CHEMICAL MODIFICATION OF LIVER ALCOHOL DEHYDROGENASE WITH α-BROMO-β-(5-IMIDAZOLYL) PROPIONIC ACID

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1. Introduction

Imidazol is known to form complexes with liver alcohol dehydrogenase, which will stimulate inactivation and inhibition of the enzyme by several reagents [1–5]. Crystallographic studies have confirmed that one imidazol is bound per subunit [5]. The zinc atom at the catalytic centre has three protein ligands, cysteine-46, cysteine-174 and histidine-67 [6]. The fourth ligand is a water molecule/hydroxyl ion, which can be replaced by imidazole [1,6].

Specific modifications of the cysteine residues at the active centre in the horse liver enzyme or the related [7] yeast enzyme, have been performed with different reagents. Among them are iodoacetate [8,9] dibromoacetone [10] and reactive coenzyme analogues [11,12]. Specificity depends on the nature of the reagent and is influenced by other factors than just binding at the active site. In this context, α-bromo-β-(5-imidazolyl) propionic acid (BIP) was considered to be of particular interest, since its imidazol group could be expected to bind specifically to the active site zinc atom. This would give a direct correlation between the primary binding site and the secondary chemical modification. BIP has been used previously to modify cysteine residues in papain [13]. The rate of inactivation of liver alcohol dehydrogenase with BIP is not proportional to the inhibitor concentration, but follows Michaelis-Menten type kinetics [14], again supporting specific binding of the reagent.

The reaction of BIP with horse liver alcohol dehydrogenase has therefore been studied in the present work, and the modified residue determined. The results show that only one residue, cysteine-46, is selectively modified in the reaction.

2. Materials and methods

Horse liver alcohol dehydrogenase was obtained from Boehringer Mannheim GmbH. The crystalline suspension was centrifuged, and the pellet dissolved in 0.1 M Na_2HPO_4, pH 9.0. The solution (10 mg/ml) was recentrifuged, and the supernatants dialyzed against six changes of phosphate buffer, pH 7.0, 0.1 μ, 4°C, and then used directly for the labelling experiments.

Radioactive D,L-α-bromo-β-(5-imidazolyl)-propionic acid ([*carboxy*-^{14}C]), was prepared [15] by diazotization of D,L-[*carboxy*-^{14}C]-histidine obtained from New England Nuclear Chemicals GmbH. The product synthesized had a specific activity of 1.9 mCi/mmol. The reagent turned out to have the same mobility in thin-layer chromatography (Butanol/acetic acid/water, 12:3:5, v/v/v) as the nonradioactive product (Pierce), and the value was exactly as that reported for BIP [15].

The labelled protein was studied after 50% and 85% inactivation, respectively. The reaction was followed by activity measurements (25 μl samples) on a Gilford Model 2000 spectrophotometer [2], and stopped by addition of 10 mg cysteine. The labelled protein was dialyzed against three changes of phosphate buffer, and one of water.

The freeze-dried protein was carboxymethylated, for 4 h at 37°C (in 6 M Guanidine–HCl, 0.1 M Tris–HCl, 5 mM EDTA, pH 8.1) with iodoacetate at a tenfold excess over the cysteine content of the enzyme.

Reagents were removed by dialysis against 10^{-3} M HCl. The protein was then digested with TLCK chymotrypsin (1% by weight) in 0.1 M ammonium bicarbonate, at 37°C for 4 h, under constant stirring. This treatment resulted in a soluble peptide mixture, which was lyophilized, and separated by exclusion chromatography on Sephadex G-50 (fine, 2.5 × 100 cm) in 0.1 M ammonium bicarbonate. Radioactive fractions were pooled, and ^{14}C-labelled peptides purified by high voltage electrophoresis and chromatography on paper [11], all steps being followed with autoradiography. Sequences were determined using the dansyl-Edman method, and amino acids with a Beckman 121 M Analyzer.

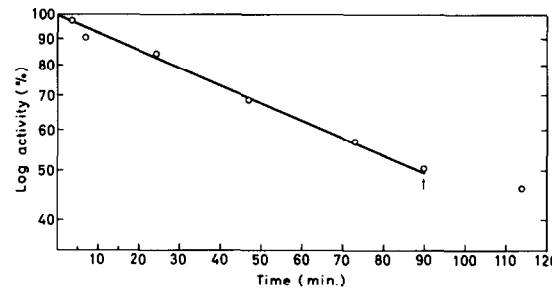

Fig.1. Inactivation of horse liver alcohol dehydrogenase with [^{14}C]BIP. The reaction was carried out in a 50 ml stopped glas flask at 23.5°C. Enzyme concentration was 30 μM and BIP 520 μM. The reaction was stopped at the arrow by the addition of 10 mg cysteine.

