METAL-DIRECTED AFFINITY LABELLING

Inactivation and inhibition studies of two zinc alcohol dehydrogenases with twelve imidazole derivatives

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1. Introduction

The role of metals in metalloproteins is the object of much research. Metal-directed affinity labels can provide a new way of studying such metals and their near surroundings, as well as the specific inactivation of metalloenzymes. The general claims for such a reagent are the ability to ligand a metal atom, and the capacity to modify an amino acid residue. A selective modification of amino acid residues in the metal-binding region of the protein may thus be attained.

Each subunit of alcohol dehydrogenase from liver contains one structural and one catalytic zinc atom [1]. The former has four protein ligands while the latter has three to the protein and one free. For the yeast enzyme, the exact number of zinc atoms is the subject of discussion [2,3].

The monodentate chelate-binding molecule, imidazole, binds as a free ligand to zinc in the active site of the liver enzyme, and can promote activation or inhibition of the enzyme [4,5]. Imidazole inhibits the yeast enzyme competitively with ethanol (unpublished results). It has been shown that (*R,S*)-2-bromo-3-(5-imidazolyl) propionic acid (BIP), prior to selective and irreversible alkylation of cysteine-46, binds reversibly at the active site zinc atom of liver alcohol dehydrogenase through the imidazole ring [6,7].

The aim of this work has been to survey how a series of metal-directed affinity labels react with two metalloenzymes. Twelve imidazole derivatives are surveyed for inhibition and inactivation of two zinc enzymes, the alcohol dehydrogenases from liver and yeast. Position 5 of the imidazole ring has a side chain, containing a chlorine or bromine atom, which promotes alkylation of cysteine residues. Some side chains also contain a carboxylic acid (carboxylate anion), methylester or alcohol group. In three of the compounds the ring is 1-methylated. Two of the labels have also been available as enantiomers, and the following paper deals with the stereospecific role of configuration [8].

Five of the derivatives were found to inactivate the liver enzyme and ten to inhibit; with yeast enzyme three inactivated and eight inhibited.

2. Materials and methods

The alcohol dehydrogenases were obtained from Boehringer Mannheim GmbH. The horse liver enzyme (crystalline suspension), was assayed as in [9]. The yeast enzyme (lyophilized) was dissolved (10 mg/ml) in phosphate buffer (pH 7.0), $I = 0.1$ M, dialyzed against 4 changes of the same buffer and when assayed as in [6], the specific activity was 270 U/mg.

(−)-(*S*)-2-chloro-3-(5-imidazolyl) propionic acid (CIP), (−)-(*S*)-methyl 2-chloro-3-(5-imidazolyl) propionate (CIPME), and (−)-(*S*)-2-chloro-3-(5-imidazolyl) propanol (CIPOH) were synthesized according to [10], (−)-(*S*)-2-chloro-3-(1-methyl-5-imidazolyl) propionic acid (CMIP), (−)-(*S*)-methyl 2-chloro-3-(1-methyl-5-imidazolyl) propionate (CMIPME) and (−)-(*S*)-2-chloro-3-(1-methyl-5-

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imidazolyl) propanol (CMIPOH) according to [11], (+)-(*R*)-2-chloro-3-(5-imidazolyl) propionic acid (CIP) and (+)-(*R*)-methyl 2-chloro-3-(5-imidazolyl) propionate (CIPME) were synthesized according to [10], but starting from D-histidine instead of L-histidine. (*R,S*)-methyl 2-bromo-3-(5-imidazolyl) propionate (BIPME) was synthesized according to [12], 5-(2-bromoethyl)-imidazole (BEI) according to [13] and 5-chloromethyl-imidazole (CMI) according to [14]. (*R,S*)-2-bromo-3-(5-imidazolyl) propionic acid (BIP) was purchased from Sigma Chemical Co.

2.1. *Inactivations*

Yeast enzyme (0.2 mg/ml) was inactivated in phosphate buffer (pH 7.0) $I = 0.1$ M with 5 or 10 mM inactivator. Aliquots (50 μl) were withdrawn, diluted 20 times with ice-cold phosphate buffer and assayed immediately. Liver enzyme (0.6 mg/ml) was inactivated in the same buffer with 1 or 25 mM inactivator. Aliquots (25 μl) were withdrawn and assayed. All inactivations were performed at 23.5°C in stopped tubes, as also were the controls. Results were plotted on semi-log paper and half-times ($t_{1/2}$) estimated from the inactivation curves.

