Yeast Uridine Diphosphate Galactose 4-Epimerase Coenzyme Complexes. Fluorescence and Energy Transfer*

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ABSTRACT: The relative protein fluorescence intensity of yeast UDP galactose 4-epimerase apo enzyme in 0.01 M Tris-HCl buffer, pH 7.4, 10^{-4} M EDTA, 10^{-4} M mercaptoethanol at 25° is 50 ± 5% more than the holo enzyme, which contains bound NAD. The conversion of bound NAD to bound NADH, by 5′-UMP and L-arabinose modification, results in no further loss in protein fluorescence. However, the number of quanta lost from protein fluorescence due to bound NADH are now recovered in NADH fluorescence. Employing fully corrected fluorescence spectra and low absorptions, energy transfer has been shown directly by the actual number of quanta per unit band width transferred to the acceptor as related to the number of quanta lost from the donor. The efficiency of energy transfer in this system is 50 ± 1%.

UDP galactose 4-epimerase[1] isolated from induced yeast catalyzes the reversible conversion of UDP galactose into UDP glucose (Le Loir, 1951). It has also been isolated from mammalian sources (Maxwell, 1957) and from *Escherichia coli* (Wilson and Hogness, 1964). Efforts to elucidate the mechanism of this important enzymatic reaction, the inversion of the hydrogen and hydroxyl group on carbon 4 of the hexose, are still unsuccessful.

Our yeast enzyme is prepared from *Saccharomyces fragilis* (*Candida pseudotropicalis*) A.T.C.C. 1002. The purification and assay procedures are methods of Darrow and Rodstrom (1968), which is a modification of the earlier method of Maxwell *et al.* (1958). The purified enzyme has a molecular weight of approximately 125,000, with approximately 1 mole of NAD or NADH bound per mole of enzyme (Bhaduri *et al.*, 1965). There are 14–16 available sulfhydryl groups, determined with *p*-mercuribenzoic acid (Creveling *et al.*, 1965). Amino acids analysis shows 3.4 tryptophan, 40 tyrosine, and 45 phenylalanine residues per 10^5 g of protein (Darrow and Rodstrom, 1968).

The present preparation of the enzyme, 90–95% pure (Darrow and Rodstrom, 1968), emits a strong blue fluorescence, with emission maximum at 450 mμ and excitation maximum at 340 mμ, first demonstrated in relatively crude preparations in 1958 by Maxwell *et al.* The enzyme can be subjected to a specific modification by 5′-UMP and highly specific free sugars. The sugars are related to *in vivo* induction and repression of the biosynthesis of UDP galactose 4-epimerase; they are D-galactose, D-fucose, or L-arabinose, their optical isomers L-fucose, D-arabinose being inactive (Bertland *et al.*, 1966). This protein, biochemically modified, by what one might call substrate analogs, increases the fluorescence emission at 450 mμ to a quantum yield of almost 90% and is assumed to be the result of reduction of bound NAD (Bhaduri *et al.*, 1965). The fluorescence is retained by the protein after Sephadex column chromatography that removes the sugar indicating that it is only loosely bound. The [^{14}C]uridine 5′-monophosphate remains, however, strongly bound, approximately mole/mole of enzyme (Bertland *et al.*, 1966). The increase in fluorescence of the modified enzyme is accompanied by a loss of catalytic activity to about 5% of the original. The modification can, however, be made reversible, but it is dependent on removal of excess sugar, low protein concentration, and storage at 4°. The result is a return of catalytic activity, even higher than the original native enzyme, accompanied by a loss in fluorescence. The modification and the reversal are a cycle which can be repeated several times (Bertland *et al.*, 1968).

The fluorescence studies of UDP galactose 4-epimerase upon 5′-UMP and sugar modification suggested to us conformational changes of the protein. Accordingly, the native and the modified enzymes were investigated by methods of optical rotatory dispersion and circular dichroism, as well as infrared spectroscopy. This study showed that there was considerable change in protein conformation upon modification (Bertland and Kalckar, 1968; Bertland *et al.*, 1968).

