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Closely related isozymes of alcohol dehydrogenase

Carboxymethylation: $\gamma_1\gamma_1$ differs widely from both $\beta_1\beta_1$ and its equine equivalence EE

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Human $\gamma_1\gamma_1$ alcohol dehydrogenase is quite insensitive to inactivation by iodoacetate, its equine counterpart EE highly sensitive, and the human $\beta_1\beta_1$ form intermediately sensitive. Imidazole hardly influences the iodoacetate inactivation of $\gamma_1\gamma_1$, enhances that of EE and decreases that of $\beta_1\beta_1$. In all isozymes, metal-binding Cys residues are the most reactive, but the patterns for those binding the active site zinc atom differ. In phosphate, Cys-46 is most sensitive in EE and $\gamma_1\gamma_1$, Cys-174 in $\beta_1\beta_1$. This difference appears to correlate with the absence or presence, respectively, of an extra methyl group in the side-chain at position 48 (Ser in EE and $\gamma_1\gamma_1$, Thr in $\beta_1\beta_1$). In imidazole, the reactivity in $\beta_1\beta_1$ is shifted to Cys-46, while the specificity is enhanced in EE and decreased in $\gamma_1\gamma_1$. Thus, the inactivations illustrate large differences among structures closely related.

Carboxymethylation; Protein thiol; Isozyme; Radiosequence analysis

1. INTRODUCTION

Liver alcohol dehydrogenase contains two zinc atoms per subunit, one catalytic at the active site, the other non-catalytic in a loop structure [1–4]. The loop constitutes a hyper-variable segment [5,6]. Its variability in related proteins (sorbitol dehydrogenase, threonine dehydrogenase and ξ-crystallin) extends to the zinc-binding residues, the metal atom itself, and the whole loop [5–10]. The two Cys residues binding the active site zinc atom (Cys-46 and Cys-174) are variably reactive as a function of conditions or species examined [11–19]. Iodoacetate preferentially labels Cys-46 in the model horse liver EE enzyme [2,18–22]. In the presence of imidazole, the reaction can be highly specific and has been correlated with an anion-binding site and accessibility [11–14, 18–20]. Arg or His at position 47 were for some time considered typical for this site [20], but characterized α-chains and sorbitol dehydrogenase lack this basic residue, although they have a second basic site at position 369 [9]. Also, a different labelling pattern has been reported for the human ββ isozymes (both the β_1 and β_2 allelic variants), where Cys-174 was found to be specifically labelled [17].

The reactivity of Cys residues in further isozymes is unknown. We therefore studied the carboxymethylation and compared the properties of the three common isozymes of two species, human $\beta_1\beta_1$ and $\gamma_1\gamma_1$, and equine EE. The differences in inactivation, labelling patterns, and effect of buffers are large. While several observations can be related to the crystallographic structure of the carboxymethylated horse enzyme others cannot. The results complement previous conclusions of reactivity of metal-liganding cysteine residues [8] and show that minor structural differences in closely related isozymes may have large effects on the labelling pattern and sensitivity to inactivation.

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2. MATERIALS AND METHODS

2.1. *Enzymes*

Horse liver alcohol dehydrogenase (isozyme EE) was obtained from Boehringer-Mannheim, whereas the human isozymes $\beta_1\beta_1$ and $\gamma_1\gamma_1$ were purified from liver [23]. The activity of the enzymes (EE 6.6 U/mg; $\beta_1\beta_1$ 0.19 U/mg; $\gamma_1\gamma_1$ 0.76 U/mg) closely resembled those reported [23,24].

2.2. *Labelling reactions*

Labelling with iodoacetate was performed in two buffers, 5 mM imidazole/HCl, pH 7.8 [19], and 0.1 M sodium phosphate, pH 7.5 [17], both at 4°C. The iodoacetate solution was extracted with chloroform before use to remove traces of free iodine. The specific activity of the reagent was 480–2400 cpm/nmol. Iodoacetate was added either directly to the enzyme solution [17,18], or dialyzed in [19]. Enzyme activity was monitored at 24°C by the absorbance at 340 nm in 0.1 M Gly/NaOH, pH 10.0, 33 mM ethanol, 2.5 mM NAD^+. When the residual enzymatic activity was about 20% (50% for $\gamma_1\gamma_1$, because of the slow inactivation), the carboxymethylation was stopped by addition of mercaptoethanol. The samples were dialyzed extensively to remove reagents (15 times against 500 ml),

