REACTIVE LYSINE RESIDUES IN HORSE LIVER ALCOHOL DEHYDROGENASE

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Summary. Horse liver alcohol dehydrogenase was modified under various conditions with ^{14}C-labelled formaldehyde in the presence of sodium borohydride. Changes in the enzymatic activity were correlated with incorporated label and modified residues were characterized. It is shown that most of the lysine residues react and that many are affected by the binding of coenzymes and inhibitors to the protein. Reactive residues are reported and possible structural and functional interpretations given.

Modifications of horse liver alcohol dehydrogenase (EC 1.1.1.1) with reagents specific for amino groups indicate that lysine residues may occur at the "active site" of the enzyme (1,2,3). In all cases, however, several lysine residues are modified and differential reactivities estimated from the total label of the whole protein. Different parts of the bound coenzyme have also been concluded to be close to lysine residues (2,3).

The present work aimed at identifying the reactive lysine residues directly in the primary structure (4) of the protein. ^{14}C-Labelled formaldehyde, in the presence of sodium borohydride, was used to modify (5) the enzyme under various conditions. Modifications were correlated with changes in the enzymatic activity and labelled residues were characterized after proteolytic digestion of the enzyme.

MATERIALS AND METHODS

Horse liver alcohol dehydrogenase (Boehringer Mannheim GmbH, Germany) in 0.05 M N-ethylmorpholine/phosphate buffer, pH 8.5 (1 mg/ml) was incubated at 0°C with ^{14}C-labelled HCHO (4-870 mole/mole protein subunit). After 30 sec $NaBH_4$ was added (1000-2000

mole/mole protein subunit). To some samples AMP, NAD, NAD + pyrazole or NADH were added before labelling to yield binary and ternary enzyme complexes. After dialysis against the same buffer enzymatic activity was determined (6) and radioactivity measured. In some cases repeated labellings were performed, the first with non-radioactive HCHO. All samples were then carboxymethylated and digested with chymotrypsin (7). Radioactive peptides were purified by high-voltage electrophoresis and chromatography on paper, detected by autoradiography and analysed for total compositions, end-groups and partial amino acid sequences, as previously described (4,7).

RESULTS

Total labelling and enzymatic activity. Treatment with formaldehyde and sodium borohydride methylates (5) horse liver alcohol dehydrogenase and enhances its enzymatic activity (Table 1). The specificity was checked by acid hydrolysis of the modified protein, followed by paper chromatography (8) or electrophoresis

Table 1.

The effect of variation of formaldehyde excess during modification with ^{14}C-labelled formaldehyde and sodium borohydride. Radioactivity in labelled atoms per protein subunit, enzymatic activity in % of an un-modified control; both values are relative and without corrections for volume changes, denaturation or other losses.

HCHO/protein subunit	Without NAD during labelling		0.2 mM NAD during labelling	
	Radioactivity	Enzymatic activity	Radioactivity	Enzymatic activity
4	0.03	90	0.01	90
16	0.2	90	0.2	90
170	3	115	2	90
320	7	130	4	90
430	7	135	5	105
870	12	150	7	105

at pH 1.9 and autoradiography, which revealed radioactivity only at the positions of methylated lysine residues.

Both labelling and increase of enzymatic activity are prevented by the presence of coenzymes during modification, while the presence of AMP yields an even greater activation (Table 2).

Table 2.

The effect of the presence of coenzymes and inhibitors during methylation.
Units as in Table 1.

Compound present during labelling	870 molar excess of HCHO		200 molar excess of HCHO	
	Radio-activity	Enzymatic activity	Radio-activity	Enzymatic activity
-	12	140	2	110
0.9 mM AMP	10	155	2	125
0.2 mM NAD	7	95	1	85
2 mM NAD	6	105	1	105
0.2 mM NAD + 0.9 mM pyrazole	8	90	2	80
0.2 mM NADH	7	90	1	80

Methylation, therefore, causes effects that are similar to both picolinimidylation (1,2), which suggested a lysine residue to be close to the nicotinamide part of the coenzyme; and to the modification with pyridoxal phosphate and sodium borohydride (3), which suggested a role of a lysine residue in binding the phosphate group of the AMP part of the coenzyme. Methylation may therefore be useful to study the function of lysine in the enzyme.

Many groups of lysine residues seem to be involved. Some, protected by AMP, yield the most active enzyme when they are non-modified (Table 2). NAD protects additional lysine residues, which apparently yield the most active enzyme when modified. The effects of a ten-fold increase in NAD-concentration suggest that still

more lysine residues are affected when NAD, at high concentrations, binds to secondary sites. The increased ^{14}C-incorporation in the presence of the inhibitor pyrazole (Table 2) suggests involvement of additional lysine residues. The existence of several groups of residues protected by the presence of different compounds, is also supported by the results of differential labelling after repeated treatments (Table 3).

Table 3

Values obtained after treatment with ^{14}C-labelled formaldehyde and sodium borohydride of an already modified enzyme.

Primary modification with sodium borohydride and non-radioactive formaldehyde (left part) or pyridoxal phosphate (right part) in the presence of the indicated compounds, which were absent during second modification. 870-molar excess of aldehyde in all cases. Units as in Table 1.

