UDP-glucose 4-epimerase from *Saccharomyces fragilis*

PRESENCE OF AN ESSENTIAL ARGININE RESIDUE AT THE SUBSTRATE-BINDING SITE OF THE ENZYME

(Received for publication, June 10, 1985)

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UDP-glucose 4-epimerase from *Saccharomyces fragilis* was inactivated by the arginine-specific reagents phenylglyoxal, 1,2-cyclohexanedione, and 2,3-butanedione following pseudo first order reaction kinetics. The reaction order with respect to phenylglyoxal was 1.8 and that with respect to the other two diones was close to unity. Protection afforded by substrate and competitive inhibitors against inactivation by phenylglyoxal and the reduced interaction of 1-anilinonaphthalene 8-sulfonic acid, a fluorescent probe for the substrate-binding region after phenylglyoxal modification, suggested the presence of an essential arginine residue at the substrate-binding region. Experiments with [7,14C]phenylglyoxal in the presence of UMP, a ligand known to interact at the substrate-binding region, showed that only the arginine residue at the active site could be modified by phenylglyoxal. The characteristic coenzyme fluorescence of the yeast enzyme was found to be enhanced three times in phenylglyoxal-inactivated enzyme suggesting the incorporation of the phenyl ring near the pyridine moiety of NAD.

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UDP-glucose 4-epimerase (EC 5.1.3.2) is an obligatory enzyme of the galactose metabolic pathway. This enzyme catalyzes a freely reversible epimerization between UDP-glucose and UDP-galactose. Detailed and intensive study of this epimerase from *Escherichia coli* and from *Saccharomyces fragilis* by several groups of workers has clearly identified UDP-4-ketohexose and NADH as enzyme-bound intermediates in the catalytic process, and the enzyme is, therefore, mechanistically classified as an oxidoreductase (1,2). In contrast to this extensive knowledge about the reaction mechanism of this enzyme, much less is known regarding its active site. There are, however, certain interesting features of this enzymatic reaction that warrant closer attention to the problem of the mapping of the active site. Both the yeast (1,3,4) and the *E. coli* enzymes (5) have only one coenzyme molecule for the dimeric apoenzyme and hence only one active site for the two subunits. Important questions of symmetry at the active site and assembly of the holoenzyme remain unanswered. Moreover, the epimerase is the prototype of a class of oxidoreductases that include such apparently dissimilar enzymes as TDP-glucose oxidoreductase and UDP-glucuronic acid dehydrogenase. Mechanistically, these enzymes are all very similar, since catalysis in each case proceeds through an initial enzyme-bound nucleotide 4-ketosugar and NADH complex (6).

Nothing is known regarding the possible common and distinguishing features at the active site of these enzymes. A systematic study of the amino acid residues constituting the active site or participating in the catalytic process for this enzyme may throw some light on some of these questions. We have recently demonstrated the presence of two closely spaced and essential sulfhydryl groups at the active site, each residing on the two different subunits that can be cross-linked via the formation of a disulfide bond under suitable oxidizing conditions (7). Employing a reconstituting system, elements of a dinucleotide fold, and a hydrophobic pocket for the adenosine subsite for the pyridine nucleotide-binding site of this enzyme has been recently detected (8,9).

With the advent of specific arginine-modifying reagents such as phenylglyoxal (10), 2,3-butanedione (11), and 1,2-cyclohexanedione (12) a number of enzymes have been examined for the involvement of arginine residues in the catalytic mechanism (13-17). One plausible function of active site arginines is their participation in electrostatic binding of the anionic ligand to the active site. Since the substrate of UDP-glucose 4-epimerase is negatively charged at cellular pH we have attempted to explore the possible involvement of arginine residues in the function of this enzyme by using 1,2-cyclohexanedione, 2,3-butanedione, and phenylglyoxal as modifying reagents. The results described herein strongly suggest the presence of at least one essential arginine residue at the substrate-binding region of the active site of UDP-galactose 4-epimerase.

