Evidence that NADP\(^+\) Is the Physiological Cofactor of ADP-L-glycero-d-mannoheptose 6-Epimerase

Received for publication, March 13, 2001, and in revised form, April 19, 2001
Published, JBC Papers in Press, April 19, 2001, DOI 10.1074/jbc.M102258200

YiSheng Ni\(^*\), Peter McPhie\(^\dagger\), Ashley Deacon\(^\#\), Steve Ealick\(^\&\), and William G. Coleman, Jr.\(^\|\)

From the \(^\text{*}\)Laboratory of Biochemistry and Genetics, NIDDK, National Institutes of Health, Bethesda, MD 20892, \(^\text{\#} Stanford Linear Accelerator Center, Stanford University, Stanford, CA 94309, and \|^\)Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853

ADP-L-glycero-d-mannoheptose 6-epimerase is required for lipopolysaccharide inner core biosynthesis in several genera of Gram-negative bacteria. The enzyme contains both fingerprint sequences Gly-X-Gly-X-X-Gly and Gly-X-X-Gly-X-Gly near its N terminus, which is indicative of an ADP binding fold. Previous studies of this ADP-L-glycero-d-mannoheptose 6-epimerase (ADP-hep 6-epimerase) were consistent with an NAD\(^+\) cofactor. However, the crystal structure of this ADP-hep 6-epimerase showed bound NADP (Deacon, A. M., Ni, Y. S., Coleman, W. G., Jr., and Ealick, S. E. (2000) Structure 5, 453–462). In present studies, apo-ADP-hep 6-epimerase was reconstituted with NAD\(^+\), NADP\(^+\), and FAD. In this report we provide data that shows NAD\(^+\) and NADP\(^+\) both restored enzymatic activity, but FAD could not. Furthermore, ADP-hep 6-epimerase exhibited a preference for binding of NADP\(^+\) over NAD\(^+\). The \(K_v\) value for NADP\(^+\) was 26 \(\mu\)M whereas that for NAD\(^+\) was 45 \(\mu\)M. Ultraviolet circular dichroism spectra showed that apo-ADP-hep 6-epimerase reconstituted with NADP\(^+\) had more secondary structure than apo-ADP-hep 6-epimerase reconstituted with NAD\(^+\). Perchloric acid extracts of the purified enzyme were assayed with NAD\(^+\)-specific alcohol dehydrogenase and NADP\(^+\)-specific isocitric dehydrogenase. A sample of the same perchloric acid extract was analyzed in chromatographic studies, which demonstrated that ADP-hep 6-epimerase binds NADP\(^+\) in \(v\)ivo. A structural comparison of ADP-hep 6-epimerase with UDP-galactose 4-epimerase, which utilizes an NAD\(^+\) cofactor, has identified the regions of ADP-hep 6-epimerase, which defines its specificity for NADP\(^+\).

ADP-L-glycero-d-mannoheptose 6-epimerase catalyzes the interconversion of ADP-D-glycero-d-mannoheptose and ADP-L-glycero-d-mannoheptose (Fig. 1). This epimerization reaction is the last of four enzymatic steps required for the biosynthesis of ADP-L-glycero-d-mannoheptose. ADP-L-glycero-d-mannoheptose is the precursor of the aldohexose, 1-glycero-d-mannoheptose (or heptose), which is a highly conserved component of the lipopolysaccharide core domain among several genera of enteric and nonenteric Gram-negative bacteria (1–3). The epimerization at carbon 6 of the heptose involves an oxidation-reduction process. In previous studies of the Escherichia coli K-12 ADP-hep 6-epimerase\(^1\) it was observed that the N terminus contained the fingerprint sequence Gly-X-Gly-X-X-Gly or Gly-X-X-Gly-X-X-Gly, which is characteristic of the ADP binding \(\beta\beta\beta\beta\) fold (or Rossmann fold) associated with NAD(P) binding and also FAD-binding proteins (4, 5). In addition, it was observed that one nicotinamide coenzyme was tightly bound to each ADP-hep 6-epimerase subunit; enzymatic analyses suggested that NAD\(^+\) was the cofactor used by ADP-hep 6-epimerase (6). ADP-L-glycero-d-mannoheptose 6-epimerase showed 24\% sequence identity with UDP-galactose 4-epimerase (UGE) based on calculation with BLAST (7). The crystal structure of UGE showed two NAD molecules bound to the dimeric enzyme (8, 9). However, the crystal structure of ADP-hep 6-epimerase (10) indicated that NADP\(^+\) is the more likely natural cofactor for this enzyme, because only NADP was found on each of the five subunits of ADP-hep 6-epimerase. The conformation of NAD bound to ADP-hep 6-epimerase is less extended than that of NAD bound to UGE as judged by the distance between adenine C6 and C2 of the nicotinamide ring.

