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Two Tryptophans at the Active Site of UDP-glucose 4-Epimerase from *Kluyveromyces fragilis**

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Efficient fluorescence energy transfer from aromatic residues to the pyridine moiety of the bound coenzyme (NAD) of UDP-glucose 4-epimerase from *Kluyveromyces fragilis* had been reported earlier (Mukherji, S., and Bhaduri, A. (1992) *J. Biol. Chem.* 267, 11709–11713). We have employed *N*-bromosuccinimide (NBS) to identify tryptophan as the exclusive aromatic donor in the energy transfer. The characteristic UV absorption spectrum associated with Trp oxidation is observed during NBS modification of two of the four Trp residues of native epimerase along with concomitant inactivation of the enzyme. Excellent correlation between the observed inactivation and abolition of fluorescence energy transfer to coenzyme from Trp in epimerase upon treatment with NBS implicates the involvement of the same two tryptophans in both catalytic activity and fluorescence energy transfer. SDS-polyacrylamide gel electrophoresis and fluorescence data preclude gross structural/conformational changes in epimerase due to NBS oxidation. The susceptible tryptophans do not reside at the substrate binding site as substrates and UMP fail to protect against NBS modification. However, failure of sodium borohydride to reduce the bound NAD in the NBS-inactivated epimerase suggests that the reactive tryptophans are close to the coenzyme. Tryptophan fluorescence lifetime values of 1.9 and 3.9 ns for the native and 3.5 ns for the NBS-modified epimerase, complemented by a linear Stern-Volmer plot (effective Stern-Volmer constant = 2.85 M^{-1}) of acrylamide quenching, suggest that the two key tryptophans are buried close to an intrinsic quencher, presumably NAD.

UDP-glucose 4-epimerase (EC 5.1.3.2), an obligatory enzyme of the galactose metabolic pathway, catalyses a freely reversible transformation between UDP-Glc and UDP-Gal (1). Extensive studies on the mechanism of epimerization have demonstrated that the tightly bound pyridine nucleotide NAD is involved in a transient oxidation/reduction reaction in which a hydride is removed from C_4 of the hexose moiety of the nucleotide sugar substrate and subsequently returned to the opposite face of the enzyme-bound 4-ketopyranose intermediate (2, 3). Thus epimerase has emerged as the prototype of a new class of oxidoreductase that, unlike the classical dehydrogenases, utilize enzyme-bound NAD as a true cofactor rather than a cosubstrate (3). Besides epimerase, some typical examples of apparently dissimilar enzymes belonging to this class are dTDP-glucose oxidoreductase (EC 4.2.1.46), UDP-glucuronate decarboxylase (EC 4.1.1.35), *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1), and dehydroquinate synthase (EC 4.6.1.3) (4, 5). The striking similarity in the mechanism of the enzymes of this class of oxidoreductase raises several questions of considerable interest for contemporary enzymology. Do these enzymes have a common evolutionary relationship or do they have a common conserved tertiary structure at the active site that emerges from totally different types of amino acid sequences? Is there a commonality between the amino acid residues that may participate directly in catalysis for these oxidoreductases and the dehydrogenases?

In contrast to the detailed knowledge about the mechanistic pathway of these oxidoreductases, little is known about the structural features at their active site. Hence, mapping of active sites of these enzymes may be the initial step in addressing some of the fore mentioned critical questions. Our consistent efforts in this direction have provided a partial map of the structural organization at the active site of epimerase from *Kluyveromyces fragilis*. The yeast enzyme is a homodimer containing 1 mol of NAD noncovalently bound per mol of the apoenzyme. Thus, the enzyme has only one active site per mol of the functional dimer. One of the most intriguing features of the *K. fragilis* epimerase is its unique NADH-like fluorescence (excitation λ:353 nm, emission λ:435 nm) due to the pyridine moiety of its bound coenzyme NAD (6). Although its exact chemical nature is not known, this characteristic coenzyme fluorophore serves as a direct monitor of environmental changes at the active site. This property has been exploited by us to reveal an essential arginine at the substrate binding site, a histidine and two thiols at the coenzyme binding site as well as other relevant features of the enzyme (7–13).

Apart from this well documented coenzyme fluorescence, an efficient fluorescence excitation energy transfer from aromatic residues to the coenzyme fluorophore of epimerase had been accidently discovered during the course of the work related to establishing the presence of an essential histidine at the active site of epimerase (13). This observed energy transfer phenomenon provides a second delicate intrinsic monitor of events occurring at the active site of the enzyme as well as demonstrates the presence of one or more aromatic amino acid residues within the Forster distance of the bound coenzyme NAD. The involvement of tryptophan in the reported fluorescence energy transfer was suspected, although detailed investigations in this regard were not attempted at that stage. In the present work, it was our aim to explore the possibility of the location of tryptophan(s) at the active site of the enzyme and subsequently elucidate their functional role. Chemical modification and fluorescence studies, which were used as an initial tool to achieve this, have revealed the presence of two Trp residues which are involved in maintaining catalytic activity as well as are the exclusive aromatic donors of fluorescence excitation energy to the coenzyme fluorophore. In addition, we have

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exploited the environmentally sensitive, intrinsic fluorescence characteristics of tryptophans, such as fluorescence lifetime and quenching, to probe the neighborhood and approximate location of these reactive residues of epimerase.

MATERIALS AND METHODS

All biochemicals, unless otherwise mentioned, were purchased from Sigma. NBS[1] was recrystallized from water before use. Essentially homogenous UDP-glucose 4-epimerase was prepared by following the method of Darrow and Rodstrom (14) up to stage III, and this was further purified by a Waters Protein Pak 300 SW HPLC column (7.8 × 300 mm) when microheterogenity was encountered. The specific activity of the enzyme batches, measured by the coupled assay method (14), ranged between 40 and 60 units mg^{-1} of protein. UDP-glucose dehydrogenase was used as the coupling enzyme, and stoichiometric formation of NADH was monitored at 340 nm. Protein was estimated by the method of Lowry *et al.* (15) using bovine serum albumin as standard or by measuring absorption at 280 nm (14). The molecular weight of epimerase was determined to be 150,000 on a Sephadex G-200 column. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (16). Centrifuge spin columns containing Sephadex G-50 were used to remove excess unreacted modifying reagents according to the method of Maniatis *et al.* (17).

NBS Oxidation of Epimerase—The procedure employed for NBS modification was adapted from the methods of Patchornik *et al.* (18) and Spande and Witkop (19).

