RECONSTITUTION OF THE ELECTROPHORETIC COMPLEXITY OF MOUSE LACTATE DEHYDROGENASE

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Summary:

The residues which are covalently joined by disulphide bonds to mouse lactate dehydrogenase, and which affect the electrophoretic behaviour of the enzyme, have been isolated and identified as cysteine and glutathione. Oxidation of partially purified and previously reduced mouse muscle lactate dehydrogenase, in a solution containing a mixture of cysteine and glutathione, results in an electrophoretic pattern of the enzyme apparently identical with that of the native enzyme. The possibility that the polypeptide subunits of lactate dehydrogenase may exert a selective effect in their reactions with cysteine and reduced glutathione is discussed, and the likelihood that complex electrophoretic patterns of enzymes other than mouse lactate dehydrogenase may be caused by covalently bound simple metabolites is noted.

Drastic simplification of the electrophoretic band patterns of mouse LDH (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27) can be effected by treating the enzyme with thiols prior to electrophoresis, as shown by Dudman and Zerner (1969). They concluded that the mouse isoenzymes, as extracted from the tissues, bind varying numbers of anionic residues by disulphide bonds. In the present communication the bound anions are shown to be glutathione and cysteine.

This result appears of more than trivial importance owing to its wide implications. Thus low molecular weight metabolites, covalently bound to an enzyme, can change its physical properties without markedly affecting its catalytic activity. Further, Riley and Snell (1968) have demonstrated that such a metabolite (pyruvate), covalently bound to histidine decarboxylase, is essential for enzymatic activity, where it presumably acts as a "primitive" coenzyme. D-Proline reductase also appears to require covalently bound pyruvate for activity (Hodgins and Abeles, 1967). Consequently it seems reasonable to anticipate that a variety of such metabolites may be found covalently bound to a number of proteins, affecting their physical properties, or biological activity, or both. For example, in a great many of the reported systems of isoenzymes, electrophoretic

complexity has been ascribed to the existence of conformational isomers of the enzyme, or to genetic variations in its structure (Markert and Whitt, 1968). This complexity in some instances may be caused by covalently bound small ions, a possibility which at this stage has not been adequately investigated.

The preparation of mouse tissue extracts, the electrophoresis of LDH preparations on polyacrylamide gels, and the staining of the gels for enzymatic activity have already been described (Dudman and Zerner, 1969). Unless otherwise specified, all manipulations of mouse LDH were carried out in 10 mM Tris-20 mM glycine buffer (pH 8.8). Treatment of the enzyme with thiols generally increased the specific activity slightly, to a maximum of 7%. Paper chromatograms on Whatman No. 1 paper were eluted with one of three solvent systems, n-butanol-acetic acid-water (40:6:15, by vol.), n-butanol-pyridine-acetic acid-water (15:10:3:12, by vol.), and ethanol-water (70:30, by vol.). Compounds with sulphydryl or disulphide groups were located by staining the dried chromatograms with an alkaline nitroprusside solution containing potassium cyanide (Greenstein and Winitz, 1961); and chromatograms were stained with ninhydrin to indicate spots of amino acids and peptides (Miller and Rockland, 1952).

Mouse liver extract, treated with 0.098 M dithiothreitol, was fractionated on a column of Sephadex G-100 at 25°. LDH activity in the effluent was pooled and aliquots (0.1 ml) were added to samples (0.25 ml) of effluent collected after the enzyme. Following intermittent oxygenation of the mixtures at 25° for four hours, electrophoresis showed that a later fraction of effluent was capable of reversing the effects of thiols on mouse LDH. However, reoxidation of the pooled enzyme in the presence of dithiothreitol, alone, did not reverse the electrophoretic pattern. This result suggests that the enzyme, after reduction with thiols, can be separated from small molecules which affect its electrophoretic behaviour.

