Eur. J. Biochem. 70, 319–323 (1976)

Uridine-Diphosphate-Glucose 4-Epimerase from *Saccharomyces fragilis*

Inactivation by Heat and Reconstitution of the Inactive Enzyme

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(Received May 10/July 26, 1976)

UDP-glucose 4-epimerase from *Saccharomyces fragilis* is rapidly inactivated by heating at 42 °C for 7 min and at 45 °C for 4 min. The effector site, specific for sugar phosphates, is destroyed still earlier. The enzyme is inactivated by the dissociation of NAD from it leaving the dimeric structure unaffected. It can be reactivated by mercaptoethanol and NAD, both of which are essential for reactivation, and NAD becomes associated with the dimeric protein moiety.

UDP-glucose 4-epimerase catalyses a freely reversible reaction between UDP-glucose and UDP-galactose. We have recently reported that the epimerase from *Saccharomyces fragilis* is activated by specific sugar phosphates like glucose 6-phosphate when it is assayed in the presence of low concentrations of UDP-galactose as substrate. The sugar phosphates interact with the enzyme at a site away from the active site [1]. Further, with UDP-glucose as the substrate, the enzyme exhibits distinct allosteric kinetics, which are partially abolished in the presence of glucose 6-phosphate [2]. In an effort to desensitize preferentially the effector site by heat treatment, we have observed that mild heating rapidly inactivates the enzyme, which can be partially reactivated in presence of NAD and mercaptoethanol. Yeast epimerase undergoes inactivation and reactivation was previously reported on treatment with *p*-chloromercuribenzoate [3,4].

Recent studies have demonstrated the essential requirement of NAD in the enzymic epimerasation through an oxidation-reduction mechanism [5–8]. The mode of attachment of NAD to the protein, however, varies widely depending on the source from which the enzyme has been isolated [9]. The epimerase from *S. fragilis* contains 1 mol of tightly bound NAD/mol of the dimeric apoenzyme, which leads to two monomeric units with the release of bound NAD, when titrated with *p*-chloromuribenzoate [4]. The present work is concerned with nature of NAD attachment and molecular aggregation for the yeast enzyme under heat treatment.

Enzyme. UDP-glucose 4-epimerase (EC 5.1.3.2).

MATERIALS AND METHODS

All the biochemicals, unless otherwise mentioned, were purchased from Sigma Chemical Co. Acrylamide, bis-acrylamide, riboflavin were products of Kodak (U.S.A.). Ammonium persulphate and mercaptoethanol were purchased from E. Merck (Germany). UDP-glucose 4-epimerase was isolated from *S. fragilis* and purified up to stage III by the method of Darrow and Rodstrom [10]. The specific activity of the enzyme at this stage was usually between 5–8 units/mg protein, where 1 unit of the epimerase was defined as the amount of the enzyme that could convert 1 μmol of UDP-galactose to UDP-glucose/min under standard assay conditions. The highly purified UDP-glucose 4-epimerase from galactose-adapted *S. fragilis* was also occasionally purchased from Sigma Co. The specific activities of these preparations were usually about 10 units/mg protein. The lyophilized enzyme was taken into solution with 0.1 M sodium citrate, pH 7.0. The enzyme was assayed by coupling the reaction with UDP-glucose dehydrogenase and NAD. The assay mixture contained in a total volume of 1 ml, 100 μmol glycine/sodium hydroxide buffer, pH 8.8, 0.5 μmol NAD, 0.02 unit UDP-glucose dehydrogenase and the requisite amount of the epimerase. 1 unit of the dehydrogenase was defined as the amount of the enzyme needed to oxidize 1 μmol UDP-glucose/min. The reaction was started by the addition of UDP-galactose (0.023 mM) and the progress of the reaction was followed by the increase in absorbance at 340 nm. Readings were taken between the second and the fifth minutes. Protein was estimated by the method of Lowry *et al.* [11].

The polyacrylamide gel electrophoresis was done according to Davis [12].

RESULTS

Heat Inactivation of the Epimerase

Fig. 1 shows the time course for inactivation at 42 °C and 45 °C, which is more or less complete in 7 min and 3 min respectively. The effector site for glucose 6-phosphate was desensitized still earlier. Thus on heating for 3 min at 42 °C the enzyme retained about 70% of the activity but the stimulatory effect of glucose 6-phosphate was completely abolished, and at 45 °C this was so in less than 2 min.