Inactivation Experiments—NBS was found to have a strong inhibitory effect on the coupling enzyme UDP-glucose dehydrogenase. Hence, to examine the effect of NBS on the activity of epimerase by the convenient coupled assay procedure, aliquots from the incubation mixture were first diluted with a suitable volume of a 0.1% bovine serum albumin solution, and aliquots from this diluted mixture were transferred to the assay medium. Bovine serum albumin is known to consume large excesses of NBS (19) and hence can quench any residual NBS in the incubation mixture.

Titration of Epimerase with NBS—Oxidation of epimerase was accomplished at 25 °C by stepwise addition of 5-μl aliquots of a freshly prepared aqueous solution of NBS (10 mM) to 0.9 ml of epimerase (5.33 μM, in 20 mM phosphate buffer, pH 6.7). The absorption spectrum of the treated enzyme solution was recorded on a Hitachi U3200 spectrophotometer. The absorbance at 280 nm was corrected for dilution, and the number of modified tryptophan residues per mol of native epimerase was calculated as described by Spande and Witkop (19).

Steady State Fluorescence Measurements—Steady state fluorescence data were taken on a Hitachi Spectrofluorimeter, model F-4010 (Hitachi Ltd., Tokyo, Japan) using a 0.5-cm path length, 1-ml quartz cuvette. Dilute enzyme solutions (1–2 μM) in the desired buffer at 25 °C were used for fluorescence measurements.

Fluorimetric Estimation of NAD in the Modified Enzyme—NAD content of epimerase (1 mg/ml, 0.2 ml), inactivated by NBS (2 μl, 5 mM), was estimated by converting it to a highly fluorescent product in strong alkali in the usual manner (13, 20).

Acrylamide Quenching Studies—For quenching studies, the absorbance of epimerase solution taken was always less than 0.1 at the excitation wavelength. Fluorescence intensities were determined by continuous monitoring of the emission at 330 nm after excitation at 295 nm. The final intensity at any given acrylamide concentration was taken as a time averaged value after correcting for dilution and inner filter effects (21). A correction for absorption of acrylamide itself at 295 nm was made according to McClure and Edelman (22) using a molar extinction coefficient of 0.29 M^{-1} cm^{-1} at 295 nm.

Fluorescence Lifetime Measurements—Fluorescence lifetime measurements were made using a time-correlated single photon counter (model 199, Edinburgh Instruments, Edinburgh, Scotland) equipped with a thyratron-gated nitrogen flash lamp as a light source. Excitation and emission wavelengths were set at 297 and 330 nm, respectively. Peak counts were typically 10,000. The decay data were analyzed by a nonlinear least squares method (23), assuming that the fluorescence decay follows a multiexponential law, $It = \Sigma_i \alpha_i \cdot e^{-t/\tau_i}$, where It is the fluorescence emission intensity at a time t after withdrawing the excitation pulse and the relative amplitudes α_i and the decay constants τ_i are the numerical parameters to be recovered. The best fit between the theoretical curve and the decay data was evaluated from the plot of residuals, the autocorrelation function of the residuals and the reduced chi square. The decay of *N*-acetyltryptophan, which served as the standard sample, yielded a lifetime value of 2.95 ns.

RESULTS

The Role of Tryptophan as the Exclusive Donor of Fluorescence Excitation Energy to the Coenzyme Fluorophore of Epimerase from K. fragilis

Fluorescence Properties of Native Epimerase—The main features of the fluorescence properties of the native enzyme from *K. fragilis* are shown in Fig. 1*A*. When the native enzyme was excited at 353 nm, a characteristic, NADH-like fluorescence emission spectrum with a maximum at 435 nm was observed (24). When the enzyme was excited at 280 nm, the fluorescence emission spectrum exhibited two maxima with peaks at 330 and 435 nm, respectively. The peak at 330 nm is the typical protein fluorescence due to the tryptophan and tyrosine residues, whereas the second peak is characteristic of coenzyme fluorescence. In the present study, the appearance of the second peak in the region of the coenzyme fluorescence clearly demonstrated the transfer of fluorescence excitation energy from one or more aromatic amino acid residues to the coenzyme fluorophore. Evidently one or more aromatic amino acid residues reside within Forster distance (25) of the coenzyme binding site. In a preliminary attempt to identify the aromatic donor residue(s) involved in the energy transfer phenomenon, we selectively excited the tryptophan residues of epimerase by exciting the protein at 295 nm (26). At this position of excitation, tyrosine residues of proteins exhibit a negligible absorbance and hence do not contribute to protein fluorescence. The resultant fluorescence emission spectrum (Fig. 1*A*), displayed peaks at 330 and 435 nm due to the tryptophans and the coenzyme fluorophore of the enzyme, respectively, suggesting the involvement of one or more tryptophan residues in the energy transfer process. Moreover, when the selective excitation of tryptophans was carried out in the presence of the substrate UDP-Gal or 5′-UMP, a powerful competitive inhibitor of the enzyme, an increase in the energy transfer to the coenzyme fluorophore was observed. Apparently, both substrate and UMP binding brought the tryptophan(s) closer to the coenzyme binding site, thereby increasing the efficiency of tryptophan to coenzyme fluorescence energy transfer.

Effect of NBS on the Energy Transfer Phenomenon—The participation of tryptophan residues as donors in the fluorescence energy transfer process was confirmed by chemical modification studies. NBS is a commonly used modifying reagent for tryptophans in proteins (19, 27, 28). The reagent is found to be fairly selective in its action when used in carefully controlled amounts under acidic conditions (19). The effect of NBS on epimerase was examined under mildly acidic conditions of 20 mM phosphate buffer, pH 6.7, since the strongly acidic conditions (pH 3–4) generally recommended for NBS modification studies resulted in inactivation of epimerase. Upon treatment with NBS under the above-mentioned nondenaturing condition, epimerase was found to undergo a parallel loss of tryptophan fluorescence and fluorescence energy transfer (Fig. 1*B*), clearly indicating that one or more tryptophans act as donor residues in the observed energy transfer phenomenon.

In addition to tryptophan, the contribution of tyrosine residues to the energy transfer process was also assessed by treating the enzyme with *N*-acetylimidazole, a specific modification reagent for tyrosine residues in proteins (29). The reagent was found to have only a minor effect on the protein fluorescence and no discernible effect on the fluorescence energy transfer (data not shown). These experimental findings implicated the exclusive involvement of one or more tryptophans as the aro-

[1] The abbreviations used are: NBS, *N*-bromosuccinimide; HPLC, high performance liquid chromatography; ϵ_M, molar extinction coefficient; pCMB, *p*-chloromercuribenzoate.

