THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 267, No. 17, Issue of June 15, pp. 11714-11720, 1992
Printed in U.S.A.

Distinct Functional Roles of Two Active Site Thiols in UDPglucose 4-Epimerase from *Kluyveromyces fragilis**

(Received for publication, June 3, 1991)

Hiranmoy Bhattacharjee‡ and Amar Bhaduri

From the Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Calcutta 700 032, India

UDPglucose 4-epimerase from *Kluyveromyces fragilis* was earlier shown to have two conformationally vicinal thiols at the active site. Upon treatment with diamide, these thiols form a disulfide linkage across the subunits that results in coordinated loss of catalytic activity and coenzyme fluorescence (Ray, M., and Bhaduri, A. (1980) *J. Biol. Chem.* 255, 10777–10786). Employing a number of thiol-specific reagents, we now suggest discriminatory and nonidentical roles for these two thiols. Kinetic and statistical analysis of 5,5′-dithiobis-(2-nitrobenzoic acid) and *N*-ethylmaleimide modification reaction of epimerase show that only one thiol is essential for activity. Consecutive modification experiments clearly show that the same active thiol is modified in both cases. However, significant differences are observed when the reactivity of these reagents is monitored in terms of coenzyme fluorescence. Treatment with *N*-ethylmaleimide leads to a form of inactive enzyme that fully retains its fluorescent properties whereas modification with 5,5′-dithiobis-(2-nitrobenzoic acid), on the other hand, results in the loss of both activity and fluorescence. The closely spaced nonessential second thiol, which is not modified by *N*-ethylmaleimide is therefore involved in generating and maintaining the coenzyme fluorescence. Modification studies with a series of spin-labeled maleimide shows that only 3-(maleimidomethyl)proxyl causes partial quenching of coenzyme fluorescence. This suggests that the active thiol is situated at a distance of 4.5 Å approximately from the coenzyme fluorophore.

UDPglucose 4-epimerase (EC 5.1.3.2) catalyzes a freely reversible epimerization between UDPglucose and UDPgalactose. The enzyme isolated from *Kluyveromyces fragilis* is a dimer of apparently identical subunits. The holoenzyme contains 1 mol of NAD^+ as an obligatory coenzyme that is tightly but noncovalently bound per mol of the dimeric apoenzyme (1–4). In addition to the protein fluorescence, the yeast enzyme possesses a characteristic coenzyme fluorescence with an excitation maximum at 353 nm and emission maximum at 435 nm, which closely resembles the fluorescence of native NADH, except that a significant blue shift is observed in this case (5). The exact chemical nature of the fluorophore is not known, but since the pyridine moiety of the coenzyme is involved, this has been exploited by us as a direct fluorimetric monitor for the changes at the catalytic region of the active site of the enzyme (6–8).

In a classic paper in 1958, Maxwell *et al.* (9) showed that the partially purified fluorescent enzyme could be completely inactivated by the addition of excess *p*-chloromercuribenzoate (pCMB).[1] The inactivated enzyme regained part of its activity only when both 2-mercaptoethanol and exogenous NAD were added to the medium. This observation implicated both NAD and one or more thiols in the catalytic activity of the enzyme. Since then, the catalytic role of NAD as a true enzyme-bound cofactor has been established through the efforts of several groups of workers. UDP-4-ketohexose and NADH have been identified unambiguously as enzyme-bound reaction intermediates (10–14). The role of thiols in the activity of the enzyme, however, remained to be elucidated. The native dimer has a total of 14 free thiols that are titratable by pCMB (15). Since addition of pCMB resulted in the collapse of the quarternary structure leading to mercurated monomers and free NAD, the functional role of the thiols, if any, remained inconclusive (2, 4). Using DTNB as the modifying reagent that retains the quarternary structure, Ray *et al.* (16) could get some preliminary evidence to suggest the presence of essential thiol(s) at the active site of the enzyme. This work was further extended with diazene dicarboxylic acid bis(*N,N*-dimethylamide) (diamide), a compound that can oxidize suitably placed vicinal thiols on the protein surface to disulfides. Excellent correlation between the loss of activity and the loss of fluorescence and parallel regeneration of both of the properties followed by titration of the available free thiols at various stages established the presence of two conformationally vicinal thiol residues in the catalytic region of the active site of the enzyme (6). The specific role of these two thiols in the activity of the enzyme and also in generation of coenzyme fluorescence, however, remained unexplored. Using a wide range of modifying reagents, we have now been able to show that only one thiol is involved in activity whereas modification of the other thiol invariably leads to the loss of coenzyme fluorescence. Furthermore, introduction of spin-labeled and fluorescent maleimide derivatives has revealed some detailed features of the neighboring region around the active thiol.

MATERIALS AND METHODS

All biochemicals, unless otherwise stated, were purchased from Sigma. [^{14}C]NEM (4 mCi/mmol) was purchased from Amersham Corp. Highly purified and essentially homogeneous UDPglucose 4-epimerase with specific activity of 40–60 units/mg of protein was prepared from *K. fragilis* according to the method of Darrow and Rodstrom (3). Details of the assay and other relevant information are given in the preceding paper (8).

* This work was funded by Project SP/SO/D43/85 from the Dept. of Science and Technology, Government of India. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Senior Research Fellow, Dept. of Science and Technology.

