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UDP-GLUCOSE 4-EPIMERASE FROM *SACCHAROMYCES FRAGILIS* INVOLVEMENT OF SULFHYDRYL GROUP(S) AT THE ACTIVE SITE

MANJU RAY, KUNTALA KAR and AMAR BHADURI

Division of Biochemistry, Department of Pharmacy, Jadavpur University, Calcutta 700 032 (India)

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Summary

UDPglucose 4-epimerase (EC 5.1.3.2) from *Saccharomyces fragilis* is inactivated by 0.1 mM 5,5′-dithiobis-(2-nitrobenzoate) in 6 min. Unlike *p*-chloromercuribenzoate-inactivated or heat-inactivated enzymes, the dithiobisnitrobenzoate-inactivated enzyme retains the dimeric structure and NAD is not dissociated from the protein moiety. Inactivation of the enzyme by dithiobisnitrobenzoate can not therefore be attributed to any subsequent loss of structural integrity or to the detachment of the cofactor from the apoenzyme. The inactivated enzyme can be almost fully reactivated in the presence of mercaptoethanol and characteristic properties of native enzyme are regained. The inactivation by dithiobisnitrobenzoate can be substantially protected by UDPglucose or UDPgalactose indicating a possible critical involvement of one or more sulfhydryl groups at the active site.

UDPglucose 4-epimerase (EC 5.1.3.2) catalyzes a freely reversible reaction between UDPglucose and UDPgalactose in a wide variety of cells. The epimerisation proceeds through an oxidation-reduction mechanism [1, 2]. The reaction mechanism has been partially elucidated for the yeast and *Escherichia coli* enzymes and in both cases enzyme-bound NAD obligatorily participates in the oxidation-reduction process. UDP-4-ketoglucose and NADH had been identified as stable enzyme-bound intermediates of the reaction [3—6]. In contrast to this somewhat detailed knowledge regarding reaction intermediates, nothing is known about the amino acid residues constituting the active site or participating in the catalytic process of this reaction.

The epimerase from *Saccharomyces fragilis* is a dimeric protein of 120 000 daltons and has 1 mol of bound NAD per mol of the dimeric apoenzyme [7]. The pyridine nucleotide is apparently non-covalently but rather

tightly held to the dimeric protein structure and dissociation of NAD from the apoenzyme renders it completely inactive. Thus, though the enzyme is highly sensitive to *p*-chloromercuribenzoate, the inactivation is accompanied by a collapse of the dimeric structure of the holoenzyme and a simultaneous release of NAD in the medium [8]. It is therefore not certain whether the inactivation is specifically due to any critical presence of sulfhydryl group(s) at the active site or is due to the loss of NAD from the catalytic site. A similar situation exists when the enzyme is subjected to heat treatment. We have recently observed that on controlled heating the enzyme is rapidly inactivated [9]. The activity can be partially restored in presence of mercaptoethanol and NAD. The obligatory requirement of mercaptoethanol suggested the possible formation of one or more disulphide bonds during the process of inactivation. However, in this case also, even though the dimeric structure is maintained, NAD is released in the medium and the inactivation may simply be due to the loss of bound nucleotide from the catalytic site. Obviously, the role of specific amino acid residues can be evaluated only when modifications of such residues will not result in the release of the cofactor from the apoenzyme and the dimeric quarternary structure of the apoenzyme is retained. Employing 5,5′-dithiobis(2-nitrobenzoate) we have now been able to show the involvement of one or more sulfhydryl groups at the active site of the enzyme.

UDPglucose 4-epimerase from *Saccharomyces fragilis* was purified upto Stage III of the method of Darrow and Rodstrom [10]. The specific activity of the enzyme at this stage was usually between 3—6 units/mg protein where 1 unit of the enzyme could convert 1 μmol of UDPgalactose to UDPglucose/min under standard assay conditions [9]. The highly purified UDPglucose 4-epimerase from galactose-adapted *S. fragilis* was also purchased from Sigma Co., St. Louis, Mo., U.S.A. Only those batches which showed single band by polyacrylamide gel electrophoresis were used. The coupled assay and the two-step assay for the determination of epimerase activity were performed by methods described previously [11]. The two-step assay was employed to exclude the role of any exogenous NAD that might interfere during the coupled assay system.