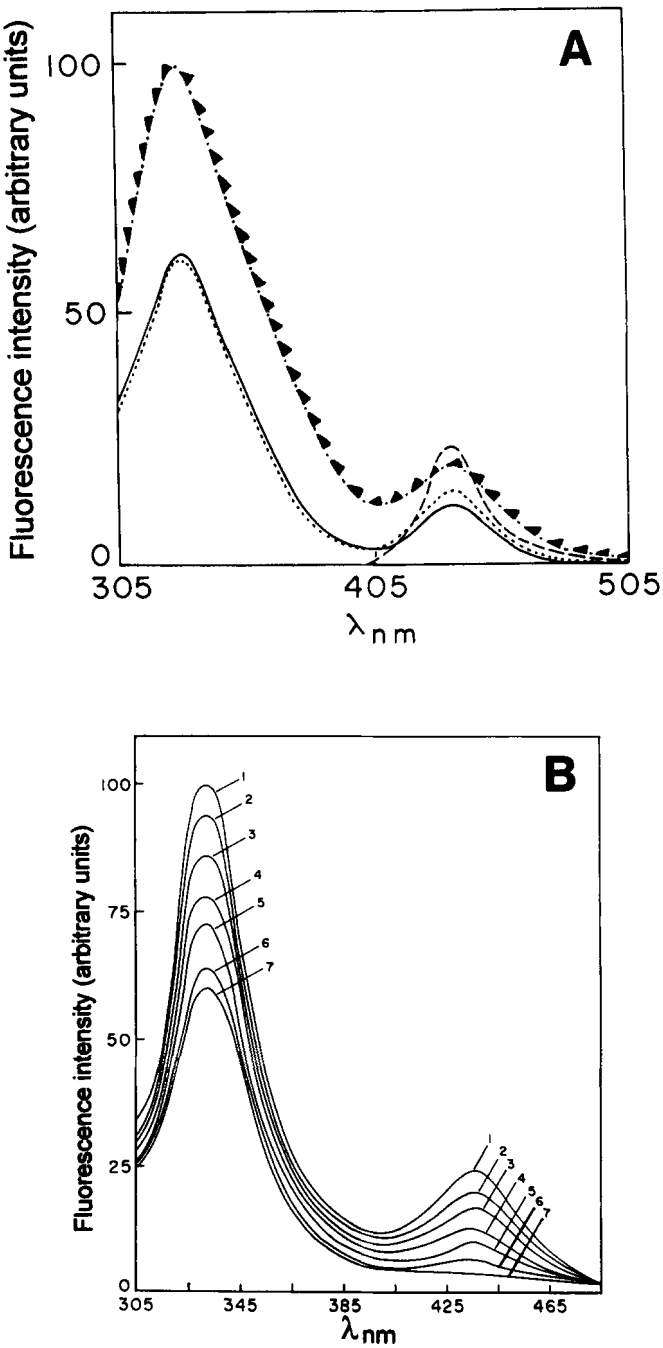

FIG. 1. **Tryptophan as the donor of fluorescence excitation energy to the coenzyme fluorophore of epimerase from *K. fragilis*.** *A*, fluorescence properties of native epimerase from *K. fragilis*. A 1 μM solution of epimerase in 20 mM phosphate buffer, pH 6.7, at 25 °C was used for recording the following spectra: (– – –) coenzyme fluorescence emission spectrum of native epimerase on excitation at 353 nm, (▲.▲.▲) fluorescence emission spectrum of native epimerase on excitation at 280 nm, (——) tryptophan fluorescence emission spectrum of native epimerase on excitation at 295 nm, (·····) tryptophan fluorescence emission spectrum of epimerase in the presence of UMP (1 mM) on excitation at 295 nm. *B*, effect of NBS on tryptophan fluorescence and tryptophan to coenzyme fluorescence energy transfer. Tryptophan fluorescence and tryptophan to coenzyme fluorescence energy transfer in epimerase was monitored as a function of incremental additions of an aqueous solution of NBS. 2-μl aliquots of an aqueous solution of NBS (4.5 mM) were added to 900 μl of epimerase solution (1 μM, in 20 mM phosphate buffer, pH 6.7). After 2–3 min of incubation following each addition, the fluorescence emission spectrum was recorded by exciting the protein at 295 nm. The molar ratio of NBS to enzyme for the *curves* 2–7 were 10 (*2*), 20 (*3*), 30 (*4*), 40 (*5*), 50 (*6*), and 60 (*7*), respectively. *Curve 1* represents the native enzyme.

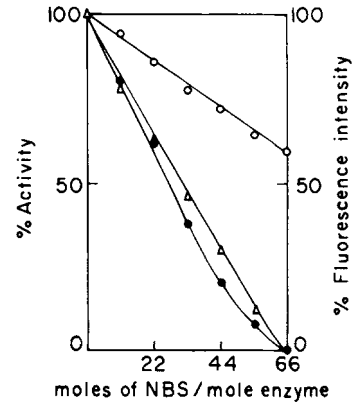

FIG. 2. **Correlation between loss of activity, loss of energy transfer fluorescence, and tryptophan fluorescence upon NBS modification of epimerase.** Epimerase activity was monitored as a function of the molar ratio of NBS:epimerase. To 200 μl of epimerase solution (1 μM, in 20 mM phosphate buffer, pH 6.7), a 2-μl aliquot was added from an appropriate aqueous stock solution of NBS so as to maintain an NBS:epimerase molar ratio of 11:1, 22:1, 33:1, 44:1, 55:1, and 66:1, respectively, in six separate tubes. After 2–3 min of incubation, aliquots were withdrawn from each tube for measurement of activity. The corresponding intensities of tryptophan fluorescence emission at 330 nm and the tryptophan to coenzyme energy transfer fluorescence at 435 nm were also recorded for each tube after excitation at 295 nm and expressed as a percentage of the intensities for the unmodified control epimerase. These percent fluorescence intensities (at 330 and 435 nm) and the corresponding activities were plotted as a function of the molar ratio of NBS:epimerase. (△——△), (○——○), and (●——●) represent the intensities of coenzyme fluorescence at 435 nm, tryptophan fluorescence at 330 nm (excitation at 295 nm), and epimerase activity, respectively.

matic donor residues in fluorescence energy transfer to the coenzyme fluorophore. Thus, it was of interest to investigate their possible role in the catalytic activity of the enzyme.

Inactivation of Epimerase by NBS

NBS was found to completely inactivate epimerase (Fig. 2) at a NBS:epimerase molar ratio of 66:1 under nondenaturing conditions of 20 mM phosphate buffer, pH 6.7. In order to ascertain whether the tryptophan residue(s) associated with activity are the same tryptophans that are involved in the energy transfer, we simultaneously monitored relative loss of activity, tryptophan fluorescence and energy transfer after each successive addition of NBS. As shown in Fig. 2, there was a parallel loss of activity and energy transfer. When the enzyme became completely inactive the energy transfer process was also completely abolished, suggesting the involvement of the same tryptophan residues in both catalytic activity and energy transfer of the enzyme. Loss of tryptophan fluorescence, however, did not correlate with the loss of activity and even after essentially complete inactivation of the enzyme nearly 60% of the total tryptophan fluorescence was retained. This was possibly due to the contribution of other tryptophan residues that are not related either to activity or to the energy transfer process. These observations prompted us to determine the number of tryptophans contributing to activity and energy transfer.