[1] The abbreviations used are: pCMB, *p*-chloromercuribenzoate; BAL, 2,3-dimercaptopropanol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; MMTS, methyl methanethiolsulfonate; Me_2SO, dimethyl sulfoxide, NBD-CL, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; PM, *N*-(1-pyrenyl)maleimide.

Rate measurements were carried out using a Hitachi U3200 spectrophotometer. Fluorescence measurements were carried out with a Perkin-Elmer Cetus Instruments spectrofluorimeter, model MPF-44B. Before determination of fluorescence of modified epimerase, modifying reagents and their reaction products were removed by passage through a spin column as described by Maniatis *et al.* (17).

Determination of Sulfhydryl Groups—The number of sulfhydryl groups reacting with DTNB was determined by the method of Ellman (18). The number of sulfhydryl residues modified by NEM was determined by the following procedure. Epimerase, 7.5 nmol, was incubated with 2.54 μmol of [^{14}C]NEM (1×10^7 cpm/μmol) in 1.0 ml of 0.02 M sodium phosphate buffer, pH 7.0. For measurement of the [^{14}C]NEM incorporated, aliquots were removed at appropriate intervals, and the reaction was quenched by adding 50 mM 2-mercaptoethanol. The aliquots were next passed through a spin column, and the radioactivity incorporated in epimerase was determined in a Beckman LS 5000 TD scintillation counter.

Inactivation Experiments—Fresh solution of various thiol modifying reagents were prepared as outlined below. DTNB was dissolved in 0.2 M sodium phosphate buffer, pH 7.0; NEM, MMTS, and sodium arsenite/BAL were dissolved in water, while *o*-iodosobenzoate, NBD-CL, spin labels, and PM were dissolved in Me_2SO. At the concentrations employed, Me_2SO had no effect on epimerase alone in the control tube.

About 0.3–0.5 nmol of epimerase was incubated with varying amounts of thiol reagents, in 0.1 ml of 0.02 M sodium phosphate buffer, pH 7.0 (or 0.02 M sodium phosphate buffer, pH 8.0, or 0.05 M glycylglycine buffer, pH 8.8) at 25 °C. Aliquots were thereafter assayed for epimerase activity. Reactivation experiments were performed by adding 50 mM DTT to the reaction mixture and incubating for 30–40 min.

RESULTS

Modification of Epimerase with DTNB—Ray and Bhaduri (6) had earlier shown, with diamide as the modifying reagent, the presence of two closely spaced thiols in the neighborhood of the pyridine fluorophore of *K. fragilis* enzyme. Formation of a disulfide bond between the subunits and coordinated loss of activity and coenzyme fluorescence led to such a conclusion. In order to further explore the specific roles of these thiols in catalysis and in maintenance of coenzyme fluorescence, modification studies with some established monospecific thiol reagents were carried out. The yeast epimerase was found to be rapidly inactivated by DTNB at higher alkaline pH. The rate of inactivation was dependent on pH of the medium and increased significantly with increasing pH (data not shown). Thus, incubation of 10 μM enzyme with 2 mM DTNB in 50 mM glycylglycine buffer, pH 8.8, resulted in a near complete loss of activity within 30 min. Fig. 1*a* shows that inactivation with DTNB at any particular concentration follows a pseudo-first order kinetics. The reaction order for inactivation with DTNB was calculated from a plot of log *k* against log reagent concentration (19). The reaction order obtained from the slope of the plot was approximately 1 (Fig. 1*a*, *inset*), indicating that modification of 1 thiol residue on the enzyme surface could be related with the loss of catalytic activity. When the number of thiol groups reacting during the course of inactivation was determined by following the increase in absorbance at 412 nm, 4 ± 0.2 thiol residues were found to be titrated during the course of inactivation (data not shown).

The monophasic pseudo-first order inactivation kinetics (Fig. 1*a*) and titration kinetics suggested that the statistical method of Tsou (20), as further elaborated by Horiike and McCormick (21), can be employed in this case to determine the number of catalytically essential residues. The number of essential groups is determined simply from quantitation of the number of groups modified and the residual activity in samples of partially modified protein. The simplest case is of an enzyme with a total of *n* residues of type A, all equally reactive toward the reagent, but only *i* of which are essential for activity. The fraction of each essential group unmodified