Reactivation of the Heat-Inactivated Enzyme

Rapid inactivation of the enzyme on mild heating prompted us to investigate whether the activity of the enzyme could be restored. Maxwell and Sjulmajster [3] had shown that NAD is tightly bound to the yeast enzyme and its release by treatment with *p*-chloromercuribenzoate leads to the inactivation of the enzyme. The inactive enzyme could be partly reactivated when preincubated with 2-mercaptoethanol and NAD. The heat-inactivated enzyme could also be partially reactivated on incubation with mercaptoethanol and NAD (Table 1). Both the components are essential for reactivation. The reconstituted enzyme recovered about 40% of the native activity. NAD or mercaptoethanol in higher concentration failed to restore the activity any further. The requirement of NAD for reactivation suggested that NAD was released in the medium. This was confirmed by the following experiment. 1 ml of the enzyme solution containing 1.8 mg of the enzyme was inactivated by heat treatment at 45 °C for 4 min. A similar control was run at 25 °C for 4 min. 0.8 ml of the inactive enzyme solution was transferred to 1-ml cuvette and NAD was measured by oxidation of alcohol in presence of alcohol dehydrogenase. NAD could be detected in the experimental tube at 45 °C but none in the control tube at 25 °C. About 80% of the NAD could be detected by this procedure.

Glaser and coworkers had previously shown that ADP-ribose, an analogue of NAD, would bind very tightly to the apoenzyme of UDP-glucose 4-epimerase from *Escherichia coli* [9], liver [13] and to the mechanistically related dTDP-D-glucose oxidoreductase [14] and thus render these proteins inactive. In these cases ADP-ribose directly competes for the binding site of NAD on the enzyme surface. With the heat-inactivated yeast enzyme, addition of ADP-ribose during the process of reactivation could terminate the reconstitution process (Table 1). The activity of the partially reconstituted enzyme was assayed with an aliquot in a

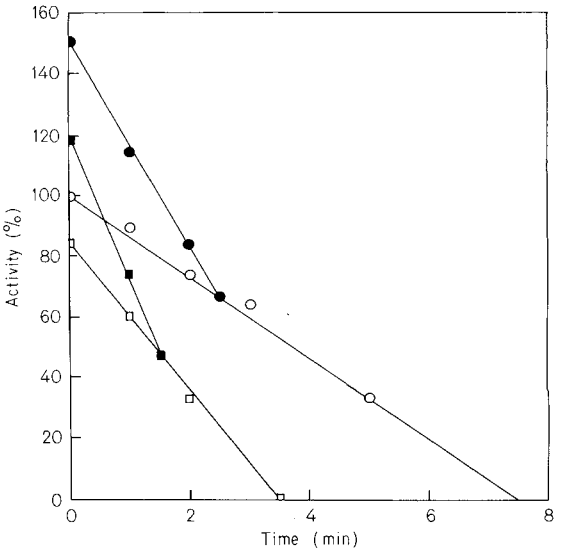

Fig. 1. *Inactivation of UDP-glucose 4-epimerase by heat.* 1 ml UDP-glucose 4-epimerase solution in 0.1 M sodium citrate, pH 7.0, containing 1.2 mg protein was placed on a water-bath at 42 °C or 45 °C. When the enzyme solution reached the temperature of the bath, an aliquot of 0.2 ml was removed and placed in crushed ice. This served as the zero-time control. 0.2-ml aliquots were removed at different time intervals as indicated in the figure. The enzyme activities in the aliquots were measured by our standard assay procedure. (○ ····○) (□——□) The loss of activity of epimerase; (● — ·····●) (■ — ■) the abolition of stimulatory effect of glucose 6-phosphate at 42 °C and 45 °C respectively

Table 1. *Reactivation of inactive enzymes obtained by heat*
Inactive enzyme was prepared by heating 1 ml enzyme solution in 0.1 M sodium citrate, pH 7.0, containing 1.2 mg protein for 4 min at 45 °C. Each experimental tube contained in a total volume of 100 µl, 5 µmol glycine/sodium hydroxide buffer, pH 8.8, 50 µg inactive enzyme and other components. The final concentrations of these components are indicated in the table. The incubation period was 30 min at 30 °C