Number of Tryptophan Residues Involved in Activity and Energy Transfer

The oxidation of indole to oxindole by NBS exhibits a characteristic absorption spectrum with a decrease in the absorbance at 280 nm (ϵ_M = 5500) and the appearance of a major peak at 261 nm (ϵ_M = 10,300) and a minor peak at 309 nm (ϵ_M = 1630) (30). The difference absorption spectra induced (as described under "Materials and Methods") during the stepwise

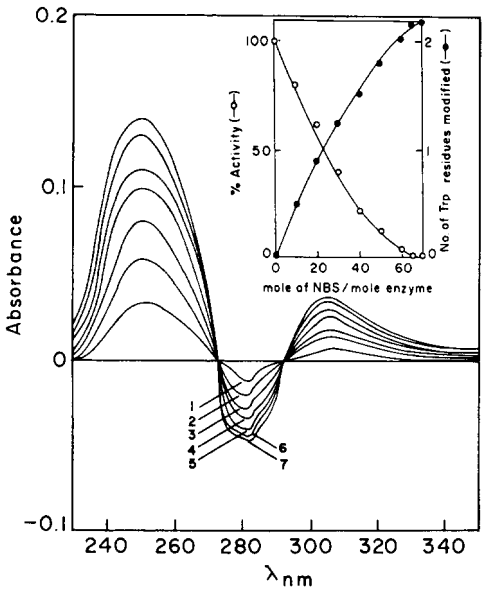

FIG. 3. **Difference absorption spectra of epimerase induced by NBS oxidation.** The difference spectra were induced by NBS oxidation as described under "Materials and Methods." *Curves 1–7* correspond to a final NBS:epimerase molar ratio of 10 (*1*), 20 (*2*), 30 (*3*), 40 (*4*), 50 (*5*), 60 (*6*), and 70 (*7*), respectively, in the incubation medium. *Inset*, correlation between the number of tryptophans modified by NBS and loss in activity of epimerase. The residual activity and the number of tryptophan residues modified was determined at each stage of NBS titration (*curves 1–7* in Fig. 3) and plotted as a function of the molar ratio of NBS:epimerase.

modification of epimerase with NBS showed a similar characteristic pattern with the appearance of a negative peak at 283 nm and two positive peaks at 252 and 305 nm, indicating the modification of tryptophan residues (Fig. 3). The *inset* in Fig. 3 shows the correlation between the loss of activity and the number of tryptophan residues modified. The oxidation of two tryptophan residues per mol of enzyme was found to result in complete inactivation of the enzyme at a NBS:Trp molar ratio of ~30:1. These results indicated that two tryptophan residues were involved in maintaining the activity of epimerase from *K. fragilis*.

As only two tryptophan residues of the native enzyme were modified by NBS under nondenaturing conditions, NBS titration was carried out under various denaturing conditions to estimate the total number of tryptophan residues present per mol of epimerase. Four tryptophans were titrated by NBS when epimerase was oxidized, either in the presence of the strongly acidic conditions (50 mM acetate buffer, pH 4) generally recommended for the reaction (19) or after complete denaturation with urea (8 M). In both cases, the NBS:Trp molar ratio was estmated to be ~15:1. Thus, two classes of tryptophan with significantly different reactivities toward NBS were identified for native epimerase.

Exclusive Modification of Tryptophan Residues by NBS

In addition to tryptophan, NBS may occasionally oxidise other amino acid residues such as tyrosine, histidine, and cysteines in proteins (19), although, except for the sulfydryl group, the rate of oxidation of these residues is very small compared with the indole group (31). NBS oxidation of these residues, however, does not interfere with the detection of tryptophan modification as none of these residues or their oxidation products exhibit the characteristic absorption spectrum observed during tryptophan oxidation (19). In the present study, modification of residues other the tryptophans seems unlikely upon considering the linear relationship between the number of tryptophans oxidized and the moles of NBS consumed (*inset*, Fig. 3). Moreover, under the neutral conditions we used for NBS oxidation, reaction with side chains other than tryptophans and cysteines becomes negligible since the rate of oxidation by NBS falls dramatically above pH 5 (31). As two cysteines had earlier been identified at the active site of epimerase (7, 12), the possibility of sulfydryl oxidation by NBS with concomitant loss of activity had to be considered. Direct quantitative estimation of free sulfydryl groups in the native and NBS-modified enzyme, according to the previously tested spectrophotometric method involving pCMB titration of free thiols (7, 32), clearly demonstrated that the sulfydryl groups of epimerase were not modified by NBS. For this experiment, 0.6 mg of the enzyme in 0.5 ml of 20 mM phosphate buffer, pH 6.7, was first inactivated with NBS in a molar ratio of 70:1 for 3 min. The inactivated enzyme was passed through a Sephadex G-50 spin column, previously equilibriated with 50 mM glycylglycine buffer, pH 7.8. Appropriate aliquots of the eluted enzyme were taken in a final volume of 1 ml of the same buffer and titrated stepwise with pCMB (2.3 nmol/μl). Average of two such titrations showed that 25.4 nmol of pCMB was consumed for 0.24 mg of the inactivated enzyme. The unmodified, native enzyme, treated in the same fashion recorded an average value of 25.2 nmol of pCMB for the same amount of protein.

Structural Integrity of the NBS Modified Enzyme

The active holoenzyme from *K. fragilis* is a dimeric protein containing 1 mol of bound NAD essential for catalytic activity. Unlike treatment with pCMB or exposure to heat (33, 34), inactivation of epimerase by NBS does not stem from the dissociation of the dimeric holoenzyme or the loss of the bound NAD. A direct fluorimetric estimation of the enzyme bound NAD as described under "Materials and Methods," for the native and NBS-modified epimerase clearly demonstrates that the modified enzyme retains more than 90% of the bound NAD as compared to the control enzyme. Large excess of NBS in the presence of strongly acidic conditions (pH 3–4) can cause scission of peptide bonds at tryptophan, tyrosine or histidine residues (30). Under the conditions employed (20 mM phosphate buffer, pH 6.7), reactivity of NBS is usually restricted to the oxidation of tryptophan residues. Identical bands obtained for the native and NBS-modified enzymes in SDS-polyacrylamide gel electrophoresis (data not shown) as well as identical elution volumes (5.5 ml, with 20 mM phosphate buffer, pH 6.7) on a protein Pak HPLC column (7.8 × 300 mm) ruled out the possibility of peptide cleavage and subunit dissociation. Moreover, modification with NBS does not introduce a bulky group into the primary structure of the enzyme that might cause inactivation due to steric disturbances to the active tertiary structure.