first against the labelling buffer with 0.1% mercaptoethanol, then against the labelling buffer only, and finally against 0.1 M Tris-HCl, pH 8.0, all at 4°C. The enzymes were then homogeneously carboxymethylated with [^{12}C]iodoacetate in 8 M urea/0.2 M Tris-HCl/2 mM EDTA, pH 8.1, after reduction with dithiothreitol. After extensive dialysis against distilled water, the enzymes were lyophilized.

2.3. *Extent of Cys modification*

The labelled isozymes were dissolved in 1 M ammonium bicarbonate, diluted to 0.1 M, and digested with trypsin or Staphylococcal Glu-specific protease (enzyme:substrate ratios 1:100, by weight) for 6 h at 37°C. Peptides produced were fractionated on Sephadex G-50 in 30% acetic acid (0.25 ml/min) and purified further by reverse phase HPLC (Ultropac C-18 in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile; 1 ml/min). Radioactive peptides were quantitated by amino acid compositions (hydrolysis with 6 M HCl/0.5% phenol for 24 h at 110°C; analysis on a Beckman 121M amino acid analyzer) and ^{14}C-labels. Peptides containing more than 1 Cys were subjected to sequencer degradation (Beckman 890D or Applied Biosystems 470A sequencers) and the relative radioactivities of the Cys(Cm) positions were determined.

3. RESULTS

3.1. *Effect of buffer*

Imidazole was found to inhibit the ethanol oxidizing capacity of all three isozymes to roughly the same extent, about 40% of the values in phosphate under the conditions tested. For EE and $\beta_1\beta_1$ but not $\gamma_1\gamma_1$, imidazole influenced the inhibition by iodoacetate, markedly enhancing it with the EE isozyme, and markedly decreasing it with $\beta_1\beta_1$ (Table I). Although the active site zinc atom to which imidazole binds [18,19] is conserved and the structural relationships at the active sites are very similar in all three isozymes [3,18], EE and $\beta_1\beta_1$ differ in sensitivity to iodoacetate in imidazole, but in phosphate they are equally sensitive. The $\gamma_1\gamma_1$ isozyme is the one least sensitive in phosphate, and imidazole hardly affects it. With the exception of the initial phases, all inactivations follow a regular pattern. However, a distinction of the reaction kinetics cannot be made from the present data. Hence, values are given as inactivation times (Table I) establishing the great differences. In imidazole an initial activation of the EE and $\gamma_1\gamma_1$ isozymes is noticed, but at the times measured no such activation is noticed of the $\beta_1\beta_1$ isozyme.

3.2. *Labelling of the cysteine residues*

All three isozymes inactivated with ^{14}C-labelled iodoacetate and subsequently homogeneously carboxymethylated with [^{12}C]iodoacetate, were digested with trypsin and Glu-specific Staphylococcal protease in separate batches. The resultant peptides were purified and their ^{14}C incorporation determined. For peptides with more than one Cys, the residues were distinguished by sequence degradations and determination of radioactivity released in each cycle. The extent of labelling (Fig. 1) differs both among the isozymes in the same buffer and for one isozyme in the two types of buffer. Details should not be overinterpreted, but the patterns clearly divide the Cys residues into three groups. For the EE isozyme, the group distinction and some of the residues have been noticed before [18]. They are now shown to apply to all three isozymes. One group encompasses the highly reactive Cys residues (Cys-46 or Cys-174) binding the active site zinc atom, another the Cys residues of intermediate reactivity (Cys-97, -100, -103, -111, and -132) binding the second zinc atom (plus Cys-132, not involved in zinc binding), and the third the remaining Cys residues (7–8 positions, non-liganding Cys residues only), which are essentially unreactive. The large group of unreactive Cys residues is noticeable in all three isozymes.

