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Half-Sites Oxidation of Bovine Liver Uridine Diphosphate Glucose Dehydrogenase

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6,6′-Dithiodinicotinate shows half-of-the-sites reactivity towards the six catalytic-site thiol groups of bovine liver UDP-glucose dehydrogenase. The reagent introduces three intrasubunit disulphide linkages between catalytic-site thiol groups and non-catalytic-site thiol groups and abrogates 60% of the catalytic activity of the hexameric enzyme; excess 2-mercaptoethanol rapidly restores full catalytic activity. These results show the half-of-the-sites behaviour of the enzyme with the reagent and the presence of a non-catalytic-site thiol group capable of forming a disulphide linkage with a catalytic-site thiol group on the same subunit without irreversible denaturation.

UDP-glucose dehydrogenase (EC 1.1.1.22) from bovine liver consists of six identical subunits of mol.wt. 52000 and contains ten to twelve thiol groups per subunit (Gainey *et al.*, 1972; Uram *et al.*, 1972; Franzen *et al.*, 1976). One thiol group on each subunit is present in the catalytic-site region (Gainey *et al.*, 1972; Uram, 1971; Uram *et al.*, 1972) and is involved in a covalent linkage with partially and fully oxidized substrate during each turnover of the enzyme (Ordman & Kirkwood, 1977; Ridley *et al.*, 1975). The enzyme displays half-of-the-sites behaviour with respect to both substrate binding (Franzen *et al.*, 1973; Gainey & Phelps, 1974) and reaction of the catalytic-site thiol group with iodoacetic acid and iodoacetamide (Franzen *et al.*, 1976). Three of the catalytic-site thiol groups are alkylated rapidly, whereas the remaining thiol groups react much more slowly, and activity loss during the first rapid phase of the reaction exceeds the extent of alkylation. Gainey *et al.* (1972) state that the enzyme contains two thiol groups that react rapidly with 5,5′-dithiobis-(2-nitrobenzoate). Some of their data, however, appear to be consistent with the presence in the enzyme of three, rather than two, highly reactive thiol groups. Taken together, these findings indicate either that unmodified or unchanged catalytic sites on each enzyme subunit are not identical, i.e. they can be assigned to two different classes, or that they are identical but interact with each other antagonistically after modification.

We report here that UDP-glucose dehydrogenase displays half-of-the-sites reactivity with 6,6′-dithiodinicotinate, an analogue of the reagent used by Gainey *et al.* (1972). The reaction modifies the enzyme by introducing three disulphide linkages per hexameric molecule, with each disulphide linkage connecting the catalytic-site cysteine residue to a non-catalytic-site cysteine residue on the same subunit.

Materials and Methods

Materials

UDP-glucose dehydrogenase was prepared and assayed as described by Zalitis & Feingold (1969) with the modifications described by Franzen *et al.* (1976). Polyacrylamide-gel electrophoresis showed that the enzyme was at least 95% pure.

Iodo[^{3}H]acetate (207mCi/mmol) and *N*-[ethyl-1^{14}C]ethylmaleimide (9.5mCi/mmol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A. Iodo[^{3}H]acetate was recrystallized with non-radiolabelled iodoacetate from n-hexane before use. 5,5′-Dithiobis-(2-nitrobenzoic acid) and 6,6′-dithiodinicotinic acid were purchased from Aldrich, Milwaukee, WI, U.S.A.

Methods

Incorporation assays and enzyme assays were carried out as described by Franzen *et al.* (1976) with minor modifications. Reactions of the enzyme with 5,5′-dithiobis-(2-nitrobenzoate) and 6,6′-dithiodinicotinate were followed by monitoring the A_{412} of solutions ($\varepsilon = 14100$ litre·mol^{-1}·cm^{-1}; Collier, 1973) and 344nm ($\varepsilon = 10000$ litre·mol^{-1}·cm^{-1}; Grassetti *et al.*, 1969) respectively. Stock solutions of 6,6′-dithiodinicotinate were prepared by dissolving the reagent in dimethylformamide and diluting with 20–40vol. of the appropriate buffer.