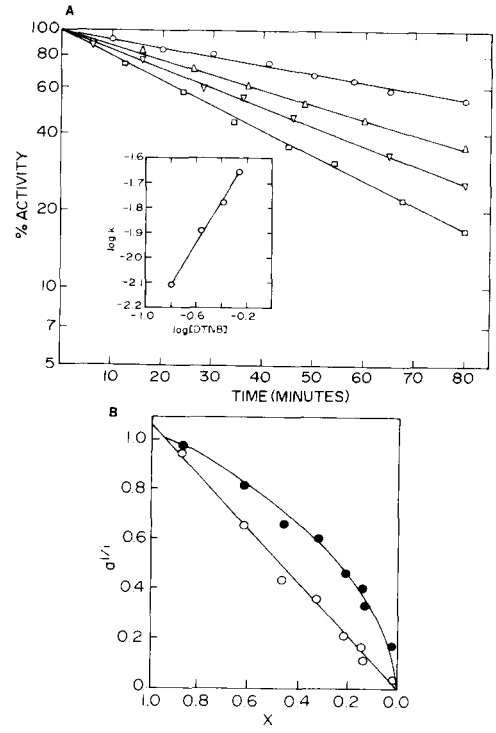

FIG. 1. *a*, inactivation of UDPglucose 4-epimerase by DTNB. The enzyme (0.05 mg/ml) was incubated in different tubes with varying concentrations of DTNB in 0.05 M glycylglycine buffer, pH 8.8, at 25 °C. At the indicated time, aliquots were removed for measurement of enzyme activity. The DTNB concentrations in various runs were: ○——○, 0.16 mM; △——△, 0.27 mM; ▽——▽, 0.42 mM; □——□, 0.55 mM. *Inset*, plot of the log of pseudo-first order rate constant (*k*) of DTNB inactivation reaction *versus* log of DTNB concentration. *b*, the relation between the fraction of activity remaining (*a*) and the fraction of thiol remaining (*X*) for the reaction of DTNB with epimerase. The data are plotted assuming one essential thiol ($i = 1$) (○) and two essential thiols ($i = 2$) (●). For this experiment, epimerase (0.6 mg/ml) in 20 mM sodium phosphate buffer, pH 8.0, was incubated with 2 mM DTNB. The modification of thiols was followed by monitoring the increase in absorbance at 412 nm. The loss of enzymatic activity was measured by coupled assay.

is equal to the total fraction, *X*, of type A residues remaining. The relation between *X* and the fraction of the activity remaining, *a*, is given by $a^{1/i} = X$. The value of *i* is found from a plot of *X versus* $a^{1/i}$ which gives the best straight line. Fig. 1*b* shows the result of such an analysis. Clearly, this study shows that although 4 thiol residues are reactive to DTNB, only 1 thiol residue could be directly correlated with the catalytic activity of the enzyme.

Interestingly, the presence of substrate or UMP, a powerful competitive inhibitor for the enzyme, failed to afford any protection against inactivation by DTNB. The enzyme inactivated with DTNB could be partially reactivated (45–65%) on incubation with DTT. Unlike in the case of inactivation with pCMB (2, 4), no exogenously added NAD was needed for the reactivation process, suggesting that the cofactor was retained on the enzyme surface. This was further confirmed by direct fluorimetric estimation of NAD on the inactivated enzyme (22). Inactivation with DTNB resulted in a progressive and nearly parallel loss of coenzyme fluorescence (data not shown). When the inactivated enzyme was reactivated with DTT, coenzyme fluorescence was also partially restored along with regeneration of partial activity. Fig. 2*a* shows that, in a typical experiment, the enzyme regained about 50% of its native activity along with 50% of its native fluorescence. These results led us to conclude that formation of a disulfide

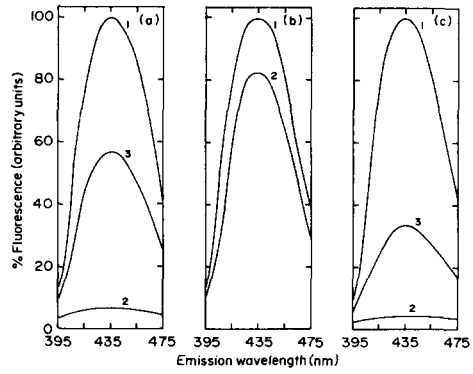

FIG. 2. **Effect of thiol-modifying reagents on coenzyme fluorescence.** *a*, DTNB-inactivated epimerase; coenzyme fluorescence of native epimerase (*1*), DTNB-inactivated epimerase (*2*), and after reactivation of (2) with DTT (*3*). *b*, NEM-inactivated epimerase; coenzyme fluorescence of native epimerase (*1*) and NEM-inactivated enzyme (*2*). *c*, *o*-iodosobenzoate-inactivated epimerase; coenzyme fluorescence of native enzyme (*1*), after *o*-iodosobenzoate inactivation (*2*), and after reactivation of (2) with DTT (*3*). Excitation wavelength, 353 nm.

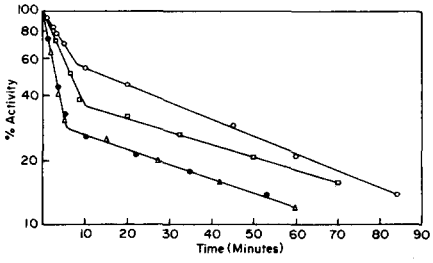

FIG. 3. **Inactivation of epimerase by NEM and PM.** Epimerase (0.3 mg/ml) in 20 mM sodium phosphate, pH 7.0, was incubated with 10 mM NEM (○——○) or 2 mM PM (△——△). For protection against PM inactivation, 1.0 mM UMP (●——●) or 1.2 mM UDPGal (□——□) was employed.

bond as seen earlier with diamide experiments (6, 23) is not necessary for loss of activity or coenzyme fluorescence. Modification of the catalytically essential thiol alone or simultaneous modification of other accessible thiol(s) also results in a coordinated loss of activity and fluorescence of the enzyme.

