An Arginine Residue Is Essential for Stretching and Binding of the Substrate on UDP-glucose-4-epimerase from *Escherichia coli*

USE OF A STACKED AND QUENCHED URIDINE NUCLEOTIDE FLUOROPHORE AS PROBE*

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Uma Bhattacharyya‡, Gautam Dhar§ and Amar Bhaduri¶

From the Indian Institute of Chemical Biology, Jadavpur, Calcutta, India 700032

In the previous paper we demonstrated that uridine-5′-β-1-(5-sulfonic acid) naphthylamidate (UDPAmNS) is a stacked and quenched fluorophore that shows severalfold enhancement of fluorescence in a stretched conformation. UDPAmNS was found to be a powerful competitive inhibitor (Kᵢ = 0.2 mM) for UDP-glucose-4-epimerase from *Escherichia coli*. This active site-directed fluorophore assumed a stretched conformation on the enzyme surface, as was evidenced by full enhancement of fluorescence in saturating enzyme concentration. Complete displacement of the fluorophore by UDP suggested it to bind to the substrate binding site of the active site. Analysis of inactivation kinetics in the presence of α,β-diones such as phenylglyoxal, cyclobutanedione, and 2,3-butanedione suggested involvement of the essential arginine residue in the overall catalytic process. From spectral analysis, loss of activity could also be directly correlated with modification of only one arginine residue. Protection experiments with UDP showed the arginine residue to be located in the uridyl phosphate binding subsite. Unlike the native enzyme, the modified enzyme failed to show any enhancement of fluorescence with UDPAmNS clearly demonstrating the role of the essential arginine residue in stretching and binding of the substrate. The potential usefulness of such stacked and quenched nucleotide fluorophores has been discussed.

UDP-glucose-4-epimerase (EC 5.1.3.2, henceforth called epimerase) catalyzes a freely reversible reaction between UDP-glucose and UDP-galactose. This enzyme of the galactose metabolic pathway is essential for the biosynthesis of numerous galactoconjugates in all cell types studied so far. This epimerase has emerged as the prototype of a new class of oxidoreductases. Extensive chemical modification studies with the yeast enzyme revealed the requirement of one essential thiol (8, 9) one histidine (10), and an arginine (11) in the overall catalytic process. The primary sequence and the three-dimensional structure of this enzyme are not known. The homodimeric 79-kDa *E. coli* holoenzyme, on the other hand, has recently been cloned, expressed, and crystallized in various catalytically inactive forms (12–14). Although no modification work has been reported with the enzyme, a reasonably clear picture of the active site has emerged at 1.8 Å resolution (13, 14). The substrate analog UDP-benzene seems to be in a stretched conformation, and amino acid residues involved in various binding interactions can be tentatively identified. It is imperative that modification studies be carried out with the *E. coli* enzyme to specify and confirm the tentative roles of these amino acid residues residing at the active site.

In the previous paper we demonstrated that uridine-5′-diphosphoro-β-1-(5-sulfonic acid) naphthylamidate (UDPAmNS) in aqueous solution behaves as a quenched fluorophore because of its predominantly stacked conformation. Quenching is fully released when complete destacking takes place (15). To explore the possibility of whether such designed fluorophores can be used to probe conformational transitions of a putative ligand as it interacts with its target protein, we used UDPAmNS as the probe and the *E. coli* epimerase as the model target enzyme for this purpose. We first show that UDPAmNS is indeed a substrate site-directed probe for this enzyme. Furthermore, the probe assumes a fully stretched conformation on the enzyme surface, as is evidenced by total dequenching of fluorescence on interaction with the enzyme. Finally, we demonstrate the essential requirement of at least one arginine residue in this binding process. The reliability of these results is confirmed from the available x-ray data.

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‡Present address: Dept. of Molecular Pharmacology, Albert Einstein College of Medicine, New York, NY.

§Research Associate for the CSIR. Present address: Dept. of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, CA 90095.

¶Emeritus Scientist for the CSIR. To whom correspondence should be addressed: Indian Institute of Chemical Biology, 4, Raja S. C. Mallick Rd., Jadavpur, Calcutta, India 700032. Tel. and Fax: 91-33-4735197; E-mail: IICHBII0@IASCLO1.VSNL.NET.IN.

