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Presence of Two Conformationally Vicinal Sulfhydryl Groups at the Active Site of UDP-Glucose 4-Epimerase from *Saccharomyces fragilis**

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UDP-glucose 4-epimerase from *Saccharomyces fragilis* was inactivated by diazene dicarboxylic acid bis-*N,N*-dimethylamide or diamide, a compound that can specifically oxidize conformationally vicinal sulfhydryl groups on protein surfaces. The inactive enzyme was shown to retain the original dimeric structure and NAD, which is a coenzyme for this reaction, was not dissociated from the apoenzyme. The loss of activity was due to the direct modification of sulfhydryl groups and could not be attributed to any subsequent loss of structural integrity. The activity of the enzyme could be regained almost completely on incubation with mercaptoethanol alone and no exogenous NAD was needed for reactivation. The reactivated enzyme showed most of the characteristic properties of the native enzyme like activation by cations or inhibition by UMP. Presence of substrate provided partial protection against inactivation by the reagent. Formation of disulfide bond(s) across the subunits was demonstrated by sodium dodecyl sulfate gel electrophoresis in absence of mercaptoethanol. Titration of native and diamide-inactivated enzyme with *p*-chloromercuribenzoate revealed that only two sulfhydryl groups were involved in the formation of the disulfide cross-linkage across the subunits. The above results indicate the possible presence of two conformationally vicinal sulfhydryl groups at two different subunits of the enzyme that constitute part of the active site.

UDP-glucose 4-epimerase (EC 5.1.3.2) catalyzes a freely reversible reaction between UDP-glucose and UDP-galactose in a wide variety of cells. In recent years, considerable effort has been given by several groups of workers to elucidate the mechanism of epimerization for this enzyme (1, 2). Most of these works have been carried out with highly purified *Escherichia coli* and *Saccharomyces fragilis* enzymes and these works have been concerned mainly with the identification and characterization of the reaction intermediates of this catalytic pathway. In the case of this enzyme, epimerization proceeds through an oxidation-reduction mechanism and protein-bound NAD is an obligatory participant for this process. UDP-4-ketohexose and NADH have been demonstrated to be stable enzyme-bound intermediates of this catalytic process. The initial controversies regarding the formation of the 4-keto compound have been resolved satisfactorily (3-6). In contrast to this rather detailed knowledge regarding the reaction intermediates, virtually nothing is known about the amino acid residues constituting the active site or participating in the catalytic process.

The epimerase from *S. fragilis* is a dimeric protein of 125,000 daltons, and has a mole of NAD bound per mol of the dimeric apoenzyme (7-9). The pyridine nucleotide is held tightly to the dimeric protein structure, although the nature of the forces involved in the binding of the prosthetic group to the apoenzyme is not clear at the moment. Removal of the cofactor from the dimer or the dissociation of the dimer into monomers, which simultaneously releases the cofactor into the medium, renders the protein completely inactive. This has complicated the study of the involvement of the amino acid residues in the catalytic process. Thus, although 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 (7, 9). The inactivation with *p*-chloromercuribenzoate may therefore be due to the critical presence of one or more sulfhydryl groups at the active site or may simply be due to the loss of the quaternary structure of the holoenzyme. A similar situation exists when the yeast enzyme is subjected to controlled heat treatment. We have observed that the activity of the heat-inactivated enzyme can be partially restored in presence of 2-mercaptoethanol and NAD (10). However, in this case also, even though the dimeric structure is maintained, NAD is dissociated from the apoenzyme 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 quaternary structure of the apoenzyme is retained.

Diamide or diazenedicarboxylic acid bis-*N,N*-dimethylamide was introduced originally by Kosower and his group as a specific reagent for the oxidation of red blood cell glutathione (11, 12). Subsequent studies have, however, indicated that diamide or other diazene derivatives in many cases can be utilized in locating reactive sulfhydryl groups in protein molecules that are capable of forming disulfide linkages (13, 14). Employing diamide, we now find that two conformationally close sulfhydryl groups are located at or near the domain of the active site of the enzyme molecule and these are possibly involved in the overall catalytic process. More interestingly, formation of the disulfide bond leads to the cross-linking of the subunits, indicating that the two sulfhydryl groups, even though they are close conformationally and essential for overall catalysis are present in two different subunits.

