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# *Escherichia coli* Uridine Diphosphate Galactose 4-Epimerase: Circular Dichroism of the Protein and Protein Bound Dihydronicotinamide Adenine Dinucleotide†

Shan S. Wong, Joseph Y. Cassim, and Perry A. Frey*

ABSTRACT: The circular dichroism spectra of *E. coli* UDP-galactose-4-epimerase in its native (epimerase·$NAD^+$) and reduced (epimerase·NADH·UMP) forms between 190 and 400 nm are presented. The reduced form exhibits a large positive circular dichroism band at 340 nm attributed to NADH in the complex. Relative to the small negative band exhibited at this wavelength by free NADH itself, the rotational strength of enzyme-bound NADH is some 50 times larger than that of free NADH, while the oscillator strengths and other spectral characteristics are similar. This enhancement reflects dissymetric interactions involving the 340-nm transition and is most consistent with the dihydropyridine ring of NADH being highly immobilized in the reduced complex. In the 200- to 230-nm region both enzyme forms exhibit a negative band at 220 nm and a negative shoulder at 208 nm. The ellipticities of the reduced form are minimally 7% greater at both band positions than those of the native form. The spectra are interpreted to indicate that conversion of the native to the reduced form is accompanied by an increase in $\alpha$-helix structure at the expense of unordered structure.

The UDP-galactose 4-epimerase catalyzed interconversion of UDP-galactose and UDP-glucose has been shown to proceed by the following pathway

$$\begin{aligned} &\text{E·NAD}^+ \\ &\quad + \quad \rightleftharpoons \text{E·NAD}^+\text{·UDP-Gal} \\ &\text{UDP-Gal} \\ &\qquad \rightleftharpoons \text{E·NADH·UDP-4-ketopyranose} \\ &\qquad\qquad\qquad\qquad\qquad\qquad \text{E·NAD} \\ &\qquad\qquad \rightleftharpoons \text{E·NAD}^+\text{·UDP-Glc} \rightleftharpoons \quad + \\ &\qquad\qquad\qquad\qquad\qquad\qquad \text{UDP-Glc} \end{aligned}$$

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in which E·NAD+ symbolizes the homogeneous *E. coli* enzyme. This is a 79 000 mol wt dimer of identical subunits containing one molecule of tightly bound NAD+ per dimer (Wilson and Hogness, 1964, 1969). The UDP-4-ketopyranose intermediate is converted to either UDP-glucose or UDP-galactose upon reduction by NADH in the central complex (Nelsestuen and Kirkwood, 1971; Maitra and Ankel, 1971; Wee and Frey, 1973; Adair et al., 1973). In this process it is exclusively the *pro-S* hydrogen at C-4 of the dihydronicotinamide ring in NADH that is involved in hydrogen transfer; however, this hydrogen is transferred to either face of the ketone group in the substrate-derived intermediate. This aspect of the action of UDP-galactose 4-epimerase is unusual because enzymatic reactions are in general stereospecific; yet nonstereospecific hydrogen transfer is the essence of the epimerization of UDP-glucose and UDP-galactose. This mechanism is of interest because it is likely to involve enzyme–substrate interactions unlike those characteristic of stereospecific reactions. With reference to the broader problem of the mechanisms of action of enzymes, the elucidation of the mechanism of nonstereospecific enzyme action may give insights not available from studies on the usual stereospecific reactions.

It is evident that the central complex must react through two structurally different transition states to produce the two Michaelis complexes which contain either UDP-galactose or UDP-glucose. The structural difference must involve the spatial orientations of NADH and UDP-4-ketopyranose. In order to reach two such transition states NADH and UDP-4-ketopyranose must undergo spatial reorientation involving either motion of one relative to the other or of both relative to each other. An understanding of this reorientation process is what is needed to understand nonstereospecific action by this enzyme.