MATERIALS AND METHODS
All biochemicals unless otherwise stated were purchased from Sigma. [1-14C]Phenylglyoxal (2.17 mCi/mmol) was purchased from...
Bhaba Atomic Research Center. Common chemicals were of analytical grade and purchased from Merck.

Absorption measurements were done in a Hitachi U-3200 spectrophotometer, and fluorescence measurements were done in a Hitachi F-4010 spectrofluorimeter.

**Enzyme and Assays—**Highly purified and essentially homogenous UDP-glucose-4-epimerase was prepared from *E. coli* according to the method of Wilson and Hogness (3). The specific activity of the enzyme was 150–160 units/mg of protein, where 1 unit of enzyme could convert 1 μmol of UDP-galactose to UDP-glucose in 1 min. Epimerase activity was routinely determined by the use of the coupled assay system of Wilson and Hogness (3). In this case, UDP-glucose, the product of epimerization, is immediately converted to UDP-glucuronic acid by coupling the reaction with UDP-glucose dehydrogenase and NAD. The assay mixture consisted of 0.1 m glycglycine buffer, pH 8.8, 0.25 mM NAD, 0.16 units of UDP-glucose dehydrogenase, and the requisite amount of epimerase. The reaction was initiated by addition of UDP-galactose, and increase in absorbance attributable to formation of NADH was measured at 340 nm for second and fifth minutes. Because the synthetic fluorophore itself had a significant absorbance at 340 nm, a two-step assay system was used whenever necessary. This assay has been described in detail earlier (17). Briefly, in a total volume of 200 μl containing 0.2 m glycglycine buffer, pH 8.8, and 30 μg of bovine serum albumin, the requisite amount of epimerase was taken, and reaction was started with UDP-galactose. The reaction was terminated after 5 min by rapid addition of chloroform and vigorous shaking. The mixture was then centrifuged. In the second stage, an appropriate aliquot from the top aqueous layer was taken out to estimate the amount of UDP-glucose formed by adding it in a medium containing UDP-glucose dehydrogenase and NAD and observing the change in absorbance at 340 nm. A control blank without epimerase was parallelly run. Protein was estimated by the method of Lowry et al. (18). For all stoichiometric calculations, the molecular weight for the epimerase was assumed to be 79,000 (12).

**Synthetic Fluorophores and Fluorescence Measurements—**UDPAmNS and other AmNS derivatives were synthesized and purified according the methods described in our preceding paper (15). Wherever necessary, excess ligands and reagents were removed from reaction mixtures by Sephadex G-50 spin column centrifugation as described by Maniatis et al. (19).

**Modification Experiments—**Modification with phenylglyoxal was carried out in 0.05 M potassium phosphate buffer, pH 8.0. The reagent was prepared by mixing an equal volume of the reagent with 0.05 m sodium borate buffer, pH 8.8. Borate had no inhibitory effect on the enzyme. All experiments, unless otherwise stated were carried out at 28 °C. All kinetics of inactivation were followed by measurement of residual activities of reaction mixtures, withdrawn at intervals and suitably diluted. The modifying reagents at the concentrations used in the assay medium did not have any effect on UDP-glucose dehydrogenase, the coupling enzyme of the coupled assay procedure. In reactivation experiments the inactivated epimerase was treated with neutralized 0.5M hydroxylamine, which on dilution during assay had no effect on the activity of the control enzyme.

**Incorporation of t14C]Phenyglyoxal—[1-14C] Phenylglyoxal incorporation studies were performed to determine the stoichiometry of the reaction of phenylglyoxal with arginine residues of the epimerase. The enzyme (0.6 mg/ml) in 0.5 m potassium phosphate buffer, pH 8.0, was incubated with 2.5 m [1-14C] phenylglyoxal (1.2 × 106 cpm/μmole) for 45 min. Excess reagent and ligand were removed by spin column centrifugation, and the eluates were measured for enzyme activity, protein concentration, and radioactivity.