Conformational Status of the NBS-inactivated Enzyme

The intrinsic fluorescence of proteins due to aromatic residues serves as a sensitive index of environmental and conformational changes. A comparison of the fluorescence properties of the NBS-modified enzyme with those of the native and other modified states of the enzyme did not reflect any gross conformational change in the NBS-inactivated epimerase. Fluorescence emission spectra were recorded after various treatments (described below), each of which led to a modified enzyme. The results are shown in Fig. 4. *Curve 1* represents the native enzyme. NBS modification of epimerase was found to elicit a 40% depletion of the tryptophan fluorescence of the native enzyme without any change in the emission maximum (*Curve 2*). On the other hand, upon incubation with 3 M urea, a condition under which the enzyme is known to become inactive and lose tryptophan to coenzyme fluorescence energy transfer but

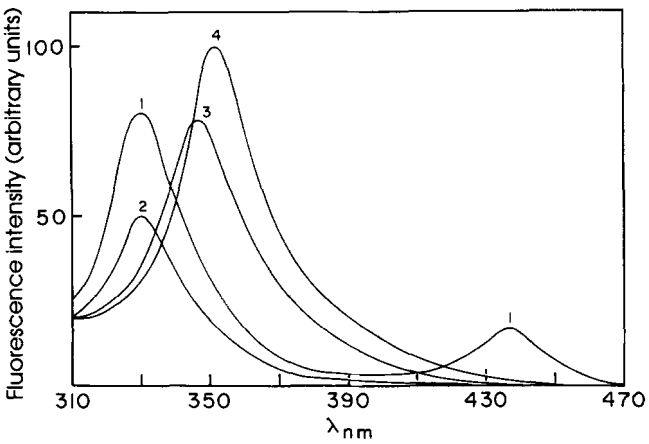

FIG. 4. **Fluorescence emission spectra of epimerase in various modified states.** *Curves 1–4* are the fluorescence emission spectra of epimerase solution (1 μM, in 20 mM phosphate buffer, pH 6.7) taken in the native state (*1*), NBS-inactivated state (*2*), after 30 min of incubation with 3 M urea (*3*), and after 30 min of incubation with 8 M urea (*4*) after excitation at 290 nm.

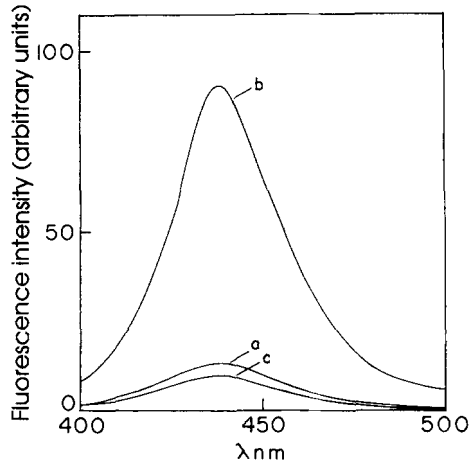

FIG. 5. **Effect of NBS on the chemical reduction of the enzyme bound NAD.** Epimerase (1 μM in 20 mM phosphate buffer, pH 6.7) was inactivated with NBS followed by incubation with UMP (1 mM) and $NaBH_4$ (10 mM) for 15 min. In the control experiment, the unmodified enzyme was similarly incubated with UMP and $NaBH_4$. The fluorescence emission spectra of the two enzyme solutions were recorded after excitation at 353 nm. *Curve a* represents the fluorescence emission spectrum of the native epimerase and *curves b* and *c* represent the fluorescence emission spectra of the unmodified native and the NBS-modified epimerase, respectively, after treatment with UMP and $NaBH_4$.

retain the tightly bound coenzyme,[2] a 15-nm red shift of the emission maximum was observed (*Curve 3*). Evidently, a large conformational change occurred in this state of epimerase which dramatically increased the exposure of tryptophan residues and also disturbed the critical relative orientation required to maintain the tryptophan to coenzyme fluorescence energy transfer. Further, upon complete unfolding of epimerase by treatment with 8 M urea, there was an additional 5-nm red shift of emission maximum accompanied by a significant increase in the fluorescence quantum yield of the tryptophan residues (*Curve 4*). On the basis of these observations we inferred that NBS modification of tryptophans only altered the microenvironment around these residues without affecting the overall structure of epimerase.

Assessment of the Approximate Location and Microenvironment of the Reactive Tryptophans of Epimerase

The physical location of the reactive tryptophan residues on the enzyme surface was next investigated. Neither of the substrates, UDP-galactose or UDP-glucose, nor the powerful competitive inhibitor 5′-UMP could protect epimerase against inactivation by NBS (data not shown). Such protection had been earlier observed for yeast epimerase during modification studies with sulfydryl reagents (12) and arginine-modifying reagents (9). Apparently, the key tryptophans of epimerase were located outside the substrate binding region of the active site. The characteristic coenzyme fluorescence of epimerase from *K. fragilis* was exploited by us to assess the approximate location of the reactive tryptophans. NBS modification resulted in a partial diminution (20–25%) of fluorescence emission intensity of the bound coenzyme fluorophore (data not shown). NAD estimation experiments discussed under "Structural Integrity of the NBS-modified Enzyme" had proven that the NBS-modified enzyme fully retained the bound coenzyme NAD. Hence, a plausible explanation for the observed dimunition of the coenzyme fluorescence intensity was the partial quenching of the coenzyme fluorophore due to the generation of a polar carbonyl group in the indole ring of the proximal tryptophans by NBS oxidation. Polar carbonyl groups and charged groups are well known quenchers of nearby fluorophores (35). The location of the key tryptophans in the catalytic region in close proximity to the pyridine moiety of the bound coenzyme was also evident when attempts were made to reduce the coenzyme chemically after the modification of the holoenzyme with NBS. Creveling *et al.* (36) had earlier shown that NAD bound to yeast epimerase can be reduced by sodium borohydride to a stable enzyme-NADH complex provided the reduction is carried out in the presence of UMP. The formation of enzyme-NADH complex is accompanied by greatly enhanced fluorescence emission (λ_{max} at 435 nm). Unlike the native enzyme, the coenzyme fluorescence emission intensity of the NBS-modified epimerase remained unchanged upon treatment with UMP and $NaBH_4$ (Fig. 5). Apparently, modification of proximal tryptophan residues made the pyridine moiety of the bound coenzyme insensitive to reduction by sodium borohydride.