Results and Discussion

Reaction of UDP-glucose dehydrogenase with 6,6′-dithiodinicotinate

The reaction of UDP-glucose dehydrogenase with 5,5′-dithiobis-(2-nitrobenzoate) has been described by Gainey *et al.* (1972). We have examined the progress curves of the reaction of the enzyme with concentrations of 6,6′-dithiodinicotinate ranging from 0.5 to 16 molar equivalents of reagent per subunit of enzyme. Although the reactions of either reagent with thiol groups are essentially identical, leading to the formation of a mixed disulphide and the release of either 2-nitro-5-thiobenzoate (Ellman, 1959) or 5-carboxy-2-thiopyridone (Grassetti *et al.*, 1969), 6,6′-dithiodinicotinate was chosen for the present study because non-enzyme blank values were much lower than those obtained with 5,5′-dithiobis-(2-nitrobenzoate).

Fig. 1 shows that the enzyme contains a set of thiol groups that reacts very rapidly with 6,6′-dithiodinicotinate. These results, which are very similar to those obtained with iodoacetamide (Franzen *et al.*, 1976), are consistent with half-of-the-sites attack of the reagent on the catalytic-site thiol groups of UDP-glucose dehydrogenase. All phases of the production of 5-carboxy-2-thiopyridone in this system are exponential, and therefore the magnitude of the burst size evaluated by a linear extrapolation of any part of the latter phase of the progress curve will be overestimated unless all of the latter-phase reactions are much slower than the initial-phase reaction. Since the latter-phase reactions are relatively much slower at 1°C, the half-sites effect is most clearly evident at that temperature.

Fig. 2 shows that the initial reaction between 0.5 molar equivalent of 6,6′-dithiodinicotinate per subunit of enzyme and enzyme was followed by a reaction that caused slower generation of 5-carboxy-2-thiopyridone. The total quantity of the latter eventually approached a full molar equivalent of 6,6′-dithiodinicotinate. As shown, the reaction was potently inhibited by UDP-xylose; NAD^+ had no effect. The non-diffusible material ('half-sites-oxidized enzyme') obtained by exhaustive dialysis of the reaction mixture against 0.5M-Tris/HCl buffer, pH8.0, had the same absorption spectrum as the original untreated enzyme ('non-oxidized enzyme'), indicating the absence of mixed disulphide between enzyme thiol groups and 5-carboxy-2-thiopyridine. The catalytic activity of half-sites-oxidized enzyme was 40% of that of the control. The stoicheiometry of the reaction requires that all the 6,6′-dithiodinicotinate be consumed during the initial phase, with formation of a mixed disulphide between 5-carboxy-2-thiopyridine and enzyme. The observed inhibition of this reaction by UDP-xylose suggests that a catalytic-site thiol group was involved in mixed-disulphide formation. The second, slow, phase of the reaction must have involved an attack on the mixed disulphide by an appropriately situated thiol group on the enzyme. This would release a second half-equivalent of 5-carboxy-2-thiopyridone and introduce three disulphide linkages into the enzyme hexamer.

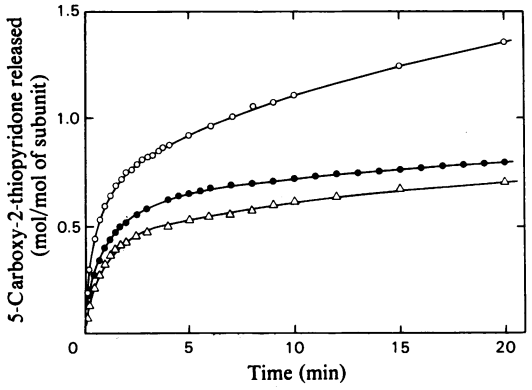

Fig. 1. *Progress curves of the reaction with 6,6′-dithiodinicotinate*

The reactions were performed in 0.1M-phosphate buffer, pH7.5, under the following conditions, where *A* and *B* represent the concentrations of enzyme subunits and 6,6′-dithiodinicotinate respectively. ○, $A = 16.6\,\mu M$, $B = 66.6\,\mu M$, 20°C; ●, $A = 17.4\,\mu M$, $B = 138\,\mu M$, 5°C; △, $A = 13.9\,\mu M$, $B = 233\,\mu M$, 1°C. All data are corrected for background absorbance due to unchanged reagent and to the mixed disulphide of protein and reagent.