In light of this change in the polypeptide backbone structure of UDP galactose 4-epimerase, we have returned to reexamination of fluorescence, with respect to prosthetic group and protein fluorescence, including energy transfer.

Materials and Methods

Reagents. UDP glucose dehydrogenase, NAD, NADH, 5′-UMP, galactose, and D- and L-fucose were purchased from Sigma Chemical Co.; L-arabinose and UDP galactose from Calbiochem. All other reagents were obtained from general commercial sources.

Enzyme. Epimerase was prepared from *Saccharomyces fragilis* (*Candida pseudotropicalis*) A.T.C.C. 10022. The purification and assay procedures are methods of Darrow and Rodstrom (1968). Large-scale preparations from 150-gal. fermentations have been made at the Tufts New England Enzyme

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[1] Abbreviations used are: UDP galactose 4-epimerase, UDP glucose 4-epimerase (EC 5.1.3.2); UDP galactose, uridine 5′-(α-D-galactopyranosyl pyrophosphate); PMB, *p*-mercuribenzoic acid.

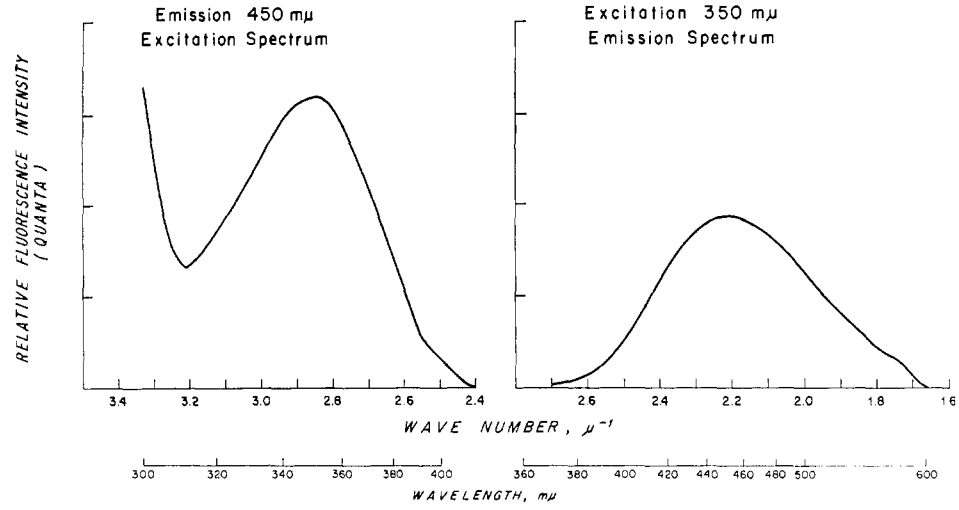

FIGURE 1: Fluorescence excitation spectrum and fluorescence emission spectrum of native UDP galactose 4-epimerase in 0.01 M Tris-HCl buffer (pH 7.4)–10^{-4} M EDTA–10^{-4} M mercaptoethanol at 25°.

Center. Enzyme units are reported as international units, 1 unit being equivalent to the transformation of 1 μmole of UDP galactose to UDP glucose per min (25°) by 1 mg of epimerase. Protein was determined by absorption at 280 and 260 mμ, or by the Biuret reaction.

Absorbance. Absorbance was obtained by a Zeiss PMQ II spectrophotometer and a Cary Model 15 recording spectrophotometer with a 1-cm path length cell.

Fluorescence. Corrected fluorescence emission and excitation spectra were measured with a Turner Model 210 "Spectro" absolute spectrofluorometer. The instrument and methods of correction are described by Turner (1964). Quartz cells 1 × 1 cm path length were used. Fluorescence units were standardized with quinine sulfate. The resolution in all figures presented is 100 Å for both excitation and emission wavelength. All the fluorescent spectra are reproducible at 25-Å resolution with about 1% error.