Here we report that the enzyme ADP-L-glycero-d-mannoheptose 6-epimerase is an NAD\(^+\)-dependent enzyme. NAD\(^+\) can substitute for NADP\(^+\), but enzymatic activity is reduced. Several important structural differences between ADP-hep 6-epimerase and UGE give rise to the different cofactor specificities.

EXPERIMENTAL PROCEDURES

Materials—The natural substrate of ADP-hep 6-epimerase, ADP-D-glycero-d-mannoheptose, was extracted and purified from an rfaD mutant (CL515), as described previously (1). Octyl-agarose resin, NAD\(^+\), NADP\(^+\), FAD, isocitric acid, isocitric dehydrogenase, and alcohol dehydrogenase were purchased from Sigma. Blue 2-Sepharose CL-6B resin was purchased from Amersham Pharmacia Biotech.

Expression and Purification of ADP-hep 6-Epimerase—Plasmid pJPS containing the ADP-hep 6-epimerase gene was transformed into the E. coli strain BL21 (DE3) pLysS. The transformed bacteria were cultured in LB medium. Cells were harvested 3 h after induction with 0.4 mM isopropyl-\(\beta\)-D thiogalactopyranoside. Buffers and crude extracts from bacterial cell pellets were prepared as described previously by Pegues et al. (4). ADP-hep 6-epimerase was purified to homogeneity employing the following three-step purification protocol: a hydrophobic interaction and an affinity chromatographic step as described (6), followed by an additional ion exchange step (11).

ADP-hep 6-Epimerase Activity Assay—The activity of ADP-hep 6-epimerase was assayed as described previously (2, 6). The reaction mixture containing 0.1 M Tris acetate, pH 8.5, ADP-L-glycero-d-mannoheptose (5 mM), and enzyme in a final volume of 50 ml was incubated at 37 °C for 30 min. The reaction was terminated by heating for 5 min at 100 °C.

Enzyme activity was determined by monitoring the formation of ADP-L-glycero-d-mannoheptose by high performance liquid chromatography (2, 6). 1 unit of enzyme activity is defined as the ADP-hep 6-epimerase activity capable of producing 1 nmol of ADP-L-glycero-d-mannoheptose in 30 min at 37 °C in 0.05 ml of reaction mixture.

Preparation and Reconstitution of Apoenzyme—ADP-hep 6-epimerase was treated with saturated acidic ammonium sulfate (adjusted to pH 2.7 with concentrated sulfuric acid) containing 5 mM diethiothreitol on ice, as described by Gomi et al. (12). For reconstitution of ADP-hep 6-epimerase, the acidic ammonium sulfate-treated apo-ADP-hep 6-epi-
NADP Binding Specificity of ADP-hep 6-Epimerase

