STEREOSELECTIVE METAL-DIRECTED AFFINITY LABELLING

A model inactivation study with alcohol dehydrogenase from liver

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1. Introduction

Enzymes, being asymmetric, have chiral active centers, are stereospecific and discriminate between different configurations of substrates, coenzymes and products. Stereoselective alkylation with asymmetric or chiral reagents can likewise result; this has been reported with ribonuclease [1,2], papain [3], yeast alcohol dehydrogenase [4] and yeast glyceraldehyde-phosphate dehydrogenase [5]. With these enzymes inactivation showed different alkylation rates with the two enantiomers of haloacids and amides, but absolute discrimination between any of the enantiomers was not attained.

The metal-directed affinity label (*R*,*S*)-2-bromo-3-(5-imidazolyl)propionic acid (BIP), has been shown to label cysteine-46 of liver alcohol dehydrogenase in a two step reaction [6,7]. A reversible complex is first formed as an intermediate by ligand binding to the active-site zinc atom through the imidazole ring. Labelling is no doubt highly dependent upon the configuration of BIP, but thus far, this racemic label has not been resolved into its enantiomers. However, it has been possible to investigate the stereospecific role of configuration by synthesis of the pure enantiomers of the corresponding chloro-compounds; (*R*)- and (*S*)-2-chloro-3-(5-imidazolyl)propionic acid (CIP), and the methylesters; (*R*)- and (*S*)-methyl 2-chloro-3-(5-imidazolyl)propionate (CIPME).

In this work inactivation of liver alcohol dehydrogenase has been attempted with the two enantiomers of CIP and CIPME. The reaction was totally stereoselective or stereospecific. No reaction was observed with the (*R*)-enantiomers, while the (*S*)-enantiomers inactivated the enzyme in reactions where reversible complexes were formed as intermediates. Consequences result in the interpretation of the enzyme active-site chirality in solution.

2. Materials and methods

Source and assay of horse liver alcohol dehydrogenase were as in [8]. The labelling reagents (*R*)- or (*S*)-2-chloro-3-(5-imidazolyl)propionic acid (CIP) and (*R*)- or (*S*)-methyl 2-chloro-3-(5-imidazolyl)-propionate (CIPME) were synthesized from D- and L-histidine [9]. Inactivations were performed as previously, except that phosphate buffer pH 7.8, instead of pH 7.0 was used [10]. Inactivation results were treated by logarithmic regression and plotted on semi-log paper. The apparent first-order rate constant (k'), was determined from the half-time of inactivation using $k' = (\ln 2)/t_{1/2}$. This relation also gave 'the minimum inactivation half-time' using the intercept on the $1/k'$-axis of the double reciprocal plot of k' versus inactivator concentration.

3. Results

In fig.1, activity is plotted against time in a semi-log plot. It is evident that (*S*)CIP inactivates the enzyme at ≥ 0.20 mM, while 25 mM (*R*)CIP cannot be distinguished from the control. Reaction with (*S*)CIP is first order with respect to enzyme, as shown