In all fluorescence spectra the ordinates are proportional to quanta per unit frequency interval (the excitation spectra are multiplied by wavelength and the emission spectra by wavelength squared) and the abscissa are in wave numbers, μ^{-1}, and in wavelength, mμ. All measurements were performed at 25°, and the temperature was controlled by circulating water bath.

TABLE I: Fluorescence Intensity of Native UDP Galactose 4-Epimerase.[a]

Preparation Number	Enzymatic Activity	Absorbance 280/260 mμ	Fluorescence Intensity: Excitation at 350 mμ/Emission at 450 mμ
I	42	1.26	30–36
II	68	1.53	23–36
III	34	1.32	20–40
IV	62	1.53	30–37
V	51	1.53	30–40

[a] Fluorescence was measured in 0.1 M Tris-HCl buffer (pH 7.4) containing 0.014 M mercaptoethanol and 10^{-3} M EDTA at 25° with a 1-cm light path. The intensity of fluorescence was based on the milligrams of epimerase protein and is linear over a range of 0.05–10 mg/ml. Enzymatic activity is in units equivalent to the transformation of 1 μmole of UDP galactose to UDP glucose per min (25°) by 1 mg of epimerase.

Results

Fluorescence of Native Epimerase. Different preparations of yeast UDP galactose 4-epimerase contain variable amounts of tightly bound NAD as measured by the alkali fluorescence method of Lowry *et al.* (1957) and reported by Darrow and Rodstrom (1968). NADH, the other tightly bound nucleotide, is also present in various amounts and accounts for the characteristic blue fluorescence of the native enzyme. Quantitative determinations have shown the stoichiometric relationship of 1 mole of NAD or NADH per mole of enzyme of molecular weight of approximately 125,000 (Darrow and Rodstrom, 1968). The blue fluorescence attributed to NADH on native enzyme, however, is never more than one-third of the total nicotinamide–adenine dinucleotide content (Creveling *et al.*, 1965).

The fluorescence of various preparations of highly purified epimerase are shown in Table I. The yields of fluorescence intensity are based on milligrams of epimerase protein. The yields vary from 20 to 40 specific fluorescence units/mg of protein. While there is no apparent correlation between catalytic activity and fluorescence, one was unable to measure native fluorescence in earlier preparations with specific catalytic activity below 20.0 using 1.57 mg of protein/ml (Creveling *et al.*, 1965). In the present system one can measure native epimerase fluorescence to 0.05 mg of protein/ml. The fluorescence excitation and emission spectra at their maximum are shown in Figure 1.

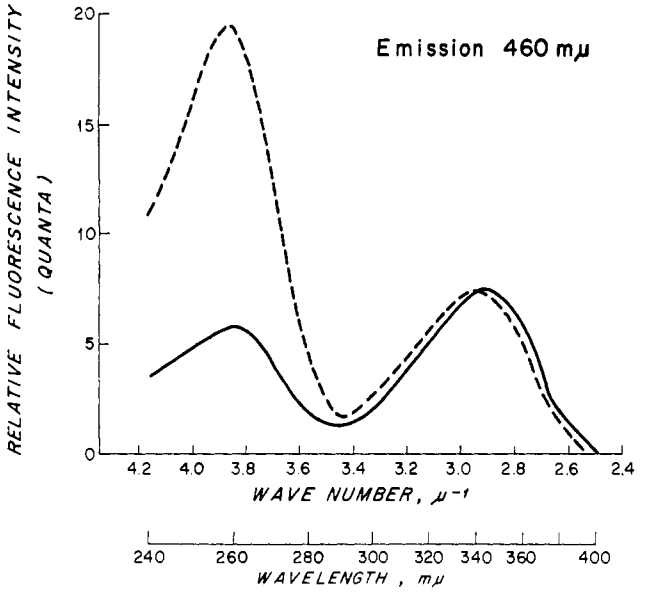

FIGURE 2: Fluorescence excitation and absorption spectra of reducted nicotinamide-adenine dinucleotide 1.65×10^{-6} M in 0.01 M Tris-HCl buffer (pH 7.4)-10^{-4} M EDTA-10^{-4} M mercaptoethanol at 25°. Absorption and excitation were normalized at 340 mμ.