Incubation medium	Conditions for incubation	$10^3 \times$ Rate
		ΔA/min
1. Native enzyme		24.6
2. Heated enzyme		0.0
3. Heated enzyme + mercaptoethanol (5 mM)		2.1
4. Heated enzyme + NAD (0.5 mM)		0.0
5. Heated enzyme + NAD (0.5 mM) + mercaptoethanol (5 mM)		10.2
6. Heated enzyme + mercaptoethanol (5 mM) + ADP-ribose (0.5 mM)	Pre-incubated for 20 min and then NAD (0.5 mM) added	2.0
7. Heated enzyme + mercaptoethanol (5 mM) + NAD (0.1 mM)	Incubated for 10 min and then ADP-ribose (1 mM) added	5.8

coupled assay system where the concentration of ADP-ribose was reduced to 0.02 mM by dilution. In control experiments ADP-ribose at this concentration failed to show any inhibition of the native enzyme. Further, the heat-inactivated enzyme failed to reconstitute itself when preincubated for 20 min with ADP-ribose. These experiments indicated that during the process of reactivation NAD or ADP-ribose, as the case may be, was tightly incorporated into the reconstituted enzyme.

Reductive Inactivation of the Reconstituted Enzymes

Kalckar and coworkers had shown that in presence of low concentrations of UMP, galactose or a few other monosaccharides could slowly but irreversibly reduce the enzyme-bound NAD to NADH directly on the enzyme surface. The gradual reduction of NAD resulted in a slow inactivation of the enzyme and the process was termed as reductive inhibition or inactivation [15, 16]. In contrast, the liver enzyme, which needs exogeneous NAD for its activity, could not be inactivated by such a method [17]. The phenomenon of reductive inactivation may therefore be utilized as a test for the strong and non-dissociable binding of NAD with the apoenzyme. When the reconstituted enzyme, obtained after inactivation by heat, was exposed to the combination of UMP and galactose, the enzyme was slowly deactivated, indicating that the incorporated NAD was bound to the reconstituted enzyme (Fig. 2). The binding characteristics of the enzyme with NAD were studied. The heat-inactived enzyme was fully (40% of the initial activity) reactivated by NAD at a concentration of 2 μM when incubated for 25 min. Increasing the concentration of NAD and prologing the incubation time did not result in restoration of activity any more. In contrast the *p*-chloromercuribenzoate-treated enzyme was fully reactivated at 0.01 mM NAD. Thus at 1 μM NAD, when the heat-treated enzyme was 50% active, only about 10% of the activity of *p*-chloromercuribenzoate-treated enzyme could be restored. This result suggests an easier binding of NAD to the heat-treated enzyme and prompted us to carry out experiments to see whether the molecular state of aggregation or conformation of the heat-treated enzyme was different from that of *p*-chloromercuribenzoate-treated enzyme.

Properties of the Reconstituted Enzyme

The reconstituted enzyme, obtained after heat inactivation, behaved in general like the native enzyme. Both the forms had similar K_m values with UDP-galactose as substrate. The reconstituted enzyme was activated by glucose 6-phosphate, indicating the restoration of the effector site which was desensitized during heat treatment. UMP acted as a competitive

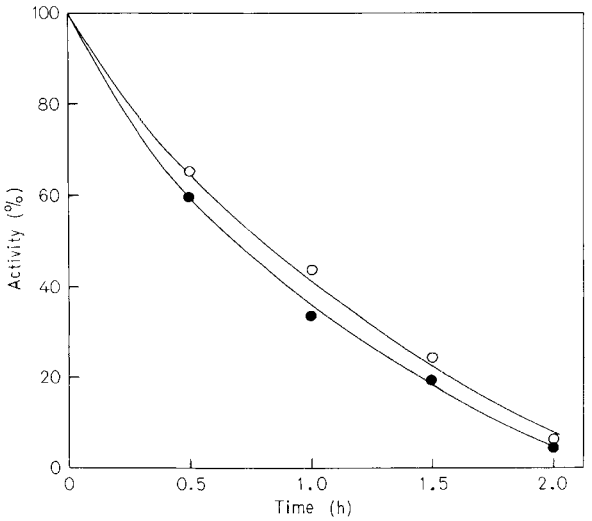

Fig. 2. *Reductive inactivation of the reconstituted enzymes.* The heat-inactivated and the *p*-chloromercuribenzoate-inactivated enzymes were prepared by the procedure described in the legend of Table 1. The inactive enzymes were reconstituted by incubation in the presence of 0.5 mM NAD and 5 mM mercaptoethanol at 30 °C for 30 min. For the final experiment, in a total volume of 100 μl, containing glycine/sodium hydroxide buffer, pH 8.8, 0.2 μmol UMP and 50 μl reconstituted enzyme, 1 μmol galactose was added. Incubation temperature was 28 °C. Aliquots were taken out at indicated time intervals to check for the epimerase activity. Control tubes, without galactose, showed no loss of activity over the period of the experiment. (●— ●) (○ ○) The reductive inhibition of reconstituted enzymes after heat inactivation and *p*-chloromercuribenzoate inactivation respectively