Compound present during primary modification with non-radioactive aldehyde	Primary modification with non-radioactive HCHO		Primary modification with non-radioactive pyridoxal phosphate	
	Radioactivity after ^{14}C-HCHO	Enzymatic activity after ^{14}C-HCHO	Radioactivity after ^{14}C-HCHO	Enzymatic activity after ^{14}C-HCHO
-	8	155	7	100
0.9 mM AMP	10	195	9	165
0.2 mM NAD	9	130	9	110
2 mM NAD	8	110	5	115
0.2 mM NAD + 0.9 mM pyrazole	10	125	9	125
0.2 mM NADH	8	110	7	95

The results of peptide mapping show that in no case is a specific label obtained. Many of the lysine residues are always partly modified. The decrease in ^{14}C-label (Tables 1 and 2) upon protection of the enzyme is to a large extent due to a general decrease in reactivity of many lysine residues. The value obtained for the total label in each case does therefore not reflect the

number of separate residues that are modified but is largely influenced by the aldehyde excess (Tables 1 and 2). Hence, it is important to control protein labelling with peptide mapping and not only with total incorporation.

Labelled residues. The autoradiographs of the protein labelled in the absence of coenzymes or inhibitors showed 14 spots with different intensities. Variation of the aldehyde excess (Table 1) had no effect on the relative intensities of the different spots, and not even the lowest amount of aldehyde gave a more specific labelling. The four peptides corresponding to the strongest spots on the autoradiographs were purified, analysed and found to be, in order of decreasing radioactivity, the following peptides in the primary structure (4) of the protein: residues 159-166 (with Lys-159), residues 247-254 (with Lys-247 and -248; Edman degradation revealed most of the label to be on the latter), residues 210-229 (with Lys-212, -226 and -228) and residues 323-335 (with Lys-323, -325 and -330). Remaining radioactive peptides were judged, from the autoradiographic intensities, to be less labelled.

The autoradiographs of the enzyme labelled in the presence of coenzymes or inhibitors showed patterns similar to those obtained from the non-protected enzyme. The most striking difference was the reduction in intensity of most spots. The reduction seemed to be roughly proportional to the general decrease in incorporated radioactivity (Table 2) and was somewhat more conspicuous in those spots that were intense in case of the non-protected enzyme.

Peptide mapping of enzyme that was ^{14}C-labelled after premodification (Table 3) again revealed all peptide spots in the autoradiographs. Corresponding cases of the two different types

of pre-modification (Table 3) gave similar pictures and lysine residues could be grouped, regarding susceptibility to the first treatment and protection by coenzymes, as given above. Fluorescence in the peptides, due to labelling with pyridoxal phosphate, was weak and difficult to detect but seemed to indicate a similar order of reactivity of lysine residues. No protection by coenzymes of Lys-248 and -247 against pyridoxal phosphate was, however, noticed.

It may thus be concluded that nearly all lysine residues are labelled to different extents during methylation. Their susceptibility is variable but many are highly reactive. Among these are Lys-159, -248 and -247. The presence of coenzymes and inhibitors cause a general decrease in labelling; Lys-159 is then much affected but no residues are completely and selectively protected. Repeated labellings confirm the results but also indicate the low selectivity of the modifications.

DISCUSSION

Differences in enzymatic activity and total label indicate that lysine residues are of importance for the activity of the enzyme. Complex mechanisms are, however, probable due to the multiple labelling and the different changes in enzymatic activity that are obtained. Indirect effects of lysine modifications, affecting the tertiary structure of the protein or the conformational changes accompanying coenzyme binding (9), are possible apart from direct effects (cf. 1,2,3) on specific binding. Such indirect influences may explain the common activation, under certain circumstances, of the different lysine-substituted enzyme derivatives as compared to the native enzyme; activation is achieved by methylation, picolinimidylation (1,2) and pyridoxal phosphate treatment in the presence of AMP (3), although groups introduced differ

greatly in size and charge. Different structurally and functionally important lysine residues are thus concluded.

Both the total label and the peptide mapping indicate that restricted reactions with particular lysine residues are not obtained. This is largely independent of aldehyde excess and of coenzyme amounts, which affect total label more than the sensitivity of particular lysine residues. A strict correlation with structure and function is therefore prevented. Lys-159, -248 and -247, however, seem to be among the most reactive in the native enzyme. The reactivity of Lys-159 is also affected by the binding of the coenzymes. This residue is, furthermore, inbetween two hydrophobic residues in the primary structure (4). The role of Lys-159 is unknown, but it may be noticed that all its properties fit those expected for a lysine residue of particular importance for the enzymatic activity.

Lys-248 and -247 are slightly less reactive and less affected by coenzyme binding as judged by pyridoxal phosphate labelling. They are also in a more hydrophilic region in the primary structure of the protein (4). They may therefore be superficially exposed but without function for the enzymatic activity. Such an interpretation is supported by the results of iodination of the enzyme (10), which showed the neighbouring Tyr-246 to be reactive but without direct correlation with enzymatic activity.

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