3. Results

3.1. *Incorporation of [^{14}C]BIP*

The irreversible inactivation of liver alcohol dehydrogenase with BIP is a pseudo first-order reaction. This is shown in fig.1. A 50% inactivation was reached after 90 min. Addition of cysteine stopped the reaction. This gave a radioactive protein with a specific activity of 2.1 mCi/mmol, corresponding to 0.55 molecules of BIP per subunit. With different enzyme and inhibitor concentrations, another preparation reached 85% inactivation after 180 min. This gave a protein with a specific activity of 3 mCi/mmol, corresponding to about one molecule of BIP per subunit.

3.2. *Isolation of radioactive peptides*

Exclusion chromatography of the chromatographic peptides gave one clear peak of radioactivity. This is shown in fig.2. Five fractions were pooled as indicated, and nearly all the radioactivity was accounted for by fractions II, III and IV, which contained 12, 28 and 56%, respectively, of the original ^{14}C-content of the protein. It is obvious that about 90% of the radioactivity is present in the peak corresponding to fractions III and IV. The radioactive peptides were

Table 1
Data for radioactive peptides from horse liver alcohol dehydrogenase labelled with [^{14}C]BIP

Total composition	B: Asp 2.0, Thr 1.7, Ser 2.1, Gly 2.2, Ala 1.2, Val 1.8[a], Ile 0.7, Leu 1.0, His 0.9, Arg 0.9 C: Asp 2.1, Thr 1.1, Ser 2.1, Gly 1.9, Val 1.1[a], Ile 0.7, Leu 1.1, His 0.9, Arg 1.0
Recovery	B: 18% C: 33%
Sequence	41 45 M 50 55 57 B: Val-Ala-Thr-Gly-Ile-Cys-Arg-Ser-Asp-Asp-His-Val-Val-Ser-Gly-Thr-Leu 44 M 50 55 57 C: Gly-Ile-Cys-Arg-Ser-Asp-Asp-His-Val-Val-Ser-Gly-Thr-Leu

[a]Recovery of Val is low due to incomplete hydrolysis of the Val–Val bond in the amino acid sequence

For total compositions, values given are molar ratios after hydrolysis in 6 M HCl, 1% phenol, at 110°C for 24 h. Recoveries are calculated from the radioactivities. For the sequence results, arrows (→) indicate residues analyzed by the dansyl-Edman method, and the positions in the primary structure of the protein are from [16]. M denotes the label from BIP.