In earlier work from this laboratory the binding affinities of epimerase·NAD+ and epimerase·NADH for uridine nucleotides and glucose were studied as well as the epimerase–NAD+ and epimerase–NADH interactions (Wee and Frey, 1973; Kang et al., 1975; Wong and Frey, 1977). The results led us to propose that the structural reorganization does not involve motion by the dihydronicotinamide moiety of NADH. We postulated that nonstereospecific hydrogen transfer can be explained and understood on the basis of rotational freedom on the part of the 4-ketopyranose moiety of the substrate-derived intermediate. The evidence on the immobility of the dihydronicotinamide ring, while extensive, could not be accepted as conclusive. Therefore, we have now undertaken to obtain further information on this point by investigating the degree of dissymmetry in the environment of the dihydronicotinamide ring in epimerase·NADH·UMP complex, as revealed in the circular dichroism spectrum.

The epimerase·NAD+ complex can be converted to inactive epimerase·NADH·uridine nucleotide complexes by reduction with sugars such as glucose, galactose, arabinose, fucose, etc. and with $NaBH_3CN$. These reducing agents react with epimerase only in the presence of a uridine nucleotide (Bhaduri et al., 1965; Bertland and Kalckar, 1968; Kalckar et al., 1969, 1970; Blackburn and Ferdinand, 1976). The kinetics of UMP-dependent reduction by glucose indicate that UMP activation involves the induction of a conformational change in the epimerase·NAD+ complex (Kang et al., 1975). Epimerase·NADH complexes are known to bind uridine nucleotides with 100–1000 fold greater affinity than epimerase·NAD+ complex. This is also indicative of a structural difference involving more than simply the difference between NAD+ and NADH, which itself should not lead to tighter binding of negatively charged ligands by epimerase·NADH than by epimerase·NAD+. We have also measured the CD spectra of these complexes in the peptide region and detected a difference in secondary structure.

## Experimental Procedure

Homogeneous UDP-galactose-4-epimerase was purified from *Escherichia coli*, ATCC 27797, by the procedure of Wilson and Hogness (1964). Samples of epimerase·NADH·UMP and epimerase·NAD+ were prepared for circular dichroism (CD) measurements. The reduced complex was prepared by reacting epimerase·NAD+ with 0.7 M glucose and 0.8 mM UMP for 2 to 3 h. Epimerase·NADH·UMP complex was isolated by passing the solution through a Bio-Gel P-2 column equilibrated and eluted with a buffer consisting of 35.5 μM UMP in 10 mM potassium phosphate buffer at pH 8.5. Samples of epimerase·NAD+ for comparison were prepared at the same time and in exactly the same way, with the exception that glucose was excluded. CD spectra were measured at 25 °C in the foregoing elution buffer on a Cary Model 60 spectropolarimeter with 6003 CD attachment (Varian Instrument Division, Palo Alto, Calif.). The CD mode was calibrated with a standard solution of *d*-camphor-10-sulfonic acid (Aldrich Chemical Co., Milwaukee, Wis.) by the method of Cassim and Yang (1969). Absorption spectra were measured with a Cary Model 118C low ultraviolet double-beam recording spectrophotometer with scattered transmission accessory (Varian). Details of the instrumental procedures used have been presented in detail elsewhere (Becher and Cassim, 1975, 1976; Cassim and Lin, 1975; Rafferty et al., 1977). Protein concentrations were determined by the micro-Kjeldahl technique. The mean ellipticity, $[\theta]$, was calculated in deg cm$^2$ per dmol of amino acid residues unless otherwise indicated, using the mean residue weight 127.4 calculated from the amino acid analysis published by Wilson and Hogness (1969).

## Results and Discussion

The near ultraviolet CD spectra of *Escherichia coli* epimerase·NADH·UMP and epimerase·NAD+ complexes as well as of a comparable concentration of NADH are given in Figure 1. The spectrum of epimerase·NADH·UMP consists of a well-defined intense positive nearly Gaussian band at ca. 340 nm and less well-defined positive and negative bands at ca. 296 and 280 nm, respectively. The 340-nm CD band is of particular interest, since it corresponds to the 340-nm band attributed to NADH in the absorption spectrum of epimerase·NADH·UMP. Also shown in Figure 1A is the spectrum of free NADH, which exhibits a weak negative CD band at about the same wavelength. This characteristic 340-nm band has been attributed to electronic transition of the dihydronicotinamide moiety of NADH. Although the transition assignment for this band has as yet not been established, the $N-V_1$ transition has been suggested (Evleth, 1967; Miles and Urry, 1968; Miles et al., 1968).