MATERIALS AND METHODS

Diamide and other biochemicals, unless otherwise mentioned, were purchased from Sigma Chemical Co. St. Louis, Mo. Diamide was taken freshly into water solution before start of the experiment. UDP-glucose 4-epimerase from *S. fragilis* was purified and assayed accord-

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ing to the method of Darrow and Rodstrom (8). The specific activity of the enzyme was usually between 7 to 12 units/mg of protein, where 1 unit of the enzyme could convert 1 μmol of UDP-galactose to UDP-glucose/min. The highly purified UDP-glucose 4-epimerase from galactose-adapted *S. fragilis* was also purchased from Sigma Chemical Co. Only those batches that showed single band by polyacrylamide gel electrophoresis were used. The two-step assay for the determination of epimerase activity was performed by the method described previously (15). The two-step assay was employed to exclude the role of any exogenous NAD that might interfere during the coupled assay analysis.

Protein was estimated by the method of Lowry *et al.* (16) or by measuring the absorbance at 280 nm with albumin as the standard (17). Polyacrylamide gel electrophoresis was carried out according to the method of Davis (18) and sodium dodecyl sulfate gel electrophoresis was carried out according to the method of Weber and Osborn (19). For some specific experiments 2-mercaptoethanol was excluded from the denaturing medium.

RESULTS

Inactivation of Epimerase with Diamide—UDP-glucose 4-epimerase from *S. fragilis* could be slowly but completely inactivated by millimolar concentration of diamide. UDP-glucose dehydrogenase, the coupling enzyme for the coupled assay system was found to be highly sensitive to diamide. Diamide at a concentration of 0.03 mM and above could partially affect the activity of the dehydrogenase. To avoid inactivation of dehydrogenase with diamide during the coupled assay, epimerase was preincubated separately with diamide and aliquots were then taken in such a manner that the concentration of diamide in the final coupled assay medium was reduced to a level below 0.01 mM. Fig. 1 shows the kinetics of inactivation of epimerase with varying concentrations of diamide. Increased concentration of diamide resulted in a faster rate of inactivation of the enzyme. Thus, in 30 min with 1.2 mM diamide, the enzyme was inactivated to about 50%, and with 3.6 mM diamide, the extent of inactivation at the same time was about 90%. For some experiments, where more concentrated enzyme solutions were used, the rate of inactivation was found to be somewhat slower, and to obtain complete inactivation within 2 h, higher concentrations of diamide were used.

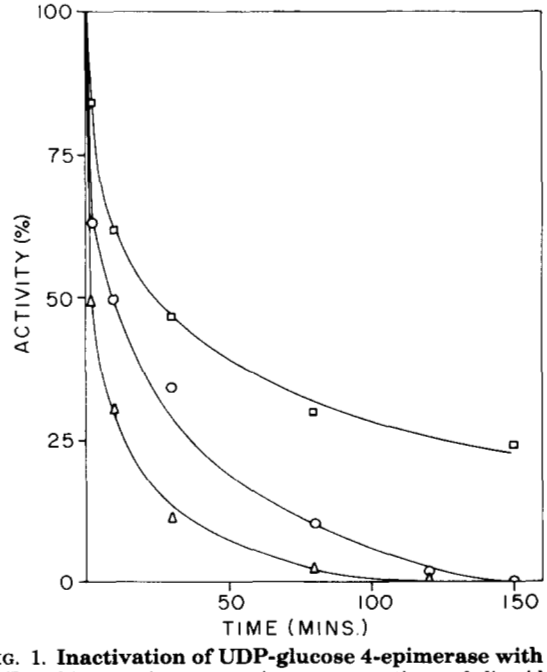

FIG. 1. **Inactivation of UDP-glucose 4-epimerase with diamide.** For this experiment, varying concentrations of diamide were added to different tubes, each containing 0.2 unit of the enzyme in 200 μl of 100 mM glycylglycine buffer, pH 7.8. Aliquots were then removed at indicated times and activities were measured by the coupled assay method. Concentrations of diamide were 1.2, 2.4, and 3.6 mM for □—□, ○—○, and △—△, respectively.

