Reaction with Iodoacetamide and the Number of Active Centres in Xanthine Oxidase

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Active milk xanthine oxidase has 2 moles of FAD, 2 g.atoms of Mo and 8 g.atoms of Fe/mole of protein of mol.wt. 275 000 (Hart, McGartoll, Chapman & Bray, 1969). There has been a tendency to assume (cf. Massey, Brumby, Komai & Palmer, 1969) that all of these constituents would not be likely to function as a single catalytic unit in the molecule and that there must be two presumably equivalent active centres (Bray, 1963). We now present evidence based on FAD loss (Bray, Chisholm, Hart, Meriwether & Watts, 1966) during inactivation of the reduced enzyme by iodoacetamide (Bray & Watts, 1965) indicating that xanthine oxidase operates, during aerobic xanthine oxidation at least, with a single active centre.

The interpretation of earlier quantitative studies on reaction of xanthine oxidase with iodoacetamide, by measuring iodide liberation (Bray & Watts, 1965) and following FAD loss (A. J. Chisholm, A. M. Scott & R. C. Bray, unpublished work), was complicated by the use as starting material of preparations with non-stoichiometric FAD/Mo ratios. These problems were overcome for the present work by the use of the salicylate method for preparing the enzyme, this giving samples free from demolybdo-xanthine oxidase (Hart *et al.* 1969). The enzyme used was also free from detectable impurities in the ultracentrifuge and in recycling gel filtration on Sephadex G-200. Techniques and analytical methods were as described by Hart *et al.* (1969). Reaction of enzyme, reduced with xanthine, with iodo[¹⁴C]acetamide was carried out at pH 5.5 to minimize side reactions (Bray & Watts, 1965) and was terminated by the addition of cysteine. The product from the reaction was dialysed, then purified by recycling gel filtration on Sephadex G-200. After three cycles it showed a single symmetrical peak, radioactivity (detected with a flow scintillation counter from Packard Instruments Ltd., Wembley, Middx.), residual xanthine oxidase activity and E_{450} all being accurately coincident with one another. E_{254} coincided also, apart from a minor tail accounting for no more than 10–15% of the total material, this being eliminated when peak tubes from the final cycle were combined.

In Table 1 activities and analytical data on the starting material and product are compared. The inactivation process is first-order (Bray & Watts, 1965) and in this case was stopped at about 85%

Table 1. *Effect of reaction with iodoacetamide on xanthine oxidase*

	Before treatment	After treatment
Activity/Mo*	4.6	1.0
FAD/Mo	1.03	0.61
FAD/protein	1.9	0.9
¹⁴ C/protein	—	0.9
¹⁴ C/Mo	—	0.62
$E_{1cm}^{1\%}$ (450 nm.)†	2.4	1.8
$E_{550}/E_{450}\ddagger$	0.32	0.41

* Activity (units)/ μ g. atom of Mo in the assay with xanthine and oxygen as reducing and oxidizing substrates respectively (Hart *et al.* 1969).

† Based on protein content (Hart *et al.* 1969).

‡ The corresponding value calculated from the data of Komai *et al.* (1969) for xanthine oxidase from which all FAD had been removed is 0.51.

completion. It is clear that the main changes accompanying this inactivation were loss of one of the two molecules of FAD and binding of a single amidocarboxymethyl residue to the enzyme. The spectrum of the product (cf. Bray *et al.* 1966), and in particular the E_{550}/E_{450} ratio, was consistent with the partial loss of FAD and with the spectrum of xanthine oxidase from which all FAD had been removed by treatment with CaCl_2 , as reported by Komai, Massey & Palmer (1969). Little loss of molybdenum relative to protein occurred and, further, the gel-filtration results make it clear that no gross change in molecular weight accompanied inactivation.

The results indicate the two FAD molecules in the enzyme to be non-equivalent, loss of most of the enzymic activity being accompanied, under the conditions employed, by loss of only one FAD. Additional experiments (A. M. Scott & R. C. Bray, unpublished work) showed that during reaction with iodoacetamide the time-courses for similar

partial loss of FAD and loss of enzymic activity paralleled one another, thus indicating the two processes to be closely interrelated. If the possibility of inactivating conformational changes induced by loss of one FAD is excluded, then the results are clearly incompatible with the existence of two independent catalytic centres (or catalytic chains) for xanthine oxidation in the enzyme molecule. A single active site seems more likely and would be in keeping with the reaction mechanism proposed elsewhere for the enzyme (Bray, Knowles & Pick, 1968). The function of the group of the enzyme reacting with iodoacetamide, assumed (Bray & Watts, 1965) to be a thiol, and its apparent involvement in FAD binding require further study.

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