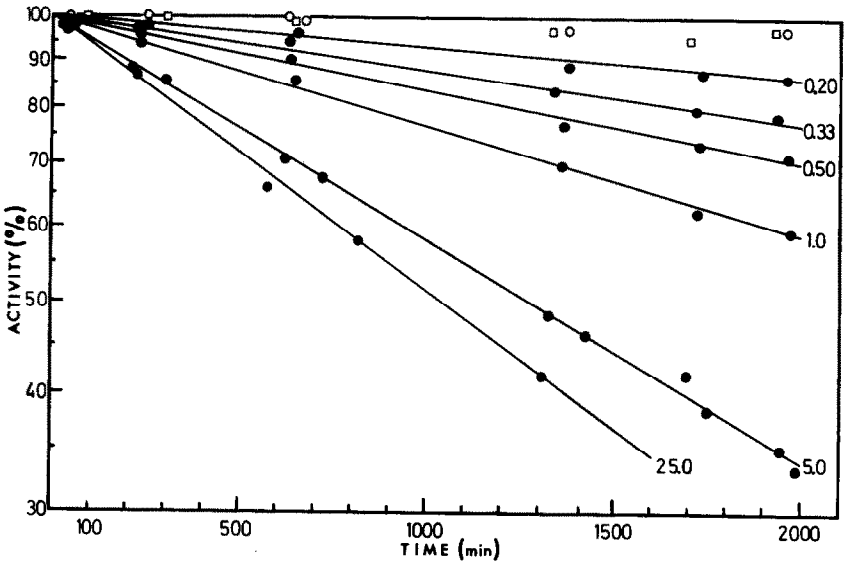

Fig.1. Semi-log plots of inactivation versus time for liver alcohol dehydrogenase. Buffer was phosphate (pH 7.8) $I = 0.1$ M, temp. 23.5°C and enzyme 0.6 mg/ml. (□) Control; (○) 25 mM (*R*)CIP; (●) (*S*)CIP (mM conc.).

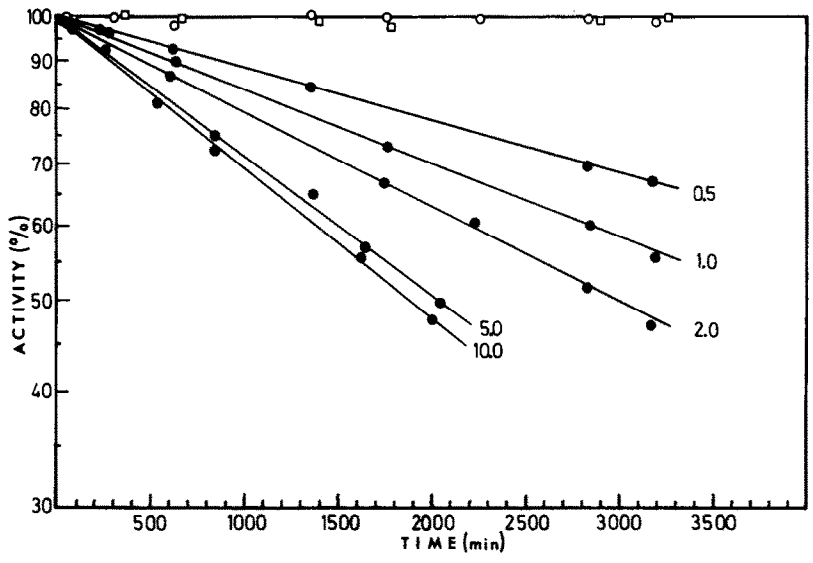

Fig.2. Semi-log plots of inactivation versus time for liver alcohol dehydrogenase. Buffer was phosphate (pH 7.8) $I = 0.1$ M, temp. 23.5°C and enzyme 0.6 mg/ml. (□) Control; (○) 25 mM (*R*)CIPME; (●) (*S*)CIPME (mM conc.).

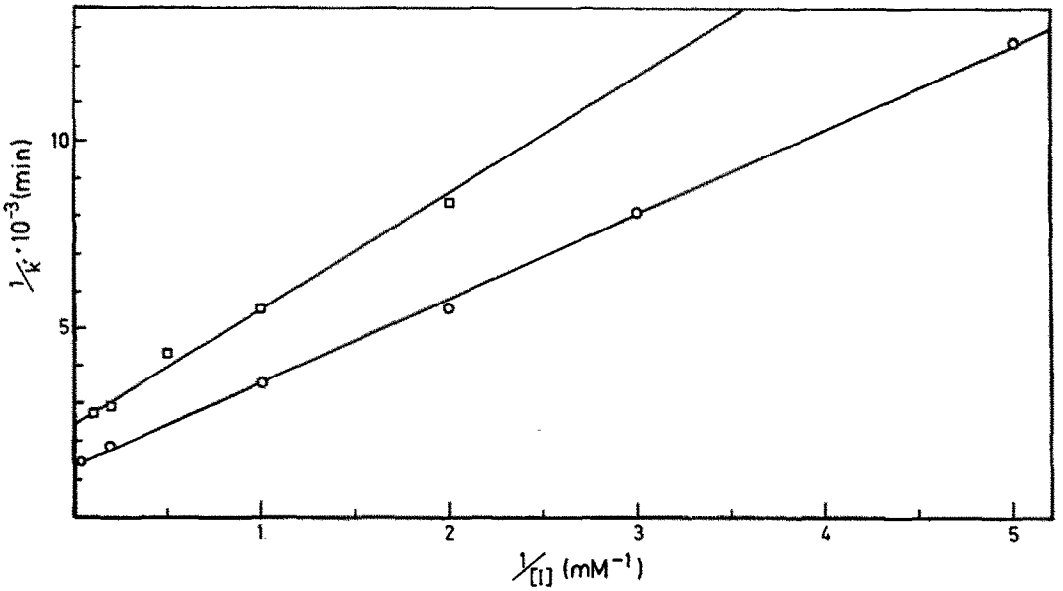

Fig.3. Double-reciprocal plots of rate constants (k') versus inactivator concentrations (I). (○) (*S*)CIP (from fig.1); (□) (*S*)CIPME (from fig.2).

