MODIFICATION OF ALCOHOL DEHYDROGENASES WITH TWO NAD$^+$-ANALOGUES CONTAINING REACTIVE SUBSTITUENTS ON THE FUNCTIONAL SIDE OF THE MOLECULE

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1. Introduction

A coenzyme analogue with a reactive bromoacetyl group on the non-functional side of the molecule has been used previously to identify different cysteine residues at the active sites of alcohol dehydrogenases [1]. These cysteine residues are known to be ligands to the zinc atom at the catalytic centre [2]. Analogues with reactive substituents corresponding to other positions on the coenzyme molecule are potentially of interest for mapping the active sites of this and other dehydrogenases. In the present work, therefore, labelled residues in yeast and horse liver alcohol dehydrogenases have been identified after modifications with two other NAD analogues, ^{14}C-labelled [3-(3-bromoacetylpyridinio)-propyl] -adenosine pyrophosphate and [3-(4-bromoacetylpyridinio)-propyl] -adenosine pyrophosphate.

Both analogues inactivated the yeast enzyme under conditions suggesting binding at the coenzyme-site [3,4], and led to covalent incorporation of 1 molecule of analogue per protein subunit [5,6]. The horse enzyme reacted similarly with the 4-bromoacetyl-pyridinio derivative but was not inactivated by the other analogue. Labelled residues in the proteins were identified by analysis of radioactive peptides after digestion with chymotrypsin. A limited variability in the labelling with different analogues was established, showing that the active sites of both alcohol dehydrogenases contain few reactive residues, in agreement with data from crystallographic studies of the horse enzyme [2] and results of structural comparisons [7].

2. Materials and methods

^{14}C-carbonyl-labelled [3-(3-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate and ^{14}C-methylene-labelled [3-(4-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate were synthesized [5,6] with specific radioactivities of 1.18 mCi/mmol and 2.1 mCi/mmol, respectively. Alcohol dehydrogenase was prepared from yeast [8] and horse liver [9], the latter preparation containing mainly the EE isozyme. Both proteins were inhibited (5–50% of native activity, as indicated) with the coenzyme analogues, as previously described [5,6]. Reactions were stopped by additions of cysteine to a final concentration of 0.035 M. The incorporated label was stabilized by reduction with sodium borohydride [1] in the presence of a few drops of octanol to reduce foaming. The hydride was added at 0°C in small portions to a final amount of 10 mg per 100 mg protein; pH was maintained at pH 6 with 1 N HCl. Optical properties and radioactivity of modified proteins were measured as previously described [1].

Labelled proteins (110–180 mg) were carboxymethylated in 9 M urea, 0.1 mM iodoacetate at 4°C for 16 hr and reagents were removed by dialysis against distilled water. The freeze-dried protein samples were then suspended (5 mg/ml) in 1% ammonium bicarbonate, and digested at 37°C for 4 hr after each of two additions of TLCK-chymotrypsin in an amount of 1% of the total protein. This treatment resulted in soluble peptide mixtures which were freeze-dried and separated by exclusion chromatography on Sephadex

G-50 fine (Pharmacia, Uppsala, Sweden), 2.5 X 100 or 5 X 100 cm, in 1% ammonium bicarbonate. Radioactive fractions were pooled and ^{14}C-labelled peptides purified by high-voltage paper electrophoresis [1]. Structures of peptides and positions of labels were determined as previously described [1].

3. Results

3.1. *Modification of yeast alcohol dehydrogenase with [3-(3-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate*

Samples of the enzyme were treated with the analogue to almost complete inactivation (5% remaining enzymatic activity) or to about half this extent (49% activity) and chymotryptic peptides were fractionated as shown in figs. 1 and 2. Only one clear peak of radioactivity was obtained in each case. The corresponding material was pooled as indicated and shown by electrophoretic purification to correspond to only one ^{14}C-labelled peptide. It was acidic with an electrophoretic mobility [10] at pH 6.5 of 0.25. Analysis for composition and sequence showed that the peptide was Ser–Gly–Val–X–His–Thr–Asp–Leu. From the structure of yeast alcohol dehydrogenase [11] X is known to correspond to a labelled derivative of Cys-43 in the protein. It was not stable during sequence analysis and the label was successively lost in solution (cf.). In the step removing the unknown cysteine derivative, however, excess label (about twice the background loss) was recovered. All other residues were recovered unmodified in full yield. Thus Cys-43 in yeast alcohol dehydrogenase is specifically labelled. Background labelling is highly limited at incomplete (fig.2) or even full (fig.1) inactivation.