Inactivation with 5,5′-dithiobis(2-nitrobenzoate) and reactivation of the inactive enzyme: The epimerase was found to be highly sensitive to dithiobisnitrobenzoate. The enzyme was completely inactivated in 5—7 min in the presence of 0.1 mM dithiobisnitrobenzoate and in 30 min in the presence of 0.05 mM dithiobisnitrobenzoate. When attempts were made to reactivate the inactive enzyme, about 70—85% of the initial activity could be restored in presence of mercaptoethanol alone (Table I). Presence of NAD along with mercaptoethanol did not stimulate the reactivation process any further. This is in sharp contrast to the situation with *p*-chloromercuribenzoate-inactivated and heat-inactivated enzymes that are not at all reactivated in absence of exogenously added NAD. The lack of any requirement for exogenous NAD suggested that during inactivation by dithiobisnitrobenzoate, the coenzyme is retained on the enzyme surface. Experiments with ADPribose, which is a powerful competitive inhibitor of NAD for the nucleotide binding site [1, 12] also substantiated this conjecture. Thus, preincubation with ADPribose had no effect on the reactivation process. Under identical conditions, reactivation of

TABLE I

REACTIVATION OF DITHIOBISNITROBENZOATE-INACTIVATED ENZYME

For this experiment, in each tube 0.1 unit of the enzymes was taken in 250 μl of 0.1 M glycylglycine buffer, pH 7.6 (Tubes A and B). Tube B was incubated at room temperature for 20 min in the presence of 0.075 mM dithiobisnitrobenzoate. 35-μl aliquots from Tube B were transferred to several tubes, each containing in a total volume of 50 μl, 5 μmol of glycylglycine buffer, pH 7.6, and other reagents as indicated in the table. A 35-μl aliquot from Tube A was also transferred under identical conditions. At the end of the incubation period, the extent of reactivation was measured by the two-step assay procedure. The incubation period was 20 min at 30°C.

	Conditions of incubation	10^3 × rate (ΔA/min)
1. Native enzyme (Tube A)		25.4
2. Dithiobisnitrobenzoate-enzyme (Tube B)		0.0
3. Dithiobisnitrobenzoate-enzyme + NAD (0.5 mM)		0.0
4. Dithiobisnitrobenzoate-enzyme + mercaptoethanol (8 mM)		18.6
5. Dithiobisnitrobenzoate-enzyme + mercaptoethanol (8 mM) + NAD (0.5 mM)		17.1
6. Dithiobisnitrobenzoate-enzyme + mercaptoethanol (8 mM) + ADPribose (0.5 mM)		19.0
7. Dithiobisnitrobenzoate-enzyme + ADPribose (0.5 mM) + mercaptoethanol (8 mM)	incubated for 20 min and then NAD (0.5 mM) added and incubated for 20 min	18.2

p-chloromercuribenzoate- and heat-inactivated enzymes were completely blocked [9].

When the dithiobisnitrobenzoate-inactivated enzyme was passed through a Sephadex G-50 column, it could be reactivated by mercaptoethanol alone and no exogenous NAD was needed. This experiment also showed that the NAD needed for catalytic activity in the regenerated enzyme must have been retained by the inactive apoenzyme.

Finally, to show the persistent attachment of NAD to the apoenzyme after inactivation with dithiobisnitrobenzoate the following experiment was carried out: 3.6 mg (30 nmol) of epimerase in 1 ml of 0.1 M glycylglycine buffer, pH 7.6, was completely inactivated with 0.2 mM dithiobisnitrobenzoate in 30 min. An aliquot containing 1.2 mg (10 nmol) of this inactivated enzyme was then spectrophotometrically analysed for the presence of free NAD with excess alcohol dehydrogenase and alcohol. No free NAD could be detected. Under identical conditions, NAD from the heat-inactivated enzyme was accessible to this enzymatic assay system. The rest of the dithiobisnitrobenzoate-inactivated enzyme (20 nmol) was now passed through a Sephadex G-50 column and the effluent protein fractions were collected. The protein was denatured with 70% alcohol and the alcohol extract was concentrated under reduced pressure at 45°C. When the concentrate was estimated for free NAD with alcohol dehydrogenase, 15 nmol of NAD could be detected indicating that the cofactor was bound to the apoenzyme even after inactivation with dithiobisnitrobenzoate.

The regenerated enzyme showed some of the characteristic properties of the native enzyme; for example, it could be activated by cations and sugar phosphates [13, 14] and inhibited by UMP [15].

