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Cysteine reactivity in sorbitol and aldehyde dehydrogenases

Differences towards the pattern in alcohol dehydrogenase

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In sorbitol dehydrogenase only one cysteine residue, Cys-43, is reactive in both anionic buffer (phosphate) and zinc-liganding buffer (imidazole) upon carboxymethylation. This is in contrast to the situation in the structurally related liver alcohol dehydrogenase, with either of two alternative Cys residues being reactive, and is compatible with differences in zinc-binding and active site relationships between these two metalloenzymes. Unrelated aldehyde dehydrogenase, upon carboxamidomethylation, shows a third pattern, now less well defined but confirming the presence of a thiol function of Cys-302 close to the active site.

Dehydrogenase differences; Reactive Cys residue; Carboxymethylation; Active site; Sorbitol dehydrogenase; Aldehyde dehydrogenase

1. INTRODUCTION

Cysteine reactivity in several dehydrogenases is well known since long [1–11]. A cysteine residue, known to be zinc-liganding at the active site of both alcohol and sorbitol dehydrogenases, and one presumed to be at the active site of the non-metalloenzyme aldehyde dehydrogenase, selectivity reacts in each case. The positions in the primary structures were early identified, Cys-46 or Cys-174 in liver alcohol dehydrogenase [1,2,5,12,13], Cys-43 in sorbitol dehydrogenase [7], and Cys-302 in aldehyde dehydrogenase [14]. The Cys reactivities offer important tools for studying the active sites and have established differential reactivities of the two separate cysteine residues in alcohol dehydrogenase, the best known of these three enzymes, where the residues shift in reactivity upon minor differences in buffer, reagent or isozyme [4,11–13]. Similar shifts have not been reported with the sorbitol and aldehyde dehydrogenases, for which active site relationships are not equally well established.

In the case of sorbitol dehydrogenase, the third zinc-liganding residue (in addition to the ones corresponding to Cys-46 and His-67 of liver alcohol dehydrogenase) has been ascribed to Glu-154 (for positional numbering cf. [15]), constituting a shift from a Cys, Cys-174, in alcohol dehydrogenase [16]. Further proof for this interpretation of a lack in sorbitol dehydrogenase of a Cys-equivalent to the Zn-liganding Cys-174 in liver alcohol dehydrogenase would be desirable. Similarly, in aldehyde dehydrogenase, which is unrelated to the other two enzymes, active site relationships are not conformationally established, and assignment of other than Cys residues at the active site have been reported, including Glu-268 [17]. Carboxymethylation of sorbitol dehydrogenase and carboxamidomethylation of aldehyde dehydrogenase were now studied under buffer variations (anionic or zinc-liganding), and presence of ligands (coenzymes, substrates), respectively, that have proven useful for establishing the differential reactivities in alcohol dehydrogenase [13]. Considerable differences were found in labelling patterns between the three enzymes, confirming conclusions of differences at the active sites.

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2. MATERIALS AND METHODS

Sheep liver sorbitol dehydrogenase from Boehringer Mannheim was carboxymethylated in either 5 mM imidazole–HCl, pH 7.8, or 100 mM sodium phosphate, pH 7.5. The original specific activity of the enzyme preparation was 1.5 U/mg in both buffers and inactivations were monitored at 24°C by the absorbance at 340 nm in 20 mM sodium phosphate, pH 7.5, 100 mM sorbitol, 2 mM NAD^+, 90 mM dithioerythritol. Cytosolic aldehyde dehydrogenase was purified from human liver [4] and was carboxamidomethylated in the absence/presence of coenzymes (final concentrations $10 \times K_m$) and/or propanal (final concentration $100 \times K_m$) in 100 mM phosphate, pH 7.8, containing 1 mM EDTA. The original specific activity of the enzyme was 0.6 U/mg and inactivations were monitored at 24°C by the absorbance at 340 nm in 120 mM pyrophosphate buffer, pH 9, 7 mM propanal, 0.1 mM NAD^+. For both the sorbitol and aldehyde dehydrogenases, effects of incubation at 4°C with ^{14}C-labelled iodoacetate and ^{14}C-labelled iodoacetamide, respectively (final concentration 1000-fold molar excess over enzyme subunit), were evaluated by enzyme assays.

At 20% remaining enzyme activity, further modifications of Cys residues were stopped by addition of excess β-mercaptoethanol (10-fold over iodoacetate/iodoacetamide concentration). All reagents were removed by extensive dialysis against first the labelling buffer, then 0.2 M Tris–HCl, pH 8.1, 2 mM EDTA, 8 M urea. After that, the proteins were reduced with dithiothreitol and homogeneously carboxymethylated [7,13] with [^{13}C]iodoacetate or [^{13}C]iodoacetamide. Total modification of Cys residues was monitored by scintillation counting, and carboxymethylated/carboxamidomethylated residues were identified by peptide analysis after digestion with trypsin (protease/protein ratio of 1:20–50, 37°C, 4 h). Peptides obtained were fractionated by exclusion chromatography on Sephadex G-50 and reverse phase HPLC on Ultropac TSK ODS-120T [7,13–15]. For peptides with more than one Cys residue, the relative labelling of each Cys was determined by sequencer degradations in an ABI 470A peptide sequencer and monitoring of radioactivity in the extract of each cycle.