2.2. *Inhibitions*

Initial rate inhibitions were carried out in quartz cuvettes at 23.5°C. For the yeast enzyme these contained 1 mM NAD^+, 10 mM ethanol, 10 mM pyrophosphate–HCl buffer (pH 9.0) and from 1–5 mM inhibitor. Enzyme was 0.08 μg/ml. Inhibitions of the liver enzyme were performed in phosphate buffer (pH 7.8) $I = 0.1$ M, with 2.4 mM NAD^+, 0.5 mM ethanol and from 0.33–20 mM inhibitor. Enzyme was 5 μg/ml. Ethanol concentration was such that for both enzymes, it was limiting so that ethanol competitive inhibition emerged. Reaction was started by adding either enzyme to give 3.025 ml final vol. The initial rate of NADH production determined at 340 nm, was used as a measure of enzyme activity.

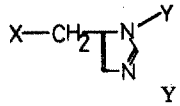
3. Results

3.1. *Liver enzyme*

BIP and BIPME rapidly inactivated the liver enzyme (table 1). The results in [6] with BIP are confirmed. The corresponding chloro-compounds (*S*)CIP and (*S*)CIPME also gave significant inactivation. Essentially no inactivation occurred with the third bromo-compound BEI. All inactivations of the liver enzyme gave linear semi-log inactivation curves, and thus pseudo first-order kinetics.

BEI followed by CMI were the most potent

Table 1
The structure of the compounds and the inactivation results at pH 7.0

Compound			Liver enzyme		Yeast enzyme	
	X	Y	[I] (mM)	$t_{1/2}$ (min)	[I] (mM)	$t_{1/2}$ (min)
(*S*)CIP	$-CHClCOOH$	$-H$	25	1350	10	∞
(*R*)CIP	$-CHClCOOH$	$-H$	25	∞	10	
(*S*)CIPME	$-CHClCOOCH_3$	$-H$	25	1650	10	∞
(*R*)CIPME	$-CHClCOOCH_3$	$-H$	25	∞	10	
(*S*)CIPOH	$-CHClCH_2OH$	$-H$	25	∞	10	∞
(*S*)CMIP	$-CHClCOOH$	$-CH_3$	25	∞	5	∞
(*S*)CMIPME	$-CHClCOOCH_3$	$-CH_3$	25	∞	10	∞
(*S*)CMIPOH	$-CHClCH_2OH$	$-CH_3$	25	∞	10	∞
CMI	$-Cl$	$-H$	25	∞	see text	
BEI	$-CH_2Br$	$-H$	25	3050	10	26
(*R,S*)BIP	$-CHBrCOOH$	$-H$	1	18	10	3750
(*R,S*)BIPME	$-CHBrCOOCH_3$	$-H$	1	40	10	710

[I] (mM) is inactivator concentrations, $t_{1/2}$ (min) the half-time of inactivation and '∞' corresponds to $t_{1/2} > 6000$ min.

