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# 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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**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 ($K_i$ = 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 presence of α,β-diones such as phenylglyoxal, cyclohaxanedione, and 2,3-butadione 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 in which the coenzyme NAD, noncovalently but firmly bound to the apoenzyme, acts as a true cofactor and not as a cosubstrate as in the case of classical dehydrogenases. Several apparently unrelated enzymes such as *S*-adenosylhomocysteinase (EC 3.3.1.1), dTDPglucose oxidoreductase (EC 4.1.1.46), and UDP-glucuronate decarboxylase (EC 4.1.1.35) mechanistically belong to this new class of oxidoreductases. In each case catalysis is initiated by reduction of enzyme-bound NAD. The oxidized substrate then undergoes chemical transformation, if necessary, on the enzyme surface before the hydride is stereospecifically returned back and the product is released (1, 2).

In case of the epimerase, UDP-4-ketohexose and NADH have been unambiguously demonstrated to be the reaction intermediates on the enzyme surface. Stereospecific return of the hydride from NADH to the opposite face of the oxidized hexose moiety completes the catalytic process (1–7). Most of the studies on mechanism, quaternary structure, and active site have been carried out with the *Kluyveromyces fragilis* and *Escherichia coli* enzymes. 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× 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 (UDP-AmNS)[1] 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.

### MATERIALS AND METHODS

All biochemicals unless otherwise stated were purchased from Sigma. [1-$^{14}$C]Phenylglyoxal (2.17 mCi/mmol) was purchased from

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[1]The abbreviation used is: UDPAmNS, uridine-5′-diphosphoro-β-1-(5-sulfonic acid) naphthylamidate.

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.

*Enzymes 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 glycylglycine 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 glycylglycine buffer, pH 8.8, and 30 μg of bovine serum albumin, the requisite amount of epimerase was taken, and reaction was initiated 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 parallely 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*—UDP-AmNS and other AmNS derivatives were synthesized and purified according the methods described in our preceeding 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 phenyglyoxal was carried out in 0.05 M potassium phosphate buffer, pH 8.0. The reagent was dissolved in dimethyl sulfoxide, which had no effect on the stability of the enzyme. Modification with 1,2-cyclohexanedione and 2,3-butanedione was carried out in 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 0.5 M hydroxylamine, which on dilution during assay had no effect on the activity of the control enzyme.

*Incorporation of [$^{14}$C]Phenylglyoxal*—[1-$^{14}$C] 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 mM [1-$^{14}$C] phenylglyoxal ($1.2 \times 10^6$ cpm/μmol) 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: $1/(F/F_0 - 1) = K_d/Q{\cdot}n{\cdot}P + 1/Q$, where $F_0$ and $F$ represent the fluorescence intensity of UDPAmNS in the absence and in presence of the *E. coli* epimerase 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*-nitrophenyluridyl pyrophosphate (0.21 mM) (24, 25).

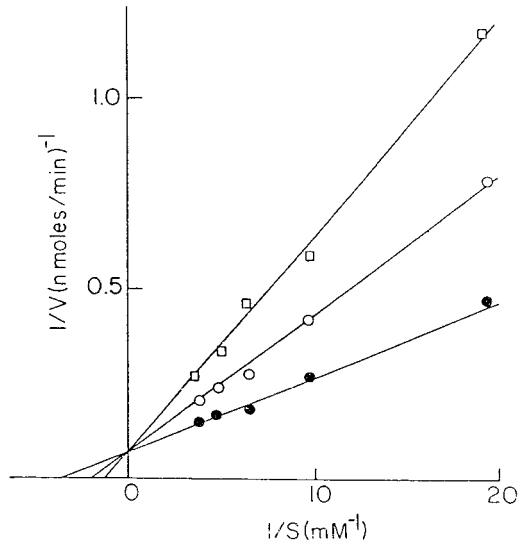



FIG. 1. **Inhibition kinetics of epimerase with UDPAmNS.** The inhibition kinetics was carried out by two-step assay method as described under "Materials and Methods." UDPAmNS concentrations for the different sets are no UDPAmNS (●), 0.23 mM (○), and 0.46 mM (□). Blank was also done containing no epimerase. The $K_i$ for UDPAmNS was 0.20 mM.