The microenvironment around the active site tryptophan residues was assessed by determining the fluorescence lifetimes of the tryptophan residues of native and NBS-modified enzyme (37). The calculated decay parameters are listed in Table I. For native epimerase, tryptophan fluorescence was assumed to decay biexponentially on the basis of the accuracy of the fit between the theoretical curve and the actual decay curve for the native enzyme in the plot of the residuals (data not shown) and the value of reduced chi square (χ^2 = 1.285) (Table I). The lifetime components of tryptophan fluorescence in native epimerase were calculated to be 1.9 and 3.9 ns (Table I). The existence of two widely different lifetimes for the four tryptophans of the native enzyme indicated the presence of two distinct environments for the tryptophans in native epimerase which may arise from their distinct locations in the enzyme molecule or two distinct conformers of the native enzyme (37). The observations were rationalized and subsequent investigations were made by initially assuming that two distinct locations of the tryptophans in the enzyme accounted for their distinct environments in the native epimerase. This assumption was based on the fact that NBS had identified two distinct classes of tryptophans (reactive and inert) for the native state of the enzyme, both of which contributed significantly to the total tryptophan fluorescence of epimerase (Fig. 2). Unlike the native enzyme, tryptophan fluorescence of the NBS-modified

[2] S. Ray and A. Bhaduri, unpublished observation.

TABLE I

Fluorescence lifetime of tryptophans of native epimerase and NBS-inactivated epimerase

Dilute solutions of native and NBS-inactivated epimerase (0.6 μM in 20 mM sodium phosphate buffer, pH 6.7) were used for lifetime measurements so that absorbance was less than 0.1 at the excitation wavelength (295 nm). Emission was measured at 337 nm. Decay parameters were obtained using a nonlinear least squares method of analysis as described under "Materials and Methods."

Sample	Monoexponential analysis		Bioexponential analysis					Average lifetime[a]
	τ	χ^2	α_1	τ_1	α_2	τ_2	χ^2	$\langle\tau\rangle$
	ns			*ns*		*ns*		*ns*
Native epimerase	3.28 ± 0.05	1.73 ± 0.02	0.57 ±0.05	3.96 ± 0.05	0.43 ± 0.05	1.93 ± 0.05	1.28 ± 0.02	3.41
NBS-inactivated epimerase	3.52 ± 0.05	1.2 ± 0.02	ND[b]	ND	ND	ND	ND	ND

[a] The average lifetime $\langle\tau\rangle$ was determined from the relative amplitudes (α_1 and α_2) and the corresponding lifetime components (τ_1 and τ_2) using the formula (38): $\langle\tau\rangle = [\alpha_1/(\tau_1)^2 + \alpha_2(\tau_2)^2] \div [\alpha_1\tau_1 + \alpha_2\tau_2]$.

[b] ND, not determined.

enzyme exhibited monoexponential decay with a lifetime of 3.5 ns (Table I). Since NBS oxidation rendered the catalytically relevant tryptophans nonfluorescent, this single lifetime must arise from a homogenous environment of the fluorescent but inert tryptophans which remain untouched by NBS. In the absence of any gross conformational change of the enzyme, only a small shift in the value of the fluorescence lifetime of the inert tryptophans could be expected during oxidation of the two key tryptophans of native epimerase. On comparing the tryptophan lifetime values of the native and NBS-inactivated states of epimerase, we could assign the longer lifetime component of 3.9 ns to the inert tryptophans and the shorter lifetime of 1.9 ns to the catalytically relevant tryptophans of epimerase. Two possible factors could account for the presumed shorter lifetime of the reactive tryptophan residues, exposure to the polar aqueous surroundings and/or close proximity to an intrinsic quencher moiety within the protein core of the native epimerase.

Acrylamide quenching studies of the native enzyme were undertaken to determine the exposure of tryptophans in order to assess the contribution of this factor to the observed shorter lifetime of the key tryptophan residues (39). The experimental details are described under "Materials and Methods." Acrylamide quenching data for the native epimerase yielded a linear Stern-Volmer plot (Fig. 6*A*) indicating a uniformity in exposure of the tryptophans. Tryptophan fluorescence emission spectra of epimerase recorded at various stages of acrylamide quenching are shown in the inset of Fig. 6*A*. No shift in the position of emission maxima was observed during the quenching process indicating that the fluorescence of both classes of tryptophans was quenched simultaneously. Such a situation can arise when both classes of tryptophans are equally accessible to the quencher or when one class is selectively available for the quenching process (39). The possibility of the latter case was ruled out on analyzing the Lehrer plot (40) of $F_0/F_0 - F$ *versus* $[Q]^{-1}$ (Fig. 6*B*). The fraction of the total tryptophan fluorescence available for quenching (F_{eff}) is given by the *y* intercept of the Lehrer plot. The observed value of (F_{eff}) was "1" which clearly demonstrated that the entire tryptophan fluorescence intensity of the native enzyme was quenchable. Thus it appeared that both classes of tryptophans (reactive and inert) of the native epimerase had similar exposures and hence were equally accessible to the quencher.

Average exposure of the tryptophan residues was quantitatively estimated by determining the values of the effective Stern Volmer constant (K_{SVeff}) and the effective quenching rate constant (k_{qeff}). The value of K_{SVeff} which is obtained from the initial slope of the Stern Volmer plot or the slope of the Lehrer plot is a measure of the average degree of quenching. The values of K_{SVeff} generally range from 0.1 M^{-1} to 10 M^{-1} for deeply buried to highly exposed tryptophan residues of proteins

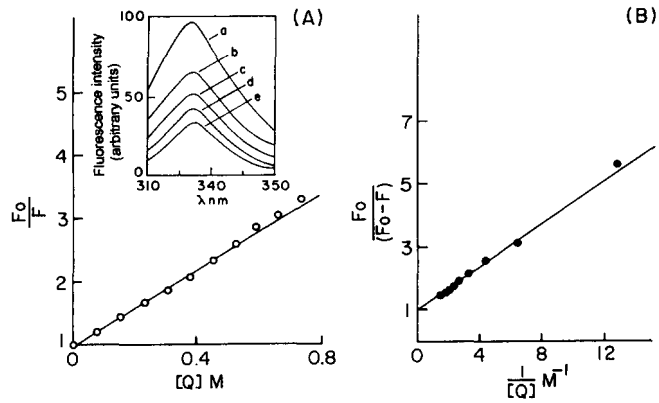

FIG. 6. **Acrylamide quenching of the tryptophan fluorescence of native epimerase.** *A*, Stern Volmer plot of the quenching data. 4-μl aliquots of acrylamide (aqueous stock = 8 M) were added each time to a 400-μl solution of native epimerase (0.6 μM in 20 mM phosphate buffer, pH 6.7), and the fluorescence intensity was measured at 330 nm on excitation at 295 nm. The ratio (F_0/F) of the fluorescence intensities at 330 nm of the enzyme in the absence (F_0) and presence (F) of the quencher are plotted against the molar concentration of the quencher [*Q*]. *Inset, curve a* represents the fluorescence emission spectrum of epimerase in the absence of quencher on excitation at 295 nm. *Curves b* to *e* represent fluorescence emission spectra of epimerase in the presence of 0.16 M, 0.3 M, 0.46 M, and 0.59 M acrylamide, respectively. *B*, Lehrer plot (modified Stern Volmer plot) of the quenching data. The quenching data were plotted as $F/(F_0 - F)$ *versus* $[Q]^{-1}$.