The two zinc-binding Cys residues at the active site are selectively labelled not only in EE and $\beta_1\beta_1$ [17,18] but also in $\gamma_1\gamma_1$ (Fig. 1). Among the isozymes, the reactivities of these two residues differ, shifting between Cys-46 and Cys-174, $\gamma_1\gamma_1$ being of the EE type (Fig. 1). Both residues are not highly susceptible to labelling at the same time, in accord with their juxtaposition at the active site [2,18,19], and the specificity for one is pronounced with $\gamma_1\gamma_1$ in phosphate (Fig. 1). The intermediately reactive Cys residues which bind the second zinc atom are known to be sensitive to partial labelling in EE [18] and to oxidation and disulfide bridge formations in the rat enzyme [25]. Consequently, their modification may reflect a background of fairly sensitive residues. The reactivity of Cys-132 is in agreement with the binding of heavy atoms in crystals of EE and correlates with its position at the surface of the dimer [2,18].

TABLE I

Inactivation of three alcohol dehydrogenase isozymes with iodoacetate

Isozyme concentration (mg/ml)	Inactivation time (min)					
	EE		$\beta_1\beta_1$		$\gamma_1\gamma_1$	
	Phosphate	Imidazole	Phosphate	Imidazole	Phosphate	Imidazole
2–3	350	<1	300	1300		
0.5	560	125				
0.05	1750	160			2800	>2800

Time required for inactivation to 20% remaining activity (EE and $\beta_1\beta_1$; 50% for $\gamma_1\gamma_1$) after addition of reagent in a 1000-fold molar excess over the amount of enzyme subunit in 0.1 M sodium phosphate buffer, pH 7.8, or 5 mM imidazole buffer, pH 7.5.