3.2. *Modification of yeast alcohol dehydrogenase with [3-(4-bromoacetylpyridinio)-propyl]-adenosine pyrophosphate*

The enzyme was labelled and analyzed in the same way as with the 3-bromoacetylpyridinio derivative. The elution pattern on exclusion chromatography of chymotryptic peptides of a partly inactivated sample (44% remaining enzymatic activity) is shown in fig.3. Completely inactivated enzyme gave still more radioactive peaks.

Fractions were pooled as indicated in fig.3, and radioactive peptides purified and analyzed. The first peak was found to correspond to labelled Cys-43 in

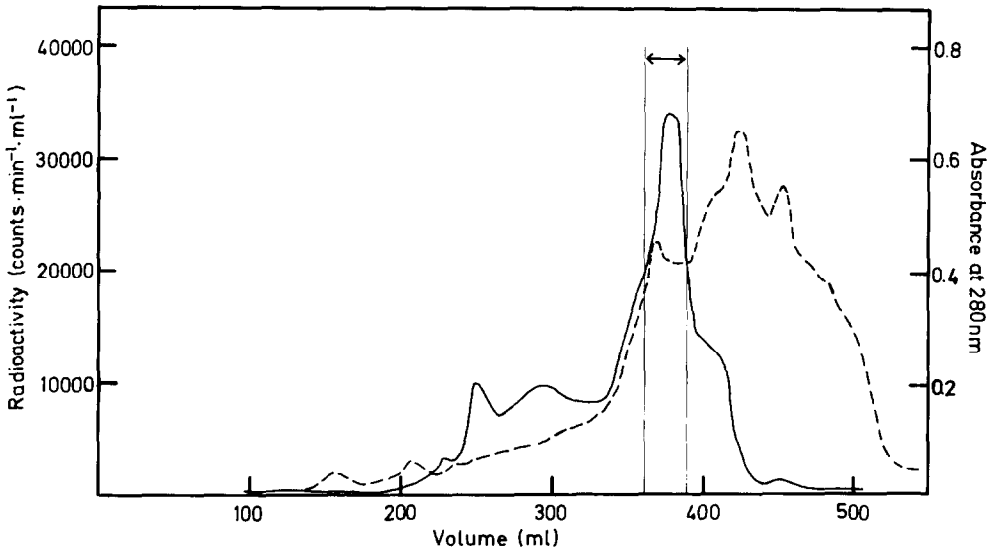

Fig.1. Exclusion chromatography of a chymotryptic digest of modified yeast alcohol dehydrogenase. Sephadex G-50 (2.5 X 100 cm) in 1% ammonium bicarbonate; 5% remaining enzymatic activity after modification with [3-(3-bromoacetyl-pyridinio)-propyl]-adenosine pyrophosphate.