TABLE I

Reactivation of diamide-inactivated enzyme

For this experiment, 0.2 unit of the enzymes was taken in 200 μl of 0.05 M glycylglycine buffer, pH 7.8 (tubes A and B). To tube B diamide was added to a final concentration of 2.5 mM and both the tubes A and B were incubated at 30°C for 120 min. At the end of the incubation period, 25-μl aliquots from tube B were transferred from diamide-treated enzyme to several tubes, each containing in a total volume of 50 μl, 5 μmol of glycylglycine buffer, pH 7.7, and other reagents as indicated in the table. An equal aliquot was also transferred from the control tube under identical condition. All these tubes were allowed to stand at 28°C for 30 min. At the end of the incubation period, the extents of inactivation and reactivation were measured by transferring 10 μl of enzyme solutions from each tube to 1 ml of medium for two-step assay procedure (16).

Tube	UDP-glucose formed in 4 min of incubation
	nmol
1. Native enzyme (tube A)	17.0
2. Diamide-enzyme (tube B)	0.5
3. Diamide-enzyme + NAD (0.5 mM)	0.5
4. Diamide-enzyme + mercaptoethanol (10 mM)	12.0
5. Diamide-enzyme + mercaptoethanol (10 mM) + NAD (0.5 mM)	12.6
6. Diamide-enzyme + mercaptoethanol (10 mM) + ADP-ribose (1 mM)	11.2

Reactivation of Diamide-inactivated Enzyme—When attempts were made to reactivate the diamide-inactivated enzyme, about 75 to 80% of the initial activity could be restored in presence of 2-mercaptoethanol alone (Table I). Higher concentrations of mercaptoethanol or longer period of incubation for reactivation did not result in any further increase in the recovery of activity (data not presented). 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 total lack of requirement of exogenous NAD for reactivation suggested that during inactivation with diamide, the cofactor was retained on the enzyme surface. Experiments with ADP-ribose, which is a powerful competitive inhibitor of NAD for the nucleotide binding site of this enzyme (1, 20), also supported this conjecture. Thus, preincubation with ADP-ribose had no effect on the reactivation process (Table I). Under identical conditions, reactivation of *p*-chloromercuribenzoate-inactivated and heat-inactivated enzymes, which were known to have lost their NAD in the medium, were blocked completely (10). When the diamide-inactivated enzyme was passed through a Sephadex G-50 column, it could be reactivated in presence of mercaptoethanol alone, excluding the possibility that the molar amount of NAD, dissociated from the apoenzyme, might be responsible for regenerating the enzyme activity. Finally, a direct and persistent binding of the coenzyme to the apoenzyme after inactivation with diamide was demonstrated by the following experiment. Two and four-tenths milligrams (20 nmol) of the enzyme was taken in 0.35 ml of 0.1 M glycylglycine buffer, pH 7.7, and was inactivated completely with 5 mM diamide in 150 min. The inactive enzyme was passed through a Sephadex G-50 column (25 × 1.5 cm) to remove excess diamide and any presumed dissociated NAD. The protein eluant was denatured with 70% alcohol and the denatured protein was removed by centrifugation. The supernatant was

concentrated under reduced pressure at 45°C to dryness, redissolved in a minimal volume of water, and then estimated for the presence of NAD with alcohol and yeast alcohol dehydrogenase. Recovery of about 80% NAD (15.6 nmol) could be demonstrated by this assay.

Quaternary Structure of the Diamide-inactivated Enzyme—The dimeric structure of the native protein remained unaffected on inactivation with diamide. This was evident by polyacrylamide gel electrophoresis and ultracentrifugal analysis.

Polyacrylamide gel electrophoresis patterns of the diamide-treated inactive enzyme and the native enzyme showed identical mobilities at different concentrations of the gel. Control samples of *p*-chloromercuribenzoate-treated enzyme and heat-inactivated enzymes showed their expected mobility patterns (10). In a typical experiment, even when 95 μg of diamide-inactivated enzyme was used on a 7.5% cross-linked gel and bands were stained with Coomassie blue, no second band other than the band corresponding in mobility to the native band could be detected. It is evident, therefore, that on treatment with diamide, no perceptible dissociation of the dimeric structure of the yeast enzyme takes place. Moreover, lack of appearance of any band indicated that intermolecular disulfide bond formations resulting in higher molecular weight components were also absent.

