Residual Activity in Carboxymethylated Liver Alcohol Dehydrogenase

By C. H. REYNOLDS and J. S. MCKINLEY-McKEE

Department of Biochemistry, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

(Received 24 July 1970)

Alcohol dehydrogenase (EC 1.1.1.1) from horse liver contains two identical subunits (Jörnvall & Harris, 1970); each subunit contains one thiol group that is reactive towards iodoacetate (Li & Vallee, 1963, 1965; Harris, 1964), and alkylation is accompanied by inactivation. We report here that the carboxymethylated enzyme was found to contain 2–2.5% residual activity, and the possible significance of this is discussed.

Horse liver alcohol dehydrogenase was purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Details of the enzyme, assay and reagents have been described previously (Reynolds & McKinley-McKee, 1969; Reynolds, Morris & McKinley-McKee, 1970). Carboxymethylation of 1 ml samples of enzyme in 40 mM-sodium phosphate buffer, pH 7.4, was carried out in stoppered cellulose acetate tubes. Imidazole was added, because it increases the rate of reaction of the reactive thiol group with iodoacetate (Evans & Rabin, 1968; Reynolds *et al.* 1970); consequently the enzyme requires a shorter exposure to iodoacetate, and this should minimize the chance of other groups being carboxymethylated. The loss of activity with time is shown in Fig. 1.

It was thought that the residual activity might be due to a minor isoenzyme component. Native enzyme was compared with carboxymethylated enzyme by starch-gel electrophoresis (McKinley-McKee & Moss, 1965) in 10 mM-sodium phosphate buffer, pH 7.4; the gel was sliced, and one half stained for alcohol dehydrogenase activity and the other for protein. Native enzyme showed one very intense band of activity, corresponding to the EE isoenzyme (Pietruszko & Theorell, 1969), with one weak basic band (probably ES) and two less basic bands (probably EE' and EE''). The carboxymethylated enzyme showed just one weak band of activity, corresponding to the main component of native enzyme. The protein staining showed one main band, of the same mobility, for both native and carboxymethylated enzyme, and this corresponded to the main activity bands (EE) described above. The more basic isoenzyme protein (in native enzyme) was also just discernible.

These results show that the residual activity is unlikely to be due to a minor isoenzyme component. The similar mobility of native and carboxymethylated enzyme is not surprising, since they have similar physical properties (Li & Vallee, 1965). The simplest explanation is that it is a genuine catalytic activity of the carboxymethylated enzyme. However, it is also possible that a small fraction of the enzyme had been carboxymethylated at some other amino acid residue, and that this does not abolish activity but does render the protein resistant to alkylation at its essential thiol group. In the presence of AMP, which protects the enzyme from iodoacetate, the residual activity in this case should be higher, if AMP does not protect this other postulated residue. Since AMP appears not to alter the amount of residual activity (Fig. 1), then this other group would also be protected from carboxymethylation by AMP. However, this result can be considered to strengthen the probability that the residual activity is a genuine property of the carboxymethylated enzyme.

Some creatine kinases, which have a very reactive thiol group, also show considerable residual activity (Kumudavalli, Moreland & Watts, 1970). In lactate dehydrogenase, which contains an essential thiol group in a similar sequence to that in alcohol dehydrogenase (Holbrook *et al.* 1967), the thiol group is 13 Å from the nearest part of the coenzyme on the enzyme surface (Rossmann, 1970). Li & Vallee (1965) showed that carboxymethylated alcohol dehydrogenase has an identical extrinsic Cotton effect with NADH at 327 nm, except that the amplitude and breadth are decreased: they interpreted this as showing that coenzyme is bound in an identical asymmetric environment, but is less firmly attached to the enzyme. The finding of residual activity would also support the view that the coenzyme site is not very close to the carboxymethylated residue.