inhibitor for this form of the enzyme, as it did for the native enzyme [17]. Activation by cations, which is a characteristic property of the yeast epimerase [18], was also demonstrated for the reconstituted enzyme. The reconstituted enzyme, obtained after inactivation with *p*-chloromercuribenzoate, similarly showed some of the basic features of the native enzyme.

Polyacrylamide Gel Electrophoresis and Sedimentation Velocity Studies

Gel electrophoretic studies revealed a definite difference in the molecular forms between the heat-inactivated and *p*-chloromercuribenzoate-treated enzymes (Fig. 3). The native epimerase gave a sharp single band with both 7.5% and 5% (not shown) polyacrylamide gels. Occasionally in some batches of the purchased enzyme a faint slower moving band, probably due to some contaminants, was observed. The *p*-chloromercuribenzoate-treated inactive enzyme moved closer to the dye front indicating a possible reduction in molecular weight. This form of the enzyme consistently showed a diffuse band. The reason for this was not clear to us. Use of different concentrations of the gel and variations of ionic concentrations failed to give a sharper band. Darrow and Rodstrom

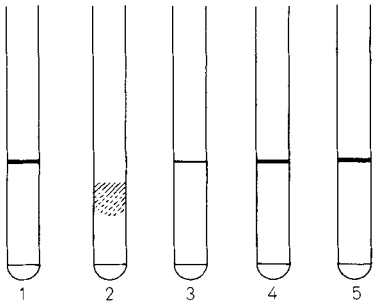

Fig. 3. *Polyacrylamide gel electrophoresis.* Polyacrylamide gel electrophoresis was carried out in Tris-glycine buffer, pH 8.5. The amount of protein applied in each tube was 25 μg. The direction of migration was from the top (cathode) to bottom (anode). Faint bands seen in each gel at the bottom represent the tracking dye. (1) Native enzyme; (2) *p*-chloromercuribenzoate-treated enzyme; (3) reconstitiuted enzyme after *p*-chloromercuribenzoate inactivation; (4) heated enzyme; (5) reconstituted enzyme after heat inactivation. This figure is the diagrammatic representation of the original electrophoretic experiment

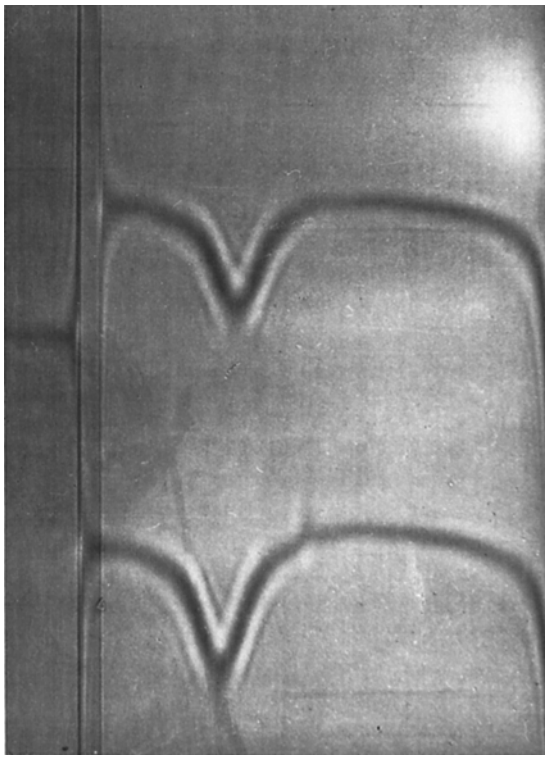

Fig. 4. *Sedimentation velocity pattern of the native and the heat-inactivated enzymes at 59870 rev./min.* 10 mg epimerase was taken in 2 ml of 0.01 M Tris-HCl buffer, pH 7.4. A portion of this sample was heated at 45 °C for 4 min to inactivate the enzyme. The centrifugation was carried out in Beckman Model E analytical ultracentrifuge. Top pattern, native enzyme. Bottom pattern, heat-inactivated enzyme. Photographs represent these at 16 min after full speed, at 55 °C phase angle. Temperature of experiment 25 °C