Sedimentation velocity studies in the analytical ultracentrifuge confirmed the conclusions drawn from the electrophoretic experiments. For this experiment, 7.2 mg of yeast epimerase was taken in 1 ml of 0.1 M glycylglycine buffer, pH 7.8. The enzyme was treated with 6 mM diamide at room temperature for 120 min. After this period, 2 mM diamide was added again and the enzyme became completely inactive in another 60 min. The diamide-inactivated enzyme was dialyzed extensively for 8 h at 4°C against 0.02 M Tris-HCl buffer, pH 7.4, with two changes of the dialyzing solution. The dialyzed inactivated epimerase (5.1 mg/ml) was then subjected to centrifugation in a Beckman model E analytical ultracentrifuge. A single symmetrical peak was observed and the apparent Svedberg constant was found to be 7.3 which agreed fairly well with the apparent Svedberg constant of 7.1 for the native enzyme (10).

Properties of the Reactivated Enzyme—The reactivated enzyme regained all the major characteristic properties of the native enzyme. Thus, the enzyme could be activated by cations (21) and by sugar phosphates (22, 23) and could be inhibited competitively by UMP (22, 24). The reactivated enzyme could be deactivated slowly by the process of "reductive inhibition" (in presence of 1 mM UMP and 10 mM D(+)-glucose), which is a very special characteristic of the yeast and *E. coli* enzymes (25, 26). Finally, the diamide-treated nonfluorescing inactive enzyme regained its characteristic native fluorescence with an emission maximum at 431 nm, on reactivation with mercaptoethanol. The study of fluorescence properties of this reconstituted enzyme will be the subject of the following report.

Substrate Protection against Inactivation by Diamide—When substrate was present in the medium, significant protection against inactivation by diamide was observed (Fig. 2). Because of the free reversibility of the reaction, UDP-glucose and UDP-galactose are equivalent in this case and the experiment obviously was carried out under equilibrium conditions. UMP, a strictly competitive inhibitor for the enzyme (22) and GDP-glucose, a control nucleotide sugar, both failed to provide any protection against inactivation with diamide under identical conditions. Presence of higher concentrations of UDP-glucose did not result in any further increase in protection. Although substantial protection by the substrate could be demonstrated, complete protection could not be achieved under varying conditions of pH and ionic strength of the medium.

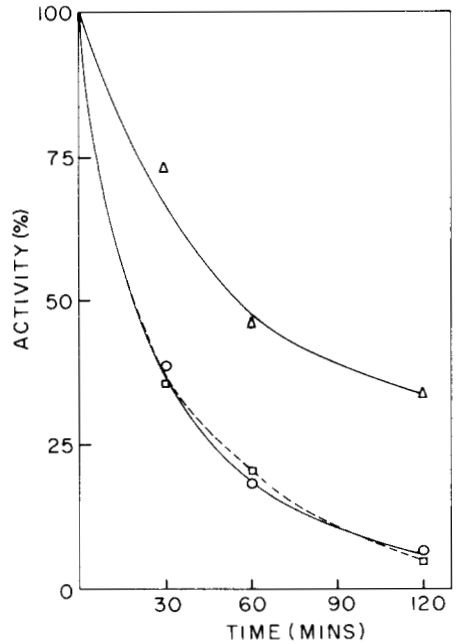

FIG. 2. **Protection of inactivation by diamide in presence of substrate.** Each experimental tube contained 0.12 unit of the epimerase in 200 μl of 0.05 M glycylglycine buffer, pH 7.7. To one set of tubes which served as the control, diamide (2.5 mM) was added, and the incubation mixtures were passed through Sephadex G-50 column (0.8 × 16 cm; equilibrated and washed with the same buffer) at the indicated times. Fractions (0.5 ml) were collected and the total recovery of the enzyme activity was measured by the coupled assay method. In one set of parallel experimental tubes, the enzyme was preincubated with 1 mM UDP-glucose for 5 min and in another set of parallel tubes, the enzyme solution was preincubated with 1 mM UMP for 5 min. Addition of diamide and subsequent operations were the same. ○——○, △——△, and □- - -□ are control, control + UDP-glucose, and control + UMP, respectively.

