity, and yet acetylate 14 of the 24 ϵ-amino groups of the protein. Sulphite also prevented reaction of an essential tyrosine residue with diazotised sulphanilic acid. A change in conformation of the protein in the presence of sulphite would account for these protective effects.

2. Materials and methods

Pig heart LDH, NAD, NADH and GSH were from Boehringer, Mannheim. DTNB was purchased from Aldrich Chemical Co. Inc., Milwaukee. N-acetylimidazole was prepared according to Wieland [5] or Boyer [6]. N-Caproyl-S-malonylcysteamine was a generous gift from Dr. H.Eggerer, Chemisches Laboratorium der Universität München. Kieselgel-S was purchased from Macherey, Nagel und Co., Düren. [^{35}S]-Sulphanilic acid was obtained from Farbwerke Hoechst AG with a specific radioactivity of 11.5 mC/mMole and was diazotised in the usual way.

The number of SH-groups was determined in 8M urea with either DTNB [7] or PCMB [8]. The specific enzymic activity was calculated from the initial rate of oxidation of 0.45 mM NADH by 0.5 mM sodium pyruvate in 67 mM phosphate buffer, pH 7.2 and the protein concentration, determined by biuret method. Acetylation and estimation of O-acetyltyrosine were carried out as described by Riordan et al. [9]. The relatively high contents of tyrosine and tryptophan in LDH [10] limited the accuracy of the method considerably. The residual amino groups were measured by ninhydrin reaction as described by Moore and Stein [11].

3. Results and discussion

Effects of acetylimidazole on LDH. According to Riordan et al. [9], acetylimidazole reacts more rapidly with tyrosine residues than with SH-groups or ϵ-amino groups of proteins. However with LDH (table 1, experiments 2 and 3) acetylimidazole caused a rapid destruction of the enzymic activity which was

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Abbreviations: LDH, L-Lactate: NAD oxidoreductase (E.C.1.1.1.27);
DTNB, 5,5'-Dithiobis (2-nitrobenzoic acid);
PCMB, *p*-Chloromercuribenzoate;
DS, Diazotised sulphanilic acid.

Table 1

Analysis of LDH acetylated with N-acetylimidazole.

Experiment No.	[LDH] (mg/ml)	[acetyl-imidazole] (mM)	[Na_2SO_3] (mM)	Residual enzyme activity (percent)	Residual SH-groups (moles/ 36 000 g protein)	Tyrosine acetylated (moles/ 36 000 g protein)	Residual NH_2-groups (moles/36 000 g protein)
. Control	12	0	0	100	4–4.6	–	22–24
Control	16.8	0	27.4	100	4–4.6	–	22–24
2. a	11	32.8	0	50	3.2	0 ± 0.5	–
b	11	55.5	0	30	2.7	0 ± 0.5	–
c	11	33	0	1	1.6	0 ± 0.5	–
3. a	17.8	14.2	0	39	–	–	18.5
b	17.8	42.6	0	12.5	1.5	–	13.4
c	17.8	85.2	0	0	0.03	–	8.9
d	17.8	170.4	0	0	0	–	5.3
4. a	16.8	23.7	27.4	97	3.88	–	11.9
b	16.8	47.5	27.4	97	3.80	–	9.9
c	16.8	95.0	27.4	75	2.80	–	8.7
5. a	12	60.7	0	1.4	1.7	–	10.5
b	12	60.7	38.4	100	4.0	–	13.6

LDH, Na_2SO_3 and acetylimidazole of the concentrations shown were incubated in 67 mM phosphate buffer, pH 7.5, for 1 hour and then analysed for residual enzymic activity, SH-groups, amino groups, and in one series, O-acetyltyrosine. Incubation mixtures containing sulphite were filtered through Sephadex prior to SH-analysis. Analytical procedures are described in section 2.
– = not measured.

not due to the modification of tyrosine residues, but rather to the acetylation of either SH-groups or amino groups. S-acyl and O-acyl derivatives are rapidly split by hydroxylamine to the corresponding hydroxamic acids. When acetylated LDH, from experiments 2a, b and c in table 1, was treated with NH_2OH at pH's 7.5, 8.6 and 10.1, there was no increase in the residual activity of the preparations. Therefore either acetylation of ϵ-amino groups or an irreversible change in conformation subsequent to S- or O-acylation was responsible for the inhibition. In order to protect essential SH-groups during acetylation we have added sulphite, a reagent which was found by Rajewsky [12] to protect LDH from inhibition by acetic anhydride. In the presence of sulphite, as can be seen from table 1 (experiments 4a and 4b), it is possible to acetylate all but 10 of the 24 amino groups of the protein subunit without destroying either SH groups or enzymic activity. The protecting effect of sulphite is most clearly demonstrated by the results of experiments 5a and 5b, where a sample of LDH was treated with acetylimidazole in the presence and in the absence of sulphite. The results of different sets of experiments are not directly comparable, since the concentration of "active" acetylimidazole differed in each case.