*Conformational Transition of UDPAmNS on the Enzyme Surface*—Fig. 2*A* 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. 2*A, 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 dimethylsulfoxide or after phosphodiesterase bond cleavage (previous paper, Fig. 1; Ref. 15).

Fig. 2*B* 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 ($\lambda_{em}$ = 453 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-

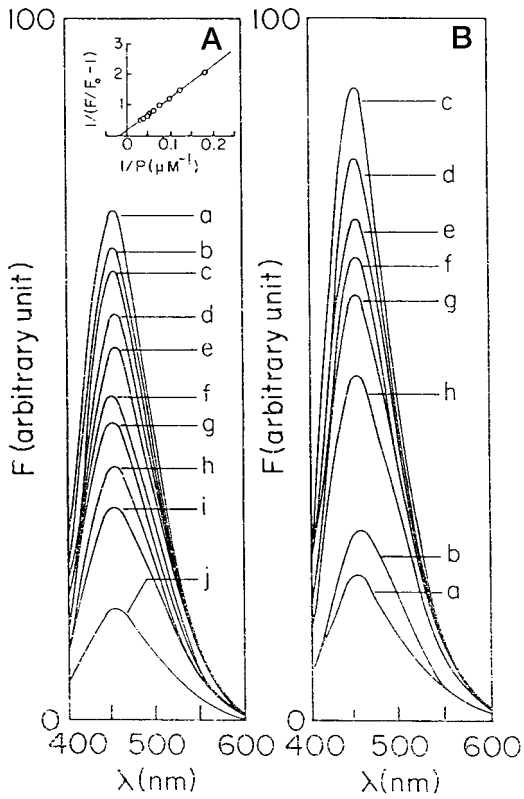



FIG. 2. *A,* destacking of UDPAmNS on interaction with epimerase. UDPAmNS (9.3 $\mu$M) and *E. coli* epimerase (27.8 $\mu$M) were taken in 100 mM potassium phosphate buffer, pH 8.0, and excited at 360 nm, and the emission spectrum was scanned. It was then serially diluted with 9.3 $\mu$M UDPAmNS in the same buffer to vary the enzyme concentration keeping the fluorophore concentration constant and similarly scanned at every step. The spectra at different epimerase concentrations ($\mu$M) in the presence of 9.3 $\mu$M UDPAmNS were: *a,* 27.83; *b,* 25.05; *c,* 22.26; *d,* 19.48; *e,* 16.70; *f,* 13.91; *g*, 11.13; *h,* 8.35; *i,* 5.57; and *j,* no epimerase. *Inset,* plot of $1/(F/F_0 - 1)$ against $1/P$. Here $F_0$ and $F$ represent the fluorescence intensity of UDPAmNS in the absence and presence of the epimerase at an excitation wavelength of 360 nm after subtracting that attributable to free protein. $P$ is the epimerase concentration. The intercept of the plot on the $1/(F/F_0 - 1)$ axis gives $1/Q$, where $Q$ is the quantum enhancement or the enhancement that would occur when all the fluorophore is bound to the protein (see "Materials and Methods). *B,* displacement of UDPAmNS from the epimerase by 5′-UDP. *E. coli* epimerase (20 $\mu$M) and UDPAmNS (9.3 $\mu$M) were taken in 200 $\mu$l of 100 mM potassium phosphate buffer, pH 8.0, and excited at 360 nm, and the emission spectrum was taken (*c*). To it were added small aliquots (1–10 $\mu$l) of concentrated stock solution of 5′-UDP, and the spectrum was similarly scanned. The final concentration of 5′-UDP are represented by 2 (*d*), 4 (*e*), 8 (*f*), and 16 (*g*) $\mu$M and (h) 1 mM. The fluorescence spectra of free enzyme and free UDPAmNS in buffer are represented by *a* and *b,* respectively.