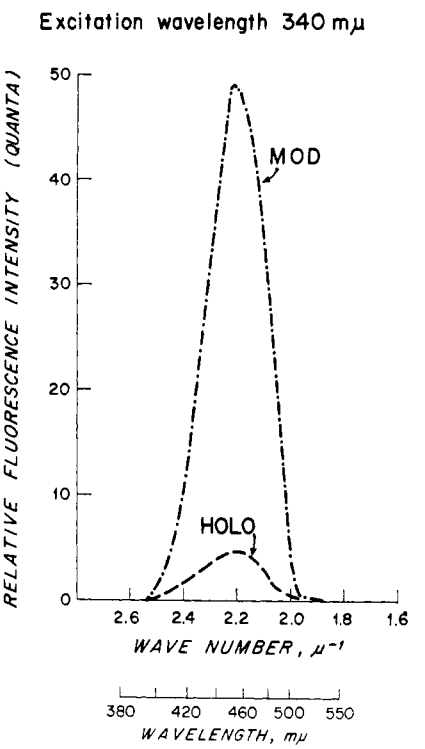

FIGURE 3: Fluorescence emission spectra of native (holo) and UMP 10^{-4} M, L-arabinose 10^{-2} M, treated (mod) UDP galactose 4-epimerase, in 0.1 M Tris-HCl buffer (pH 7.4)-10^{-3} M EDTA-10^{-2} M mercaptoethanol at 25°.

Reduced nicotinamide-adenine dinucleotide fluorescence is shown in Figure 2. The excitation spectrum of NADH on the Turner absolute spectrofluorometer shows two peaks, one at 260 mμ and one at 340 mμ, in agreement with the absorption spectrum. The excitation spectrum was obtained at an NADH concentration of 1.65×10^{-6} M, corresponding to a maximum absorption of about 0.025 optical density unit at 260 mμ. Emission intensities were proportional to concentration up to 2×10^{-5} M NADH. Fluorescence measurements are strictly linear, however, only when the sample has essentially 95–99% transmission. At higher concentrations of NADH the excitation spectrum intensity becomes gradually disproportioned, due to less transmittance at 260 mμ, while transmittance at 340 mμ is still within the 95–99% limit. The absorption spectrum was normalized at 340 mμ to the excitation spectrum. In the 260-mμ region, 80–90% of the absorption is due to the purine part of the molecule as shown in Figure 2, solid line. This indicates that 30% of the photons absorbed by the adenine portion of the NADH molecule appear as nicotinamide fluorescence, as stated by Weber (1957).

Effect of Biochemical Modification of UDP Galactose 4-Epimerase Fluorescence. It was shown (Bhaduri *et al.*, 1965) that addition of 5′-UMP and glucose or galactose will greatly increase the bound NADH fluorescence of the native enzyme. Subsequently, systematic examination of various nucleotides and sugars (Bertland *et al.*, 1966) indicated a combination of 5′-UMP and galactose or D-fucose as most effective in increasing the bound NADH fluorescence, the optical isomer L-fucose being inactive. Recently L-arabinose was found to be most effective in the rate of development of intensity of fluorescence. Figure 3 shows the fluorescence emission spectra of the holo (native) and modified (UMP, L-arabinose treated) epimerase. Fluorescence intensity increased approximately tenfold upon modification of the native epimerase. The fluorescence increase upon 5′-UMP, L-arabinose treatment of epimerase with time is shown in Figure 4. Two preparations of native epimerase with different enzymatic activities were compared for enhancement of fluorescence. The inactive optical isomer of D-fucose, L-fucose, was used as a control since D-arabinose is not completely inert, presumably because of impurities. The time course of events after addition of 5′-UMP and L-arabinose shows an initial rapid phase for about 20 min, followed by a slower rate. Enhancement is essentially complete in 60–120 min. The fluorescence of the enzyme preparation with the highest specific activity increased to a final value of at least tenfold over the native enzyme level. The enzyme with lower specific activity shows a fluorescence intensity increase also lower, about sevenfold.