**EXPERIMENTAL PROCEDURES**

**Materials**—1,2-Cyclohexanedione and 2,3-butanedione were products of Aldrich. Phenylglyoxal and other biochemicals were purchased from Sigma. [7,14C]Phenylglyoxal was purchased from Amersham Corp. Highly purified and essentially homogeneous UDP-glucose 4-epimerase from *S. fragilis* was prepared by following up to stage III of the method of Darrow and Rodstrom (3). Highly purified enzyme was also purchased from Sigma. The specific activity of the enzyme was usually between 10-15 units/mg of protein where 1 unit of enzyme could convert 1 pmol UDP-galactose to UDP-glucose/min. The enzyme activity was assayed according to the coupled assay procedure of Darrow and Rodstrom (3). Protein was estimated by the method of Lowry et al. (18). Polyacrylamide gel electrophoresis was carried out according to the method of Davis (19).

**Modification Procedures**—Pathy and Smith (12) had shown that 1,2-cyclohexanedione reacts specifically with a guanidine group of arginine residues at pH 8.9 in sodium borate buffer in the temperature range of 25-40 °C. The borate ions are necessary to stabilize the reaction product. We could not use borate buffer because of an inhibitory effect of high concentrations of borate ions on the enzyme activity. The reason for this inactivation of the enzyme was not investigated. Modification experiments using either 1,2-cyclohexanedione or 2,3-butanedione were, therefore, performed in 0.05 M borate buffer, pH 8.9.
Experimental conditions was also reactivated up to 50% of initial activity passed through a Sephadex G-50 column (0.8 M Hepes buffer, pH 8.0, at 28°C). Phenylglyoxal solution was made in dimethyl sulfoxide. Dimethyl sulfoxide did not have any effect on epimerase activity. Control experiments without modifying reagents were always run. All the reagents (1,2-cyclohexanedione, borate, 2,3-butanedione, phenylglyoxal, and dimethyl sulfoxide) were tested on UDP-glucose dehydrogenase, the coupling enzyme of the coupled assay procedure, and no effect was observed at the concentrations being transferred to the assay medium.

For stoichiometric and other calculations, the dimeric holoenzyme was assumed to have a molecular weight of 125,000 (3).

Reactivation of 1,2-Cyclohexanedione and 2,3-Butanedione-inhibiting Enzyme—For a typical reactivation experiment, 15 µg of enzyme was taken in 25 µl of 0.05 M Hepes buffer, pH 8.0, and incubated with 4 mM borate and 10 mM 1,2-cyclohexanedione for 1 h at 28°C. Then 5 µl of inactivated enzyme having 20% residual activity was transferred to 50 µl of Tris-HCl buffer and incubated for 2 h at the same temperature. A control sample containing 4 mM borate was kept under identical conditions in Hepes buffer, and the percentage of activity was calculated by comparing the activity in the control tube. The enzyme modified by 2,3-butanedione (2 mM) under similar experimental conditions was also reactivated up to 50% of initial activity from 20% residual activity by simple dilution with 0.05 M Hepes buffer of the same pH. Epimerase inactivated by phenylglyoxal, however, could not be reactivated.

Estimation of NAD in Arginine-modified Enzyme—Estimation of NAD bound to the inactivated enzyme was carried out in the following manner. One hundred fifty-six micrograms (1.3 nmol) of the enzyme in 0.15 ml of 0.02 M sodium phosphate buffer, pH 8.0, were incubated with 2 mM phenylglyoxal for 30 min at 27°C to ensure complete modification. The modified enzyme with 15% residual activity was passed through a Sephadex G-50 column (0.5 × 20 cm) to remove excess reagents and any presumed dissociated NAD. The protein eluant was denatured with 70% alcohol. The alcoholic supernatant was evaporated to dryness and redisolved in a minimal volume of water. NAD content of that solution was measured fluorimetrically after converting to a highly fluorescent product in strong alkali (20).

The control enzyme sample containing the vehicle dimethyl sulfoxide in absence of phenylglyoxal was treated in a similar way.