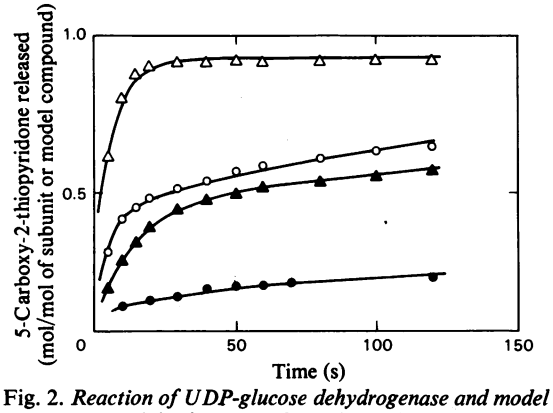

Fig. 2. *Reaction of UDP-glucose dehydrogenase and model compounds with half an equivalent of 6,6′-dithiodinicotinate*

The reactions were performed in 0.15M-Tris/HCl buffer, pH8.0, at 23°C, and the 6,6′-dithiodinicotinate concentration was 11.6 μM. The thiol-compound concentrations were: △, 23.3 μM-dithiothreitol; ○, 23.3 μM-enzyme; ▲, 23.3 μM-2-mercaptoethanol; ●, 23.6 μM-enzyme plus 138 μM-UDP-xylose.

Treatment of half-sites-oxidized enzyme with a 4000-fold excess of 2-mercaptoethanol for 5min restored catalytic activity to a value comparable with that of the control, an observation that is consistent with the presence of a readily reducible disulphide group. The circular-dichroism spectra of half-sites-oxidized enzyme in the 200–240nm (secondary structure) range and in the 250–320nm (aromatic residues) range did not differ from those of non-oxidized enzyme (Chen *et al.*, 1974). Therefore the presence of disulphide linkages did not contort the enzyme sufficiently to affect the circular-dichroism spectrum or to prevent complete and rapid restoration of catalytic activity on treatment with excess 2-mercaptoethanol.

Half-sites-oxidized enzyme incorporated 9mol of reagent per mol of subunit when reacted exhaustively with a large excess of ^{14}C-labelled *N*-ethylmaleimide in 5M-urea; under the same conditions non-oxidized enzyme incorporated 10mol per mol of subunit. This value agrees with the total thiol content of the non-oxidized enzyme sample, which also was 10mol per mol of subunit as determined with 5,5′-dithiobis-(2-nitrobenzoate) (Ellman, 1959), and is in reasonable agreement with the literature values of 10–12mol per mol of subunit. The difference in incorporation of 1equiv. of *N*-ethylmaleimide per subunit is precisely what would be expected if one disulphide linkage were formed per two subunits. Hence half-sites-oxidized enzyme must contain three disulphide groups per hexameric molecule.

The enzyme thiol group that reacts with the mixed disulphide could conceivably be: (1) on the same subunit; (2) a catalytic-site thiol group on a neighbouring subunit; (3) a non-catalytic-site thiol group on a neighbouring subunit; or (4) a thiol group from another enzyme hexamer. Since reaction of the catalytic-site thiol groups on neighbouring subunits would have abolished all catalytic activity, possibility (2) can be ruled out on the basis of the observed residual enzyme activity (40% of control) of half-sites-oxidized enzyme.

If the displacement reaction were intermolecular, dimers and higher aggregates of hexameric enzyme would be present in the reaction mixtures. However, half-sites-oxidized enzyme was eluted from a column of Sephadex G-200 in the same volume as non-oxidized enzyme. The presence of intermolecular or intersubunit disulphide bonds in half-sites-oxidized enzyme would yield products of mol.wt. 104000 in strongly denaturing solvents. Half-sites-oxidized enzyme exhaustively alkylated with *N*-ethylmaleimide migrated as a single band of mobility corresponding to 52000mol.wt. on electrophoresis in polyacrylamide gel containing sodium dodecyl sulphate. These results exclude the presence of either intermolecular or intersubunit bonds; hence the enzyme thiol group that displaces the 2-carboxy-5-thiopyridine moiety from the mixed disulphide must have been on the same subunit as the catalytic-site thiol group.

Some insight into the nature of the displacement reaction can be gained by comparing the reaction of 6,6′-dithiodinicotinate with enzyme, 2-mercaptoethanol and dithiothreitol (Fig. 2). The reaction with dithiothreitol is a model for a second-order reaction leading to formation of a mixed disulphide followed by a first-order displacement reaction. The reaction with 2-mercaptoethanol is a model for a second-order reaction, leading to formation of a mixed disulphide followed by a second-order displacement reaction. It is obvious from Fig. 2 that the displacement phase of the reaction between the reagent and the enzyme, although considerably slower than the reaction between the reagent and dithiothreitol, was at least as fast as, and possibly faster than, the second phase (displacement phase) of the reaction between 6,6′-dithiodinicotinate and 2-mercaptoethanol. These results suggest that the enzyme thiol group that takes part in the displacement is not well situated for rapid reaction with the mixed disulphide. Perhaps the rate-limiting step in the overall reaction is a change in enzyme conformation, which brings the second thiol group into position for reaction.