In the absence of added thiols, mouse LDH was purified 15-fold from a muscle extract, by ammonium sulphate fractionation and gel filtration on Sephadex G-200. The enzyme appeared to be about one-sixth pure, comparing its specific activity towards pyruvate and NADH at 25° with the activity reported for highly purified LDH from rat liver (Anderson et al., 1964), and other sources (Pesce et al., 1964). The mouse enzyme was dialysed against several changes of distilled

water, then extracted by dialysis with four changes (each of 3.5 volumes) of 20 mM sodium 2-mercaptoethanolate buffer (pH 8.98). The extract and an unused sample of the 2-mercaptoenthanolate buffer as a control were acidified to pH 7.4 with Dowex 50W-X4 ion exchange resin, lyophilized, and taken up in water. The dissolved residues were acidified to about pH 5.9 with 0.5 M acetic acid, relyophilized, and redissolved in a small volume of water. Paper chromatography revealed the presence of two components containing thiol or disulphide groups in the extract, in addition to the disulphide of 2-mercaptoethanol which appeared in the chromatographs of both extract and control. Table I shows that the two components were identified as cysteine and GSH.

A sample of material with the same R_f as authentic GSH was isolated from the extract by preparative paper chromatography, and after oxidation with a low concentration of hydrogen peroxide (Krimsky and Racker, 1952) proved to be a substrate for glutathione reductase (Racker, 1955). Since this enzyme shows specificity for its disulphide substrate (Knox, 1960), this result confirms the presence of glutathione in the extract.

An experiment was performed to see if bound cysteine and glutathione both contributed to the electrophoretic pattern of native mouse muscle LDH. Purified enzyme, which had already been extracted with 2-mercaptoethanol, was dialysed against several changes of Tris-glycine

TABLE I

Identification of Components in the 2-Mercaptoethanol Extract of Purified Mouse Muscle LDH

Sample Chromatographed	Paper chromatographic R_f values			Colour with ninhydrin
	n-Butanol-acetic acid-water	n-Butanol-pyridine-acetic acid-water	Ethanol-water	
Component 1 from extract:	0.19	0.24	0.32	Blue-purple
GSH:	0.19	0.20	0.31	Blue-purple
Component 2 from extract:	0.26	0.40		Brown
Cysteine:	0.24	0.34		Brown

buffer, after which aliquots of the enzyme were treated with 2 mM GSH, 2 mM cysteine, or a mixture of 1 mM GSH and 1 mM cysteine. Following intermittent oxygenation of these mixtures for 70 min at 25°, electrophoresis revealed that the pattern of the enzyme which had been treated with the mixture of cysteine and GSH appeared identical with that of the native enzyme, while the patterns of the enzyme treated with cysteine or GSH separately were substantially different (Fig. 1). What Fig. 1 does not show is that no fewer than 18 bands in the native enzyme pattern also appear in the pattern of the reduced enzyme which has been reoxidised in the presence of both cysteine and GSH.

This observation corroborates the previous evidence implicating covalently bound glutathione and cysteine as the causes of the electrophoretic complexity of mouse LDH. Dudman and Zerner (1968) have reported that oxidation of a mouse liver extract in the presence of GSH alone, following treatment of the extract with 2-mercaptoethanol and then dialysis, reconstituted the original electrophoretic pattern of the liver LDH exactly. The apparent disagreement between this result and the observations of the present communication is clearly resolved if the ratio of concentrations of available GSH/available cysteine is much higher in liver extract than in muscle extract, as seems to be the case for the cat (Tallan et al., 1954).

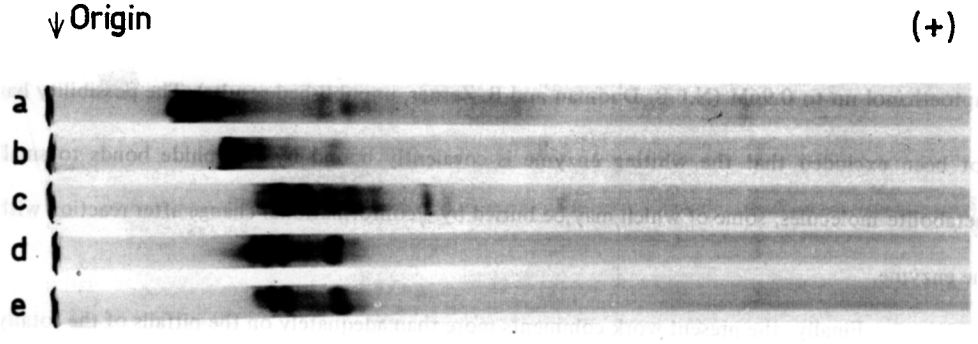

Figure 1. Polyacrylamide gel electrophoresis patterns of purified mouse muscle LDH. a, after reduction of the enzyme with 2-mercaptoethanol; b-d, after reduction, and subsequent reoxidation in the presence of (b) cysteine, or (c) GSH, or (d) both (with concentrations as specified in the text); e, the enzyme before reduction.