The excitation spectra of the native enzyme with the emission monochrometer set at 450 mμ for bound NADH fluorescence is shown in Figure 5. The 260-mμ absorption band of NADH is completely obscured by the strong protein fluorescence band at 280 mμ due to tryptophan and tyrosine residues. Addition of 5′-UMP in 10^{-4} M concentration (OD_{260} 1.00) results in a shift of the protein excitation peak from 280 to 275 mμ. This apparent shift is a concentration artifact since the Turner records artifactually high fluorescence in samples with high optical density. Figure 5 is only used to show that both bands 280 and 350 mμ increase.

The final effect (after 3-hr incubation) of sugar and UMP combination on epimerase excitation spectra with the emission monochrometer set at 450 mμ is shown in the top curve of Figure 5. The protein fluorescence excitation band at 275 mμ increased concurrently with the excitation band at 350 mμ. The increase in fluorescence intensity at 450 mμ of each band was about eightfold. The increase in fluorescence at 275–280

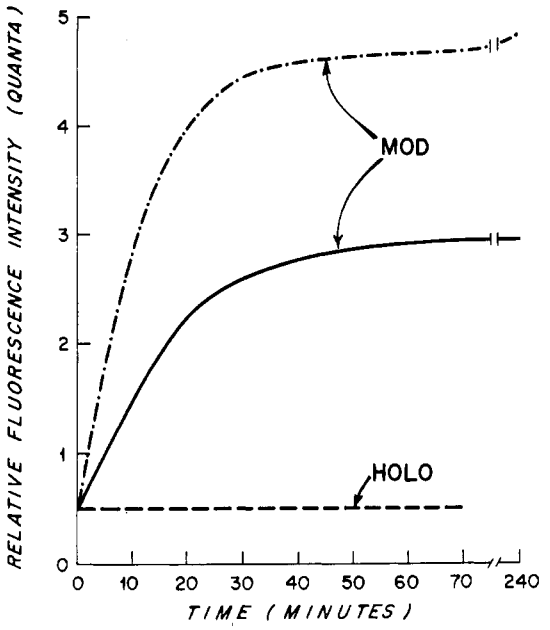

FIGURE 4: Time course of fluorescence increase of UDP galactose 4-epimerase with 10^{-4} M 5′-UMP and 10^{-2} M L-arabinose in 0.1 M Tris-HCl buffer (pH 7.4)–10^{-3} M EDTA–10^{-2} M mercaptoethanol at 25°. Two enzyme preparations are compared, one with specific activity 68 (·–·–·), one with specific activity 25 (——). Controls (holo, - - -) were treated with L-fucose 10^{-2} M–5′-UMP 10^{-2} M; excitation wavelength 340 mμ, emission wavelength 450 mμ.

mμ activation is due to NADH fluorescence, clearly shown here, by measuring emission at 450 mμ. It occurs in a region where NADH absorption is low and protein absorption is maximal. In agreement with Velick (1958), the nucleotide activation in this region arises by energy transfer from the