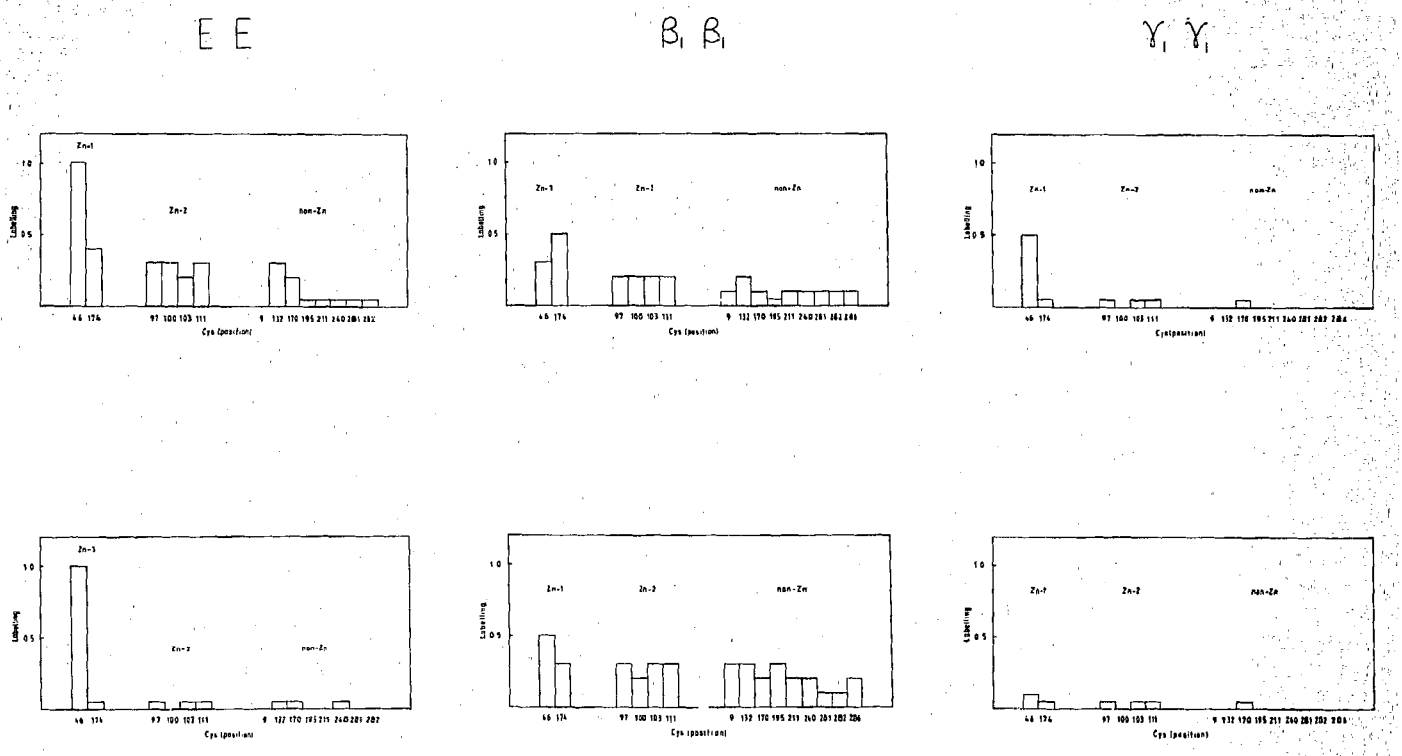

Fig. 1. Sensitivity to carboxymethylation for each of the cysteine residues in the isozymes. The columns show the labelling (nmol [^{14}C]iodoacetate/nmol Cys) of the cysteine residues liganded to the active site Zn (Zn-1), to the non-active site Zn (Zn-2), and of those not liganded to Zn (non-Zn) after inactivation in phosphate or imidazole buffer (top and bottom row, respectively). Non-recovered cysteine residues are shown without a column for the labelling, which, however, is <0.1 judging from the total incorporation.

4. DISCUSSION

The isozymes differ markedly in reactivity to iodoacetate and response to changes in the buffer ions. This is apparent both from the extent of carboxymethylation of cysteine residues and the accompanying inhibition of enzyme activity. Some of the $\beta_1\beta_1$ labelling differences from EE are known before [17] but are now studied with buffer changes, Cys identifications throughout, and inclusion of $\gamma_1\gamma_1$ (Fig. 1), establishing different sensitivities (Table I). The human $\gamma_1\gamma_1$ isozyme is quite insensitive to the effect of iodoacetate, especially in imidazole buffer when no Cys of $\gamma_1\gamma_1$ is labelled significantly above the background (Fig. 1). In contrast, the equine EE and human $\beta_1\beta_1$ isozymes are sensitive to inactivation (Table I), and the presence of imidazole influences both the inhibition and the labelling pattern. In $\beta_1\beta_1$, Cys-174 being the most reactive in phosphate buffer (Fig. 1), is apparently blocked by imidazole, such that Cys-46 then is the most reactive, presumably because of bound imidazole close to Cys-174.

The labelling patterns of the cysteine sulfur atoms correlate to some extent with estimates of accessible surface areas [18]. The group of Cys residues most reactive corresponds to the most accessible residues, those labelled to an intermediate extent are largely those partially accessible, whereas the unreactive residues are largely inaccessible. However, for the four Cys residues binding the non-active site zinc, calculations show them to differ in accessibility [18], although present data suggest them to be labelled to similar extents (Fig. 1). Overall, it would appear that accessibility represents only a first approximation of the reactivity. Similarly, anion-binding sites [18,19] from basic residues at positions 47 and 369 have been thought to favor iodoacetate-binding [18,19], and the size of the residue at position 48 to affect substrate binding [3]. However, the residues at positions 47 and 369 are all identical in the three isozymes studied. Furthermore, Ser occupies position 48 both in EE and $\gamma_1\gamma_1$, the most and least sensitive forms in the presence of imidazole, respectively, showing that specific correlations with charge and space are difficult to make. The fact that in phosphate buffer Cys-46 is labelled preferentially in EE and $\gamma_1\gamma_1$, while Cys-174 has that characteristic in $\beta_1\beta_1$ may correlate with the extra methyl group at position 48. This fits with the labelling of $\beta_2\beta_2$, where Cys-174 is also labelled prior to Cys-46 in phosphate buffer [17].

Effects of different buffers were detected early [11–14,26,27] and were found critical for the replacement of the active site and structural zinc atoms [26–28]. This circumstance has permitted selective exchanges of either of these atoms with zinc isotopes or

with cadmium or cobalt. In those cases, the metal atom itself and in the present case iodoacetate are the reagents, while acetate/phosphate and imidazole/phosphate, respectively, constitute the controlling ion pair. As shown for the present isozyme triplet, interactions are complex and subtle relationships of the zinc ligands have large effects. Obviously, the reactivity of identical residues towards the same reagents and ions can vary considerably among closely related isozymes.

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