Modification of Epimerase with NEM—A fresh insight in the functional role of the reactive thiols was obtained when *N*-ethylmaleimide was introduced as the modifying agent. NEM could fairly rapidly inactivate the enzyme. The overall kinetics of inactivation at about 10 mM NEM is presented in Fig. 3. It is evident that the plot is not linear as it would have been if the modifiable residues were alkylated at the same rate. The biphasic kinetics suggested that some of the cysteine residue(s) are much more accessible to the reagent than the rest. To analyze this result further, the total number of thiol residues modified by NEM was estimated quantitatively. We found that on complete inactivation, 2.9 nmol of [^{14}C]NEM was incorporated per nmol of dimeric enzyme (data not shown). As the two groups of modifiable thiol residues react at widely different rates, a kinetic and graphical method developed by Ray and Koshland (24) was used to analyze such a situation. The Ray and Koshland equation can be represented as:

$$x = f_1\, e^{-k_1 t} + f_2\, e^{-k_2 t}$$

where x is the total fraction of residues remaining after reaction time t, f_1 and f_2 are the fractions of the total thiols of the intact enzyme comprising each of the two reactivity groups, and k_1 and k_2 are the first order rate constants for the corresponding groups. Graphical analysis of the data in Fig. 4 resulted in the following equation,

$$x = 0.4\, e^{-1.054t} + 0.6\, e^{-0.068t}$$

The values of 0.4 and 0.6, when multiplied by the total number of modifiable thiols by NEM, *i.e.* 3, indicates that the enzyme contains one accessible thiol and two relatively inaccessible ones. The pseudo-first order rate constants are 1.054 and 0.068, respectively, indicating that the ratio of reactivity between the accessible and relatively inaccessible group of thiols is approximately 15:1.

We thus find that as in case of modification with DTNB, modification with NEM also implicates only 1 thiol residue as essential in terms of overall enzyme activity. However, a very striking and significant difference was observed when the coenzyme fluorescence of the NEM-modified enzyme retained nearly full (80–85%) coenzyme fluorescence (Fig. 2*b*). Thus, in contrast to treatment with diamide (23) and DTNB (Fig. 2*a*) that generated nonfluorescent, inactive enzyme species, treatment with NEM led to inactive but fluorescent enzyme molecules. Obviously, loss of coenzyme fluorescence is dependent upon the simultaneous modification of an additional thiol residue other than the essential thiol. Clearly, this additional thiol is not accessible to modification by NEM.

As in the case of inactivation with DTNB, the inactivation kinetics with NEM remained completely unaffected in the presence of substrate or UMP. However, when *N*-(1-pyrenyl)maleimide (PM), a substantially bulkier analogue of NEM, was used as the inactivating agent, a small but significant protection was provided by UDPGal (Fig. 3). UMP,

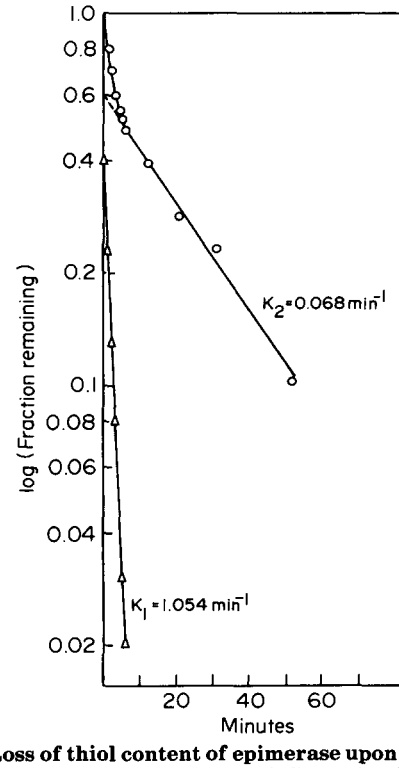

FIG. 4. **Loss of thiol content of epimerase upon modification with [^{14}C]NEM.** The enzyme (0.9 mg/ml) was incubated with 2.5 mM [^{14}C]NEM, in pH 7.0 sodium phosphate buffer. At each indicated time, the number of thiols modified was followed by incorporation of [^{14}C]NEM, and the fraction of thiols remaining (○——○) was calculated by taking the total number of such modifiable residues (2.9) as unity. The fast phase of modification (△——△) was obtained by subtracting the contribution of slow phase (– – –) and replotting the difference.

however, failed to show any protection. Implications of these results are discussed later.

To determine whether the essential thiol residue separately identified by DTNB and NEM modification studies is indeed the same thiol or not, protection experiments by consecutive modification with both reagents were carried out. In these experiments, we took advantage of the fact that in contrast with NEM, modification with DTNB is reversible in nature. In one of the experiments, the enzyme was first inactivated with DTNB and then further treated with NEM. If both the reagents were modifying the same thiol for inactivation, modification with DTNB would protect the thiol against subsequent reaction with NEM and hence activity would be at least partially restored after final incubation with DTT. If, on the other hand, the loss of activity by NEM was due to a distinctly separate thiol, initial modification with DTNB would fail to provide protection against subsequent irreversible modification by NEM. In this case, final exposure to DTT should fail to regenerate any activity. Table I summarizes the results of such an experiment. Set I and Set II are controls showing partially reversible and completely irreversible nature of inactivation by DTNB and NEM, respectively. Set III shows the experimental situation. The consecutively modified enzyme (Set III, b) regained 30% of the original activity when the enzyme was finally incubated with DTT. The control DTNB-inactivated enzyme (Set III, c) under identical conditions recovered 40% of the original activity. Obviously, in this case, DTNB has protected the same active thiol residue against modification by NEM. We therefore conclude that the loss of enzymatic activity is due to modification of a unique thiol that is accessible to both reagents. The comparatively lower reactivation in the experimental control (40%) (Set III, c) compared to the basic control (55%) (Set I, c) is due to the longer incubation period of 80 min for this experiment. The results were reproducible within ±5% in three separate experiments.