Quaternary structure of the inactivated enzyme: Inactivation with dithionitrobenzoate kept the dimeric structure of the native protein essentially unaffected. This was revealed when polyacrylamide gel electrophoresis

patterns of the nitrobenzoate-treated inactive enzyme and the native enzyme showed identical mobilities at different concentrations of the gel. In one case, using 120 μg of the inactive protein on 7.5% gel, we failed to detect a second band with coomassie blue as the staining agent. Thus, if the monomeric structure is at all present at this stage, its concentration should be less than 10% of the total protein. Control samples of *p*-chloromercuribenzoate-treated enzyme showed their expected mobility patterns [9]. Gel filtration experiments on a Sephadex G-200 (1.6 X 22 cm) column along with reference proteins also confirmed that the molecular weight of the dithiobisnitrobenzoate-inactivated enzyme essentially remained the same as the native enzyme.

Substrate protection against inactivation by 5,5′-dithiobis(2-nitrobenzoate): UDPglucose was found to afford significant protection against inactivation by dithiobisnitrobenzoate (Fig. 1). Because of the free reversibility of the reaction, UDPglucose and UDPgalactose are equivalent in this case and the experiment was, of course, carried out under equilibrium conditions. UMP, which is a strictly competitive inhibitor for the enzyme [13, 15] and also participates in combination with specific sugars in 'reductive inactivation' of the enzyme [16] failed to afford any protection. Similarly, GDPglucose which was used as a control nucleotide sugar, had no effect on the rate of enzyme inactivation. Though substantial protection by the substrate could be demonstrated, complete protection could not be achieved by varying conditions of pH and ionic strength of the medium.

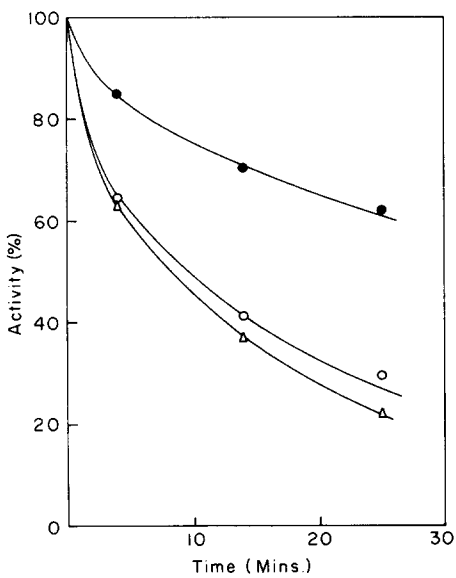

Fig. 1. Protection of inactivation by dithiobisnitrobenzoate in presence of substrate. Each experimental tube contained 0.1 unit of the enzyme in 150 μl of 0.1 M glycylglycine buffer, pH 7.7. To one set of tubes, which serves as the control, dithiobisnitrobenzoate (0.025 mM) was added and the incubation mixtures were passed through Sephadex G-50 columns (0.8 cm X 14 cm, equilibrated and washed with the same buffer) at the indicated times. 0.5 ml fractions were collected and the total recovery of the enzyme was measured by the coupled assay method. In one set of parallel experimental tubes, the enzyme was preincubated with 1 mM UDPglucose for 5 min and in another set of parallel tubes, the enzyme solution was preincubated with 1 mM UMP for 5 min. Addition of dithiobisnitrobenzoate and subsequent operations were the same. ○—○, control; ●—●, and △—△, control + UDPglucose and control + UMP, respectively.

The inactivation of the yeast enzyme by dithiobisnitrobenzoate resulted in the formation of a form of the enzyme which is different both from the *p*-chloromercuribenzoate-treated and from the heat-inactivated enzymes. In this case, the cofactor is not dissociated from the catalytic site and a minimum of 90% of the original dimeric structure of the protein moiety remains unaffected. The inactivation is therefore exclusively due to the interaction of protein sulfhydryl group(s) with dithiobisnitrobenzoate and cannot be attributed to any subsequent loss of structural integrity or loss of cofactor. It is likely that of 14 sulfhydryl groups present in the dimeric protein [17] only a limited number reacts with dithiobisnitrobenzoate. With *p*-chloromercuribenzoate, when all the sulfhydryl groups are titrated, there is a simultaneous collapse of the dimeric structure [8, 17].

The partial but significant protection afforded by the substrate indicates that at least one sulfhydryl group is critically present in or near the domain of the active site. That the protection is specific for the substrate is shown by the fact that neither UMP nor GDPglucose affords any protection under identical conditions. Since UMP, the competitive inhibitor for the substrate, fails to provide any protection, it is likely that the sulfhydryl group(s) are not essentially involved in the initial binding of the substrate to the enzyme but probably are involved at some later stage of the catalytic process. The exact number of sulfhydryl groups and their specific mode of participation, however, remains to be worked out.

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