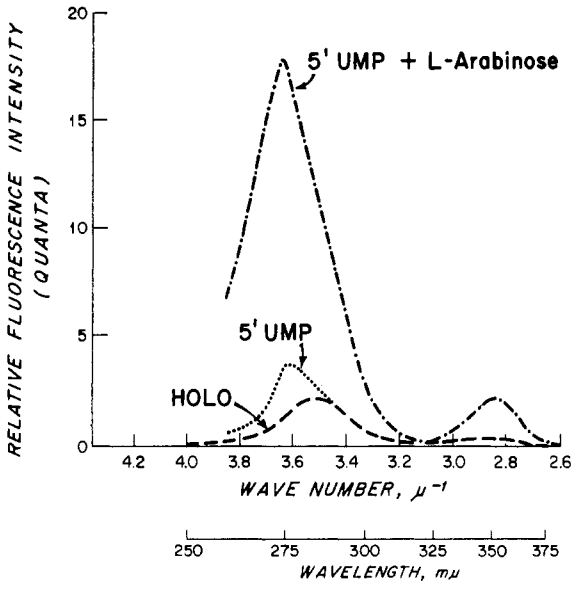

FIGURE 5: Excitation spectra are shown, emission fixed at 450 mμ: (---) native (holo) UDP galactose 4-epimerase; (.....) after addition of 5′-UMP (10^{-4} M); (·–·–·) after addition of 5′-UMP (10^{-4} M) and L-arabinose (10^{-2} M) and incubated 3 hr at 25° in 0.1 M Tris-HCl buffer (pH 7.4)–10^{-3} M EDTA–10^{-2} M mercaptoethanol.

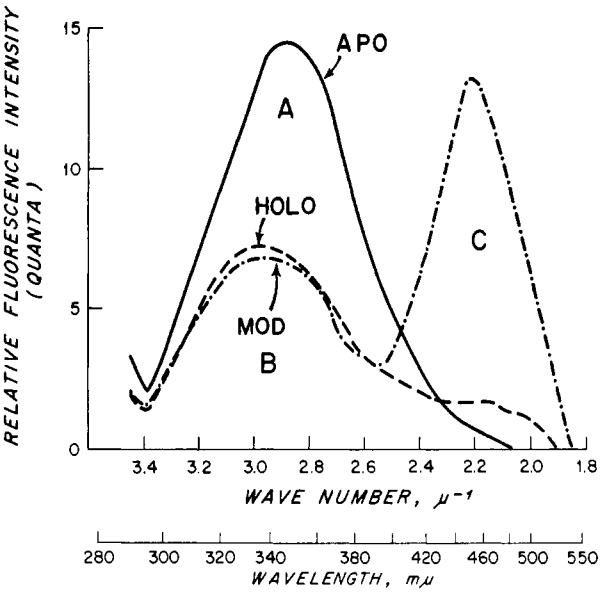

FIGURE 6: Emission spectra are shown of apo epimerase (——), holo epimerase (---), and 5′ UMP, L-arabinose modified epimerase (mod, ·–·–·), in 0.01 M Tris-HCl buffer (pH 7.4)–10^{-4} M EDTA–10^{-4} M mercaptoethanol at 25°. Protein absorption adjusted at 280 mμ to 0.08 optical density unit, 1-cm light path.

excited aromatic residues of the protein to the bound reduced nicotinamide–adenine dinucleotide. It is also evident when compared with Figure 2, free NADH excitation spectra, that photons absorbed by the protein aromatic amino acids must contribute to the fluorescence of the nicotinamide portion of the NADH molecule.

Energy Transfer, Protein Fluorescence Quenching. Evidence for energy transfer is shown by the excitation and emission spectra of the apo, holo, and modified enzymes in Figures 6 and 7. The apo enzyme is prepared by PCMB treatment of the holo (NAD) or modified (NADH) epimerase. The protein is treated in one step for 30 min at 25°, with 24–25 equiv of PCMB per mole of enzyme (mol wt 120,000), in the absence of EDTA or mercaptoethanol. The cofactor is immediately removed on a Sephadex G-50 column at 4°, equilibrated with 0.01 M Tris-HCl buffer, pH 7.5, containing 10^{-3} M mercaptoethanol.

The apo epimerase is essentially 100% resolved from PCMB and cofactor as measured by enzymatic activity and no NAD or NADH remaining could be detected by the alkali fluorescence method (Lowry *et al.*, 1957). The method was used at a sensitivity that would have detected 4% of remaining cofactor. The apo enzyme can be reactivated to 75–100% of original enzymatic activity with NAD or NADH at 10- to 50-fold excess equivalents per mole of enzyme in the presence of 0.1 M mercaptoethanol. The quantitative recovery of NAD from the holo enzyme is of the magnitude of 1 mole of NAD per mole of enzyme, and from the modified enzyme it is 1 mole of NADH per mole of enzyme. This shows that reduction does occur and that it is complete. The physical characteristics of the apo enzyme are under investigation, as well as the quantitative recovery and characterization of the cofactor resulting from the above treatment, and will be described in a subsequent publication.