Consecutive modification in the reverse order revealed the role of a second thiol in the generation and maintenance of coenzyme fluorescence. In this case, the enzyme was first inactivated with NEM. The completely inactive but fluorescent enzyme was then further treated with DTNB. This led to a near complete loss of fluorescence (>70%) (data not shown). Clearly, DTNB is modifying a second thiol in the vicinity of the pyridine fluorophore that results in quenching of the coenzyme fluorescence. Obviously, simultaneous modification of this thiol along with the active thiol either by DTNB or by diamide results in a parallel loss of both coenzyme fluorescence and activity.

TABLE I

Consecutive modification experiment with DTNB and NEM

Experiment	Reaction condition	% activity remaining
Set I		
(a) Epimerase control		100
(b) Epimerase (0.01 mM) + DTNB (2 mM) (DTNB-inactivated epimerase)	Incubated for 40 min	3
(c) I(b) above + DTT (50 mM)	Incubated for another 40 min	55
Set II		
(a) Epimerase (0.01 mM) + NEM (10 mM) (NEM-inactivated epimerase)	Incubated for 40 min	5
(b) II(a) above + DTT (50 mM)	Incubated for another 40 min	5
Set III		
(a) DTNB-inactivated epimerase (I(b) above) + NEM (10 mM)	Total incubation time 80 min	0
(b) III(a) above + DTT (50 mM)	Incubated for another 40 min	30
(c) DTNB-inactivated epimerase (I(b) above) + DTT (50 mM)	Exposure to DTNB for 80 min and further incubated with DTT for 40 min	40

Modification Studies with Other Thiol-modifying Reagents—A large number of fairly specific thiol-modifying reagents have been developed. These reagents differ from one another in size, charge, hydrophobicity, and bulk. We used quite a few of such reagents to further extend our studies with DTNB and NEM. Some of the salient features of the results are summarized in Table II. All of the reagents listed caused fairly rapid inactivation although the reagent concentration had to be varied between 0.2 and 10 mM. Analyses of the inactivation kinetics of these reagents, according to the method of Levy *et al.* (19), showed that in each case loss in activity could be related to interaction of 1 mol of the modifying reagent with the enzyme.

o-Iodosobenzoate and arsenite-BAL are commonly used reagents for detecting conformationally vicinal thiols on the enzyme surface (25). Inactivation with these reagents coupled with loss of coenzyme fluorescence (Fig. 2*c*) confirms once again that at least a pair of vicinal thiols are located close to the pyridine fluorophore.

Interestingly, sodium arsenite by itself had no significant effect on enzymatic activity. Incubation of epimerase with equimolar concentrations of arsenite and BAL considerably enhanced the rate of inactivation. Fluharty and Sanadi (26) had earlier proposed that in some cases arsenite by itself cannot reach the functional site, but complex formation with BAL neutralizes the charge and allows the more lipophilic complex to be transported across a hydrophobic barrier. Further evidence for the location of the active thiol at a hydrophobic pocket was obtained when PM was used as the probing agent (see below).

Modification with MMTS leads to a minimum change in bulk at the modified site (27). The reagent seems to be highly reactive with yeast epimerase, as even at very low concentrations (0.2 mM) it could effectively inactivate the enzyme in 20 min. It is, therefore, unlikely that the loss of enzymatic activity is due to any major structural change at the coenzyme binding region.

As regards protection by the substrate, UDPGal (1 mM) does not provide any protection against inactivation by most of the reagents. Significant differences in the rate of inactivation in the presence of substrate was observed only in the case of PM (Fig. 3) and NBD-CL. In both these cases, UMP, a powerful competitive inhibitor, failed to provide any protection. These results show that only bulkier reagents like NBD-CL and PM experience steric hindrance in the presence of substrate while reacting with the active thiol. This may be due to the partial orientation of the bulky group toward the hexose binding region of the active site.

Characterization of the Neighborhood of the Active Thiol—The strong inactivating effect of the arsenite-BAL complex suggested the presence of a hydrophobic milieu around the essential thiol. This possibility was further explored by modification studies with pyrene maleimide. PM has been used as a polarity-sensitive probe (28). On modification of epimerase with PM, there is a rapid loss in activity of the enzyme (Fig. 3) and a corresponding rapid enhancement of the fluorescence of pyrene chromophore (Fig. 5). The severalfold enhancement of pyrene fluorescence, together with protection provided by substrate against inactivation by PM, strongly suggests that

TABLE II

Effect of various thiol-specific modifiers on epimerase

Thiol modifiers	Effect on activity			Effect on coenzyme fluorescence	Reversal of activity with DTT	Substrate protection	Number of thiols essential for activity
	Reagent concentration (mM)	pH	% residual activity (20 min)				
1. DTNB	2	8.8	15	+	+	−	1.0
2. *o*-Iodosobenzoate	2	7	29	+	+	−	1.0
3. MMTS	0.2	8	10	−	+	−	1.0
4. NBD-CL	1	7	14	+	+	+	1.0
5. NEM	10	7	45	−	−	−	1.0
6. PM	2.5	7	20	−	−	+	ND[a]
7. Sodium arsenite/BAL	1	7	18	+	−	−	1.0

[a] ND, not determined.