**Estimation of Quantum Enhancement Q—**This was done according the procedure of Mas and Colman (20) using the following equation: 1/(F/F_0 - 1) = K_QP_mP, where F_0 and P represent the fluorescence intensity of UDPAmNS in the absence and in presence of the E. coli enzyme after subtracting that attributable to epimerase, and P is the epimerase concentration. The intercept of the plot of 1/(F/F_0 - 1) versus 1/P gives 1/Q, where Q is the quantum enhancement or the enhancement that would occur when all the fluorophore is bound to the protein.

**RESULTS**

UDPAmNS Is a Probe for the Active Site of *E. coli* Epimerase—We first investigated whether UDPAmNS can be used as a probe for the active site of the *E. coli* enzyme. Both the *K. fragilis* and the *E. coli* enzymes are known to be competitively inhibited by UMP and UDP (21, 22). UDPAmNS was also found to be a strict competitive inhibitor for the epimerase (Fig. 1) and hence a probe for the active site of the enzyme. Clearly, substitution of the hexose moiety by the bulky AmNS did not present any major steric problem for specific interaction of this ligand to the substrate-binding site of the enzyme. This is consistent with the reaction mechanism that assumes free rotation of the ketohexose moiety during catalysis (2). Furthermore, earlier observations had shown that considerable modification of the hexose moiety or its substitution by other moieties can be effected without hampering the binding property of the substrate (23–25). The K_i for UDPAmNS was calculated to be 0.20 mM, which compares very well with the K_i obtained with other such similar aromatic analogs of UDP such as p-bromoacetamidophenyluridyl pyrophosphate (0.21 mM) and p-nitrophenylnuridyl pyrophosphate (0.21 mM) (24, 25).

**Conformational Transition of UDPAmNS on the Enzyme Surface—**Fig. 2A shows the fluorescence spectra of UDPAmNS (9.3 μM) as the concentration of epimerase was progressively increased in the cuvette. Quite evidently, the stacked and quenched fluorophore assumes a stretched conformation on interaction with the enzyme and hence is relieved of its quenching (15). Fig. 2A, inset, shows that extrapolation at infinite enzyme concentration when all the fluorophores have interacted with the enzyme results in a 8-fold increase in fluorescence, a value that agrees excellently with the value obtained for the fully unstacked fluorophore in isopropanol and dimethyl sulfoxide or after phosphodiesterase bond cleavage (previous paper, Fig. 1; Ref. 15).

Fig. 2B shows complete displacement of UDPAmNS from the enzyme surface by UDP. This is additional evidence to show that the fluorophore is interacting with the enzyme exclusively at the substrate binding site. The native *E. coli* holoenzyme displays a weak intrinsic fluorescence (λ_em = 455 nm) when excited at 360 nm. This is probably attributable to the varying amounts of NADH that are known to be bound to the dimeric apoenzyme when purified from overexpressed *E. coli* (26). We shall now show that by using this fluorophore one can uncover very conveniently amino acid residues that are essential for the binding of the substrate.

**Modification Studies with α,β-Diones—**Because the sub-
The Essential Arginine Residue Is Critical for Stretching and Protection of the Active Site

Active Site Reserve of UDP-glucose-4-epimerase from E. coli

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**FIG. 3.** A, inactivation of epimerase by phenylglyoxal. The enzyme (7.5 μg/ml) was incubated with varying concentrations of phenylglyoxal in 0.05 M potassium phosphate buffer, pH 8.0, at 28 °C. At the indicated intervals, aliquots were withdrawn for measuring enzyme activity. The phenylglyoxal concentrations in the various runs were 0.25 mM (○), 0.5 mM (△), 1 mM (●), 2 mM (▲) and 5 mM (□). Inset, plot of pseudo-first order rate constant ($K$) versus log of phenylglyoxal concentration. The slope of the plot is 0.75. B, inactivation of epimerase by 1,2-cyclohexanediol. The enzyme (6 μg/ml) was incubated with increasing concentrations of cyclohexanediol in 0.05 M sodium borate buffer, pH 8.8. Aliquots were withdrawn at the indicated intervals for measuring residual activity of the enzyme. The cyclohexanediol concentrations at various runs were 0.25 mM (○), 0.5 mM (△), 1 mM (●), 2 mM (▲) and 5 mM (□). Inset, plot of pseudo-first order rate constant ($K$) versus log of cyclohexanediol concentration. The slope of the plot is 0.8.