The enzyme by a modification of the method of Daussmann et al. (13). Enzyme solution (50 μl) containing 150 μg of purified ADP-hep 6-epimerase was extracted with 50 μl of 5% perchloric acid at 0 °C for 20 min. Saturated NaHCO₃ was then added to the extract to bring the pH value to 7.0. After neutralization, the mixture was centrifuged at 14,000 × g for 10 min, and the supernatant was used for analysis. Quantitative determination of NADP⁺ and NADPH was carried out by reversed phase HPLC (Amersham Pharmacia Biotech Smart System). The column (Hypersil ODC-5 μm; 125 × 4-mm diameter; Hypersil Inc.) was equilibrated in 20 mM sodium phosphate, pH 5.0. A gradient of 20 mM sodium phosphate, pH 5.0, and acetonitrile (13) resulted in unambiguous resolution of NADP⁺ and NADPH at 12.2 and 15.7 min, respectively. The standards used in the HPLC analysis were subjected to the same treatments as the perchloric acid-extracted materials. Ultra-FIG. 1. Reaction catalyzed by ADP-l-glycero-d-mannoheptose 6-epimerase. ADP-hep 6-epimerase catalyzes interconversion of ADP-L-glycero-d-mannoheptose and ADP-l-glycero-d-mannoheptose.

| Protein treated | Addition | Relative activity a |
|-----------------|----------|--------------------|
| Native AGME     | None     | 100                |
| Apo-AGME        | None     | 0                  |
| Apo-AGME        | 200 μM NADP⁺ | 88         |
| Apo-AGME        | 200 μM NAD⁺ | 40                |
| Apo-AGME        | 200 μM FAD | 0                  |

a A relative activity of 100% equals a specific activity of 875 units/mg.
b Assays performed at subsaturating concentrations of NAD (see Table I).

Enzymatic Identification of the ADP-hep 6-Epimerase Cofactor—NADP⁺-specific isocitric dehydrogenase and NAD⁺-specific alcohol dehydrogenase were used to define which nucleotide was the ADP-hep 6-epimerase cofactor. The cofactor was extracted by dialysis against 50 mM sodium phosphate buffer, pH 7.0, at 4 °C for 3 h or longer. The standards used in the HPLC analysis were subjected to the reconstituted enzyme by dialysis against 50 mM sodium phosphate buffer, pH 7.0, at 4 °C for 3 h or longer.

K₅ Determinations—The values of K₅ for NAD⁺ and NAD⁺ were determined using freshly prepared apoenzymes. The reconstituted enzyme samples, following dialysis for 5 h at 4 °C (as described above), were then added to the standard assay system, which contained 2.7 nmol of ADP-D-hep 6-epimerase-bound cofactor, NADP(H), was identified by a modification of the method of Daussmann et al. (13). Enzyme solution (50 μl) containing 150 μg of purified ADP-hep 6-epimerase was extracted with 50 μl of 5% perchloric acid at 0 °C for 20 min. Saturated NaHCO₃ was then added to the extract to bring the pH value to 7.0. After neutralization, the mixture was centrifuged at 14,000 × g for 10 min, and the supernatant was used for analysis. Quantitative determination of NADP⁺ and NADPH was carried out by reversed phase HPLC (Amersham Pharmacia Biotech Smart System). The column (Hypersil ODC-5 μm; 125 × 4-mm diameter; Hypersil Inc.) was equilibrated in 20 mM sodium phosphate, pH 5.0. A gradient of 20 mM sodium phosphate, pH 5.0, and acetonitrile (13) resulted in unambiguous resolution of NADP⁺ and NADPH at 12.2 and 15.7 min, respectively. The standards used in the HPLC analysis were subjected to the same treatments as the perchloric acid-extracted materials. Ultra-

The reaction mixture consisted of 60 mM NAD, 150 mM isocitrate, 10 mM Tris-HCl buffer, pH 7.0. After standing on ice for 20 min, the supernatant was used for assay the nature and amount of released coenzyme. To assay for NADP the reaction mixture consisted of 60 mM NAD, 150 mM isocitrate, and 5 mM NADP⁺ or FAD was removed from the reaction mixture by dialysis against 50 mM sodium phosphate buffer, pH 7.0, at 4 °C for 3 h or longer.