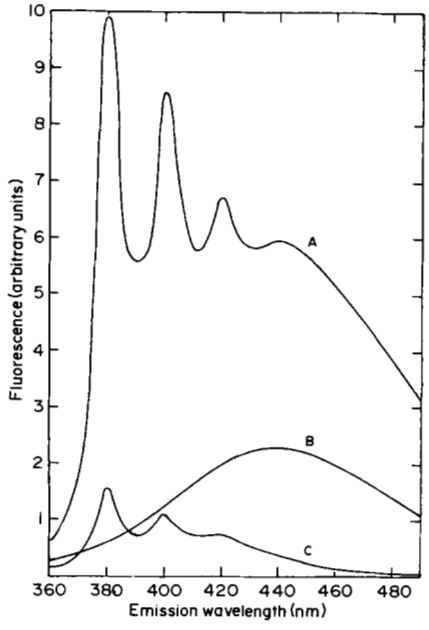

FIG. 5. **Emission spectrum of epimerase inactivated by PM (*A*), native enzyme (*B*), and free PM in sodium phosphate buffer, pH 7.0 (*C*).** Excitation wavelength = 342 nm.

catalytic thiol resides in a hydrophobic environment.

Effect of Spin-labeled Maleimide on Coenzyme Fluorescence—Spin-labeled maleimides are compounds containing stable nitroxide radical attached at different distances from the maleimide moiety. Their use stems from the observation that species with electron spin greater than zero can quench nearby excited electron states which fall within 4–6-Å range (29). An example of this quenching effect in biological systems has been demonstrated by Morrisett *et al.* (30) for the intrinsic tryptophan fluorescence of bovine serum albumin by spin-labeled fatty acid analogoues.

The effect on coenzyme fluorescence of epimerase after labeling with various spin-labeled maleimides are summarized in Table III. The fluorescence was recorded after the enzyme was completely inactivated by spin labels. It is evident from Table III that only 3-(maleimidomethyl)-proxyl causes partial quenching of coenzyme fluorescence compared to other spin labels, considering NEM as the reference standard. The distance, *d*, in Angstroms between the maleimide nitrogen and the nitrogen of the nitroxide radical has been calculated using a Dreiding model and considering the molecule in stretched conformation. Since this distance amounts to 4.5 Å for 3-(maleimidomethyl)-proxyl, it is reasonable to assume that the essential cysteine residue is located at an approximate distance of 4.5 Å from the fluorophore structure.

TABLE III

Quenching of coenzyme fluorescence by spin-labeled maleimides

Modifiers	Residual coenzyme fluorescence (F/F_o)	Distance, $N \rightarrow N$
		Å
1. None	1.0	
2. *N*-Ethylmaleimide	0.8	
3. 3-(Maleimido)-proxyl	0.84	3.6
4. 3-(Maleimidomethyl)-proxyl	0.60	4.5
5. 3-(2-Maleimidoethylcarbamoyl)-proxyl	0.90	8.4
6. 3-(2-(2-maleimidoethoxy)-ethyl carbamoyl)-proxyl	0.90	11.7

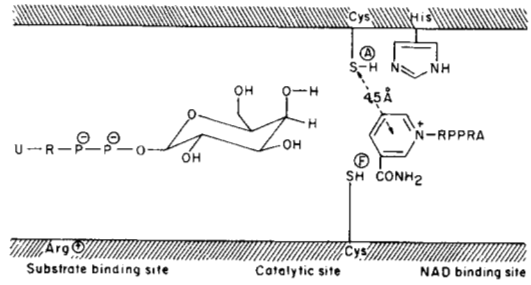

FIG. 6. **Schematic representation of the active site of UDPglucose 4-epimerase.** The thiols responsible for activity and fluorescence are denoted by Ⓐ and Ⓕ, respectively.

DISCUSSION

UDPglucose 4-epimerase needs NAD^+ as an obligatory coenzyme for catalysis. The active site for *K. fragilis* holoenzyme may, therefore, be conveniently divided in three regions, the substrate binding, coenzyme binding, and catalytic regions. This is shown in Fig. 6. The hexose moiety of the nucleotide sugar and the pyridine moiety of the coenzyme are located adjacently at the catalytic site for the hydride transfer and epimerization to take place. The pyridine fluorophore acts as a reporter for chemical changes at the catalytic region.

Our earlier work with diamide showed that formation of one disulfide bond across the subunits results in a coordinated loss of activity and coenzyme fluorescence. Both the activity and the fluorescence could be substantially restored on regeneration of the thiols (6, 23). Results obtained with *o*-iodosobenzoate (Table II) are also consistent with these earlier observations. Disulfide bonds are known to act as general quenchers of fluorescence on protein surfaces (31). The abolition of coenzyme fluorescence in this case is, however, not due to the formation of the disulfide bridge. This became evident when DTNB and NBD-CL were introduced as the modifying agents. These reagents do not normally generate

disulfide bridges. Still, coordinated loss of activity and fluorescence could be observed in both the cases (Fig. 2*a* and Table II). Obviously, disulfide bond formation is not essential either for loss of activity or for quenching of fluorescence. There are reports in literature that in some special cases, where thiols are closely spaced, reaction with DTNB may give rise to disulfide bond formation (25, 32). Employing sodium dodecyl sulfate-gel electrophoresis in the absence of mercaptoethanol, we could not find any evidence for formation of the disulfide bridge across the subunits.

