Turkey Liver Xanthine Dehydrogenase

RELATION BETWEEN NICOTINAMIDE-ADENINE DINUCLEOTIDE OXIDOREDUCTASE ACTIVITY AND THE CONTENT OF FUNCTIONAL ENZYME

By ÍDE NÍ FHAOLÁIN and MICHAEL P. COUGHLAN

Department of Biochemistry, University College, Galway, Ireland

(Received 13 April 1976)

The turkey liver xanthine dehydrogenase-catalysed oxidation of NADH by Methylene Blue, by ferricyanide or by O_2 is not dependent on the integrity of the active-centre persulphide groups. By contrast, the NADH-dichlorophenol-indophenol oxidoreductase and NADH-trinitrobenzenesulphonate oxidoreductase activities are directly proportional to the content of functional enzyme. These findings help to identify the sites of egress of electrons to oxidizing substrates.

Xanthine oxidase (EC 1.2.3.2), xanthine dehydrogenase (EC 1.2.1.37) and aldehyde oxidase (EC 1.2.3.1) contain molybdenum, flavin and iron-sulphur prosthetic groups (see, e.g., Bray, 1975). In addition, at the active centres of the fully functional enzymes, there is an essential sulphur atom, perhaps part of a persulphide group (Massey & Edmonson, 1970; Cleere & Coughlan, 1974; Nishino, 1974; Branzoli & Massey, 1974b). Loss of this sulphur atom, during isolation and storage or as result of CN^- treatment, gives rise to non-functional enzyme devoid of catalytic activity with reducing substrates such as xanthine. By contrast the NADH-ferricyanide oxidoreductase activity of milk xanthine oxidase is completely insensitive to the integrity of the active-centre persulphides (Edmonson *et al.*, 1972; Massey, 1973). Moreover, the NADH-Methylene Blue activity of this enzyme is not inhibited by CN^- treatment (Corran *et al.*, 1939). However, the chicken liver xanthine dehydrogenase-catalysed oxidation of NADH by 2,6-dichlorophenol-indophenol or by trinitrobenzenesulphonate is decreased by such treatment (Rajagopalan & Handler, 1967). Such reports prompted a closer investigation on the relation between the content of functional active sites and NADH oxidoreductase activities of turkey liver xanthine dehydrogenase.

Experimental

Materials and reagents

Fresh-frozen turkey livers were from Castlemahon Co-op, Newcastle West, Co. Limerick, Ireland, and stored at $-20^\circ C$ until required. Enzyme, having the full complement of molybdenum, flavin and iron-sulphur groups, was isolated from this source by a slight modification (Cleere, 1976) of a previously published method (Cleere & Coughlan, 1975). Samples of different degrees of functionality were prepared by mixing native enzyme and cyanide-inactivated enzyme in the appropriate proportions (Cleere *et al.*, 1974).

Enzyme assays

All reactions were carried out at $30^\circ C$ in 0.05M-potassium phosphate buffer, pH 7.8, containing 0.1mM-EDTA, in a final volume of 1.0ml. The oxidation of NADH ($50\mu M$) by the following acceptors (and the wavelengths at which the reactions were monitored) was investigated: 2,6-dichlorophenol-indophenol ($50\mu M$, 600nm); ferricyanide (2.0mM, 420nm); trinitrobenzenesulphonate ($500\mu M$, 340nm); Methylene Blue ($20\mu M$, 340nm); O_2 air-saturated buffer, 340nm. Turnover numbers were calculated by using the following molar extinction coefficient differences ($\text{litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$) between oxidized and reduced substrate: dichlorophenol-indophenol (21000; Gurtoo & Johns, 1971); ferricyanide (2080; Krenitsky *et al.*, 1972); NADH (6200). The oxidation of xanthine ($150\mu M$) by NAD^+ ($500\mu M$) was followed at 340nm. This activity is expressed as the $AFR_{340}^{30^\circ}$ value (see Avis *et al.*, 1955), i.e. the activity:flavin ratio, obtained by dividing the change in E_{340}/min by the E_{450} of the enzyme used in the assay. Fully functional enzyme has a value of 325 under these conditions (Cleere *et al.*, 1974). Assays were carried out with a Unicam SP.1800 spectrophotometer equipped with thermostatically controlled cuvette holder and continuous chart recorder.

Protein determination

Enzyme concentration was determined by the previously determined ϵ_{450} value of $36550\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ per equivalent of enzyme-bound flavin (Cleere & Coughlan, 1975). This value was also used in the calculation of turnover numbers (see above).

Results and Discussion

Fig. 1 shows the relation between the content of functional active sites and various NADH diaphorase activities of turkey liver xanthine dehydrogenase. Clearly, the oxidation of NADH by Methylene Blue, by ferricyanide or by O_2 is not affected by the degree of functionality. By contrast, NADH-dichloro-

Fig. 1. Relation between turkey liver xanthine dehydrogenase and NADH oxidoreductase activities and the content of functional active sites

Turnover numbers (mol of NADH oxidized/mol of active site per min) and the degree of enzyme functionality were determined as described in the Experimental section. The oxidation of NADH by the following acceptors is illustrated: 2,6-dichlorophenol-indophenol (○); trinitrobenzenesulphonate (△); Methylene Blue (□); ferricyanide (▲); O₂ (●).

phenol-indophenol oxidoreductase and NADH-trinitrobenzenesulphonate oxidoreductase activities are directly proportional to the content of functional enzyme. The turnover numbers for the latter reactions catalysed by fully functional enzyme may be calculated to be 1790 and 1520 mol/mol of enzyme-bound flavin per min respectively. One may also calculate that complete destruction of the persulphide groups of fully functional enzyme would result in the loss of 91% of the activity with dichlorophenol-indophenol and of 97% of the activity with trinitrobenzenesulphonate as acceptor. If the same situation obtains in the case of chicken liver xanthine dehydrogenase one may conclude that the preparations of this enzyme used by Rajagopalan & Handler (1967) contained about 20% of non-functional active sites. Although one cannot carry comparisons too far, such conclusions are consistent with a report by Nishino (1974) on the presence of non-functional enzyme in chicken liver xanthine dehydrogenase preparations.