Determination of Molecular Weight of Modified Enzyme—Three hundred micrograms (2.4 nmol) of the enzyme in 0.25 ml of 0.02 M sodium phosphate buffer, pH 8.0, were incubated with 2 mM phenylglyoxal for 1 h at 27°C to ensure complete modification. The modified enzyme with 15% residual activity was applied to a previously calibrated Sephadex G-50 column (1.5 × 54 cm) pre-equilibrated with 0.02 M sodium phosphate buffer, pH 8.0, and eluted with the same buffer. The enzyme eluted in the same position as the native enzyme.

RESULTS

Inactivation of UDP-glucose 4-Epimerase by Phenylglyoxal—Incubation of epimerase with phenylglyoxal resulted in a progressive loss of enzyme activity. The inactivation followed a pseudo first order kinetics to 15% of the initial activity (Fig. 1). The enzyme could not be inactivated completely even after prolonged incubation with much higher concentrations of the reagents. The second order rate constant for the inactivation was 12.5 min⁻¹ M⁻¹. A plot of log k (pseudo first order rate constant) versus log [phenylglyoxal] (21) resulted in a straight line with a slope of 1.8 (Fig. 1, inset) indicating that at least 2 mol of phenylglyoxal/mol of enzyme was required to produce inactivation.

Inactivation of Epimerase with 1,2-Cyclohexanedione and 2,3-Butanedione—Both 1,2-cyclohexanedione and 2,3-butanedione inactivated the epimerase activity (Figs. 2 and 3). But as with phenylglyoxal inactivation, the enzyme always had some residual activity even after prolonged incubation with much higher concentrations of these reagents and also of borate ions (8 mM). Inactivation by these reagents also followed pseudo first order kinetics. The second order rate constant for inactivation by 1,2-cyclohexanedione was 3.4 min⁻¹ M⁻¹ and that of 2,3-butanedione was 25 min⁻¹ M⁻¹. Hence reactivity toward epimerase is much higher for 2,3-butanedione than phenylglyoxal, and phenylglyoxal reactivity is again much higher than 1,2-cyclohexanedione. Since the kinetic order of inactivation was close to one in both cases (1.2 for 1,2-cyclohexanedione and 1.0 for 2,3-butanedione (Figs. 2 and 3, insets)), the minimal number of arginine residues that are involved in the inactiv-
that other uridine phosphates can also inhibit the enzyme. The concentration of borate in the control enzyme was also adjusted to 5 mM. The concentration of borate in each tube was adjusted to 0.37 mM (○), 0.63 mM (●), 0.73 mM (■), and 1.12 mM (▲) 2,3-butanedione at 28 °C. The concentration of borate in each tube was adjusted to 5 mM by adding sodium borate buffer of the same pH. At the indicated times aliquots were removed for measurements of enzyme activity. Control enzymes were incubated under identical conditions without 2,3-butanedione.

The kinetic parameters of the dimeric holoenzyme were determined by us by employing the assay procedure of Hayman and Colman (17). The dimeric structure of the native protein remained intact on incubation with 0.05 mM 1,2-cyclohexanediom and 2,3-butane-dione. The rate of inhibition was followed by transferring the 1-pl aliquot to the assay medium.

Structural Integrity of the Inactivated Enzyme—In order to find out whether the inhibition of epimerase activity due to the modification of arginyl residue was the result of detachment of NAD from the enzyme, direct estimations of NAD through the fluorimetric procedure were carried out. More than 90% NAD was estimated to be present in phenylglyoxal-inhibited enzyme. The enzyme (5.4 mg) in 0.1 ml of 0.02 M sodium phosphate buffer, pH 8.0, was incubated with 1 mM competitive inhibitor and 2 mM phenylglyoxal at 28 °C. The rate of inactivation was followed by transferring 10-pl aliquots to the assay medium at various times and the rate constant calculated. For the substrate protection experiment 6 μg of enzyme in 15 μl of 0.02 M sodium phosphate buffer, pH 8.0, was preincubated with 1.1 mM UDP-galactose for 10 min. 2 mM phenylglyoxal was then added and again incubated at 28 °C. The rate of inactivation was followed by transferring the 1-pl aliquot to the assay medium at various times and the rate constant calculated. The Km for UDP-galactose and Ki for the uridine phosphates were independently determined by us by employing the assay procedure of Darrow and Rodstrom (3).