respectively (39). In case of native epimerase, the value of K_{SVeff} was determined to be 2.85 M^{-1}. The value of the effective quenching rate constant (k_{qeff}), which is a measure of the average exposure of tryptophans of proteins, is calculated from the values of K_{SVeff} and the average lifetime ($<\tau>$) using the following relationship, $k_{qeff} = K_{SVeff/<\tau>}$ (39).

The values of k_{qeff} generally rage from $40 \times 10^8 M^{-1}s^{-1}$ to $5 \times 10^8 M^{-1} s^{-1}$ for fully exposed to deeply buried tryptophans in proteins, respectively (39). In case of native epimerase, the value of k_{qeff} was determined to be 8.35×10^8 $M^{-1}s^{-1}$ using values of 2.85 M^{-1} (Fig. 6*B*) and 3.41 ns (Table I) for the effective Stern Volmer constant (K_{SVeff}) and the average lifetime of tryptophan fluorescence ($<\tau>$), respectively. From these results we inferred that both classes of tryptophans were buried within the hydrophobic protein core of native enzyme. This was also confirmed by studies with a surface quencher, KI.

DISCUSSION

Oxidation of tryptophan with NBS is a multistep reaction which occurs specifically at the 2,3-double bond of the indole moiety (31). The reactivity of tryptophans with NBS is dependent upon their exposure in the protein molecule, the orientation

of the indole ring as this affects the accessibility of the 2,3-double bond, neighboring group effects, as well as pH of the reaction medium (31). In the present case, the results of acrylamide quenching studies clearly indicate that the observed difference in reactivity between the two classes of tryptophan does not arise from a wide difference in their exposure in the protein molecule. The buried nature of the reactive tryptophans as well as the neutral conditions we used for the NBS reaction (rather than the recommended acidic pH) could account for the observed large molar ratio of NBS:Trp (~30:1) required for their modification under nondenaturing conditions. This molar ratio dramatically reduces to half its value (15:1) on carrying out titrations under denaturing conditions where the tryptophans are fully exposed.

The two reactive tryptophans appear to be located near the coenzyme binding region of the the active site of epimerase. Partial quenching of the coenzyme fluorescence (20–25%) and the apparent inaccessibility or insensitivity of the bound coenzyme (NAD) to reduction by UMP and $NaBH_4$ upon modification of the relevant tryptophans (Fig. 5) provides evidence in this direction. This view is further supported by the widely different lifetime values obtained for the two classes of tryptophan (reactive and inert). In absence of a major difference in their exposure to surroundings (Fig. 6), the presence of an intrinsic quencher of fluorescence in close proximity to the two active site tryptophans provides an alternative explanation for their short lifetime. Pyridine nucleotide coenzymes as well as certain amino acid residues, such as cysteines, cystines, and methionines, are well established intrinsic quenchers of tryptophan fluorescence in proteins (41). In the present case, the bound coenzyme NAD appears to be the most likely candidate for quenching of fluorescence of the catalytically relevant tryptophan residues. The observed fluorescence energy transfer from these reactive residues to the bound coenzyme fluorophore provides evidence for the mechanism by which the coenzyme accomplishes quenching. Similar observations have been reported for epimerase related enzymes like *S*-adenosylhomocysteine hydrolase and glyceraldehyde-3-phosphate dehydrogenase (42, 43). In addition to the results of fluorescence lifetime measurements, the presence of a natural quencher of tryptophan fluorescence in the native enzyme was also implicit in the fluorescence data shown in Fig. 4. The 20-nm red shift in emission maximum of tryptophan fluorescence upon denaturation of epimerase with 8 M urea was due to a shift from a hydrophobic protein environment to polar aqueous surroundings. This was expected to be accompanied by a partial decrease in quantum yield rather than the observed increase in quantum yield of tryptophan fluorescence. These paradoxical observations could be rationalized by assuming that the key tryptophans were located in close proximity to a protein bound intrinsic quencher, presumably the coenzyme NAD, in the native folded state of epimerase. Upon denaturation, the release of the noncovalently bound NAD into the medium on collapse of the dimeric holoenzyme structure abolished the quenching process so that the red shift in emission maximum was accompanied by an increase in quantum yield of tryptophan fluorescence relative to the native enzyme. The above mentioned findings are strong indirect evidence of the proposed role of NAD as the natural quencher of fluorescence of the catalytically relevant tryptophans. Direct evidence in this direction could not be provided since the coenzyme NAD is an essential constituent of the active holoenzyme and its detachment from the enzyme molecule leads to an inactive state which represents a large deviation from the native state of the enzyme.

At this stage it is worth mentioning that none of the fore mentioned observations precludes the possibility of the existence of two conformers in a state of dynamic equilibrium for the native epimerase, analogous to the "open" and "closed" conformations of glyceraldehyde 3-phosphate (43) or horse liver alcohol dehydrogenase (44). Future studies in this direction are expected to address this issue.