[4] had carefully determined the molecular weight of this form of the enzyme by using sucrose density gradient and gel chromatographic techniques and have shown that on treatment with *p*-chloromercuribenzoate, the 125000 M_r yeast enzyme dissociated into two equal subunits of M_r 60000. Incubation with mercaptoethanol and NAD resulted in a reassociation of the subunits to give a species with the molecular weight of the original enzyme. Consistent with this observation, we found that the *p*-chloromercuribenzoate-treated enzyme after reconstitution gave a sharp band with exactly the same mobility as the native enzyme. In contrast to the results obtained with *p*-chloromercuribenzoate treatment, both the heat-inactivated enzyme and the reconstituted enzyme obtained after heat treatment showed mobilities identical with those of native enzyme. At no stage of heat treatment, therefore, was there a change in the molecular weight or the dimeric state of the protein molecule.

Sedimentation velocity studies in the analytical ultracentrifuge further confirmed the conclusions drawn from the electrophoretic experiments. The enzyme inactivated by exposure to heat did not show any change in the aggregation state of the molecule (Fig. 4). The apparent Svedberg constants for the native and the heat-inactivated enzymes were 7.4 and 7.2 respectively.

DISCUSSION

The mode of attachment of the pyridine nucleotide and its effect on the substrate binding to the epimerase apoenzyme have been the subjects of considerable studies in recent years [13, 19, 20]. In the case of the yeast and the *E. coli* enzymes [21] NAD is tightly and practically irreversibly bound to the protein moiety. The calf liver enzyme, on the other hand, uses NAD as a freely dissociable cofactor and the binding constant of NAD is about 5×10^{-7} M [22]. The strong binding of NAD to the protein moiety in the case of the yeast enzyme probably does not involve any covalent linkage and the nucleotide is held together by weaker forces between the two subunits of the yeast enzyme. This was previously speculated by Kalckar *et al.* [20] primarily on the basis of release of NAD on *p*-chloromercuribenzoate treatment. The detachment of NAD from the enzyme surface on mild heating substantiates the possibility of such non-covalent attachment. However, an alternative possible mode of binding is suggested by the obligatory requirement of mercaptoethanol for reconstitution of the heat-inactive protein (Table 1). The release of NAD from the enzyme may be accompanied by the formation of a critical disulphide linkage, which has to be reduced by mercaptoethanol before the enzyme is reactivated. The binding of NAD to the enzyme surface thus needs the presence of at least two cysteine residues near the

active site, one of which could be responsible for holding the NAD in position. Such binding of NAD through cysteine residue has been well-documented in the case of glyceraldehyde-3-phosphate dehydrogenase [23].

The inactivation of the enzyme with heat and *p*-chloromercuribenzoate treatment resulted in the formation of two different forms of the inactive protein molecule. In contrast to *p*-chloromercuribenzoate treatment, exposure to heat did not affect the dimeric structure of the protein molecule (Fig. 3 and 4). Binding of NAD to the apoenzyme is, therefore, not essential for the stability of the dimeric form of the enzyme. On reconstitution, both the forms incorporate NAD tightly into the fold of the dimeric protein, as was evidenced by the reductive inactivation of the reconstituted enzyme (Fig. 2).

The reconstituted enzymes showed many of the characteristics of the native enzyme, *e.g.* activation by cations and by sugar phosphates and inhibition by UMP. However, in some important respects they differed from the native enzyme. For example, we could never restore the full activity for these reconstituted enzymes. Further, the reconstituted enzyme after inactivation by *p*-chloromercuribenzoate, failed to emit fluorescence, so characteristic a property of the native enzyme [24]. We were unable to check the situation with the heat-reconstituted enzyme because of lack of facilities. It is therefore likely that though the reconstituted enzymes have the same dimeric state as the native enzyme, they probably fail to recover the original conformation. Such subtle difference in conformation between the reconstituted apoenzyme after *p*-chloromercuribenzoate treatment and the native enzyme was suggested by Kalckar and his group [20] on the basis of hydrogen-exchange studies.

This work was supported by grants obtained from the Department of Atomic Energy, Government of India. Ultracentrifugation studies were carried out with the kind help of Dr A. Sen of Bose Research Institute, Calcutta.

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