Protection against Inactivation by Phenylglyoxal—Pal and Bhaduri (22) had shown that UMP is powerful competitive inhibitor for the yeast enzyme. These authors further showed that other uridine phosphates can also inhibit the enzyme competitively but less powerfully. The kinetic parameters were independently determined by us. All these competitive inhibitors and substrates were found to protect epimerase activity against inactivation by phenylglyoxal approximately according to their affinity for the active site (Table I). The results further showed that uridine did not act as a competitive inhibitor for the enzyme with UDP-galactose as the substrate and hence probably had no binding interaction at the substrate-binding region of the active site. Consistent with this observation, it failed to provide any protection to the enzyme against inactivation by phenylglyoxal (Table I). When the concentration of 5'-UMP was raised to 5 mM, effectively complete protection of the enzyme was observed even on prolonged incubation with phenylglyoxal. Because of the free reversibility of the reaction, UDP-glucose and UDP-galactose are equivalent, and protection by substrate was tested under equilibrium conditions. The little amount of substrate transferred along with the aliquot to the assay media had no additional effect on enzymic rate. Protection experiments could not be performed in case of 1,2-cyclohexanediione and 2,3-butanedione inactivation due to the reaction of borate ion with the sugar portion of substrates and competitive inhibitors.

Experiments with [7-14C]Phenylglyoxal—Once we could demonstrate that complete protection was afforded by 5'-UMP (5 mM), it provided us with a method to specifically label the arginine residues at the active site and also to estimate quantitatively the total number of arginine residues modified by phenylglyoxal. Surprisingly, we found that even after complete modification only 1.7 nmol of [7-14C]phenylglyoxal was incorporated per nmol of the dimeric enzyme (Table II). When the enzyme was protected with 5'-UMP, very little radioactivity was incorporated, indicating that no arginyl residue outside the UMP-binding region reacted with phenylglyoxal. In epimerase preincubated with cold phenylglyoxal in the presence of 5'-UMP and then treated with [7-14C]phenylglyoxal after making it free from UMP and excess cold phenylglyoxal, about 19 nmol of radiolabeled phenylglyoxal was incorporated per nmol of the dimeric enzyme (Table II). These results further confirmed that the arginine residue(s) that reacted with 2 mol of phenylglyoxal could be completely protected in the presence of 5'-UMP and are, therefore, possibly located at the substrate-binding region of the dimeric holoenzyme.

**Location of the Arginine Residue on the Enzyme Surface—**

**Table I**

<table>
<thead>
<tr>
<th>Protecting agents (1 mM)</th>
<th>Ki of competitive inhibitors</th>
<th>Inhibition rate constants, K</th>
<th>min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>0.032</td>
</tr>
<tr>
<td>Substrate (UDP-galactose)</td>
<td>0.010</td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>5'-UMP</td>
<td>0.015</td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>5'-UMP (5 mM)</td>
<td>0.015</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>UDP</td>
<td>0.37</td>
<td></td>
<td>0.015</td>
</tr>
<tr>
<td>UTP</td>
<td>0.6</td>
<td></td>
<td>0.024</td>
</tr>
<tr>
<td>Uridine</td>
<td></td>
<td></td>
<td>0.030</td>
</tr>
</tbody>
</table>
TABLE II
Incorporation of [7-14C]phenylglyoxal to epimerase in absence and presence of 5'-UMP