Based on the Gaussian assumption for the 340-nm band shape, the rotational strengths for bound and free NADH in Figure 1A were approximated as

$$R = \frac{1.23 \times 10^{-42}[\theta_{max}]\Delta}{\lambda_{max}}$$

where $[\theta]_{max}$ is the maximum molar ellipticity of the band, $\lambda_{max}$ is the wavelength of the maximum ellipticity, and $\Delta$ is the half band width at $[\theta_{max}]/e$. The oscillator strengths were approximated as

$$f = \frac{7.5 \times 10^{-2}\epsilon_{max}\Delta}{\lambda_{max}}$$

TABLE I: Spectral Parameters for Bound and Free NADH.

| | CD spectrum | | | | Absorption spectrum | | | |
|---|---|---|---|---|---|---|---|---|
| | Molar ellipticity of extremum $\times 10^{-3}$ (deg cm$^2$ dmol$^{-1}$) | Half band width (nm) | Wavelength of extremum (nm) | Rotational strength $\times 10^4$ (erg cm$^3$) | Molar extinction coefficient $\times 10^{-3}$ of extremum (mol$^{-1}$ cm$^{-1}$) | Half band width (nm) | Wavelength of extremum (nm) | Oscillator strength |
| NADH | 1.03 | 29.0 | 340 | 1.08 | 6.2 | 33.5 | 338 | 0.136 |
| Epimerase·NADH·UMP | 43.75 | 34.0 | 340 | 53.8 | 6.2 | 36.0 | 343 | 0.142 |

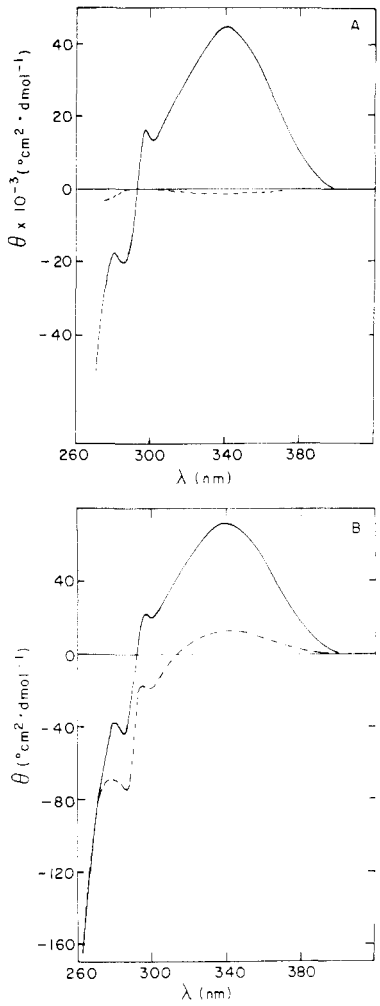


FIGURE 1: Near ultraviolet circular dichroism of epimerase·NADH·UMP and free NADH. (A) The visible and near ultraviolet circular dichroic spectra of epimerase·NADH·UMP and free NADH. Ellipticities are calculated as deg cm$^2$ per dmol of NADH, assuming one molecule of NADH per dimer of epimerase and 79 000 as the molecular weight of epimerase dimer (Wilson and Hogness, 1964). The solid line is the spectrum of epimerase·NADH·UMP and the dashed line is that of free NADH. (B) The visible and near ultraviolet circular dichroic spectra of epimerase·NADH·UMP (solid line) and of epimerase·NAD$^+$ (dashed line) calculated as deg cm$^2$ per dmol of amino acid residue.

where $\epsilon_{max}$ is the maximum molar extinction coefficient of the absorption band at about 340 nm (spectra not shown), $\lambda_{max}$ is the wavelength of the maximum extinction coefficient, and $\Delta$ is the half band width at $\epsilon_{max}/e$.