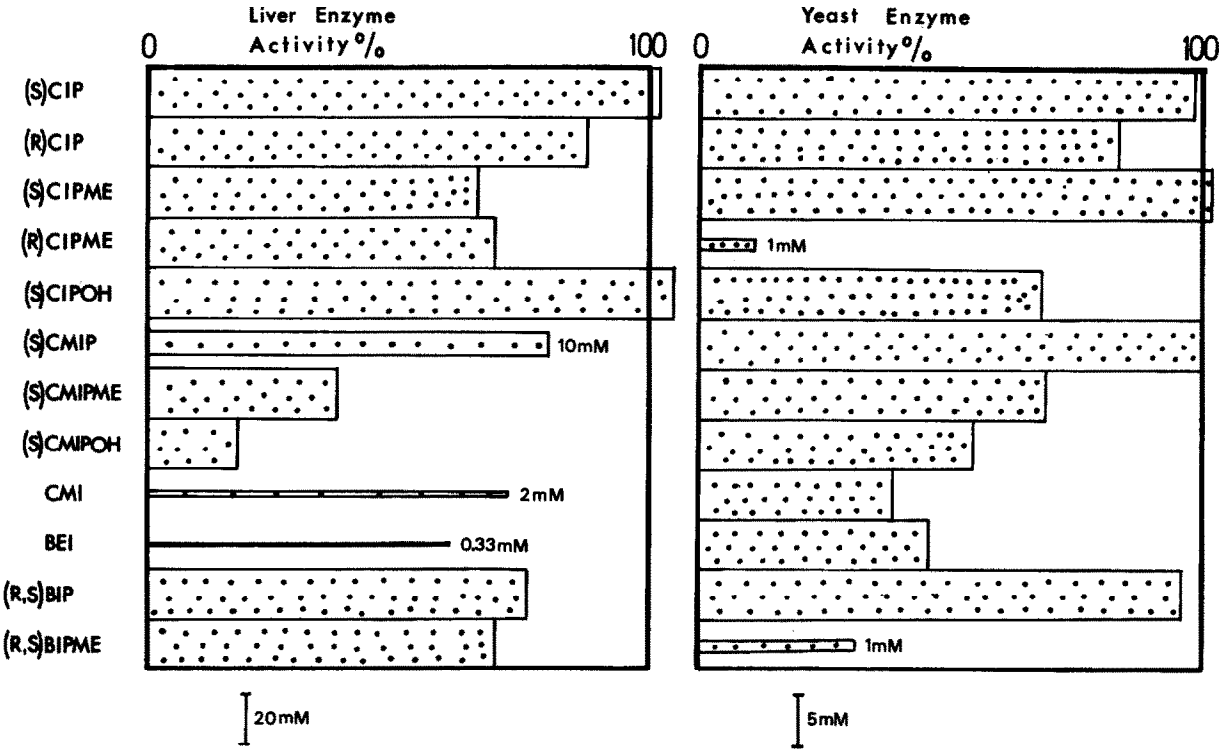


Fig.1. Inhibition with the imidazole derivatives at pH 7.8 for the liver and at pH 9.0 for the yeast enzyme. Activity with inhibitor is shown as a % of activity without. Column width is proportional to inhibitor concentration. This was normally 20 mM for the liver enzyme and 5 mM for the yeast enzyme. When different, the concentration is indicated.

inhibitors of the liver enzyme (fig.1). (*S*)CIP and (*S*)CIPOH gave no inhibition. (*S*)CMIPOH inhibited, and the two alcohols are also substrates for the liver enzyme. With 10 mM alcohol, the relative activities for ethanol, (*S*)CIPOH and (*S*)CMIPOH were 1.0, 0.01 and 0.005, respectively.

3.2. *Yeast enzyme*

Only one chloro-compound CMI, had any significant inactivation effect on the yeast enzyme (table 1). CMI inactivation was different from other inactivations studied. After ~3 min, the inactivation curve reached a plateau, which with 2.5, 5.0 and 10 mM CMI corresponded to 85%, 65% and 11% of the original activity, respectively. Of the bromo-compounds, BEI was found to be very reactive, BIPME far less reactive while BIP confirmed the results in [6] by giving essentially no inactivation. With BEI and BIPME a pseudo first-order reaction was established from linear semi-log inactivation curves.

The methyl esters BIPME and (*R*)CIPME were the most potent inhibitors of the yeast enzyme (fig.1). No significant inhibition was achieved with (*S*)CIP, (*S*)CIPME and BIP. The other compounds gave intermediate values.

4. Discussion

Metal-directed affinity labelling is based on reversible binding to the metal. The work with BIP and the liver enzyme in [6] did not fully establish whether imidazole binding to zinc or the carboxylate group to the general anion-binding site, was the more important for formation of a reversible BIP–enzyme complex. Since BIPME and (*S*)CIPME, which bear a neutral side chain, show the same mode of inactivation as BIP and (*S*)CIP, ligand binding to zinc is obviously the more important.

Comparison of BEI and BIPME inactivation of the liver enzyme shows selective discrimination of an active site. When the methylester is replaced by hydrogen, the molecule is no longer able to inactivate or modify the enzyme. On the other hand, structural change from a carboxylate to a methyl ester group, which means from a negative to a neutral side chain, does not change the ability to inactivate. These results imply that the carboxylate and the methyl ester groups force the side chain into a position where alkylation can occur.

