Purification and Properties of the Escherichia coli K-12 NAD-dependent Nucleotide Diphosphosugar Epimerase, ADP-\(\text{L-glycero-\(D\)-mannoheptose} \) 6-Epimerase*

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Li Ding, Belinda L. Seto, S. Ashraf Ahmed*, and William G. Coleman, Jr.§

From the Section on Pharmacology and §Section on Enzyme Structure and Function, Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland 20892

The Escherichia coli K-12 NAD-dependent nucleotide-diphosphosugar epimerase, ADP-\(\text{L-glycero-\(D\)-mannoheptose} \) 6-epimerase, catalyzes the conversion of ADP-\(\text{r-glycero-\(D\)-mannoheptose} \) to ADP-\(\text{L-glycero-\(D\)-mannoheptose} \). ADP-\(\text{L-glycero-\(D\)-mannoheptose} \) is a key intermediate of lipopolysaccharide inner core biosynthesis in several genera of Gram-negative bacteria. Sedimentation equilibrium and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified epimerase revealed that the native enzyme has a molecular mass of 240 kDa and a subunit molecular weight of 37,000 ± 3,000. Lectin binding studies of the purified epimerase indicated that the protein is glycosylated. There was 1 mol of tightly bound NAD* per enzyme subunit. Variable but small fractions of purified preparations of epimerase are highly fluorescent and contain NADH. The native enzyme can be resolved into apoenzyme and NAD* by acidic ammonium sulfate precipitation. The catalytic activity can be reconstituted with the addition of NAD* to the apoenzyme. Optimum pH range for enzyme activity is broad, between 5.5 and 9.5. It exhibits a temperature optimum at 42 °C. The \(K_m\) and \(V_{\text{max}}\) for the substrate is 0.1 ms and 46 mmol min\(^{-1}\) mg\(^{-1}\), respectively. The native enzyme displays UV and fluorescence spectra that are consistent with the presence of enzyme bound NAD*. CD spectra of the holoenzyme indicate 11% \(\alpha\)-helical and 36% \(\beta\)-sheet structures.

ADP-\(\text{L-glycero-\(D\)-mannoheptose} \) 6-epimerase (EC 5.1.3.-), hereafter referred to as epimerase, catalyzes the interconversion of ADP-\(\text{D-glycero-\(D\)-mannoheptose} \) to ADP-\(\text{L-glycero-\(D\)-mannoheptose} \) (Fig. 1). This is the last step in the ADP-L-glycero-o-mannoheptose synthetic pathway (Fig. 1), as proposed by Eidel and Osborn (1). The proposed pathway has been further elaborated by results from several laboratories (2-7). ADP-L-glycero-o-mannoheptose is the precursor of the aldohexose, L-glycero-D-mannoheptose. L-glycero-D-mannoheptose is a typical component of the lipopolysaccharide core region of several genera of enteric and nonenteric Gram-negative bacteria. The epimerase is encoded by a gene designated, \(rfad\). Escherichia coli K-12 and Salmonella typhimurium strains with the \(rfad\) mutation exhibit the classical heptoseless phenotype, which includes mucoidal colonies, reduced growth rates and viability, a truncated lipopolysaccharide structure, and increased permeability to a large number of hydrophobic agents, most importantly, antibiotics. Wild type strains usually exclude these hydrophobic agents, rendering them refractory to antibiotic treatments. The common occurrence of the epimerase in several genera of Gram-negative bacteria provides a viable strategy for targeting this protein in antibiotic therapy (5, 6). We previously reported the cloning and sequencing of the \(rfad\) gene from \(E.\ coli\), the identification of the promoter region and the transcription start site (7). We also reported the preliminary purification and characterization of the gene product. The \(N\) terminus of each monomer has the fingerprint sequence Gly-X-Gly-X-Gly, which is characteristic of the ADP-binding \(\beta\)-fold of FAD-binding and NAD-binding proteins (8). In this study, we investigated the catalytic properties as well as the quaternary structure of the epimerase. These studies were facilitated by the development of a rapid and simple purification procedure to obtain large quantities of the homogeneous enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**

The substrate, ADP-\(\text{D-glycero-\(D\)-mannoheptose} \), was extracted and purified from a \(rfaD\) mutant (CL515), which accumulates this nucleotide, as described previously by Coleman (2). Blue 2 Sepharose CL-6B resin, phenylmethylsulfonyl fluoride, DNase I, RNase, \(\alpha\)-galactose, streptomycin sulfate, lactate dehydrogenase kit, and NADH were purchased from Sigma. Oxytetracycline was obtained from ICN ImmunoBiological and Sigma. The Lectin-Link kit was from Genzyme. \([\text{35S\text{-Methionine}}]\) was purchased from DuPont. Nucleotide sugars were obtained from Calbiochem.