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nmol of [7-14C]phenylglyoxal incorporated per nmol of dimeric holoenzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Completely modified epimerase in absence of 5'-UMP</td>
<td>1.7</td>
</tr>
<tr>
<td>(b) Epimerase modified by [7-14C]phenylglyoxal in presence of 5'-UMP</td>
<td>0.18</td>
</tr>
<tr>
<td>(c) Epimerase specifically labeled at UMP-binding region</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Wong and Frey (23) were the first to demonstrate that 1-anilino-8-naphthalene sulfonic acid (ANS) could react with the E. coli epimerase to show an enormously enhanced fluorescence and a blue shift of 40 nm. Further, the extrinsic fluorophore was specifically directed toward the substrate-binding site, and the interaction could be prevented by UMP and other uridine nucleotides. This observation was extended to the S. fragilis enzyme by Samanta and Bhaduri (24). These results suggest that the extrinsic fluorophore can be utilized to monitor the substrate-binding region of the active site of the S. fragilis enzyme. When such an experiment was carried out with the enzyme, inactivated by phenylglyoxal, a very marked reduction in the enhancement of fluorescence was observed (Fig. 4). This was evidently due to the impaired interaction of the fluorophore with the substrate-binding region of the enzyme as protection by UMP during treatment with phenylglyoxal could show a full enhancement of fluorescence for the probe (Fig. 4). ANS interaction was also found to be decreased after modification by 1,2-cyclohexanedione (data not shown).

Epimerase from S. fragilis is unique in that it exhibits an intense and characteristic NADH-like fluorescence with an apparent blue shift having an emission maxima at 435 nm on excitation at 353 nm. The exact chemical nature of the fluorophore is not known. Apparently, the pyridine moiety of NAD in combination with some amino acid residues at the catalytic region of the active site acts as the fluorophore (25). Whatever is the nature of this intrinsic fluorophore, it provides a direct monitor of the changes at the active site of the enzyme. In phenylglyoxal-inactivated enzyme, the native fluorescence was found to be enhanced three times that of the control enzyme and if the enzyme was protected by UMP against inactivation by the reagent, no detectable change in the nature and extent of fluorescence could be observed (Fig. 5). It is possible that the incorporation of the phenyl ring near the pyridine moiety of NAD makes the environment more hydrophobic and hence the enhancement. Whatever is the exact cause of this enhancement, it is clear that at least one modified arginine residue is physically located near the catalytic region of the active site.

**DISCUSSION**

The kinetic method of Levy et al. (21) has been utilized by several groups of workers to determine the minimal number of functionally essential arginine residues for different enzymes (15, 26-28). For the yeast epimerase, all three α-dicarbonyl reagents were effective, but in each case the final product was an enzyme species which retained 15% of the original activity. Though phenylglyoxal occasionally lacks in specificity, the other two reagents are fairly specific for argi-
nine (12, 29). The partial but definite regeneration of activity of 1,2-cyclohexanedione-inactivated enzyme in nucleophilic buffer and of 2,3-butanedione-inactivated enzyme on removal of borate by dilution is consistent with modification of arginine residues on the enzyme surface. Such reversibilities had earlier been demonstrated for some other enzymes (12, 15). As regards the number of arginine residues that are essential for catalytic activity there exists some uncertainty. Stoichiometric experiments with radiolabeled phenylglyoxal (Table II) show that 2 mol of the reagent react with the active dimeric holoenzyme. If 1:1 stoichiometry is assumed for phenylglyoxal to arginine, as has been shown for many enzymes (13–15), there are two essential arginine residues on the enzyme surface. In contrast, if 2:1 stoichiometry is assumed, as was originally shown to occur with free arginine by Takahashi (10) and was subsequently shown to occur for several enzymes (30, 31), only one essential arginine residue appears to be involved in the catalytic activity of the enzyme. Notwithstanding these difficulties, the simplest interpretation of the data obtained from experiments with radiolabeled phenylglyoxal and also from kinetic analysis with all the three dicarboxyl reagents would be to assume the presence of a minimum of one arginine residue that is essential for the full catalytic activity of the enzyme. Direct estimation of NAD content and also determination of molecular weight of the phenylglyoxal-modified enzyme by gel filtration clearly showed that the structural integrity of the holoenzyme remained unaffected, and the loss of activity was exclusively due to the modification of at least one arginine residue on the enzyme surface. When such modifications lead to a change in tertiary structure, the direct cause for inactivation remains uncertain. The S. fragilis enzyme was earlier shown to become inactive, either due to ejection of the coenzyme from the dimeric epoxygenzyme on controlled heating (32) or due to dissociation of the dimer on treatment with p-chloromercuribenzoate (32, 33).