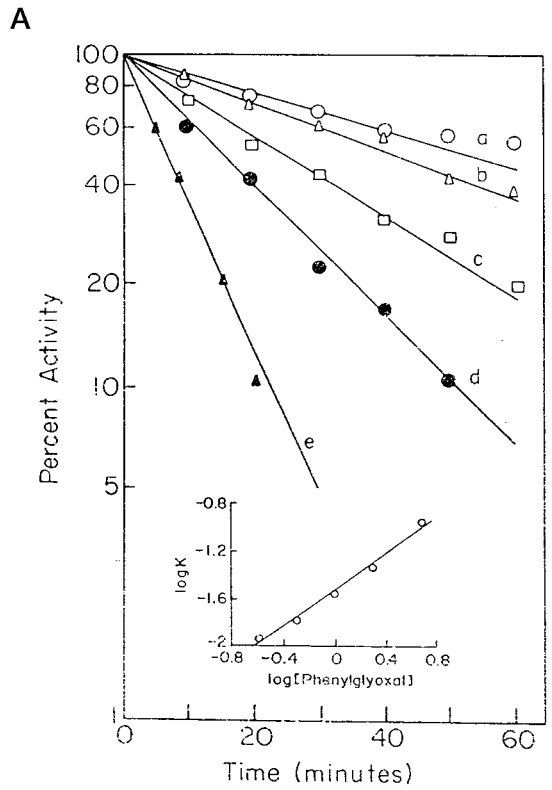
strate for epimerase is negatively charged at the cellular pH, we decided to explore the possible involvement of cationic arginine residue(s) in the binding of the substrate. We had earlier shown the presence of an essential arginine residue at the substrate binding site of the *K. fragilis* enzyme (11). Incubation of epimerase with phenylglyoxal led to a progressive loss of enzyme activity. The inactivation followed a pseudo-first order kinetics. A plot of log$K$ *versus* log of reagent concentration (27) resulted in a straight line with a slope close to unity (Fig. 3*A*), indicating that at least 1 mol of phenylglyoxal/mol of enzyme was required to produce inactivation. Because phenylglyoxal has occasionally been shown to react with residues other than arginine (28), two highly selective arginine-modifying reagents, cyclohexanedione (29) and butanedione (30), were also used to modify the enzyme. In both cases phosphate buffer was replaced by borate to stabilize the adducts (28). With cyclohexanedione also, epimerase was completely inactivated following a pseudo-first order kinetics, and a reaction order of 1 was obtained (Fig. 3*B*). Similar results were obtained with 2,3-butanedione (data not shown). Notwithstanding some limitations in this kinetic method (31), because the kinetic order of inactivation was close to 1 in all cases, the minimal number of arginine residue(s) that were involved in the inactivation process could be taken to be 1.

A specific test for modification of essential arginine residue(s) is the regeneration of activity in presence of hydroxylamine (29). The enzyme modified by cyclohexanedione and inactivated to <10% of its original activity could be completely reactivated when the inactivated enzyme was diluted 20-fold in 0.5 M neutralized hydroxylamine in 50 mM potassium phosphate buffer, pH 7.0 (data not shown). In contrast, when the enzyme was inactivated with phenylglyoxal, it could not be significantly reactivated. Such irreversible modification is indicative of the formation of a stable diphenylglyoxal derivative (32). To establish the stoichiometry of phenylglyoxal to arginine also as the number of arginine residues that are modified during the process of inactivation, both spectrophotometric analysis and radiolabeling with [1-$^{14}$C]phenylglyoxal were carried out. For this purpose, epimerase (0.8 mg/ml) was incubated with phenylglyoxal (2.5 mM) in 0.05 mM potassium phosphate buffer, pH 8.0, for 45 min at 28 °C. The inactivated enzyme (>95%) was freed of excess reagent in a spin column and a differential spectrum was obtained with the untreated enzyme as the control. The differential spectrum with $\lambda_{max}$ at 250 nm was characteristic of a diphenylglyoxal derivative (data not shown). Using a molar absorption coefficient of 11,000 M/cm for the diderivative (32), 3.1 arginine residues were found to be modified in the process. In a parallel experiment with [1-$^{14}$C]phenylglyoxal, 5.8 mol of phenylglyoxal was found to be incorporated per mole of the enzyme under identical conditions, confirming the stoichiometry of phenylglyoxal to arginine to be 2:1.

The spectral signal at 250 nm for the formation of the diphenylglyoxal derivative provided a handle to directly relate the extent of arginine modification with loss of activity. Fig. 4 shows that although all the three arginine residues are reactive, the loss of activity *versus* number of arginine residues modified extrapolates to 1 at zero activity of the enzyme. The excellent correspondence between this direct method and the kinetic method leaves little doubt that at least 1 arginine residue is intimately associated with the overall catalytic process. The usefulness of such a combination of methods was first demonstrated by Hayman and Coleman (31) for isocitrate dehydrogenase.