We confirmed the observation of Rajewsky [12] that acetylated LDH migrates more rapidly to the anode than native LDH during electrophoresis at pH 8.6, as would be expected from the conversion of $-NH_3^+$ to $-NH-CO-CH_3$. The acetylated enzyme gave a more diffuse band than the native indicating the presence of many different, active, acetylated species of enzyme. The pH-optimum at 0.5 mM pyruvate for the acetylated enzyme was 5.3 as compared to 7.15 for the native enzyme. Thus none of the 14 easily acetylated amino groups of LDH appear to be necessary for the enzymic activity of the

Table 2
Effect of sulphite on the inhibition of LDH by diazotised sulphanilic acid.

Minutes after addition of $[^{35}S]$-DS	Control LDH *	LDH + $[^{35}S]$-DS *		LDH + $[^{35}S]$-DS + Na_2SO_3 *	
	Percent residual enzyme activity	Percent residual enzyme activity	Percent DS remaining	Percent residual enzyme activity	Percent DS remaining **
0	100	100	100	100	100
15	100	0	–	100	–
30	–	–	52	–	30
60	100	0	–	100	–
90	–	–	36	–	25
120	100	0	–	100	–
150	–	–	25	–	12.5

* 5.8×10^{-5} M LDH was incubated at 0°C in 0.1 M sodium pyrophosphate-HCl buffer, pH 8.5, either with no additions or with the addition of 1.7×10^{-2} M Na_2SO_3 and 5×10^{-2} M $[^{35}S]$-DS as shown.
** The amount of DS remaining was measured by coupling a portion of the incubation mixture with resorcinol.

protein. Acetylated LDH was as stable as the native enzyme at 25°C at pH 7.5 in phosphate buffer. It seems that some 14 amino groups are more easily acetylated than the remaining 10. This could be a reflection of the fact that the majority of the polar groups of LDH are in contact with the solvent as has been found, for example, with myoglobin [13] and other proteins. It has been known for some time that it is possible to acetylate completely the amino groups of trypsin without destroying its enzymic activity [14]. Polyanovskii [15] was able to prepare succinylated (ϵ-amino groups) glutamine-oxaloacetic acid transaminase with full activity.

The different results in absence of sulphite (experiment 2b: 2.7 residual SH-groups and 30% residual activity) and presence of sulphite (experiment 4c: 2.8 residual SH-groups and 75% residual activity) could be interpreted as different acetylation rates of essential and non-essential SH-groups.

Recently we have reported the coupling of an essential tyrosine residue in LDH with diazotised $[^{35}S]$-sulphanilic acid [16]. Table 2 shows the same protecting effect of sulphite against inactivation of LDH by this reagent, even after incubation for 150 min. Although the sulphite accelerated the decomposition of diazotised sulphanilic acid, there was still a great excess of active reagent remaining at the end of the experiment. In the absence of sulphite 5 moles $[^{35}S]$-DS were incorporated into each subunit; in the presence of sulphite we could not detect any incorporation.

At this time we have no explanation for the protecting effect of sulphite ions. Since LDH preparations which have been treated with Na_2SO_3 regain their enzymic activity after the sulphite has been dialysed out, no irreversible complex of sulphite with the enzyme could account for these results. In $SO_3^=$ buffered electrophoresis we were unable to detect any charged complex of reduced glutathione or thioglycollic acid with sulphite. Another explanation of the protective effect of sulphite would be that S-acetyl LDH is formed, but that sulphite ions, being good nucleophiles, rapidly hydrolyse these bonds. However, model experiments with N-caproyl-S-malonylcysteamine show that this thioester is stable both in the absence and presence of sulphite at concentrations which protect the SH-group of LDH. It might also be thought that Na_2SO_3 caused so rapid a destruction of N-acetylimidazole that this was the reason for the lack of acetylation. However, as will be noted from table 1, even in the presence of sulphite, there was always sufficient acetylating agent present to acetylate more than 50% of the amino groups of the protein. Therefore it is most likely that sulphite ions cause the enzyme to take up such a conformation that the essential SH- and tyrosine groups are no longer available for reaction.

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