Intersubunit Disulfide Cross-linking of the Diamide-inactivated Enzyme—An indication that intersubunit covalent bonding might be taking place on inactivation with diamide was obtained when the diamide-treated enzyme was treated further with *p*-chloromercuribenzoate. The native dimeric yeast enzyme is dissociated into monomers of identical molecular weights either by treatment with *p*-chloromercuribenzoate (7, 9) or by treatment with guanidine hydrochloride (8). However, when the enzyme was first inactivated with diamide and then treated with *p*-chloromercuribenzoate, no dissociation of the subunits took place. This was revealed by polyacrylamide gel electrophoresis pattern of this form of the enzyme, which in contrast to the control *p*-chloromercuribenzoate-treated enzyme (10) showed identical mobility with the native enzyme (results not shown).

Formation of one or more intersubunit disulfide cross-links was confirmed when the diamide-treated inactive enzyme was subjected to sodium dodecyl sulfate-polyacrylamide gel treatment in absence of mercaptoethanol. For this experiment, 100 μg of the enzyme in 10 μl of 0.05 M glycylglycine buffer, pH 7.8, was almost completely inactivated (92%) with 4 mM diamide. Thirty microliters of the inactive enzyme was reactivated with 10 mM mercaptoethanol. Twenty-microliter aliquots of the native, inactivated, and reactivated enzymes were then subjected to analysis on sodium dodecyl sulfate-polyacrylamide gels. A schematic representation of this experiment, along with the original, is given in Fig. 3. Phosphorylase

A and γ-globulin were used as marker enzymes. Fig. 3 shows that the native enzyme was dissociated completely into identical subunits of presumably about M_r = 60,000. In contrast, treatment with sodium dodecyl sulfate failed to dissociate the subunits of the diamide-treated inactive enzyme. The inactivated enzyme showed a band of approximately M_r = 125,000 (between phosphorylase A and γ-globulin). Obviously, reaction with diamide results in the formation of covalent cross-links across the subunits which prevent dissociation of the subunits on treatment with sodium dodecyl sulfate. Unlike the inactivated enzyme, the reactivated enzyme can be dissociated again into subunits on treatment with sodium dodecyl sulfate (Lane 3). This shows that disulfide cross-link(s) across the subunits which were formed during inactivation, were reduced to free sulfhydryl groups of the native enzyme during reactivation with mercaptoethanol. The reactivated enzyme showed a second minor band on the gel in some experiments including this one. The reason for the formation of this band is not clear at the moment.

Titration of Diamide-inactivated Enzyme with p-Chloromercuribenzoate—Creveling *et al.* (27) had shown previously that quantitative titration of the native yeast epimerase with