**TABLE I** Activity of apo-AGME reconstituted with dinucleotide cofactors

| Protein treated | Addition | Relative activity b |
|-----------------|----------|--------------------|
| Native AGME     | None     | 100                |
| Apo-AGME        | None     | 0                  |
| Apo-AGME        | 200 μM NADP⁺ | 88         |
| Apo-AGME        | 200 μM NAD⁺ | 40                |
| Apo-AGME        | 200 μM FAD | 0                  |

a A relative activity of 100% equals a specific activity of 875 units/mg.
b Assays performed at subsaturating concentrations of NAD (see Table I).

Enzymatic Identification of the ADP-hep 6-Epimerase Cofactor—NADP⁺-specific isocitric dehydrogenase and NAD⁺-specific alcohol dehydrogenase were used to define which nucleotide was the ADP-hep 6-epimerase cofactor. The cofactor was extracted by dialysis against 50 mM sodium phosphate buffer, pH 7.0, at 4 °C for 3 h or longer. The standards used in the HPLC analysis were subjected to the reconstituted enzyme by dialysis against 50 mM sodium phosphate buffer, pH 7.0, at 4 °C for 3 h or longer.

K₅ Determinations—The values of K₅ for NAD⁺ and NAD⁺ were determined using freshly prepared apoenzymes. The reconstituted enzyme samples, following dialysis for 5 h at 4 °C (as described above), were then added to the standard assay system, which contained 2.7 nmol of ADP-D-hep 6-epimerase-bound cofactor, NADP(H), was identified by a modification of the method of Daussmann et al. (13). Enzyme solution (50 μl) containing 150 μg of purified ADP-hep 6-epimerase was extracted with 50 μl of 5% perchloric acid at 0 °C for 20 min. Saturated NaHCO₃ was then added to the extract to bring the pH value to 7.0. After neutralization, the mixture was centrifuged at 14,000 × g for 10 min, and the supernatant was used for analysis. Quantitative determination of NADP⁺ and NADPH was carried out by reversed phase HPLC (Amersham Pharmacia Biotech Smart System). The column (Hypersil ODC-5 μm; 125 × 4-mm diameter; Hypersil Inc.) was equilibrated in 20 mM sodium phosphate, pH 5.0. A gradient of 20 mM sodium phosphate, pH 5.0, and acetonitrile (13) resulted in unambiguous resolution of NADP⁺ and NADPH at 12.2 and 15.7 min, respectively. The standards used in the HPLC analysis were subjected to the same treatments as the perchloric acid-extracted materials. Ultra-
violet circular dichroism spectra of holo-ADP-hep 6-epimerase, apo-ADP-hep 6-epimerase, apo-ADP-hep 6-epimerase $NAD^+$, and apo-ADP-hep 6-epimerase $NADP^+$ were measured in a Jasco J715 spectropolarimeter, using a 1-mm path length quartz cuvette at 25 °C. Smoothed spectra from 4 scans were analyzed in terms of secondary structure, using the CONTIN program (14).

Structural Comparison with UGE—The structure of ADP-hep 6-epimerase was determined recently by x-ray crystallography (10). A single molecule of ADP-hep 6-epimerase (PDB code 1EQ2) was superimposed on a molecule of UGE (PDB code 1XEL) using the graphics program O (15). Only core residues from the cofactor-binding domain were used in the superposition, resulting in a root mean square deviation of 1.62 Å for 124 $\alpha$-carbon atoms.

RESULTS

Reconstitution of apo-ADP-hep 6-Epimerase with Adenine-based Dinucleotides—To identify the cofactor preference of ADP-hep 6-epimerase, we used $NAD^+$, $NADP^+$, and FAD to reconstitute apo-ADP-hep 6-epimerase. Enzyme activities of the reconstituted enzyme were detected by the standard ADP-hep 6-epimerase assay as described under “Experimental Procedures.” As shown in Table I, the apo-ADP-hep 6-epimerase is inactive. However, when 100 $\mu$M $NADP^+$ was used to reconstitute the apoenzyme, 88% of the native ADP-hep 6-epimerase activity was restored. Restoration of 40% of ADP-hep 6-epimerase activity was observed when 100 or 200 $\mu$M $NAD^+$ was used to reconstitute the apoenzyme. Thus, the reactivation of apo-ADP-hep 6-epimerase by $NAD^+$ is about 50% of that achieved by $NADP^+$. In contrast, no restoration of ADP-hep 6-epimerase activity was achieved in the presence of FAD. These results demonstrate a clear preference among the selected adenine dinucleotides for reconstituting ADP-hep 6-epimerase, which probably reflects the cofactor preference of ADP-hep 6-epimerase.