The spectral parameters for free NADH and NADH in epimerase·NADH·UMP are given in Table I, which shows that the rotational strength of bound NADH is about 50 times larger than that of free NADH, while the oscillator strengths are similar.

Three types of dissymmetric interactions have been proposed to explain the generation of optical activity of molecules. They are: (1) the interaction of an electronic transition moment of one group with the dissymetric electrostatic fields of all other groups; (2) the coupling of electronic transition moments on different groups; and (3) the coupling of a magnetic transition moment of one group with the electric transition moment of another (Schellman, 1968). In cases in which an optically active chromophore is complexed with an optically active apoprotein, as in epimerase·NADH·UMP, the intrinsic dissymmetric interactions within the chromophore molecule may differ drastically from those in the free molecule. Moreover, there may be additional dissymmetric interactions between the apoprotein and the chromophoric transitions. This can result in significent enhancement of the rotatory strength of a transition such as that responsible for the 340-nm band of NADH. This transition moment could couple with others within the chromophoric molecule and/or with transition moments of groups within the apoprotein. In general the three mechanisms cited above contribute in varying degrees to the optical activity of the molecules. In the present case it is difficult to see how that mentioned in 2 can contribute significantly to the enhanced rotatory strength of the 340-nm transition of bound NADH, in view of the relatively low oscillatory strength of this transition and the absence of other transitions of close lying energies (Miles and Urry, 1968; Miles et al., 1968). The evaluation of the contributions of the other two mechanisms remains for further investigation.

Regardless of the mechanisms involved, the enhanced rotatory strength of the 340-nm transition of NADH in epimerase·NADH·UMP relative to NADH itself is indicative of enhanced dissymmetric interactions involving this transition. To achieve strong dissymmetric interactions, the dihydronicotinamide ring of NADH in epimerase·NADH·UMP complex must be highly immobilized.

These results complement the previously published fluorescence studies showing that NADH fluorescence in epimerase·NADH·UMP is highly polarized (Wong and Frey, 1977), and they give further independent evidence that the dihydronicotinamide ring of NADH in epimerase·NADH·UMP is strongly immobilized. Thus, as pointed out before (Wong and Frey, 1977), there remains no evidence that nonstereospecific hydrogen transfer mediated by enzyme-bound NADH could be accounted for on the basis of conformational mobility on the part of the coenzyme.

Also given in Figure 1B is the CD spectrum of epimerase·NAD$^+$ compared with epimerase·NADH·UMP. Epimerase·NAD$^+$ also exhibits a weak positive band at 340 nm, which is attributed to the presence of NADH at up to 20% of the active sites in the enzyme as purified in our laboratory. This also appears in the absorption spectrum.

Figure 2 shows the ultraviolet and far ultraviolet CD spectra of epimerase·NAD$^+$ and epimerase·NADH·UMP complexes.

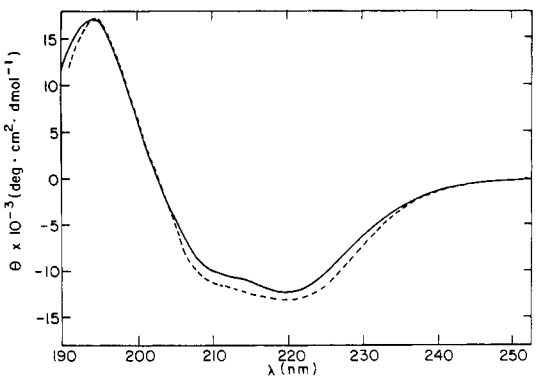


FIGURE 2: Far ultraviolet circular dichroism of UDP-galactose 4-epimerase. The dashed line curve is the spectrum of epimerase·NADH·UMP complex and the solid line is the spectrum of epimerase·NAD+.