The apo, holo, and UMP L-arabinose modified enzymes

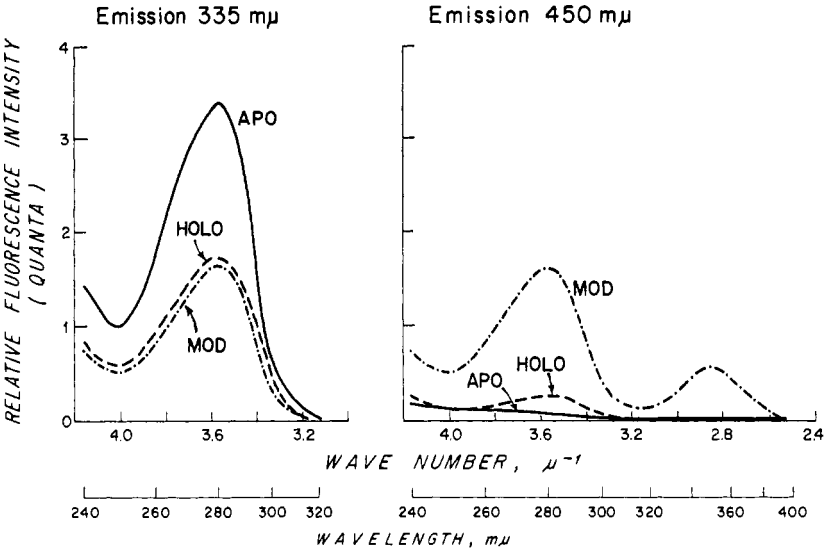

FIGURE 7: Excitation spectra of apo epimerase emission are shown at 335 mμ (——) and 450 mμ (——); holo epimerase emission at 335 mμ (---) and 450 mμ (---); 5′-UMP, L-arabinose modified epimerase emission at 350 mμ (·—·—·—·) and 450 mμ (·—·—·—·) in 0.01 M Tris-HCl buffer (pH 7.4)–10^{-4} M EDTA–10^{-4} M mercaptoethanol at 25°. Protein absorption adjusted at 280 mμ to 0.08 optical density unit, 1-cm light path.

were all chromatographed on a Sephadex G-50 column, to remove in the later case the sugar and all but approximately 1 mole of UMP/mole of enzyme (mol wt 125,000). The absorbance of all three enzyme preparations was adjusted at 280 mμ, 1-cm light path, to 0.08 optical density unit. The emission spectra in Figure 6 are proportional to concentration and have been reproduced at 0.04 and 0.02 optical density unit at 280 mμ. Absorption of 0.08 optical density unit at 280 mμ was chosen for illustration in order to include the small contribution of bound NADH fluorescence of the native enzyme.

The areas under the curves are relative quanta and it is intended only to show the relative fluorescence quantum efficiencies of the three protein preparations, which have equal tryptophan and tyrosin concentrations. The decrease of protein fluorescence of the holo epimerase when excited at 280 mμ, as compared to the apo enzyme, is indicative of quenching of protein fluorescence as in other NAD dehydrogenase systems. Nicotinamide–ademine dinucleotide is nonfluorescent, and its absorption band is considerably lower than the absorption and emission bands of tryptophan or tyrosine. Consequently, the acceptor cannot be sensitized by the donor absorption. It has been suggested that low temperature luminescence studies should be performed to show sensitization of NAD phosphorescence.

The average quenching of the apo enzyme protein fluorescence after 5′-UMP and L-arabinose modification is 50%, which is accompanied with the appearance of NADH fluorescence at 450 mμ. This indicates energy transfer from the aromatic amino acids to the reduced pyridine nucleotide like that observed in other protein–NADH interactions (Velick, 1958).