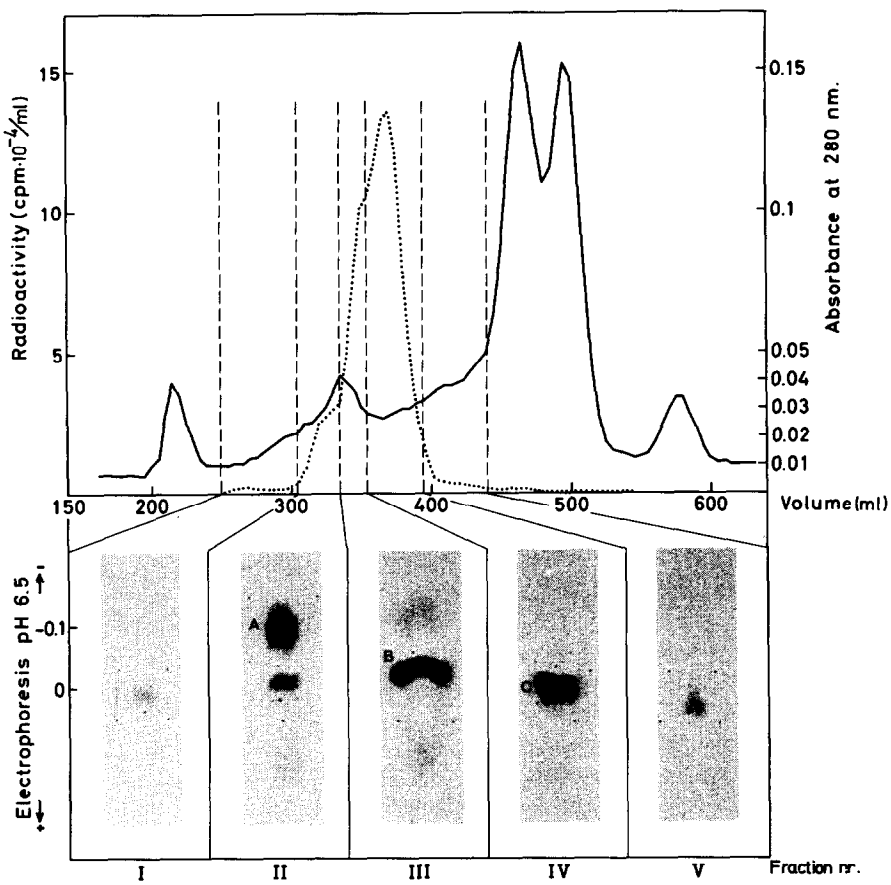

Fig.2. Exclusion chromatography of a chymotryptic digest of ^{14}C-labelled horse liver alcohol dehydrogenase (120 mg) on Sephadex G-50 fine (2.5 × 100 cm) in 0.1 M ammonium bicarbonate. Absorbance at 280 nm (———) was monitored in a Unicam spectrophotometer, and radioactivity (. . . . . .) was measured in a Packard Tri-Carb liquid scintillation counter on 0.1 ml samples from every 5 ml. Fractions were pooled as indicated and separated by high-voltage paper electrophoresis at pH 6.5. Resulting autoradiographic pictures are also shown. Electrophoretic mobility is relative to aspartate [17].

purified further on paper. In this way, three different peptides (A, B and C) were obtained. Peptide A was the main radioactive peptide of fraction II, but was recovered in small amounts. Only about 2% of the ^{14}C-content of this minor fraction was recovered, and therefore is not further considered. The other two peptides, B and C, were recovered in good yield from fractions III and IV, as shown in table 1.

3.3. *Identification of radioactive peptides*

Results of analysis for total composition and amino acid sequence of peptides B and C are given in table 1. The sequences show that the peptides are overlapping due to an incomplete sensitivity of a Thr–Gly bond to hydrolysis by chymotrypsin. The known primary structure of the protein [16] identifies the peptides as originating from positions 41–57 and 44–57, respectively. Measurements of the radioactivities of all extracts removed during the Edman degradations of peptide C showed a 15-fold increase in the ^{14}C-content corresponding to the third amino acid in the sequence. These results establish that the cysteine residue equivalent to cysteine-46 (*cf.* table 1) is modified, explaining why it is not detected in the total composition (table 1). None of the other residues in the peptide was found to be labelled. Peptide data from the enzyme preparation 85% inactivated gave identical results.

The data show that labelling of cysteine-46 is specific and accounts for over 90% of the reagent incorporated into the protein.

4. Discussion

D,L-α-bromo-β-(5-imidazolyl) propionic acid is a specific reagent for modification of liver alcohol dehydrogenase. The stoichiometry of BIP incorporation per subunit is in good agreement with the degree of inactivation obtained. Thus, on incorporation of 0.55 molecules correspond to a 50% inactivation. The reaction is of the pseudo first-order and follows Michaelis-Menten type kinetics [14]. Cysteine-46 is the only modified amino acid detected, with very little background labelling. The modified cysteine seems to be chemically quite stable, and the label is not lost by decompositions during Edman degradations. This is in agreement with other observations [15] and in contrast to the lability of other labels that have been used [11,12].

The results yield direct chemical support for the conclusions from kinetic and crystallographic studies showing that imidazole binds to the active site zinc atom [1,5]. Apparently, a reversible complex is first formed between BIP and the enzyme by imidazole-binding at the active site zinc atom, followed by an irreversible alkylation of cysteine-46. The tertiary structure of the enzyme also suggests that cysteine-46 is more exposed to hydrophilic surroundings than the other cysteine residue at the active site, cysteine-174 [5,6]. This is also in agreement with the present results, since the hydrophilic propionic acid part of BIP is probably oriented towards the hydrophilic anion binding site [5] of the active site region. Thus, labelling studies prove that BIP is a useful and specific reagent for chemical modification, and all data fit well with the three-dimensional structure of liver alcohol dehydrogenase.

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