*Location of the Essential Arginine Residue*—Protection experiments with UMP or UDP convincingly demonstrated that the essential arginine residue is indeed located in the substrate binding region of the active site. Fig. 5 shows that the rate of inactivation of phenylglyoxal is progressively decreased as the concentration of UMP is increased. A plot of $t_{1/2}$ of inactivation against the concentration of 5′-UMP was linear (Fig. 5, *inset*). The intercept on the abscissa of such a plot gives the dissociation constant ($K_d$) of the enzyme-protecting agent complex (27). The $K_d$ of the epimerase-UMP complex was calculated to be 1.9 mM, which is in excellent agreement with the $K_d$ for UMP (1.9 mM) obtained kinetically (22). The experiment with UDP also gave strong protection. Based on this protection experiment, the essential arginine residue was placed in the binding subsite for the nucleotide phosphates.

*The Essential Arginine Residue Is Critical for Stretching and Binding of the Substrate*—To ascertain the specific role of the essential arginine residue located at the uridylphosphoryl



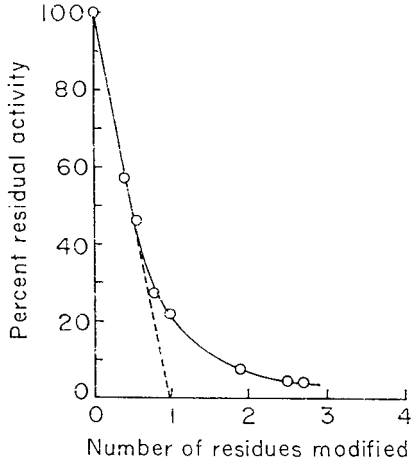




FIG. 4. **Correlation between loss of activity and number of arginine residues modified on phenylglyoxal treatment of the epimerase.** The enzyme (0.58 mg/ml) was treated with 2.5 mM phenylglyoxal in 0.05 M potassium phosphate buffer, pH 8.0, containing 1 mM EDTA, at 28 °C for 60 min. At intervals of 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 ($\epsilon$ = 11,000 M/cm).

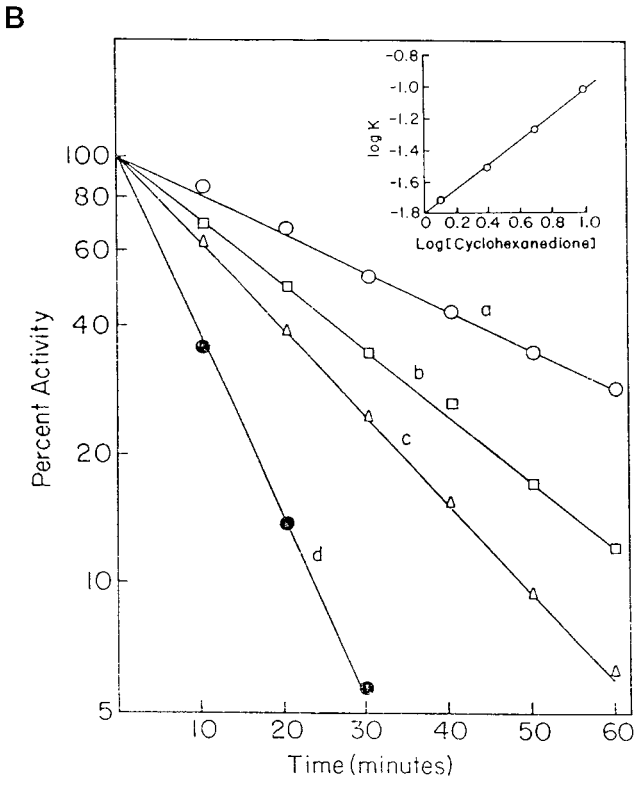



FIG. 3. *A,* inactivation of epimerase by phenylglyoxal. The enzyme (7.5 $\mu$g/ml) was incubated with varying concentrations of phenylglyoxal in 0.05 M potassium phosphate buffer, pH 8.0, at 28 °C. At the indicated intervals, aliquots were withdrawn for measuring enzyme activity. The phenylglyoxal concentrations in the various runs were 0.25 mM (○), 0.5 mM (△), 1 mM (●), 2 mM (▲) and 5 mM (□). *Inset,* plot of puedo-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-cyclohexanedione. The enzyme (6 $\mu$g/ml) was incubated with increasing concentrations of cyclohexanedione 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 cyclohexanedione concentrations at various runs were 1.25 mM (○), 2.5 mM (■), 5 mM (△), and 10 mM (●). *Inset,* plot of log psuedo-first order rate constant (*K*) of cyclohexanedione inactivation reaction *versus* log of cyclohexanedione concentration. The slope of the plot is 0.8.