The discriminatory and specific role of the reactive thiols in maintaining activity and fluorescence became apparent when NEM was used as the modifying agent. In this case, one can generate a completely inactive species that retains nearly all its coenzyme fluorescence (Fig. 2*b*). Quite clearly, catalytically essential thiol(s) have no role to play in generation and maintenance of pyridine fluorophore structure. Extensive kinetic analyses with all the modifying agents uniformly showed that modification of only one thiol group could be directly related to loss of activity (Figs. 1, *a* and *b* and 4 and Table II). Consecutive modification experiments (Table I) established the identity of this thiol as the unique thiol involved in maintaining the activity. When the second thiol in close proximity to this essential thiol is simultaneously modified by DTNB or by disulfide-forming reagents like *o*-iodosobenzoate (Table II) or diamide, a coordinated loss and regeneration of both activity and fluorescence is observed.

Based on the above evidence, we place the two thiols in close proximity to the pyridine moiety and hence in the catalytic region of the active site. These two thiols are most probably located outside the substrate binding region. UMP, the powerful but truncated competitive inhibitor analogue of substrate, failed to provide protection against inactivation by any of the modifying agents. We could demonstrate only partial protection with either of the substrates against inactivation by bulkier modifying agents like PM or NBD-CL (Fig. 3 and Table II). We infer from fluorescence signals with PM (Fig. 5), inactivation with sodium arsenite in the presence of BAL (Table II), and from quenching data with spin-labeled maleimides (Table III) that the essential thiol is situated in a hydrophobic milieu at an approximate distance of 4.5 Å from the pyridine fluorophore. Finally, these two thiols must be sufficiently close in space to generate a disulfide bond under suitable oxidizing conditions (Fig. 6). The picture of the active site, as it has emerged from other modification and reconstitution studies, is also incorporated in this figure. Reconstitution experiments in the presence of Cibacron Blue had earlier shown the presence of a classic dinucleotide fold in the binding region of the coenzyme (33). Inactivation with phenylglyoxal and other diones, that could be completely protected with UMP, allowed us to place at least 1 arginine residue at the substrate binding region (7). The presence of an active histidine residue in the neighborhood of the catalytic region has also been uncovered. Modification of this residue abolishes the fluorescence energy transfer phenomena and makes the pyridine moiety inaccessible to borohydride (8).

UDPglucose 4-epimerase has emerged as the prototype of a new class of oxidoreductase that utilized enzyme-bound NAD as a true cofactor to carry out apparently dissimilar catalytic processes such as epimerization, cyclization, aldol reactions, α,β elimination, and decarboxylations (14). The initial oxidation labilizes a scissile bond by generating a carbonyl or imine function in a suitably placed adjacent position. Although considerable work has been done on reaction pathways and reaction intermediates, little is known about the architecture of the active site of most of these enzymes. The fragmentary picture of the active site of the *K. fragilis* enzyme that has emerged is the outcome of the first systematic study on any of these enzymes belonging to this class of oxidoreductases. The *K. fragilis* enzyme is unique for its coenzyme fluorescence. The chemical origin of the pyridine fluorophore still remains an unsolved problem. It is, however, abundantly clear that the presence of a noncatalytic free thiol is essential for maintaining the fluorophore structure. In analogy with glyceraldehyde-3-phosphate dehydrogenase, the thiol in combination with the pyridine ring may give rise to a charge-transfer complex (34). Alternately, as in case of a NAD-alkali (35) or NAD-cyanide complex (36), the thiolate anion may be involved in generating this fluorophore. We have been unable to resolve this question. The role of the active thiol also remains speculative at the moment. In the case of classical dehydrogenases, the hydride transfer is facilitated by either a metal ion as in the case of yeast alcohol dehydrogenase (37) or by a suitable amino acid residue acting as a general base as in the case of lactate dehydrogenase (38). Our preliminary analysis does not give any indication that *K. fragilis* epimerase may be a metalloenzyme. Furthermore, careful neutron activation analysis of *Escherichia coli* epimerase by Arabshahi *et al.* (39) failed to demonstrate the presence of any metal ion as a cofactor. Under such a circumstance, one may speculate that the active thiol of epimerase either alone or in tandem with histidine may facilitate the formation of the oxyanion that will provide the driving force for hydride transfer. It is interesting to note that in the case of *S*-adenosyl homocysteinase, a member of this class of oxidoreductases, a critically important cysteine residue, and an essential histidine residue (14) have been implicated in catalysis. An old but preliminary report on TDPglucose oxidoreductase also indicated the presence of an essential thiol at the active site (40). Employing a suicide inhibitor like UDP-chloroacetol, Frey and his group (41) have recently gathered substantial indirect evidence for the participation of a general base in catalysis for the *E. coli* epimerase. It remains to be seen whether the essential thiol or the histidine of the yeast enzyme either alone or in combination indeed plays such a role in catalysis. The recent crystallization of the *E. coli* epimerase (42) may give more definitive answer to this question.

Acknowledgments—We are grateful to Prof. Mihir Chowdhury of IACS, Calcutta for making his spectrofluorimeter available for our use. We also thank Prof. D. Balasubramanian and Dr. E. Ali for their valuable suggestions on spin label measurements.