With regard to the functional role of these active site tryptophans, they do not appear to be directly responsible for the noncovalent binding of NAD since their modification does not detach the bound coenzyme from the holoenzyme. Instead, it is possible that these key tryptophans play a role in the activation of the bound coenzyme NAD. The binding of substrates (UDP-Glu/UDP-Gal) or the competitive inhibitor UMP to native epimerase is known to induce a catalytically important conformational transition in the enzyme which results in the increased reactivity of the bound coenzyme NAD toward reducing agents (45, 46).The proposed activation arises from a distortion of the π-electron distribution in the nicotinamide ring, thereby decreasing the activation energy for its reduction (47). Almarsson *et al.* (48) have recently demonstrated that a structural perturbation of the pyridine moiety of NAD toward a boat conformation contributes significantly toward decreasing the activation energy for reducing NAD to NADH. The driving force for such a deformation of the nicotinamide ring in the case of lactate dehydrogenase is considered to be provided by interactions between the nicotinamide ring and the hydrophobic amino acid side chains (48). In the case of the *K. fragilis* epimerase, the driving force may arise from the hydrophobic interactions between the reactive tryptophans and NAD. The observed increase in fluorescence energy transfer from tryptophans to coenzyme fluorophore upon UMP binding (Fig. 1*A*) to the native epimerase demonstrates that the reactive tryptophans move closer to the bound coenzyme as may be expected during the proposed activation process. A second line of evidence in this direction is provided by the apparent insensitivity of the bound NAD toward the UMP-dependent reduction in the NBS-inactivated enzyme. Since NBS introduces negligible change in the bulk of the reactive tryptophans and no significant change in the structure or conformation of epimerase, the dramatic loss in reactivity of NAD toward sodium borohydride cannot be explained on the basis of steric hindrance or unfavorable orientation of NAD. A plausible explanation for these observations is the modification of a residue(s) that is critical for the substrate-dependent activation of the bound NAD. Ongoing studies are expected to provide more conclusive evidence in this direction.

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REFERENCES

1. Caputto, R., Leloir, L. F., Trucco, R. E., Cardini, C. E., and Paladini, A. C. (1949) *J. Biol. Chem.* **179,** 497–498
2. Gabriel, O., Kalckar, H. M., and Darrow, R. A. (1975) in *Subunit Enzymes* (Ebner, K. E., ed) pp. 84–135, Marcel Dekker, Inc., New York
3. Frey, P. A. (1987) in *Pyridine Nucleotide Coenzymes* (Dolphin, D., Poulson, R., and Avramonic, O., eds) Vol. 2B, pp. 462–477, Wiley, New York
4. Gabriel, O. (1978) *Trends Biochem. Sci.* **3,** 193–195
5. Gabriel, O., and Van Lanten, L. (1978) *Int. Rev. Biochem.* **16,** 1–36
6. Bertland, A. U., II (1970) *Biochemistry* **9,** 4649–4654
7. Ray, M., and Bhaduri, A. (1980) *J. Biol. Chem.* **255,** 10777–10781
8. Ray, M., and Bhaduri, A. (1980) *J. Biol. Chem.* **255,** 10782–10783
9. Mukherji, S., and Bhaduri, A. (1986) *J. Biol. Chem.* **261,** 4519–4524
10. Samanta, A. K., and Bhaduri, A. (1983) *J. Biol. Chem.* **258,** 11118–11122
11. Bhattacharya, D. (1993) *Biochemistry* **32,** 9726–9734
12. Bhattacharjee, H., and Bhaduri, A. (1992) *J. Biol. Chem.* **267,** 11714–11720
13. Mukherjee, S., and Bhaduri, A. (1992) *J. Biol. Chem.* **267,** 11709–11713
14. Darrow, R. A., and Rodstrom, R. (1968) *Biochemistry* **7,** 1645–1654
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
16. Laemmli, U. K. (1970) *Nature* **227,** 680–685
17. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) in *Molecular Cloning: A*

Laboratory Manual, p. 466, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Patchornik, A., Lowson, W. G., Gross, E., and Witkop, B. (1960) *J. Am. Chem. Soc.* **82,** 5923–5927
19. Spande, T. F., and Witkop, B. (1967) *Methods Enzymol.* **11,** 498–532
20. Udenfriend, S. (1962) *Fluorescence Assay in Biology and Medicine*, Vol. 1, p. 249, Academic Press, New York
21. Helene, C., Brun, F., and Yaniv, M. (1969) *Biochem. Biophys. Res. Commun.* **37,** 393–398
22. McClure, W. O., and Edelman, G. M. (1967) *Biochemistry* **6,** 559–566
23. Grinvald, A., and Steinberg, I. Z. (1974) *Anal. Biochem.* **59,** 583–598
24. Weber, G. (1957) *Nature* **180,** 1409
25. Stryer, L. (1978) *Annu. Rev. Biochem.* **47,** 819–846
26. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, p. 342, Plenum Press, New York
27. Shanmugasundaram, T., Kumar, G. K., and Wood, H. G. (1988) *Biochemistry* **27,** 6499–6503
28. May, J. M., Zhi-chao, Qu., and Beechem, J. M. (1993) *Biochemistry* **32,** 9524–9531
29. Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963) *Biochemistry* **2,** 616–622
30. Ramachandran, L. K., and Witkop, B. (1967) *Methods Enzymol.* **11,** 283–299
31. Peterman, B. F., and Laidler, K. J. (1979) *Biochim. Biophys. Acta* **577,** 314–323
32. Darrow, R. A., and Rodstrom, R. (1970) *J. Biol. Chem.* **245,** 2036–2042
33. Darrow, R. A., and Rodstrom, R. (1966) *Proc. Natl. Acad. Sci. U. S. A.* **55,** 205–212
34. Ray, M., and Bhaduri, A. (1976) *Eur. J. Biochem.* **70,** 319–323
35. Wiget, P., and Luisi, P. L. (1978) *Biopolymers* **17,** 167–180
36. Creveling, C. R., Bhaduri, A., Christensen, A., and Kalckar, H. M. (1965) *Biochem. Biophys. Res. Commun.* **21,** 624–630
37. Beechem, J. M., and Brand, L. (1985) *Annu. Rev. Biochem.* **54,** 43–71
38. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, p. 83, Plenum Press, New York
39. Eftink, M. R., and Ghiron, C. A. (1981) *Anal. Biochem.* **114,** 199–227
40. Lehrer, S. S. (1971) *Biochemistry* **10,** 3254–3263
41. Lakowicz, J. R. (1983) in *Principles of Fluorescence Spectroscopy*, p. 259, Plenum Press, New York
42. Porter, D. J. T. (1993) *J. Biol. Chem.* **268,** 66–73
43. Clermont, S., and Branlant, G. (1993) *Biochemistry* **32,** 10178–10184
44. Eklund, H., Samama, J. P., and Jones, T. A. (1984) *Biochemistry* **23,** 5982–5996
45. Bertland, A. U., and Kalckar, H. M. (1968) *Proc. Natl. Acad. Sci. U. S. A.* **61,** 629
46. Konopka, J. M., Halkides, C. J., Vanhooke, J. L., Gorestein, D. G., and Frey, P. A. (1989) *Biochemistry* **28,** 2645–2654
47. Burke, J. R., and Frey, P. A. (1993) *Biochemistry* **32,** 13220–13230
48. Almarsson, O., Karaman, R., and Bruice, T. C. (1992) *J. Am. Chem. Soc.* **114,** 8702–8704