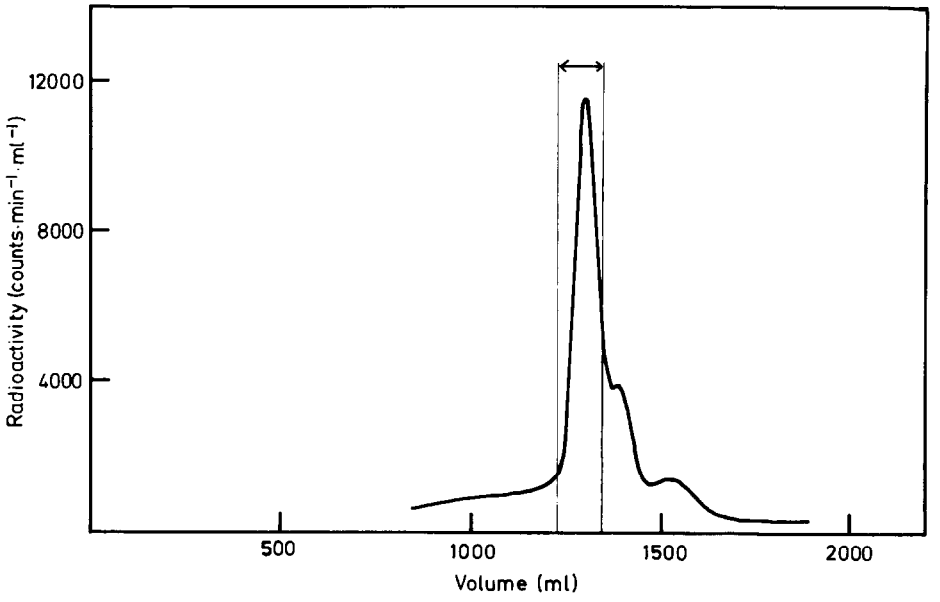

Fig.2. Exclusion chromatography of a chymotryptic digest of modified yeast alcohol dehydrogenase. Sephadex G-50 fine (5 × 100 cm) in 1% ammonium bicarbonate; 49% remaining enzymatic activity after modification with [3-(3-bromoacetyl-pyridinio)-propyl]-adenosine pyrophosphate.

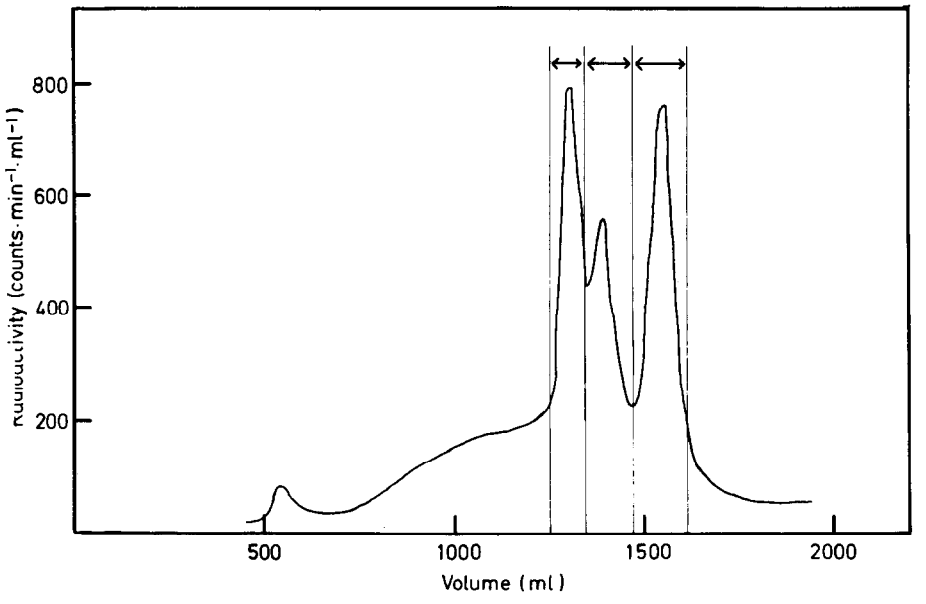

Fig.3. Exclusion chromatograpny of a chymotryptic digest of modified yeast alcohol dehydrogenase. Sephadex G-50 fine (5 × 100 cm) in 1% ammonium bicarbonate; 44% remaining enzymatic activity after modification with [3-(4-bromoacetyl-pyridinio)-propyl]-adenosine pyrophosphate.

a peptide with identical properties to that described above. The middle peak contained several weakly labelled peptides and therefore does not represent any specific modification. The last peak yielded one major radioactive compound. This was ninhydrin-negative and without detectable amino acids. It is therefore likely to represent a non-peptide or degradation product (cf. [1]) of the instable coenzyme analogue. Thus, modification is more specific than indicated by fig.3; background labelling is represented mainly by the middle peak and the only modified residue identified is Cys-43. [3-(4-Bromoacetyl-pyridinio)-propyl]-adenosine pyrophosphate therefore yields preferential label on the same cysteine residue as the 3-bromoacetylpyridinio derivative but with slightly less specificity.