**Assay of ADP-\(\text{L-glycero-\(D\)-mannoheptose} \) 6-Epimerase**

The epimerase assay was described previously by Coleman et al. (6). A typical assay mixture contained 0.1 M Tris acetate, pH 8.5, 25 mM NAD, 1.25 mM MgCl\(_2\), 5 nmol ADP-\(\text{L-glycero-\(D\)-mannoheptose} \), and enzyme, in a final volume of 50 μl. The reaction mixture was incubated at 37 °C for 30 min and was terminated by boiling for 3 min. The enzyme activity was determined by monitoring the formation of ADP-\(\text{L-glycero-\(D\)-mannoheptose} \) by high performance liquid chromatography. One unit of enzyme activity is defined as the epimerase activity capable of producing 1 nmol of ADP-\(\text{L-glycero-\(D\)-mannoheptose} \) in 30 min at 37 °C in 0.05 ml reaction mixture.

**Determination of NAD**

Epimerase-bound NAD was dissociated by perchloric acid treatment (9). The purified enzyme (63.8 μg) in 10 mm glycine-NaOH, pH 8.5 was treated with 35% perchloric acid at a ratio of 9:1, \(\text{v/v}\), and the mixture (200 μl) was incubated at 0 °C for 20 min (9). The protein precipitate was separated from the supernatant by centrifugation at 6000 x \(\text{g}\) for 5 min. NAD content in the precipitate and supernatant was determined (200 μl) was incubated at 0 °C for 20 min (9). The protein precipitate was separated from the supernatant by centrifugation at 6000 x \(\text{g}\) for 5 min. NAD content in the precipitate and supernatant was determined by three different methods. The precipitate fraction of the epimerase was resuspended in 50 μl of 0.1 M NaOH and the NAD* content was determined by a specific assay for pyridinium compounds, the methyl ethyl ketone procedure (9, 10). NAD* content was determined in the perchloric acid supernatant fractions spectrophotometrically. Estimate of the NAD* content was based on the \(A_{260}\) values (see under "Results"). NAD* content of supernatant fractions was also determined by

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§ To whom correspondence and reprint requests should be addressed: Section on Pharmacology, Laboratory of Biochemical Pharmacology, NIDDK, Bldg. 8, Rm. 2A-03, Bethesda, MD 20892. Tel.: 301-496-9108; Fax: 301-402-0240.
zyme activity were pooled, desalted, and concentrated with Amicon buffer. Table I was applied to an octyl-agarose column equilibrated with TEM buffer, pH 7.0. Proteins were first eluted with the equilibration buffer, followed by a stepwise elution in TEM buffer, pH 8.0. Proteins were then applied to a blue Sepharose CL-6B column equilibrated with TEM buffer, pH 8.0. Proteins were first eluted with the equilibration buffer, followed by a stepwise elution in TEM buffer, pH 7.0, containing 5 mM NAD^+.

**Preparation of Apoenzyme and Reconstitution with NAD**
ADP-L-glycero-D-mannoheptose 6-epimerase was resolved into apoenzyme and NAD^+ by treatment with acidic ammonium sulfate (pH 2.7) at 0 °C as described by Gomi et al. (12) and Porter and Boyd (13). In a typical experiment, the apoenzyme was reconstituted by incubation with 200 μM NAD^+ or NADH at room temperature for 20 min. Unbound NAD^+ or NADH were removed from the reconstituted enzyme by gel filtration (13) on PD-10 columns (Sephadex G-25).

**Other Methods**
Glycosyl residues on the epimerase were detected using the Genzyme Lectin-Link kit (i.e. an avidin/biotin system) and the Western blot and visualization protocols provided by the manufacturer. The cyanogen bromide procedure used to cleave the epimerase protein was described previously by Matsudaira (14). Neutral sugar analysis was performed as described previously by Coleman (3).