Reaction of half-sites-oxidized enzyme with iodoacetate

Fig. 3 shows that half-sites-oxidized enzyme was carboxymethylated at a lower rate than was the non-oxidized enzyme. As was shown previously and is confirmed here, UDP-glucose dehydrogenase is carboxymethylated only at catalytic-site thiol groups; the reaction stops and catalytic activity is abolished when one carboxymethyl group has been introduced per subunit (Franzen *et al.*, 1976). Carboxymethylation of half-sites-oxidized enzyme was not complete when one-half the subunits were substituted, but continued to some undefined limit beyond one carboxymethyl group per subunit. Because half-sites-oxidized enzyme has only three catalytic-site thiol groups available per hexamer, thiol groups other than those at the catalytic site must have been exposed to carboxymethylation by contortion of the enzyme induced by the disulphide linkages. The catalytic-site thiol groups, on the other hand, were less reactive than in non-oxidized enzyme, since 10% of the initial catalytic activity of non-oxidized enzyme was still present in half-sites-oxidized enzyme, which contained three carboxymethyl groups per hexameric molecule. It is not possible from the available data to decide whether the non-catalytic-site thiol groups of half-oxidized subunits or those of unoxidized subunits reacted differently towards iodoacetate, nor to explain the function of the non-catalytic-site thiols in enzyme activity. However, it is evident that complete integrity of non-catalytic-site thiol groups is not essential for catalytic activity, since this activity was decreased to

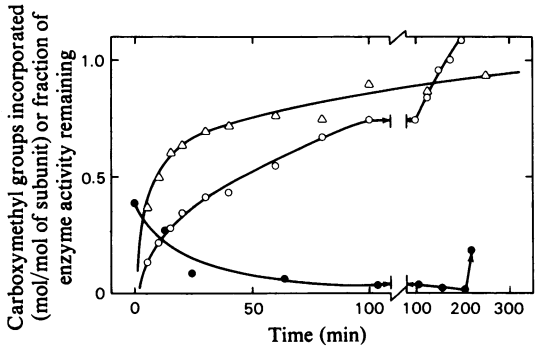

Fig. 3. *Carboxymethylation of half-sites-oxidized UDP-glucose dehydrogenase*

The enzyme (12.6 μM) was incubated at 30°C in 0.15 M-Tris buffer, pH 8.0, 775 μM in iodoacetate. The open triangles (△) show the carboxymethylation of native UDP-glucose dehydrogenase. Corresponding to this alkylation, the enzyme activity was completely lost, and was not restored by the addition of 2-mercaptoethanol. The open circles (○) show the carboxymethylation of half-sites-oxidized enzyme. The closed circles (●) show the concomitant loss of enzyme activity. The addition of 2-mercaptoethanol at 200 min restored activity to 30% of that of non-oxidized enzyme. Half-sites-oxidized enzyme had 40% of the enzymic activity of non-oxidized enzyme.

1% of that of non-oxidized enzyme by carboxymethylation of half-sites-oxidized enzyme for 200 min (1.1 carboxymethyl groups incorporated per subunit) but restored to 17% of control activity by treatment of the latter product with 2-mercaptoethanol.

UDP-glucose dehydrogenase, like glyceraldehyde phosphate dehydrogenase (Harris *et al.*, 1963), catalyses the oxidation of a thiohemiacetal to the corresponding thioester (Ordman & Kirkwood, 1977; Ridley *et al.*, 1975), and, like many dehydrogenases, the two enzymes may also share structural similarities. Treatment of glyceraldehyde phosphate dehydrogenase with 5,5′-dithiobis-(2-nitrobenzoate) causes similar formation of a disulphide bridge between the catalytic-site thiol groups, cysteine-149 and cysteine-153 located on the same polypeptide chain (Harris & Waters, 1976; Wassarman & Majors, 1969). However, glyceraldehyde phosphate dehydrogenase is irreversibly denatured by the reaction, and its catalytic activity cannot be restored (Parker & Allison, 1969), whereas half-sites-oxidized UDP-glucose dehydrogenase can be restored to full catalytic activity by reduction with 2-mercaptoethanol.

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