The actual function of the reactive thiol group in the enzyme is still obscure. Carboxymethylation does not prevent the binding of NADH (Li & Vallee, 1965), and it appears now that it is not absolutely essential for catalysis. It may be

Fig. 1. Inactivation of liver alcohol dehydrogenase. Enzyme ($13\mu\text{M}$), iodoacetate (6mM) and imidazole (base form, 3.6mM) were incubated in 40mM -sodium phosphate buffer, pH 7.4, at 23.5°C . ○, Without AMP; △, with AMP ($170\mu\text{M}$); ▽, with AMP ($340\mu\text{M}$).

concerned with a subtle stabilization of conformation, or of conformation changes during the reaction cycle. Carboxymethylation does not produce gross changes in conformation (Li & Vallee, 1965). However, we have observed a slow loss of some of the remaining thiol groups (after removal of iodoacetate with Sephadex G-25), which does not occur with native enzyme. [The method of Ellman (1959) was used, in 8M -urea.] It seems that carboxymethylation may make the enzyme more flexible, permitting thiol oxidation, presumably to cystine.

Native liver alcohol dehydrogenase follows the Theorell-Chance mechanism (Theorell & Chance, 1951), in which at maximal velocity the rate-limiting step is the breakdown of the complex between coenzyme-product and enzyme. Therefore (for ethanol oxidation) a 'burst', i.e. a rapid production of NADH (stoichiometric with the enzyme active sites present, and bound to enzyme)

would be produced. Ethanol (8mM) was added to NAD^+ (0.46mM) and carboxymethylated enzyme ($0.7\mu\text{M}$) in 62mM -glycine- NaOH buffer, pH 10, at 23.5°C . The fluorescence at 460nm (excitation at 340nm) was observed in a spectrophotofluorimeter (Farrand Optical Co. Inc., New York, N.Y., U.S.A.). No sudden production of NADH was observed, but only the expected steady increase due to residual activity. Therefore, if the residual activity is due to the bulk of the modified enzyme (and not to just a small fraction of it), it does not obey Theorell-Chance kinetics. This could perhaps be a useful method of examining the actual catalytic part of the reaction: with native enzyme, a very small proportion of the enzyme is actually catalysing the interconversion of ternary complexes at any one instant. If the carboxymethylated enzyme is a valid model system, then it may be that the proportion is much higher.

We thank the Medical Research Council for grants supporting this work.

- Ellman, G. L. (1959). *Archs Biochem. Biophys.* **82**, 70.
 Evans, N. & Rabin, B. R. (1968). *Eur. J. Biochem.* **4**, 548.
 Harris, I. (1964). *Nature, Lond.*, **203**, 30.
 Holbrook, J. J., Pfeleiderer, G., Mella, K., Volz, M., Leskowac, E. & Jeckel, R. (1967). *Eur. J. Biochem.* **1**, 746.
 Jörnvall, H. & Harris, J. I. (1970). *Eur. J. Biochem.* **13**, 565.
 Kumudavalli, I., Moreland, B. H. & Watts, D. C. (1970). *Biochem. J.* **117**, 513.
 Li, T.-K. & Vallee, B. L. (1963). *Biochem. biophys. Res. Commun.* **12**, 44.
 Li, T.-K. & Vallee, B. L. (1965). *Biochemistry, Easton*, **4**, 1195.
 McKinley-McKee, J. S. & Moss, D. W. (1965). *Biochem. J.* **96**, 583.
 Pietruszko, R. & Theorell, H. (1969). *Archs Biochem. Biophys.* **131**, 288.
 Reynolds, C. H. & McKinley-McKee, J. S. (1969). *Eur. J. Biochem.* **10**, 474.
 Reynolds, C. H., Morris, D. L. & McKinley-McKee, J. S. (1970). *Eur. J. Biochem.* **14**, 14.
 Rossmann, M. G. (1970). In *Pyridine-Nucleotide-Dependent Dehydrogenases*, Ed. by Sund, H. Heidelberg: Springer-Verlag.
 Theorell, H. & Chance, B. (1951). *Acta chem. scand.* **5**, 1127.