**Cell Extraction**
Crude extracts were prepared as described previously (7) from French pressates of E. coli strain CL627 (a K38 strain containing a plasmid-borne E. coli K-12 rfaD gene (i.e. pCG6) (7) which can be thermally induced to exclusively express, the rfaD gene product) (i.e. the epimerase protein). The in vitro expression system employed to exclusively express a cloned gene, following thermal induction, was described previously by Tabor and Richardson (15). Cells were grown in LB medium or in a defined medium (i.e. for preparation of radiolabeled protein) as described previously by Tabor and Richardson (15). Cells were grown in LB medium or in a defined medium (i.e. for preparation of radiolabeled protein) as described previously (2,7). For enzyme purification, extracts were prepared from unlabeled cells (50 g, wet weight) and [35S]methionine-radiolabeled cells (4 g, wet weight).

**Purification of ADP-L-glycero-D-mannoheptose 6-epimerase**
ADP-D-glycero-D-mannoheptose 6-epimerase Hydrophobic Interaction Chromatography—The crude extract (see Table I) was applied to an octyl-agarose column equilibrated with TEM buffer (10 mM Tris, 10 mM EDTA, 0.1 mM β-mercaptoethanol, 1 mM peptatin A, 57 μg phenylmethylsulfonyl fluoride, pH 8.0). Proteins were initially eluted with TEM buffer, pH 8.0, followed by a stepwise gradient of KCl from 0.3 M to 0.6 M in TEM buffer. The enzyme was eluted in the 0.6 M KCl fraction. The protein fractions containing enzyme activity were pooled, desalted, and concentrated with Amicon cells.

**Affinity Chromatography**—The pooled protein fractions were applied to a blue Sepharose CL-6B column equilibrated with TEM buffer, pH 8.0. Proteins were first eluted with the equilibration buffer, followed by monitoring its reduction to NADH by lactate dehydrogenase. The supernatant was adjusted to pH 8.9 with NaOH prior to lactate dehydrogenase assay. NADH formation was monitored spectrophotometrically at 340 nm.

**Protein Inhibition**
ADP-L-glycero-D-mannoheptose 6-epimerase was incubated with each inhibitor in an enzyme assay mixture of 50 μl at 25 °C for 15 min. The reaction was initiated by adding 4.4 nmol of ADP-L-glycero-D-mannoheptose. The enzyme activity was determined under standard assay conditions described above. Each determination was based on the results of triplicates.

**Spectroscopic and Analytical Methods**
Absorption spectra were recorded in a Hewlett-Packard 8452 diode array spectrophotometer at 23–25 °C. Fluorescence spectra were recorded on a Perkin Elmer MPF-3 spectrofluorimeter. Circular dichroism spectra were recorded at 25 °C, in a Jasco J-800 spectropolarimeter, using a DP-500 data processor and 1 cm length quartz cuvettes in 0.04 M sodium phosphate buffer, pH 7.2 (16). For CD experiments, the protein concentration was 0.2 mg/ml for the holo- and apoenzymes.

SDS-polyacrylamide gel electrophoresis was performed in 12% gel under reducing conditions as described by Laemmli (17). The subunit M, of epimerase was determined from the relative mobilities of protein standards. Gel filtration on an high performance liquid chromatography Zorbax GP-250 column (9.4 x 250 mm) was used to estimate the molecular weight of the native protein. The molecular mass of the native protein was determined by the sedimentation equilibrium procedure described by Attri and Minton (18). Protein concentrations were estimated using the Biorad and Bradford assay methods (19, 20). Chromatographic data were acquired and analyzed by an automated data collection system described by Minton and Attri (21).

**Effect of Substrate Concentration on Enzyme Activity**
The standard enzyme assay mixture as described was used with various concentrations of ADP-L-glycero-D-mannoheptose. Purified epimerase (65 ng) was used to initiate the reaction.

**Enzyme Inhibition**
Purified epimerase (5.2 μg) was preincubated with each inhibitor in an enzyme assay mixture of 50 μl at 25 °C for 15 min. The reaction was initiated by adding 4.4 nmol of ADP-L-glycero-D-mannoheptose. The enzyme activity was determined under standard assay conditions described above. Each determination was based on the results of triplicates.