**FIG. 4.** Correlation between loss of activity and number of arginine residues modified on phenylglyoxal treatment of the epimerase. The enzyme (0.58 mg/ml) was treated with 2.5 mM phenylglyoxal in 0.05 M potassium phosphate buffer, pH 8.0, containing 1 mM EDTA, at 28 °C for 60 min. At intervals of 5, 10, 15, 20, 30, 40, 50, and 60 min aliquots were withdrawn and subjected to spin column centrifugation to terminate the inactivation reaction. Small aliquots of the column eluates were removed for activity measurement and protein estimation. The bulk of the eluates were subjected to difference scan (230–330 nm) against control enzyme treated in an identical fashion. Fractions of arginine residues modified were determined from the molar extinction coefficient for diphenylglyoxalated arginine at 250 nm ($ε = 11,000$ M/cm).

**FIG. 5.** Protection against inactivation by phenylglyoxal by uridyl phosphates. Epimerase (10 μg/ml) in 0.05 M potassium phosphate buffer, pH 8.0, was treated with 2.5 mM phenylglyoxal in the absence or presence of increasing concentrations of 5′-UMP. a–e, UMP (mM) concentrations of 0, 1.5, 3, 6, and 9, respectively; f, kinetics in the presence of UDP (4 mM); g, untreated epimerase control. Inset, plot of half-time of inactivation ($t_{1/2}$) against concentration of UMP. The slope of the plot gives the $K_d$ of the enzyme-UMP complex.

1.25 mM (○), 2.5 mM (●), 5 mM (△), and 10 mM (□). Inset, plot of log pseudo-first order rate constant ($K$) of cyclohexanediol inactivation reaction versus log of cyclohexanediol concentration. The slope of the plot is 0.8.
binding subsite, the enzyme inactivated with phenylglyoxal (>95%) was allowed to interact with UDPAmNS. Fig. 6 shows that the quenched fluorophore failed to show significant enhancement of fluorescence with the modified enzyme. Clearly, in this case, the stacked fluorophore failed to bind and undergo transition to a stretched conformation on the enzyme surface. In contrast, interaction with the native enzyme that served as the control resulted in severalfold enhancement of fluorescence of UDPAmNS. Furthermore, UDPAmNS could be completely displaced from the enzyme surface by UDP (Fig. 6, trace e), confirming its specific interaction at the substrate binding site of the enzyme. The essential arginine residue is, therefore, critically involved not only in the binding but also in the destacking or stretching of this substrate analog on the enzyme surface. These results agree very well with the published x-ray data of the holoenzyme as it is co-crystallized with UDP-phenol or with other abortive forms of the enzyme (12–14). Because the destacking energy was calculated to be 2.3 Kcal/mol for UDPAmNS (previous paper; Ref. 15), the binding energy for the UDP moiety of the substrate may be assumed to have a minimal value of that order.

Kinetic analysis with \( \alpha, \beta \)-dicarbonyl reagents (Fig. 3), followed by estimation of arginine residues that can be directly correlated with total loss of activity (Fig. 4), clearly shows that one arginine residue is essential for the overall catalytic activity. Complete regeneration of activity of 1,2-cyclohexanedione-inactivated enzyme by hydroxylamine and of 2,3-butanediol-inactivated enzyme on removal of borate by dilution (see text) is also consistent with modification of an essential arginine residue on the enzyme surface. Nearly complete protections provided both by UMP and by UDP against modification by phenylglyoxal (Fig. 5) strongly suggest that the essential arginine residue is located in the uridylphosphoryl binding subsite of the active site and is probably involved in the productive binding of the substrate with the enzyme. Using UDPAmNS as the active site-directed designed probe, we could at this stage convincingly demonstrate that the essential arginine residue is critically needed both for binding and for stretching of the substrate (Fig. 6).