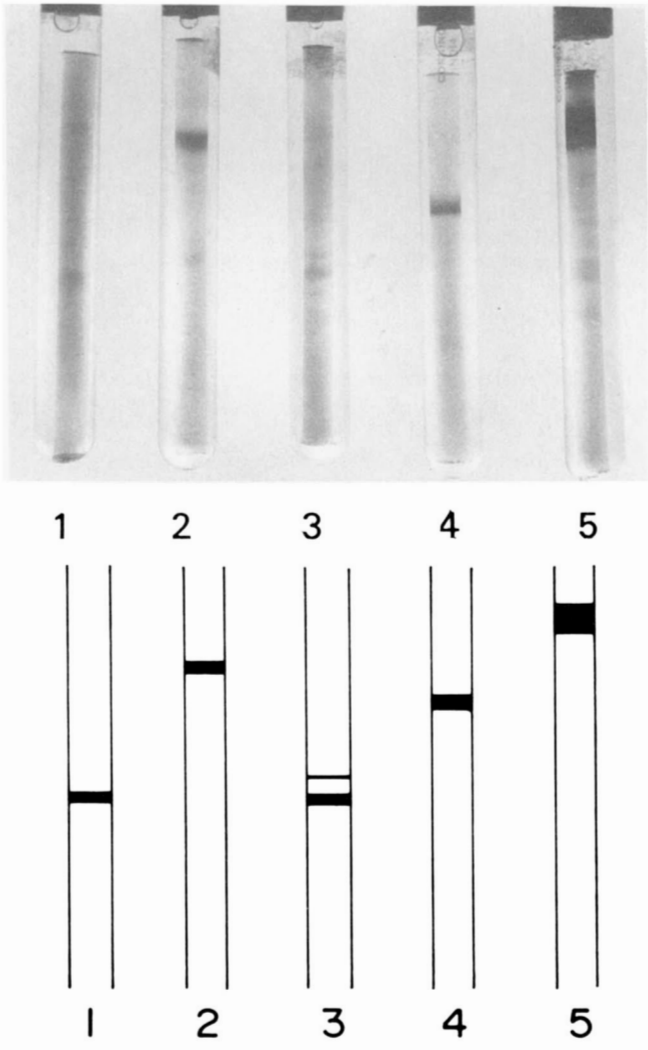

FIG. 3. **Sodium dodecyl sulfate gel electrophoresis.** The electrophoresis was carried out according to the method of Weber and Osborn (19) except 2-mercaptoethanol was omitted. The direction of migration was from top (cathode) to bottom (anode). (*1*) native enzyme, (*2*) diamide-inactivated enzyme, (*3*) reconstituted enzyme after diamide inactivation, (*4*) phosphorylase A, (*5*) γ-globulin. A diagrammatic representation of the same is also given.

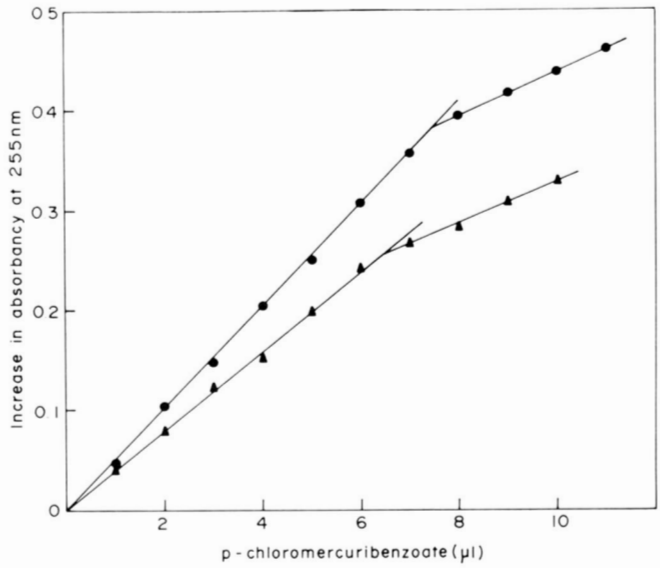

FIG. 4. **Titration of diamide-inactivated enzyme with *p*-chloromercuribenzoate.** ●——● and △——△ show the titration patterns of native and diamide-inactivated enzymes, respectively. Details of titration procedure are described in the text.