**TABLE I**

| Step | Total Protein | Specific Activity | Total Activity | Yield |
|------|---------------|------------------|----------------|-------|
| Crude extract | 150 | 1.9 | 290 | 100 |
| Octyl-agarose | 15 | 14.0 | 210 | 72 |
| Blue-Sepharose | 5 | 25.0 | 130 | 45 |

TEM buffer, pH 7.0, and finally with TEM buffer, pH 7.0, containing 5 mM NAD^+. Protein fractions containing epimerase activity were pooled and concentrated.

**FIG. 1. The biosynthesis pathway for ADP-L-glycero-D-mannoheptose (1–6). The presentation of the biosynthetic pathway using chemical formuli was adopted from a review article by C. R. H. Raetz (32).**

![Image of the biosynthesis pathway for ADP-L-glycero-D-mannoheptose](image-url)
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**Fig. 2.** Chromatographic profiles of the purification of ADP-\(L\)-glycero-\(D\)-mannoheptose 6-epimerase. Crude extract prepared from unlabeled and \([\text{\textsuperscript{\text{\textsuperscript{35}}}}\text{S}]\text{methionine-radiolabeled cells of E. coli K-12 strain CL627 was applied to octyl-agarose (Panel A) and blue Sepharose CL-6B (Panel B). Protein was monitored by absorbance at 280 nm (---) and epimerase activity was assayed as described under "Experimental Procedures." Protein fractions containing both radioactivity and enzymatic activity as indicated i---i were pooled. In Panel A, arrows indicated buffers used for the elution: a, TEM buffer; b, TEM buffer containing 0.3 M KCl; and c, TEM buffer containing 0.6 M KCl. In Panel B, the arrows indicated the following buffers: a, TEM buffer, pH 8.0; b, TEM buffer, pH 7.0; and c, TEM buffer, pH 7.0, and 5 mM NAD.

**RESULTS**

**Purification of ADP-L-glycero-D-mannoheptose 6-Epimerase—**An E. coli strain, CL627, which overproduces the epimerase (7), was used for purification of the enzyme. Following thermal induction, 12–16% of the total protein of this strain is epimerase. Purification procedure is outlined in Table I. Substantial enrichment of the epimerase was achieved by using octyl-agarose (Fig. 2), resulting in greater than 7-fold increase in specific activity. Subsequent chromatography with blue 2-Sepharose CL-6B resin resulted in a homogeneous preparation of the enzyme (Fig. 3), with an overall yield of 45%.

**Molecular Weight—**Gel filtration studies (Fig. 4A) suggest a native protein molecular weight in the range of 230,000–250,000. Sedimentation equilibrium studies with the native epimerase indicated a molecular mass of 240,000. The subunit molecular weight was estimated to be 37,000 ± 3000 (Fig. 4B). Thus, the native enzyme is composed of six identical subunits.

**Carbohydrate Content—**The purified epimerase is glycosylated as demonstrated by binding to concanavalin A (ConA), and to a lesser degree to *Datura stramonium* agglutinin and wheat germ agglutinin (Fig. 5A). The lectins used in this study have different carbohydrate binding specificities (22). ConA binding is consistent with the presence of mannose containing glycans while the binding of *D. stramonium* agglutinin and wheat germ agglutinin suggests the presence of N-acetyl-D-glucosamine. The differential binding specificities gave indication regarding possible carbohydrate moieties in the epimerase. Preliminary neutral sugar analyses of the epimerase yielded fucose, mannose and galactose. This is consistent with our observation that the epimerase was bound to ConA-Sepharose and it was selectively eluted with \(\alpha\)-methyl-D-mannopyranoside (data not shown). In order to identify the glycosylated...
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region(s) of the protein, we prepared CNBr fragments of the epimerase. A single 14,000 cyanogen bromide fragment of the epimerase showed binding with the ConA lectin (Fig. 5C, lane 2'). An analysis of the deduced amino acid sequence of the rfaD gene product, previously reported by our laboratory (71, showed a stretch between Met-81 and Met-303 that corresponds to a calculated molecular weight of 14,000. Potential glycosylation sites in this small region of the epimerase polypeptide chain include 8 asparagines, 5 serines, and 7 threonines.