3.3. *Modification of horse liver alcohol dehydrogenase with [3-(4-bromoacetyl-pyridinio)-propyl] -adenosine pyrophosphate*

Two samples of the enzyme were partly inactivated with the analogue (remaining enzymatic activities about 50%). Exclusion chromatography of chymotryptic peptides gave elution patterns with three peaks, but the relative peak heights were not reproducible. The last peak, eluted at 85% of the column's total volume, corresponded mainly to one radioactive peptide, Ile–Gly–X–Gly–Phe. From the structure of the protein [12], X is known to correspond to a labelled derivative of Cys-174. Remaining radioactivity in the eluate was divided among several weakly labelled fragments and the non-peptide material mentioned above. The weakly labelled peptides were recovered in bad yield. In those identified, label seemed to be associated with different cysteine residues.

Thus, inactivation of horse liver alcohol dehydrogenase with [3-(4-bromoacetylpyridinio)-propyl] -adenosine pyrophosphate preferentially modifies Cys-174. Background labelling is variable but more extensive than with the yeast enzyme. This is probably related to the greater thiol content of the horse protein (fourteen [12] versus eight [13] cysteine residues per subunit).

4. Discussion

4.1. *Comparisons of results with different analogues*

Modifications of horse liver and yeast alcohol dehydrogenases with reactive NAD analogues substituted on the non-functional side of the molecule [1] or on the functional, nicotinamide side (present work), show little variability. In all cases studied, Cys-43 in the yeast enzyme is labelled in a specific manner, and Cys-174 in the liver enzyme somewhat less specifically. The comparative reactivities of the analogues with the two proteins are also similar. [3-(3-Bromoacetyl-pyridinio)-propyl] -adenosine pyrophosphate does not inactivate the horse enzyme and yields little background labelling with the yeast enzyme (figs.1 and 2); nicotinamide-5-bromoacetyl-4-methyl-imidazole dinucleotide is moderately specific with both enzymes [1]; and [3-(4-bromoacetylpyridinio)-propyl] -adenosine pyrophosphate yields detectable background labelling of both proteins even with incomplete reaction (fig.3).

Labelled derivatives are unstable (cf. [1]). No evidence for additional specific modifications, degraded before analysis was, however, obtained as judged from the distribution (figs.1–3) and quantitative analysis [1] of total radioactivity. A suggestion of selective labelling of histidine with one of the analogues [6] is therefore not supported but oxidation and acid hydrolysis was previously used [6]. It is concluded that modified proteins should be treated mildly and preferably stabilized by reduction.

4.2. *Structural conclusions*

The two preferentially reactive cysteine residues occur in both of the homologous enzymes at similar numerical positions [1,11]. Corresponding residues in either enzyme are close together in space, as shown by chemical modifications of both proteins [1,13–16] and crystallographic studies of the horse enzyme [2]. It is concluded that all coenzyme analogues studied affect similar regions at the active sites of both proteins. Minor differences between the two enzymes are also indicated, since alternative cysteine residues are consistently labelled.

The small variation in modification with different NAD analogues demonstrates a limitation in the possibility of mapping particular residues, in spite of specific binding [1,3–6] of the analogues. Positions of substituents in analogues and proteins may not always be exactly correlated because of lack of sensitive residues correctly positioned for direct substitution. The limited variability therefore gives

strong evidence that both dehydrogenases contain few reactive residues close to either end of the bound coenzyme, apart from the centre containing the two cysteine residues.

These results are in full agreement with crystallographic studies of the horse enzyme, showing that its adenine and nicotinamide sites at the coenzyme-binding region are hydrophobic [2]. The labelling of Cys-174 in the horse enzyme by a nicotinamide-substituted analogue also eliminates a possible inconsistency in structural correlations by confirming that alkylation with the adenine-substituted analogue is caused by molecules anomalously bound [1]. This is compatible with the strictly hydrophobic nature of the adenine binding site of horse liver alcohol dehydrogenase (cf. [2]). Furthermore, the labelling experiments show that the properties of the coenzyme binding region also apply to the yeast enzyme, confirming the similarities suggested from structural comparisons of particular residues in the two proteins [7].

Acknowledgements

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