The spectra of both forms consist of a negative band at ca. 220 nm, a negative shoulder at ca. 208 nm, and a positive band at ca. 194 nm. The ellipticities of the reduced form at the 220-nm and the 208-nm bands are minimally 7% greater than those of the oxidized form, with no significant band position shifts. Because of high noise to signal ratio in the 190- to 205-nm region, spectral differences between the oxidized and reduced forms were not well resolved. However, the differences in the 220-nm band and 208-nm shoulder are reproducible. The spectra shown in Figure 2 are mean spectra from three sets of data, and the differences shown in Figure 2 were observed in each of the three sets. In addition, the ellipticity change at 220 nm could be detected during the course of the reduction of epimerase·NAD+ to epimerase·NADH·UMP by glucose.

Analysis of the spectra in Figure 2 is based on the conventional assumptions that (a) the far ultraviolet CD of proteins can be attributed mainly to their secondary structures, i.e., $\alpha$ helix, $\beta$ form, and unordered form, and (b) homopolypeptides are reasonable spectral models for proteins (Sears and Beychok, 1973). The following analysis of the spectra in Figure 2 is based on the CD of poly(L-lysine) in the three (secondary structure) conformational states (Greenfield and Fasman, 1969). The presence of the shoulder at ca. 208 nm can be attributed to the presence of $\alpha$ helix in the protein, since the $\beta$ and unordered forms contribute negligibly to the spectra at that wavelength. The position of the band at ca. 220 nm rather than at 223 nm can be attributed to the presence of $\beta$ form in the protein, since $\alpha$ helix or combinations of $\alpha$ helix and unordered form would result in a band at ca. 223 nm, and only the presence of $\beta$ form would cause the blue shift to 220 nm. The low ellipticity of the positive 194-nm band relative to the ellipticities of the negative bands can be attributed to the presence of unordered form, since both the $\alpha$ helix and $\beta$ form make positive contributions in the vicinity of this wavelength. Further support for this analysis is obtained by comparing the spectra in Figure 2 with CD spectra of crystallographically determined proteins containing all the three secondary structural elements in varying degrees (Chen et al., 1974).

An analysis of the differences in the CD spectra of epimerase·NAD+ and epimerase·NADH·UMP, based on the above stated assumptions, indicates that the increased ellipticities of the 220-nm band and 208-nm shoulder in the spectrum of epimerase·NADH·UMP relative to that of epimerase·NAD+ can be attributed to an increase in the $\alpha$-helix structure at the expense of the unordered structure, since changes in the $\beta$ structure would be reflected in preferential changes in the 220-nm band relative to the 208 nm shoulder. This secondary structure difference is unlike that reported in the case of the yeast UDP-galactose-4-epimerase, which appears to involve increased $\beta$-structure in epimerase·NADH·UMP relative to epimerase·NAD+ (Bertland and Kalckar, 1968).

These CD data clearly establish a structural difference between epimerase·NAD+ and epimerase·NADH·UMP. Whether this results from the differential interactions of the protein with NAD+ and NADH or from the interactions of the protein in the reduced complex with bound UMP cannot be decided at this time. On the one hand the binding of UMP activates reduction of the bound NAD+ in such a way as to imply that it forces a structural change in the protein (Kang et al., 1975; Davis et al., 1974), while on the other hand the epimerase·NADH complex is known to bind uridine nucleotides with 100- to 1000-fold greater affinity than epimerase·NAD+, suggesting that NAD+, NADH interactions may drive the structural transition. The two effects are probably different manifestations of the structural difference between these two enzyme forms.