Catalytic Properties of the Enzyme—The epimerase activity increases with increasing concentration of ADP-n-glycero-d-mannoheptose in a typical hyperbolic fashion (Fig. 6). A Michaelis constant ($K_m$) of 0.1 mM was calculated for ADP-n-glycero-d-mannoheptose (Fig. 6, inset). The corresponding maximum velocity ($V_{max}$) of 46 μmol 30 min$^{-1}$, mg$^{-1}$ was determined from the same plot. ADP, ADP-glucose, ATP, and NADH were found to inhibit the enzyme activity (Table II). Enzyme activity was completely inhibited by 0.1 mM of ADP and ADP-glucose. Inhibition was observed even at 0.02 mM, albeit to a lesser degree. ATP and NADH were less inhibitory and enzyme activity was only partially inhibited at 1 mM and 0.02 mM levels. The optimum pH range for the enzyme (Fig. 7) is quite broad, ranging between 5.5 and 9.5. The result suggests that the enzyme activity can withstand wide fluctuation of pH. The epimerase activity exhibits a temperature optimum at 42 °C (Fig. 8), although the curve is not steep, suggesting a range of temperature stability.

Enzyme-bound NAD—Purified ADP-1-glycero-d-mannoheptose 6-epimerase was tested for the presence of NAD by several methods. The $A_{260}$/A$_{300}$ and $A_{260}$/A$_{340}$ ratios of the perchloric supernatants were 0.83 and 0.28, respectively while authentic NAD under identical condition yielded similar ratios of 0.85 and 0.26. Specifically, following perchloric acid dissociation of 17 nmol of epimerase subunit, 16.8 nmol of NAD$^+$ was recovered in the supernatant fraction. Thus, a 1:1 correlation was found for epimerase subunit and NAD. A second determination of the enzyme-bound NAD$^+$ content was performed by reduction to NADH with lactate dehydrogenase. Using this method, 31 μmol of NADH was detected in 52 μmol of epimerase subunit. As a control, treatment of the supernatant with Neurospora

![Graph A](image1.png)

**Fig. 4.** Molecular weight determination of epimerase by Zorbax GF-250 gel filtration (A) and SDS-PAGE (B). A, the position of the native protein in the Zorbax GF-250 column chromatography is indicated by the arrow. B, the arrow indicates the position of the enzyme in SDS-PAGE under reducing conditions. The protein standards used are indicated.

![Graph B](image2.png)

**Fig. 3.** SDS-PAGE analysis of protein fractions from each step of the purification procedures. Samples of 2.5 μg of proteins were applied to each lane. Proteins were stained by Coomassie blue staining method. Lanes 1-3, are protein fractions from a blue Sepharose CL-6B column, 0.6 M KCl fraction from hydrophobic chromatography, and crude extract, respectively.

![Graph C](image3.png)

**Fig. 5.** Detection of glycan residues on epimerase. Epimerase samples resolved by 10% SDS-PAGE were transferred to nitrocellulose. Glycan were detected by the use of biotinylated lectins. A, lanes 1, 3, 5, 7, and 9 contained purified epimerase and lanes 2, 4, 6, 8, and 10 contained a glycoprotein standard mixture. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10 were screened, respectively, with Sambucus nigra agglutinin, wheat germ agglutinin, Ricinus communis agglutinin, concanavalin A, and D. stramonium Agglutinin. B, cyanogen bromide fragments of epimerase resolved by 16% Tricine SDS-PAGE (lane 2). C, lane 3', Western blot of lane 3; probe biotinylated ConA. Lane 3', Western blot of lane 3; probe biotinylated ConA.
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**Fig. 6.** Effect of substrate concentration on the activity of ADP-L-glycero-D-mannoheptose epimerase. The enzyme activities were measured in 0.05 ml of standard assay mixture (see under "Experimental Procedures") containing variable amount of ADP-L-glycero-D-mannoheptose as indicated. *Inset,* double reciprocal plot of the reaction velocity versus substrate concentration.

**Fig. 7.** Effect of pH on the ADP-L-glycero-D-mannoheptose 6-epimerase activity. The enzyme activity of epimerase was determined over the pH range indicated. The reaction mixture contained 0.1 mM of the buffer for the designated pH ranges. The results are expressed as percent of maximum activity.