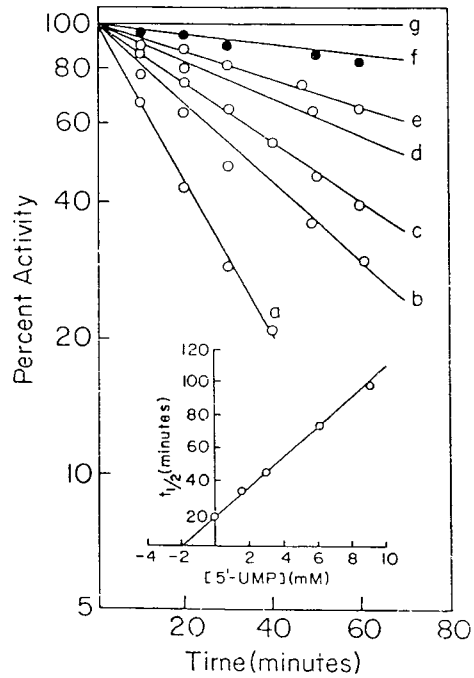



FIG. 5. **Protection against inactivation by phenylglyoxal by uridyl phosphates.** Epimerase (10 $\mu$g/ml) in 0.05 mM potassium phosphate buffer, pH 8.0, was treated with 2.5 mM phenylglyoxal in the absence or presence of increasing concentrations of 5′-UMP. *a–e,* UMP (mM) concentrations of 0, 1.5, 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.

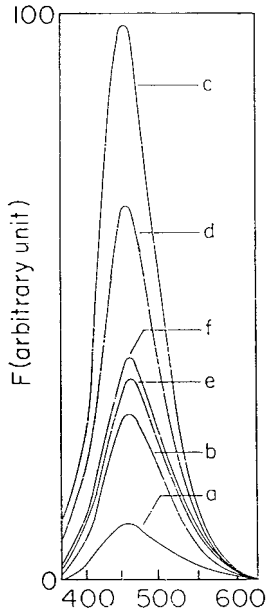



FIG. 6. **Interaction of UDPAmNS with native and modified epimerase.** *E. coli* epimerase (27 $\mu$M) was incubated with 5 mM phenylglyoxal in 100 mM potassium buffer, pH 8.0, for 2 h till complete inactivation. It was then passed through a Sephadex G-50 spin column to remove excess reagent. 27 $\mu$M unmodified enzyme was also similarly passed through the spin column. At an excitation wavelength of 360 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 mM UDP; *f,* phenylglyoxal-treated epimerase (27 $\mu$M) and UDPAmNS (9.3 $\mu$M).

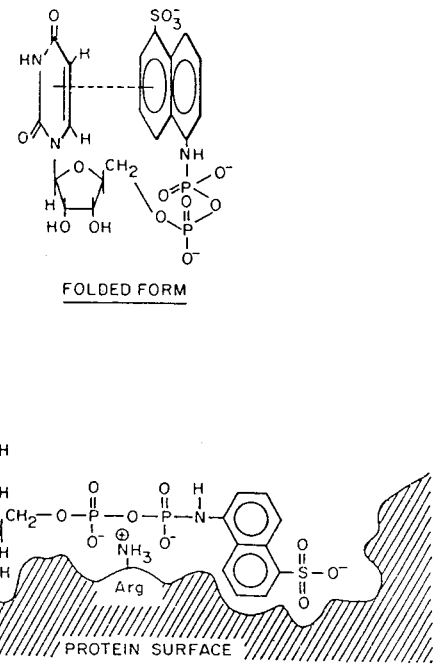



FIG. 7. **Schematic diagram depicting the binding of UDPAmNS at the substrate binding site of *E. coli* epimerase.**

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. The conformational change that the ligand underwent on the enzyme surface could be monitored quite conveniently only because this transition was accompanied by significant enhancement of fluorescence attributable to release of stacking and consequential absence of collisional quenching.