Although the results show little correlation between inactivation and inhibition of the liver enzyme, all the inactivators except (*S*)CIP show some inhibition. That the methylesters inhibit the liver enzyme more than the acids is evident from the pairs (*S*)CIP/(*S*)CIPME, (*R*)CIP/(*R*)CIPME, (*S*)CMIP/(*S*)CMIPME and BIP/BIPME. This indicates that a neutral side chain fits better into the active site of the liver enzyme than a negatively charged one. This also explains the strong inhibition with BEI. CMI is also a potent inhibitor of the liver enzyme, but gave no inactivation.

Due to the lack of inactivation of the yeast enzyme with BIP, an ability of the active-site zinc atom to bind a free ligand has been questioned [6]. In this work none of the other acids inactivated, and none except (*R*)CIP inhibited. However, BEI and BIPME gave inactivation and these and several more compounds with a neutral side chain also inhibited the yeast enzyme. Thus, ligand binding to zinc seems to occur. This implies that a negatively charged side chain prohibits reversible binding at the active site of the yeast enzyme.

Present studies confirm differences between the active sites of the two enzymes [6]. Inhibition studies show that the two methylesters (*R*)CIPME and BIPME are the strongest inhibitors of the yeast enzyme, while CMI and BEI most strongly inhibit the liver enzyme. In inactivation experiments the three bromo-compounds reaction differently with the two enzymes. BIP and BIPME give rapid inactivation of the liver enzyme, while the yeast enzyme was rapidly inactivated by BEI and slowly by BIPME. This fits in with a more narrow substrate specificity of the yeast enzyme [1].

Of the metal-directed affinity labels used in this work, some turned out to be reactive against the liver enzyme and some against the yeast enzyme. The effect of the labels was highly dependent upon structure. Methylation of the imidazole ring in position 1 does not change the chelation capacity [15], but is considered to make the ring more bulky and hydrophobic. Inactivation has not been found with any of the 1-methylated imidazole derivatives, but they turned out to be stronger inhibitors than those unmethylated. As might be expected from the better leaving group properties of bromine compared with chloride, the bromo-compounds give much faster inactivation than the chloro-compounds. Labelling reagents with carboxylate, methylester and short aliphatic side chains are seen to inactivate, while those with an alcohol side chain failed to react.

We are aware that metal-directed affinity labels have potential against other zinc-proteins and proteins containing other metals.

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References

[1] Bränden, C.-I., Jörnvall, E., Eklund, H. and Furugren, B. (1975) in: The Enzymes (Boyer, P. D. ed) 3rd edn, vol. 11, pp. 104–190, Academic Press, London, New York.
[2] Dickinson, F. M. and Berrieman, S. (1977) Biochem. J. 167, 237–244.
[3] Sytkowski, A. J. (1977) Arch. Biochem. Biophys. 184, 505–517.
[4] Theorell, H. and McKinley-McKee, J. S. (1961) Acta Chem. Scand. 15, 1811–1833.
[5] Boiwe, T. and Bränden, C.-I. (1977) Eur. J. Biochem. 77, 173–179.
[6] Dahl, K. H. and McKinley-McKee, J. S. (1977) Eur. J. Biochem. 81, 223–235.
[7] Dahl, K. H., McKinley-McKee, J. S. and Jörnvall, H. (1976) FEBS Lett. 71, 287–290.
[8] Dahl, K. H., Mckinley-McKee, J. S., Beyerman, H. C. and Noordam, A. (1979) FEBS Lett. 99, 313–316.

[9] Reynolds, C. H. and McKinley-McKee, J. S. (1969) Eur. J. Biochem. 10, 474–478.
[10] Beyerman, H. C., Buijen van Weelderen, A. W., Maat, L. and Noordam, A. (1977) Recl. Trav. Chim. Pays-Bas 96, 191–193.
[11] Beyerman, H. C., Maat, L., Noordam, A. and van Zon, A. (1977) Recl. Trav. Chim. Pays-Bas 96, 222–224.
[12] Maat, L., Beyerman, H. C. and Noordam, A. (1979) Tetrahedron, in press.
[13] Jolley, C. J. and Yankeelov, J. A. (1972) Biochemistry 11, 164–169.
[14] Pyman, F. L. (1911) J. Chem. Soc. 99, 668–682.
[15] Stability Constants of Metal Ion Complexes (1964) pp. 144, 204, The Chemical Society, London.