The data in Fig. 1 may also help to resolve the controversy (see Bray, 1975) over the site(s) of electron egress to oxidizing substrates. The persulphide groups are thought to be part of the molybdenum centres (Massey, 1973; Bray, 1975). Removal of the flavin

chromophores from these enzymes results in complete loss of ability to catalyse the oxidation of NADH or to utilize O₂ as an electron acceptor. Such treatment, however, does not decrease the enzyme-catalysed oxidation of xanthine or of *N*¹-methyl-nicotinamide by dichlorophenol-indophenol, trinitrobenzenesulphonate, ferricyanide or Methylene Blue (Komai *et al.*, 1969; Kanda & Rajagopalan, 1972; Cleere *et al.*, 1974; Branzoli & Massey, 1974a).

As shown above, NADH-O₂ oxidoreductase activity is, not unexpectedly, independent of enzyme functionality (Fig. 1). That activity with Methylene Blue or with ferricyanide is similarly independent of the state of the active-centre persulphides indicates that molybdenum is not the site of electron egress to these acceptors. One must conclude, therefore, that both Methylene Blue and ferricyanide interact with the iron-sulphur chromophores, even though Bray (1975) has argued against such a role for these centres.

The data in Fig. 1, coupled with the known effects of flavin resolution, suggest that dichlorophenol-indophenol and trinitrobenzenesulphonate do interact with the molybdenum sites. Indeed, Gurtoo & Johns (1971) have presented evidence (but see Massey, 1973) for complex-formation between dichlorophenol-indophenol and the molybdenum groups of milk xanthine oxidase. They also reported, however, that CN⁻ treatment does not affect the formation of a spectrally demonstrable complex between the dye and molybdenum. On the other hand, it was suggested (Gurtoo & Johns, 1971) that the above complex did not represent the Michaelis complex, between enzyme and dichlorophenol-indophenol, that participates in catalysis. One may speculate, therefore, that loss of the persulphides leads to a conformational change that decreases the ability of the dye to reoxidize molybdenum during turnover. In support of the conformational change proposal, one may cite the spectral change that accompanies CN⁻ treatment of these enzymes (Coughlan *et al.*, 1969; Edmonson *et al.*, 1972; Cleere & Coughlan, 1974). A less probable explanation of the data in Fig. 1 is that both dichlorophenol-indophenol and trinitrobenzenesulphonate interact with the iron-sulphur centres and that this interaction is altered following the conformational change.

Bray (1975) has suggested that enzyme lacking the essential persulphide groups be called desulpho rather than non-functional enzyme. In view of the fact that such forms still retain some, or all, of their NADH oxidoreductase activity (Fig. 1) Bray's terminology would be more suitable.

References

- Avis, P. G., Bergel, F. & Bray, R. C. (1955) *J. Chem. Soc.* 1100-1105

- Branzoli, U. & Massey, V. (1974a) *J. Biol. Chem.* **249**, 4339–4345
- Branzoli, U. & Massey, V. (1974b) *J. Biol. Chem.* **249**, 4346–4349
- Bray, R. C. (1975) *Enzymes*, 3rd edn., **12**, 299–419
- Cleere, W. F. (1976) Ph.D. Thesis, University College, Galway
- Cleere, W. F. & Coughlan, M. P. (1974) *Biochem. J.* **143**, 331–340
- Cleere, W. F. & Coughlan, M. P. (1975) *Comp. Biochem. Physiol. B* **50**, 311–322
- Cleere, W. F., O'Regan, C. & Coughlan, M. P. (1974) *Biochem. J.* **143**, 465–468
- Corran, H. S., Dewan, J. G., Gordon, A. H. & Green, D. E. (1939) *Biochem. J.* **33**, 1694–1706
- Coughlan, M. P., Rajagopalan, K. V. & Handler, P. (1969) *J. Biol. Chem.* **244**, 2658–2663
- Edmonson, D. E., Massey, V., Palmer, G., Beacham, L. M. & Elion, G. B. (1972) *J. Biol. Chem.* **247**, 1597–1604
- Gurtoo, H. L. & Johns, D. G. (1971) *J. Biol. Chem.* **246**, 286–293
- Kanda, M. & Rajagopalan, K. V. (1972) *J. Biol. Chem.* **247**, 2177–2182
- Komai, H., Massey, V. & Palmer, G. (1969) *J. Biol. Chem.* **244**, 1692–1700
- Krenitsky, T. A., Neil, S. M., Elion, G. B. & Hitchings, G. B. (1972) *Arch. Biochem. Biophys.* **150**, 585–599
- Massey, V. (1973) in *Iron-Sulphur Proteins* (Lovenberg, W., ed.), pp. 301–360, Academic Press, New York
- Massey, V. & Edmonson, D. E. (1970) *J. Biol. Chem.* **245**, 6595–6598
- Nishino, T. (1974) *Biochim. Biophys. Acta* **431**, 93–98
- Rajagopalan, K. V. & Handler, P. (1967) *J. Biol. Chem.* **242**, 4097–4107