p-chloromercuribenzoate by the spectrophotometric method of Boyer (28) yields a value of 14 sulfhydryl groups/mol of the dimeric enzyme. This was confirmed by Darrow and Rodstrom (21) when they found that 116 nmol of *p*-chloromercuribenzoate were consumed/mg of the protein. To ascertain the maximum number of sulfhydryl groups that could be involved in disulfide bond formation during oxidation with diamide, the total number of available sulfhydryl groups were determined both for the native and for the diamide-treated enzymes. In a typical experiment, 1.2 mg of epimerase in 1 ml of 0.05 M glycylglycine buffer, pH 7.8, was inactivated completely with 5 mM diamide in 150 min. Excess diamide was removed by passing the inactive enzyme through a Sephadex G-50 column (22 × 1.0 cm) which was equilibrated previously with 0.05 M glycylglycine buffer, pH 7.8. The elution was carried out in the same buffer. The final concentration of the diamide inactive enzyme was 0.3 mg/ml. Control samples of untreated epimerase was carried through the same procedure and, after elution from the column, contained 0.31 mg/ml of protein. Protein in these cases, as earlier was done by Creveling *et al.* (27) or by Darrow and Rodstrom (21), was determined by measuring the absorbance at 280 nm (17). One milliliter each of these samples were titrated stepwise with *p*-chloromercuribenzoate (4.74 nmol/μl) by the method of Boyer (28), the results of which are shown in Fig. 4. Assuming a molecular weight of 125,000 for the dimer, the native epimerase was found to have 14.1 free sulfhydryl groups, whereas the diamide-treated enzyme was found to have 11.7 free sulfhydryl groups. The difference of 2.4 sulfhydryl groups is obviously due to the formation of one disulfide linkage across the subunits. In two other separate experiments, the differences in the value of free sulfhydryl groups for the native and the diamide-treated enzymes were found to be 2.6 and 1.9. An interesting and consistent feature of these titrations was that the increment in absorbance at 255 nm for the native enzyme was slightly higher than the diamide-treated enzyme (Fig. 4), the reason for which was not explored for the present work.

DISCUSSION

The inactivation of the yeast epimerase with diamide results in the formation of an inactive form of the enzyme that maintains the original quaternary structure of the native

enzyme and retains the pyridine nucleotide on the enzyme surface. Gel electrophoresis and ultracentrifugal analysis showed that the dimer was not dissociated on treatment with diamide. Lack of involvement of exogenous NAD during reactivation (Table I) and demonstration of direct association of NAD with the apoenzyme after inactivation with diamide, clearly show that the cofactor is not dissociated from the apoenzyme on reaction with diamide. The inactivation of the enzyme must therefore be due to some direct chemical modification of amino acid residues and can not be attributed to any subsequent disruption of the quaternary structure or release of the pyridine nucleotide from the catalytic site. Such ambiguous situations were observed on inactivation with *p*-chloromercuribenzoate (7, 9) or on inactivation with controlled heat treatment (10).

In a recent study on the interaction of rat red blood cells with diamide, Kosower *et al.* (14) could observe formation of some intermolecular cross-linking between hemoglobin molecules which apparently were mediated through diamide. In our case, since electrophoresis on native gel always showed only a single band of identical mobility with the native enzyme, it was obvious that no such intermolecular cross-linking took place to any detectable extent.

The partial protection provided by the substrate (Fig. 2) strongly suggests that the sulfhydryl groups that are involved in the reaction with diamide are situated at or near the active site of the enzyme. The protection, even though partial, seems to be quite specific for UDP-glucose or UDP-galactose, since neither UMP nor GDP-glucose could provide any protection under identical conditions. Complete lack of protection in presence of the powerful competitive inhibitor UMP, suggests that the sulfhydryl groups are not probably involved in the binding of the substrate but are participating at a subsequent stage of the catalytic process. Presence of sulfhydryl groups at the active site of the enzyme has also been indicated by our recent modification studies with 2,3′-dithiobisnitrobenzoate (29). In this case also, the inactive enzyme maintains the dimeric structure and the pyridine nucleotide is retained at the catalytic site. Moreover, as in the case of inactivation with diamide, only partial protection with substrate is observed. The study with diamide, however, reveals more clearly the number and nature of the sulfhydryl groups involved at the active site of the enzyme. Only two sulfhydryl groups were found to be involved in the formation of disulfide bonds (Fig. 4). In this case, all the free sulfhydryl groups could be titrated with *p*-chloromercuribenzoate without dissociating the dimeric structure. Apparently, formation of the monomers is not needed for titration of all the sulfhydryl groups. Formation of a disulfide bond across the subunits is clearly evidenced by the fact that treatment with sodium dodecyl sulfate failed to dissociate the subunits of the diamide-treated enzyme. Dissociation could be accomplished only after the disulfide bond that was formed during oxidation with diamide was first reduced by incubation with mercaptoethanol (Fig. 3). Obviously, the two sulfhydryl groups that constitute parts of the active site are not only situated in the two different subunits but are conformationally sufficiently close to each other to form a disulfide bond under suitable oxidizing conditions.

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