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# Extracellular Hydrolases of the Lung†

Gary F. R. Hook

With the Technical Assistance of Linda B. Gilmore

ABSTRACT: A pool of acid hydrolases exists within the acellular lining material of the alveoli and distal airways of the lungs. These extracellular hydrolases, obtained using pulmonary lavage procedures, appear to be of a selected variety insofar as some hydrolases (β-*N*-acetylglucosaminidase and α-mannosidase) are highly active while others (β-glucuronidase and arylsulfatase) are barely detectable. The origins of these hydrolases were investigated. Neither leakage of serum nor cell damage can account for the presence of the extracellular hydrolases in lavage effluents. Electrophoretic mobilities on acrylamide gels indicate that the extracellular hydrolases generally differ from those found in serum. Cytoplasmic soluble enzymes such as lactate dehydrogenase were used to monitor cell damage and show that the extracellular hydrolases did not originate from cell leakage during the lavage procedure. Hydrolases similar to those found extracellularly are associated with highly purified lysosome-free lamellar bodies isolated from homogenates of lung. The extracellular hydrolases are probably secreted by the type 2 cells of the pulmonary alveolar epithelium during their secretion of lamellar bodies.

The distal airways of the lungs, that is, the bronchioles and alveoli, are lined with an acellular layer of material whose presence is essential for the maintenance of normal pulmonary function. Although this acellular lining has been the subject of numerous investigations, the composition of the lining has not been elucidated completely and many of its properties have been recognized only recently.

The lipid components have been investigated extensively, especially those surface-active phospholipids such as dipalmitoyllecithin which play a major role in the stabilizing influence of the acellular lining in the distal regions of the lungs (Macklem et al., 1970; Pattle, 1958); however, the composition of the lining is not confined to phospholipids and many other components, particularly proteins, have been detected (Bhattacharyya et al., 1975; Bignon et al., 1975, 1976; Hand and Cantey, 1974; Reynolds and Newball, 1974).

The acellular lining as an enzymatically active medium has not been considered previously. In this report evidence is presented for the existence of selected lysosomal hydrolases within the acellular lining of the alveoli and distal airways of the lungs.

## Materials and Methods

*Materials.* Adult male rabbits of the New Zealand strain (Dutchland Laboratory Animals Inc., Denver, Pa.) weighing from 2 to 2.5 kg were used throughout. Rabbits were allowed free access to food and water.

Chemicals used are listed with their sources in parentheses as follows: disodium *p*-nitrophenyl phosphate, *p*-nitrophenyl acetate, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, *p*-nitrophenyl α-D-mannoside, *p*-nitrophenyl palmitate, *p*-nitrophenyl β-D-galactopyranoside, dipotassium *p*-nitrocatechol sulfate, α-ketoglutarate, DL-aspartate, NADH, malic dehydrogenase (Sigma Chemical Co., St. Louis, Mo.); sodium pyruvate, phenolphthalein glucuronide sodium salt (Calbiochem, San Diego, Calif.); Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.); acrylamide, *N,N'*-methylenebisacrylamide (Eastman Kodak Co., Rochester, N.Y.); bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.).

*Pulmonary Lavage Procedure.* Rabbits were killed by injection of 3–4 mL of sodium pentobarbital solution (50 mg/mL) into the marginal ear vein. The trachea was exposed and clamped shut immediately after inflation of the lungs by caudad pressure on the diaphragm. The trachea and lungs were removed intact and carefully dissected free of other tissues. The external surfaces of the lungs were rinsed free of blood using Hanks' solution (Hanks and Wallace, 1949). The trachea was cut approximately 1 cm below the clamp, gently closed below the bifurcation, and tracheal airway surfaces rinsed with Hanks' solution. A plastic tube was then inserted into the trachea and the lungs were made to respire, using minimal positive air pressure via the tube, until any signs of atelectasis had gone. The lungs and the trachea were then filled with ice cold Hanks' solution. No attempts were made to force the lavage medium into the lungs. The lungs were filled to the top of the trachea which was about 5 cm long. The external surfaces of the lung were very gently massaged for approximately 30 s to assist dispersion of the Hanks solution. The lungs were then inverted and the lavage fluid allowed to drain into the collecting vessel. The lavage procedure was repeated as many times as required by experimental design. Although the volumes of lavage effluents varied among different lungs, consecutive lavages were very similar (standard deviation amounted to less than 12%). The trachea was wrapped in several layers of absorbent gauze prior to drainage to avoid

† From the Laboratory of Pharmacology, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, North Carolina 27709. *Received September 8, 1977.*