The active sites of S. fragilis enzyme can be arbitrarily but conveniently grouped into three regions, namely the substrate-binding region, the coenzyme-binding region, and the catalytic region comprising of the hexose moiety of the substrate and the pyridine moiety of the coenzyme, the two moieties that actively participate in the hydride transfer process. The characteristic pyridine fluorescence of the enzyme may be regarded as a direct monitor of the catalytic region. UMP, on the other hand, is an excellent monitor for the substrate-binding region of the active site. Extensive work with both the S. fragilis and the E. coli enzyme from several laboratories on the process of "reductive inhibition," a phenomenon in which UMP in combination with various free sugars slowly inactivates the enzyme, leaves little doubt that UMP specifically interacts with the substrate-binding region of the active site of epimerase from these sources (1, 2). The graded protection of activity with the substrate and the competitive inhibitors (Table I), the complete reversal of enhanced fluorescence of the substrate site-directed extrinsic amionic fluorophore on addition of UMP (Fig. 4), and the failure to radiolabel the enzyme in the presence of UMP (Table II) all strongly indicate that the arginine residue is physically located in the substrate-binding region of the active site. In contrast to UMP, uridine does not act as a competitive inhibitor for the enzyme (Table I) nor is it capable of replacing UMP in the "reductive inhibition" phenomenon (1). Presumably, the phosphate moiety of UMP is critically involved in the binding of the ligand to the enzyme. It is not unlikely, therefore, that the cationic arginine residue at the substrate-binding region is involved in an interaction with the α-phosphate moiety of the substrate. Such interactions of the arginine residues with the phosphate portions of the substrate or coenzyme had earlier been documented for a number of enzymes (13, 17, 34–36). However, in the absence of further evidence such interaction for this enzyme remains somewhat speculative at the moment. Interestingly, even after complete modification of the arginine residue by any of these dicarbonyl reagents, 15% of the initial enzyme activity was retained, and the K_m for the substrate for the modified enzyme increased significantly. Obviously, a weak but partially effective interaction can still take place between the substrate and the enzyme even after the arginine residue is modified. Such residual catalytic activity has been demonstrated for some other enzymes (37–39). Though the arginine residue is placed in the substrate-binding region, it must also be fairly close to the coenzyme fluorophore. Introduction of a phenyl group and increased hydrophobicity in the region after modification may be the cause of the 3-fold enhancement of this fluorescence (Fig. 5). However, the nature of this fluorescence spectrum remained unaltered, any direct role of the arginine residue in maintaining the fluorophore structure can be eliminated. Such a role for a thiolate anion was assumed by Ray and Bhaduri (25) from their fluorimetric studies with sulphydryl-modifying reagents.

We do not have any adequate explanation for the exclusive reactivity of the arginine residue at the active site that was observed with phenylglyoxal as the modifying reagent (Table II). The dimeric enzyme has a large number of arginine residues (3), and it was expected that at least a few of them would be exposed outward and would be fairly reactive. An analogous situation was earlier observed by Takata and Fujioka (31) for S-adenosyl homocysteine from rat liver when they could show that only 2 arginine residues at the active site out of the 14 available per subunit were reactive to phenylglyoxal. Interestingly, this enzyme, consisting of four identical subunits, also has a tightly bound NAD as the coenzyme at the active site of each of the monomers (40). As the active site arginine is the only arginine residue that is modified by phenylglyoxal, labeling of this residue followed by proteolytic cleavage provides for the first time a direct method to sequence portions of the active site of this model oxidoreductase.

Acknowledgment—We are grateful to Dr. Manju Ray of our laboratory for her valuable help in various ways.

REFERENCES
Active Site Arginine of UDP-galactose 4-Epimerase