## 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 strongly 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. 2*A*) 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.

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-butanedione-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

x-ray and sequence homology data (13, 14). Resolution at ×2.0 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.

REFERENCES


1. Gabriel, O., Kalckar, H. M., and Darrow, R. A. (1975) in *Subunit Enzymes: Biochemistry and Fuction* (Ebner, K. S., ed) pp 85–133, Marcel Dekker, New York
2. Frey, P. A. (1987) in *Pyridine Nucleotide Cenzymes: Chemical, Biochemical and Medical Aspects* (Dolphin, D., Poulson R., and Avromovic, O., eds) Vol. 2B, pp. 462–467, John Wiley & Sons, New York
3. Wilson, D. B., and Hogness, D. S. (1964) *J. Biol. Chem.* **239,** 2469–2481
4. Maitra, U. S., and Ankel, H. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 2660–2663
5. Nelsestuen, G. L., and Kirkwood, S. (1971) *J. Biol. Chem.* **246,** 7533–7543
6. Adair, W. L., Jr., Gabriel, O., Ullrey, D., and Kalckar, H. M. (1973) *J. Biol. Chem.* **248,** 4635–4639
7. Wee, T. G., and Frey, P. A. (1973) *J. Biol. Chem.* **248,** 33–40
8. Ray, M., and Bhaduri, A. (1980) *J. Biol. Chem.* **255,** 10782–10786
9. Bhattacharjee, H., and Bhaduri, A. (1992) *J. Biol. Chem.* **267,** 11714–11720
10. Mukherji, S., and Bhaduri, A. (1992) *J. Biol. Chem.* **267,** 11709–11713
11. Mukherji, S., and Bhaduri, A. (1986) *J. Biol. Chem.* **261,** 4519–4524
12. Bauer, A. J., Rayment, I., Frey, P. A., and Holden, H. M. (1992) *Proteins Struct. Funct. Genet*. **12,** 372–381
13. Thoden, J. B., Frey, P. A., and Holden, H. M. (1996) *Biochemistry* **35,** 2557–2566
14. Thoden, J. B., Frey, P. A., and Holden, H. M. (1996) *Biochemistry* **35,** 5137–5144
15. Dhar, G., and Bhaduri, A. (1999) *J. Biol. Chem.* **274,** 14568–14572
16. Helmriech, E. J. M., and Hofmann, K. P. (1996) *Biochim. Biophys. Acta* **1286,** 285–322
17. Ray, M., and Bhaduri, A. (1975) *J. Biol. Chem.* **250,** 4373–4375
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem* **193,** 265–275
19. Maniatis, J., Fristsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual,* pp. 466–467, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
20. Mas, M. T., and Colman, R. F. (1986) *Biochemistry* **24,** 1634–1646
21. Pal, D. K., and Bhaduri, A. (1971) *Biochim. Biophys. Acta* **250,** 588–591
22. Wong, S. S., and Frey, P. A. (1978) *Biochemistry* **18,** 3551–3556
23. Flentke, G. R., and Frey, P. A. (1990) *Biochemistry* **29,** 2430–2436
24. Wong, Y.-H., and Frey, P. A. (1979) *Biochemistry* **18,** 5337–5341
25. Wong, S. S., and Frey, P. A. (1977) *Biochemistry* **16,** 298–305
26. Vanhooke, J. L., and Frey, P. A. (1994) *J. Biol. Chem.* **269,** 31496–31504
27. Levy, H. M., Leber, P. A., and Ryan, I. M. (1963) *J. Biol. Chem.* **238,** 3654–3659
28. Takahashi, K. (1968) *J. Biol. Chem.* **243,** 6171–6179
29. Patthy, L., and Smith, E. L. (1975) *J. Biol. Chem.* **250,** 557–564
30. Rioidan, J. F. (1973) *Biochemistry* **12,** 3915–3923
31. Hayman, S., and Colman, R. F. (1978) *Biochemistry* **17,** 4161–4168
32. Takahashi, K. (1977) *J. Biochem. (Tokyo)* **81,** 403–414
33. Schulz, G. E. (1992) *Curr. Opin. Struct. Biol.* **2,** 61–67