REFERENCES

1. Gabriel, O., Kalckar, H. M., and Darrow, R. A. (1975) in *Subunit Enzyme: Biochemistry and Functions* (Ebner, K. E., ed) pp. 85–135, Marcel Dekker, Inc., New York
2. Darrow, R. A., and Rodstrom, R. (1966) *Proc. Natl. Acad. Sci. U. S. A.* **55,** 205–212
3. Darrow, R. A., and Rodstrom, R. (1968) *Biochemistry* **7,** 1645–1654
4. Bertland, L. H., and Bertland, A. U., II (1971) *Biochemistry* **10,** 3145–3151
5. Bertland, A. U., II (1970) *Biochemistry* **9,** 4649–4654
6. Ray, M., and Bhaduri, A. (1980) *J. Biol. Chem.* **255,** 10777–10781
7. Mukherji, S., and Bhaduri, A. (1986) *J. Biol. Chem.* **261,** 4519–4524
8. Mukherjee, S., and Bhaduri, A. (1992) *J. Biol. Chem.* **267,** 11709–11713
9. Maxwell, E. S., de Robichon Szulmajster, H., and Kalckar, H. M. (1958) *Arch. Biochem. Biophys.* **78,** 407–415
10. Nelsestuen, G. L., and Kirkwood, S. (1971) *J. Biol. Chem.* **246,** 7533–7543
11. Maitra, U. S., and Ankel, H. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 2660–2663

12. Wee, T. G., and Frey, P. A. (1973) *J. Biol. Chem.* **248,** 33–40
13. Adair, W. L., Gabriel, O., Ullrey, D., and Kalckar, H. M. (1973) *J. Biol. Chem.* **248,** 4635–4639
14. Frey, P. A. (1987) in *Pyridine Nucleotide Coenzymes: Chemical, Biochemical and Medical Aspects* (Dolphin, D., Poulson, R., and Avramovic, O., eds) Vol. 2B, pp. 462–511, John Wiley and Sons, Inc., New York
15. Creveling, C. R., Bhaduri, A., Christensen, A., and Kalckar, H. M. (1965) *Biochem. Biophys. Res. Commun.* **21,** 624–630
16. Ray, M., Kar, K., and Bhaduri, A. (1978) *Biochim. Biophys. Acta* **526,** 635–639
17. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual,* pp. 466–467, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77
19. Levy, H. M., Leber, P. D., and Ryan, E. M. (1963) *J. Biol. Chem.* **238,** 3654–3659
20. Tsou, C. L. (1962) *Sci. Sin.* **11,** 1535–1558
21. Horiike, K., and McCormick, D. B. (1979) *J. Theoret. Biol.* **79,** 403–414
22. Udenfriend, S. (1962) *Fluorescence Assay in Biology and Medicine,* Vol. 1, p. 249, Academic Press, New York
23. Ray, M., and Bhaduri, A. (1980) *J. Biol. Chem.* **255,** 10782–10783
24. Ray, W. J., and Koshland, D. E. (1962) *J. Biol. Chem.* **237,** 2493–2505
25. Carlson, G. M., Colombo, G., and Lardy, H. A. (1978) *Biochemistry* **17,** 5329–5338
26. Fluharty, A. L., and Sanadi, D. R. (1961) *J. Biol. Chem.* **236,** 2772–2778
27. Smith, D. J., Maggio, E. T., and Kenyon, G. L. (1975) *Biochemistry* **14,** 766–771
28. Wu, C. W., Yarbrough, L. R., and Wu, F. Y. H. (1976) *Biochemistry* **15,** 2863–2868
29. Berliner, L. J. (1978) *Methods Enzymol.* **49,** 418–479
30. Morrisett, J. D., Pownall, H. J., and Gotto, A. M. (1975) *J. Biol. Chem.* **250,** 2487–2494
31. Udenfriend, S. (1969) *Fluorescence Assay in Biology and Medicine,* Vol. 2, pp. 253–256, Academic Press, New York
32. Riddles, P. W., Blakeley, R. L., and Zerner, B. (1983) *Methods Enzymol.* **91,** 49–60
33. Samanta, A. K., and Bhaduri, A. (1982) *Biochim. Biophys. Acta* **707,** 129–132
34. Harris, J. I., and Waters, M. (1976) in *The Enzymes* (Boyer, P. D., ed) 3rd Ed, Vol. 13, pp. 1–49, Academic Press, New York
35. Kaplan, N. O., Colowick, S. P., and Barnes, C. C. (1951) *J. Biol. Chem.* **191,** 461–472
36. Hanker, J. S., Gamson, R. M., and Klapper, H. (1957) *Anal. Chem.* **29,** 879
37. Branden, C.-I., Jornvall, H., Eklund, H., and Furugren, B. (1975) in *The Enzymes* (Boyer, P. D., ed) 3rd Ed, Vol. 11, pp. 103–190, Academic Press, New York
38. Holbrook, J. J., Liljas, A., Steindel, S. J., and Rossmann, M. G. (1975) in *The Enzymes* (Boyer, P. D., ed) 3rd Ed, Vol. 11, pp. 191–292, Academic Press, New York
39. Arabshahi, A., Flentke, G. R., and Frey, P. A. (1988) *J. Biol. Chem.* **263,** 2638–2643
40. Gabriel, O., and Van Lanten, L. (1978) *Int. Rev. Biochem.* **16,** 1–36
41. Flentke, G. R., and Frey, P. A. (1990) *Biochemistry* **29,** 2430–2436
42. Bauer, A. J., Rayment, I., Frey, P. A., and Holden, H. M. (1991) *Proteins: Struct. Funct. Genet.* **9,** 135–142