The efficiency of energy transfer T is defined as the number of quanta transferred to the acceptor divided by the number of quanta absorbed by the donor. The area in Figure 6 (quanta per unit frequency interval) are: protein fluorescence of apo enzyme, A + B; protein fluorescence of holo enzyme, protein fluorescence of mod enzyme, B; loss of protein fluorescence in conversion of apo enzyme to holo enzyme, A; quanta transferred from protein fluorescence to NADH fluorescence, C. Efficiency of energy transfer (T) can also be calculated from increase in fluorescence of the acceptor (Brandt and Withold, 1967).

$$T = \frac{OD_{\text{acceptor, 340}}}{OD_{\text{donor, 280}}} \times \frac{\text{fluorescence of acceptor excited at 280 m}\mu}{\text{fluorescence of acceptor excited at 340 m}\mu}$$

$$T = \frac{(0.00414)\,(130)}{(0.00800)\,(13)} = 0.51$$

The efficiency of transfer (50%) is in excellent agreement with 50% quenching. The number of quanta lost from protein fluorescence upon NADH binding are completely accounted for in NADH fluorescence. The use of low absorption and the fully corrected fluorescence values permit such direct evidence for energy transfer.

Discussion

It is generally recognized that binding of reduced nicotinamide–adenine dinucleotide coenzyme to a dehydrogenase leads to a shift in excitation and fluorescence maxima and to an alteration in fluorescence intensity. The fluorescence intensity of the bound NADH usually increases (except in triose phosphate dehydrogenase) over that of free NADH two- to threefold.

The present epimerase system is unique in that the increase in bound NADH fluorescence is at least eight- to tenfold. This increase of NADH fluorescence suggests that NADH in the epimerase complex is shielded and thermal deactivation is restricted. The number of quanta lost from protein fluores-

cence is emitted as NADH fluorescence. The high efficiency of transfer from protein to coenzyme does suggest particularly favorable spatial or geometric factors. A change of lifetime of excited state raises, however, new problems and a detailed discussion is postponed until studies of the lifetime of excited states are examined, which are presently under way.

The UMP, L-arabinose modification of the holo enzyme results in reduction of bound NAD to bound NADH. Considerable conformational changes accompany this reduction, as was shown by ultraviolet circular dichroism and optical rotatory dispersion (Bertland and Kalckar, 1968) as well as by infrared spectra of the amide I band (Bertland *et al.*, 1968). These changes certainly could modify the environment of the bound NADH to produce the resulting high efficiency of transfer and, in fact, the studies were done because of our observations with fluorescence. The shift in emission maximum of NADH from 465 to 450 mμ when bound is similar to other dehydrogenases and suggests the hydrophobic environment of the binding site. Another unique feature, however, is the observation that no extrinsic Cotton effect could be detected in the epimerase NADH system, as opposed to a number of NADH–protein complexes (Ulmer and Vallee, 1965).

The average loss in protein fluorescence intensity due to bound NAD in the holo enzyme as compared with the apo enzyme is 50%. This loss in protein fluorescence is generally ascribed to loss of photons due to thermal deactivation. Bound NAD in a number of dehydrogenases affects protein fluorescence similarly.

Preliminary observations on the polarization of the 450-mμ emission band show a preservation of high polarization (0.4–0.42) not only through excitation at 340 mμ but also through excitation at the aromatic band at 280 mμ. It has been suggested by Dr. H. M. Kalckar that a large number of aromatic amino acids stacked in longer sequences of antiparallel β structure on each side of the NADH group oriented properly toward this group are contributing to the energy transfer and to the preservation of fluorescence polarization.

It is not possible at the present to determine which or how many aromatic amino acids are involved. Nevertheless, the protein moiety does respond to the oxidative or reductive state of the prosthetic group and provides a built-in sensitive physicochemical parameter for the study of structure–function relationships in epimerase. The sensitivity and application of protein fluorescence presented in this paper, as an indication of protein conformation, had been recently shown in the multiple forms of chicken heart aspartate aminotransferase (Bertland and Kaplan, 1970).

The molecular weight of epimerase is independent of the oxidative or reductive state of the cofactor (Linda H. Bertland and A. U. Bertland, II, unpublished).

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