**Fig. 8.** Effect of reaction temperature on the epimerase stability. The enzyme was incubated at various temperatures as described under "Experimental Procedures." The results are expressed as percent of maximum activity.

**Table II**

| Compound                  | Enzyme activity* at concentration (mM): |
|---------------------------|-----------------------------------------|
|                           | 2   | 1   | 0.1 | 0.02 |
| None                      | 100 | 100 | 100 |
| ADP                       | <1  | <1  | 73  |
| ADP-glucose               | <1  | <1  | 82  |
| ATP                       | <1  | 83  | 99  |
| AMP                       | 87  | 108 | 97  |
| GMP                       | 110 | 110 | 110 |
| GDP                       | 104 | 110 | 92  |
| GDP-glucose               | 129 | 126 | 89  |
| n-Mannose                 | 105 | 96  | 99  |
| n-Galactose               | 88  | 83  | 108 |
| n-Glucose                 | 88  | 78  | 74  |
| NADH                      | 29  | 57  | 78  | 109 |

*ADP-L-glycero-D-mannoheptose 6-epimerase was preincubated in the presence of various reagents (0.05 ml final volume) at 25°C for 15 min. See "Experimental Procedures" for standard reaction mixture. The activity is expressed as percent of control activity.

**Spectral Properties of Epimerase**—The absorption spectrum of purified epimerase (Fig. 9A) displays two major maxima centered at 272 nm and 350 nm (see inset). An absorption maximum at 272 nm instead of the usual 278 nm absorption due to protein suggests that there is absorption due to nonprotein moiety bound to the enzyme. Free NADH absorbs at 340 nm, and may shift to higher wavelengths when it is bound to a protein. The absorbance at 350 nm (Fig. 9A) may be due to NADH as well as 1 mol of NAD⁺ bound per subunit of the enzyme as determined by chemical analysis. The A_{350}/A_{272} Ratio for the epimerase is 19. A similar analysis of another NAD containing epimerase, UDP-galactose 4-epimerase, yielded a ratio of 17 (24). The addition of a reducing agent, sodium dithionite, caused the 350 absorption maximum to shift between 330 and 332 nm (Panel B). The absorption maximum at 330 nm has previously been observed when another NAD⁺ containing enzyme, S-adenosylhomocysteine hydrolase, was reduced by adenosine or NaBH₄ (13). Fluorescence emission spectra of the epimerase and NADH are shown in Fig. 10A. The emission maximum of the epimerase (curve 1) is 450 nm. The emission maximum of unbound NADH is 470 nm (curve 2). The fluorescence intensity of the epimerase-bound NADH is approximately 2-fold that of equimolar concentration of unbound NADH. The emission spectrum of epimerase-bound NADH is not only increased in intensity but the wavelength of the maximum emission is also shifted to a shorter wavelength (470 to 450 nm) relative to unbound NADH. Increased intensity and shift in wavelength for enzyme bounded NADH has been observed for beef heart muscle lactic dehydrogenase and horse liver alcohol dehydrogenase (25, 26). Excitation fluorescence spectra (Fig. 10B) were also obtained for the epimerase and
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0.3

Absorbance spectra of ADP-L-glycero-D-mannoheptose 6-epimerase. Spectra were recorded (see inset) at 25°C in solutions of enzyme (1.2 mg/ml in TEM buffer, pH 7.0) before and immediately after indicated additions. The protein alone (——) and the protein in the presence of sodium dithionite (-----).

Table III
Effect of NAD⁺ and NADH on apoenzyme activity

|            | Activity |
|------------|----------|
| Epimerase  | 100      |
| Apoenzyme  | 0        |
| ENAD⁺      | 103      |
| ENADH      | 15       |

Fig. 10. Fluorescence spectra of ADP-L-glycero-D-mannoheptose 6-epimerase. A, Emission spectrum (excitation at 345 nm) of 1 mg/ml epimerase. For comparison, the emission spectra (excitation at 365 nm) of an equimolar amount of free NADH is also shown. B, excitation spectrum (emission at 450 nm of 1 mg/ml of enzyme or equimolar NADH). The measurements were recorded on samples in TE buffer (10 mM Tris, 10 mM EDTA, pH 7.0) and corrected for background fluorescence of buffers. Curves 1 and 2 are native epimerase and NADH respectively. The inset (designations 1 and 2 are defined above) is a photograph of the fluorescence of epimerase and NADH following exposure to UV light (302 nm).