3. RESULTS

3.1. *Sorbitol dehydrogenase*

The enzyme was sensitive to carboxymethylation in both imidazole and phosphate buffers, and inactivations proceeded identically, reaching 20% residual enzymatic activity after 30 min incubation in both buffers. At that time, total incorporation was 2 mol per mol enzyme subunit. All Cys positions were positively identified and quantitated for ^{14}C-labelled carboxymethylcysteine (Table I). As expected, the sum of individually determined labellings is slightly lower than the value obtained from the total radioactivity of the protein, which includes unspecific and non-covalent incorporations. The peptide data reveal that only one Cys residue is labelled above background, Cys-43, and that this residue is distinguished more or less similarly after labelling in both the imidazole and the phosphate buffer.

3.2. *Cytosolic aldehyde dehydrogenase*

Carboxamidomethylation of aldehyde dehydrogenase in 100 mM phosphate buffer, pH 7.8, 1 mM EDTA in the absence of coenzyme or substrate, and in the presence of NAD^+, propanal, or NADH + propanal (final concentration of coenzymes $10 \times K_m$, of the substrate $100 \times K_m$) gave inactivations to about 20% remaining activity in 0.5–4 h at 4°C. Total incorporations were 1–2 carboxamidomethyl groups per enzyme subunit, with less reproducibility between different preparations than for sorbitol dehydrogenase but with lower values from the samples treated in the presence of propanal. Identification of labelled peptides revealed above-background labels in all cases only in one Cys-containing peptide, the one with the adjacent Cys-301 and Cys-302. Labelling of these two residues was quantitated by sequencer degradation and radioactivity measurements in the corresponding extracts. Unexpectedly, considerable labelling was detected at both positions. This result confirms the sensitivity of Cys-302 to carboxamidomethylation [6,14], suggests labelling also of the adjacent Cys-301 under the present conditions, and the possibility of some protection by a substrate aldehyde.

4. DISCUSSION

The results have several implications. On the one hand, they confirm previous observations of a reactive cysteine residue in both sorbitol dehydrogenase (Cys-43) and cytosolic aldehyde dehydrogenase (Cys-302). Obviously, these residues are selectively reactive under all conditions, both those previously used, with other conditions, and those now utilized. In addition, the lack of residue shift in sorbitol dehydrogenase upon buffer change is important.

Thus, for this enzyme, carboxymethylation in phosphate buffer and in imidazole buffer gave similar results, regarding both inactivation time (intermediate between the extremes obtained with the EE isozyme of liver alcohol dehydrogenase in these two buffers, cf. [13]), cysteine modification (only one residue labelled above background) and actual Cys residue labelled (Cys-43 in both cases). This result establishes that Cys modification at the active site of sorbitol dehydrogenase is not dependent on use of anionic or zinc-liganding buffers. This is in contrast to the situation with the structurally related alcohol dehydrogenase, where an anion binding site and imidazole binding to the active site zinc atom (cf. [5]) give high selectivity, differential results in the presence of different buffers, and highly different values upon slight structural changes between isozymes, with two Cys residues (Cys-46 and Cys-174) separately labelled under different conditions [13]. Buffer variations that in alcohol dehydrogenase favour a shift to labelling of the second Cys ligand do not cause a similar shift in sorbitol dehydrogenase, suggesting that sorbitol dehydrogenase indeed only has one zinc-liganding Cys residue. Consequently, the alternative with the third ligand being Glu [16] is now indirectly

Table I

Extent of Cys modification after inactivation of sheep liver sorbitol dehydrogenase in 5 mM imidazole–HCl, pH 7.8, or 100 mM phosphate, pH 7.5, by treatment with ^{14}C-labelled iodoacetate to 20% remaining enzymatic activity. Cys positions as given in [15]

Cys position	Cys modification (mol/mol) in	
	Imidazole	Phosphate
43	0.4	0.7
104	0.2	0.2
118	0.1	0.1
128	0.1	0.1
138	0.1	<0.1
163	0.1	<0.1
177	<0.1	0.1
248	<0.1	<0.1
299	<0.1	0.1
348	<0.1	<0.1

supported by labelling experiments. In sorbitol dehydrogenase Cys-43 (the equivalence of Cys-46) is labelled at any condition or buffer. These findings suggest that sorbitol dehydrogenase has no anion binding site equally well defined as in alcohol dehydrogenase. The latter conclusion is compatible with the fact that sorbitol dehydrogenase lacks a basic residue at the equivalence of alcohol dehydrogenase position 47, known to contribute an anion binding site [5].

Regarding aldehyde dehydrogenase, the carboxamidomethylation of Cys-302 is expected [6,14]. However, an active site location of Cys-302 close to the substrate binding site, has been questioned (cf. [17]), and the present finding of an apparent reduction in reactivity in the presence of a substrate constitutes a further support for Cys-302 (and adjacent Cys-301) to be close to the active site of aldehyde dehydrogenase. Presumably, a thiohemiacetal formation between the aldehyde substrate and Cys-302 could reduce carboxamidomethylation of the enzyme.

In conclusion, the thiol-containing alcohol, sorbitol, and aldehyde dehydrogenases, although in two cases structurally related, exhibit different patterns upon Cys modification, with the differences compatible with altered ligand relationships for sorbitol dehydrogenase versus alcohol dehydrogenase, and with Cys positions close to the active site for aldehyde dehydrogenase.

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