Fig. 7 provides a schematic representation of the overall process. The stacked and quenched fluorophore in the buffered aqueous solution assumes a stretched conformation on the enzyme surface that leads to dequenching of fluorescence. The essential arginine residue is obligatorily needed in this binding process. These results fit exceedingly well with the published

**Discussion**

In the previous paper we had shown that when a uridine nucleotide is derivatized with a suitable aromatic fluorophore such as AmNS via a phosphoramidate bond through the terminal phosphate, it takes a stacked conformation in aqueous solution that is in rapid equilibrium with its extended form (15). The dynamic collisional quenching of fluorescence in the stacked form should make such quenched fluorophores eminently suitable as probes for protein or enzyme studies provided such designed molecules satisfy the following conditions. First, the derivatized probe must be true structural analogs of the desired ligand so that specificity is retained, and second, the probe on interaction with the target protein should take a destacked conformation so that the resultant enhancement of fluorescence can be a direct monitor of the conformational transition of the probe and hence of its interaction with the protein. Our present work shows that UDPAmNS satisfies both the conditions when E. coli epimerase is used as the enzyme target. Although the competitive nature of inhibition (Fig. 1) suggests it to be an active site-directed analog, maximum enhancement of fluorescence when all the molecules of the fluorophores are bound to the enzyme (Fig. 2A) clearly demonstrates a destacked or stretched conformation of the substrate analog on the enzyme surface. These results agree very well with the published x-ray data of the holoenzyme as it is co-crystallized with UDP-phenol or with other abortive forms of the enzyme (12–14). Because the destacking energy was calculated to be 2.3 Kcal/mol for UDPAmNS (previous paper; Ref. 15), the binding energy for the UDP moiety of the substrate may be assumed to have a minimal value of that order.

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**Fig. 6.** Interaction of UDPAmNS with native and modified epimerase. E. coli epimerase (27 \( \mu \)M) was incubated with 5 \( \mu \)M phenylglyoxal in 100 \( \mu \)M potassium buffer, pH 8.0, for 2 h till complete inactivation. It was then passed through a Sephadex G-50 spin column to remove excess reagent. 27 \( \mu \)M unmodified enzyme was also similarly passed through the spin column. At an excitation wavelength of 380 nm the following spectra were taken in the same buffer as above: a, unmodified epimerase (27 \( \mu \)M); b, UDPAmNS (9.3 \( \mu \)M); c, unmodified epimerase (27 \( \mu \)M) and UDPAmNS (9.3 \( \mu \)M); d, displacement of UDPAmNS with 10 \( \mu \)M UDP; e, after total displacement of UDPAmNS with 1 \( \mu \)M UDP; f, phenylglyoxal-treated epimerase (27 \( \mu \)M) and UDPAmNS (9.3 \( \mu \)M).

**Fig. 7.** Schematic diagram depicting the binding of UDPAmNS at the substrate binding site of E. coli epimerase.
x-ray and sequence homology data (13, 14). Resolution at χ.2 of the holoenzyme co-crystallized with UDP had earlier shown that apart from four molecules of water, Asn-179, Leu-200, Ala-216, and Arg-292 are in a bonding distance of UDP. More importantly, the oxyanion of α-phosphate of UDP has a potential bonding interaction with the guanidino nitrogen of Arg-292. This, most likely, is the essential arginine residue uncovered by our modification studies. Interestingly, this arginine residue is conserved across the phylogenetic scale for epimerase, which includes clones from several organisms such as bacteria, yeast, and mammals (14).

Our present study with UDPAmNS as a representative compound for stacked and quenched fluorophores shows that such designed nucleotide fluorophores can possibly be of considerable use in following ligand-protein interactions in several classes of proteins of great biological interest. Several ATPases, kinases, and G-proteins in general show that nucleotides take a stretched conformation on the protein surface (16, 33). In principle, the conformational transition of such a quenched fluorophore as it interacts with its target protein can thus be used to study many aspects of the structure-function relationship of these enzymes or proteins. An anticipated advantage in terms of ligand specificity for such synthetic fluorophores may be attributable to the fact that both the base and the ribose moieties are left unaltered during the derivatization process. Like other extrinsic fluorophores, such fluorophores can also be used for energy transfer studies, because the tryptophan emission spectrum overlaps the absorption spectrum of these potential fluorophores. These fluorophores can also be uniquely useful as sensors for the formation of substrate binding or ligand binding sites in protein-folding studies. Finally, search for lead compounds for drug development may be facilitated by rapid fluorimetric monitoring of the displacement of stretched fluorophores from the binding site of target proteins, e.g. G-proteins, by an array of synthetic molecules generated by combinatorial methods or extracts from plant sources.

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