The major excitation maxima of the purified epimerase were observed at 292 and 370 nm. The excitation maximum of unbound NADH was found at 370 nm.

Reconstitution of Apoenzyme with NAD⁺ or NADH—
Results of the reconstitution studies are shown in Table III. Aпоepimerase was inactive in the standard epimerase assay but activity was restored following incubation with 200 μM NAD⁺. The specific activity of the reconstituted enzyme was consistently greater than 100% of the untreated native enzyme. It was also observed that suboptimal concentration of NAD⁺ (<0.1 mM) resulted in partial reactivation (52%) of the inactive apoenzyme. In contrast, NADH reconstituted enzyme resulted in only 15% of the activity of untreated native enzyme. However, this meager activation following the addition of NADH is probably the results of adventitious oxidation of NADH in solution. Fluorescence analysis of the NAD reconstituted enzyme, unlike the NADH and untreated native epimerase, showed no fluorescence when exposed to UV light (302–345 nm).

Secondary Structure of Epimerase—Circular dichroism spectroscopy, which is sensitive to the contribution of various secondary structural elements, was used to evaluate the overall conformation of the epimerase. Fig. 11 shows the far-ultraviolet CD spectra of holoenzyme and apoenzyme. The holoepimerase has an intense spectrum (curve 1), with double minima at 208 and 222 nm and a maxima around 190–195 nm. Analysis of the holoenzyme CD spectrum indicates a protein with 11% α-helical and 36% β-sheet structures. The CD spectrum of the apo-epimerase (curve 2) was greatly reduced in intensity with a single minimum around 215 nm; analysis of curve 2 indicates a predominant β-sheet structure (i.e. 45% β-sheet).

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DISCUSSION
The goal of these studies was to characterize the physicochemical structure of an epimerase that is required for lipopolysaccharide core biosynthesis in several genera of Gram-negative bacteria. The collective data suggest that ADP-L-glycero-
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FIG. 11. Ultraviolet circular dichroism spectra of holo- and apoepimerase. Curves 1 and 2 are, respectively, holoepimerase and apoepimerase. Spectra were digitized, downloaded and analyzed, as previously described (13), in terms of secondary structure by least-squares fits using the PC-Mlab computer program (Civilized Software, Inc., Bethesda, MD). Protein concentrations (100-200 μg/ml) were estimated by absorption at A280.

d-mannoheptose 6-epimerase is similar to a group of epimerases (27, 28) that involves a NAD+-dependent redox catalysis.

The inhibition of the epimerase by nucleotide sugars or nucleotide diphosphates and sugar mixtures is reminiscent of the reductive inactivation of UDP-epimerase by NADH, by UDP-sugars (several aldohexoses or aldopentoses) or by free sugars in the presence of UMP (11, 29-31). The observed reductive inactivation of UDP-galactose 4-epimerase has been shown to be directly related to the reduction of the tightly bound cofactor NAD+ (11, 29).

Lipopolysaccharide is reported to contribute to the pathogenicity of enteric and nonenteric Gram-negative bacteria. Previously, we have reported (6) that the epimerase from E. coli shares significant structural and functional similarities with the epimerase from Pseudomonas aeruginosa. This conclusion is based on a number of observations including enzymatic activities, electrophoretic mobility of partially purified epimerase from P. aeruginosa and its cross-reactivity to antibody raised against the purified E. coli enzyme. Kontrohr and Kocsis (5) has reported the partial purification of a similar activity in Shigella that is required for the synthesis of L-glycero-D-mannoheptose.

L-Glycero-D-mannoheptose (heptose) is a common lipopolysaccharide component of the inner core of several genera of Gram-negative bacteria. The presence of heptose in the lipopolysaccharide of Gram-negative bacteria requires ADP-L-glycero-D-mannoheptose 6-epimerase activity. Heptoseless strains are less effective pathogens than wild type counterparts. Therefore, structural and functional studies of the epimerase may lead to novel antibacterial agents based on inhibition of the epimerase activity.

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