by linear inactivation curves, in particular that corresponding to 5 mM. The double-reciprocal plot of rate constant versus (*S*)CIP concentration (fig.3) shows saturation kinetics and indicates formation of a reversible enzyme–(*S*)CIP complex as an intermediate [6]. The intercept on the 1/[I]-axis gives K_d 1.70 mM for the reversible enzyme–(*S*)CIP complex. The intercept on the $1/k'$-axis is 1320 min, which gives a minimum inactivation half-time of 915 min.

Figure 2 shows inactivation with (*S*)CIPME, while 25 mM(*R*)CIPME is indistinguishable from the control. The linear inactivation curves show that the reaction is first order with respect to enzyme. Saturation kinetics are observed in the double reciprocal plot (fig.3). The minimum inactivation half-time is 1700 min while the K_d for the reversible enzyme–(*S*)CIPME complex is 1.20 mM.

4. Discussion

The (*S*)-enantiomers of CIP- and CIPME-inactivated liver alcohol dehydrogenase, while no reaction was observed with the (*R*)-enantiomers. Thus, there is absolute stereoselectivity of the reaction, with the active site structure allowing alkylation with only one of the enantiomers. This seems to be the first stereoselective alkylation where discrimination between enantiomers is shown to be absolute.

When the liver enzyme was labelled with (*R,S*)-2-bromo-3-(5-imidazolyl)propionic acid (BIP), it was documented that a reversible complex was formed as an intermediate, prior to irreversible alkylation [6]. The reversible complex was formed by ligand binding to the active-site zinc through the imidazole ring. The saturation kinetics seen with variable concentrations of (*S*)CIP or (*S*)CIPME indicates a similar mechanism for both these reagents. Scheme 1 illustrates the mechanism for the reaction between liver alcohol dehydrogenase (E) and (R) or (*S*)CIP. E–(*S*)CIP′ denotes the irreversible complex. A corresponding scheme can be put up for (*R*)- and (*S*)CIPME:

$$E + (S)CIP \rightleftharpoons E\text{–}(S)CIP \rightarrow E\text{–}(S)CIP'$$

$$E + (R)CIP \rightleftharpoons E\text{–}(R)CIP$$

Scheme 1

The (*S*)-enantiomers form a reversible complex with the enzyme prior to irreversible alkylation, while the (*R*)-enantiomers form a reversible complex which does not react further. The mechanism in scheme 1 represents this absolute stereoselectivity, with discrimination in the irreversible alkylation. With the imidazole ring anchored at the zinc atom, the alkylating side chain orientates in the active-site cleft according to the interacting amino acid residues. Thus bound, specificity and stereoselectivity of alkylation depend upon side chain configuration.

From the present investigations it cannot be excluded that formation of the reversible complex is also stereoselective. This results if the dissociation constants for the E–(*S*)CIP and the E–(*R*)CIP complexes are different. However, reversible complex formation alone cannot account for absolute stereoselectivity or stereospecificity.

The reversible E–(*S*)CIPME is somewhat more stable than the E–(*S*)CIP complex, as shown by K_d 1.20 mM and 1.70 mM, respectively, which are of the same magnitude as that for the E–BIP complex, 1.1 mM, at pH 7.0 [6]. However, alkylation proceeds faster with (*S*)CIP than (*S*)CIPME, as shown by minimum inactivation half-times of 915 min and 1700 min, respectively. On the other hand, alkylation with BIP proceeds about 100-times faster, the minimum half-time being ~9 min [6], a difference caused by the better leaving group properties of bromide compared to chloride.

Conclusions about metal active-site chirality or asymmetry in solution can be drawn from stereoselective metal-directed affinity labelling of this and other enzymes.

Acknowledgements

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