Results obtained by Tallan et al. (1954) and by Wieland et al. (1955) suggest that the ratio of GSH/cysteine in mouse muscle extract is likely to be considerably higher than unity. However, the electrophoretic patterns of native mouse muscle LDH and of the enzyme reoxidised in the presence of the equimolar mixture of GSH and cysteine appear to be identical (Fig. 1); this could have one of the following explanations. Firstly, the technique of Tallan et al. (1954) may measure not only cysteine and GSH free to react with the LDH in the extract, but also the concentration of these compounds already bound to other cell components. Secondly, the enzyme subunits themselves, may show selectivity in their reactions with cysteine and GSH. The effects of this selectivity may be most marked with the sulphydryl compounds at relative concentrations similar to those found in the muscle extract. The constancy of migration of the sole band of LDH isoenzyme 1, and the increase in the number of sub-bands from isoenzyme 2 to isoenzyme 5, indicate that the selectivity may be confined to subunits of the "muscle" type in this instance, and suggest that isoenzyme 5 may contain more free sulphydryl groups than isoenzyme 1.

For an understanding of the possibility that enzymes show such selectivity, three factors would appear to be important: (1) the relative concentrations of the two (or more) relevant metabolites in the tissue extract; (2) the relative specific reaction rates of the metabolites with a suitable model compound, here, for example, N-acetyl cysteinamide; (3) the alteration of a specific protein functional activity by the protein itself.

The complex electrophoretic pattern of whiting (Merluccius bilinearis) LDH (Markert and Faulhaber, 1965) is changed but not fully simplified by treatment of the enzyme with 2-mercaptoethanol up to 0.96M (N.P.B. Dudman and B. Zerner, unpublished results). The possibility has not been excluded that the whiting enzyme is covalently bound by disulphide bonds to small metabolite molecules, some of which may be buried by a conformational change after reaction with the enzyme.

Finally, the present work comments more than adequately on the pitfalls of the totally uncritical interpretation of complex electrophoretic patterns.

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REFERENCES

Anderson, S.R., Florini, J.R., and Vestling, C.S. (1964), J. Biol. Chem., 239, 2991.
Dudman, N.P.B., and Zerner, B. (1968), Proc. Australian Biochem. Soc., 8.
Dudman, N.P.B., and Zerner, B. (1969), Biochim. Biophys. Acta, 171, 195.
Greenstein, J.P., and Winitz, M. (1961), Chemistry of the Amino Acids, Wiley, New York, p.1744.
Hodgins, D., and Abeles, R.H. (1967), J. Biol. Chem., 242, 5158.
Knox, W.E. (1960), in The Enzymes (Boyer, P.D., Lardy, H., and Myrbäck, K., Editors), Vol. 2, Academic Press, New York, p.263.
Krimsky, I., and Racker, E. (1952), J. Biol. Chem., 198, 721.
Markert, C.L., and Faulhaber, I. (1965), J. Exptl. Zool., 159, 319.
Markert, C.L., and Whitt, G.S. (1968), Experientia, 24, 977.
Miller, J.M., and Rockland, L.B. (1952), Arch. Biochem. Biophys., 40, 416.
Pesce, A., McKay, R.H., Stolzenbach, F., Cahn, R.D., and Kaplan, N.O. (1964), J. Biol. Chem., 239, 1753.
Racker, E. (1955), in Methods in Enzymology (Colowick, S.W., and Kaplan, N.O., Editors), Vol. II, Academic Press, New York, p.722.
Riley, W.D., and Snell, E.E. (1968), Biochemistry, 7, 3520.
Tallan, H.H., Moore, S., and Stein, W.H. (1954), J. Biol. Chem., 211, 927.
Wieland, T., Dose, K., and Pfleiderer, G. (1955), Biochem. Z., 326, 442.