Enzymatic Identification of the in Vivo ADP-hep 6-Epimerase-bound Cofactor—$NADP^+$-specific isocitric dehydrogenase and $NAD^+$-specific alcohol dehydrogenase were employed to assay the perchloric acid extracts from purified ADP-hep 6-epimerase. When the perchloric acid extract was incubated with isocitric acid and the $NADP^+$-specific isocitric dehydrogenase, there was a marked increase in absorbance at 340 nm (Fig. 2A), which reached a plateau after 30 min. As a control, either 50 $\mu$M $NAD^+$ or 50 $\mu$M $NADP^+$ was added after the initial reaction reached the plateau. When $NAD^+$ was added to the reaction mixture at 40 min there was no increase in absorbance at 340...
nm. However, when 50 μM NADP was added to the reaction cuvette, a dramatic increase in absorbance at 340 nm was observed. The opposites were observed when the released co-factor was analyzed with the NAD-specific ethanol/alcohol dehydrogenase system (Fig. 2B). There was no increase in absorbance at 340 nm when the perchloric acid extract of ADP-hep 6-epimerase was added upon addition of NADP to the reaction vessel. However, when NAD was added to the reaction vessel, a significant increase in absorbance at 340 nm was observed. These results provide evidence that NADP is the major dinucleotide cofactor present in the perchloric acid extracts.

HPLC Analysis of the Perchloric Acid Extracts of ADP-hep 6-Epimerase—To confirm the presence of NADP, the neutralized perchloric acid extracts of purified ADP-hep 6-epimerase were analyzed by HPLC as described under “Experimental Procedures” (Fig. 3). Two clearly resolved peaks (Fig. 3) were observed when the perchloric acid extracts were analyzed. The chromatographic analysis showed that the molar ratio of NADP/NADPH in the extracts was 0.4:0.6. The occurrence of both oxidized and reduced forms of the cofactor bound to ADP-hep 6-epimerase was consistent with earlier UV and fluorescence studies (4). Our study also suggested that NADP(H) is stable during perchloric acid extraction and that the coenzyme is tightly, but non-covalently, bound to the enzyme.

Secondary Structure of Native and Reconstituted ADP-hep 6-Epimerase—Circular dichroism spectroscopy, which is sensitive to secondary structural changes, was used to evaluate the secondary structure of native ADP-hep 6-epimerase and apo-ADP-hep 6-epimerase reconstituted with either NAD or NADP. The estimated secondary structures of various protein forms, apo, holo, NAD, or NADP, bound are shown in Fig. 4. These data are clearly in agreement with the enzymatic activity assays (see below). Both cofactors can partially restore the structure of the protein. However, NADP is more effective than NAD. The circular dichroism experiments showed that no more refolding occurred after the standard reactivation procedure. Consequently, the data derived from the enzymatic assays are characteristic of the reconstituted proteins. They do not reflect differences in the rate of reconstitution by the two cofactors.

K<sub>D</sub> Determinations—The determination of the K<sub>D</sub> values for NADP<sup>+</sup> and NAD<sup>+</sup> provided further convincing evidence for the coenzyme preference. As shown in Table II, the apparent K<sub>D</sub> values for NADP<sup>+</sup> and NAD<sup>+</sup> were 26 and 45 μM, respectively, showing a higher binding affinity for NADP<sup>+</sup>. These are apparent K<sub>D</sub> values, because they are determined by measurement of enzymatic activity and not by direct measurement of binding.
A comparison of the interactions between NADP with ADP-L-glycero-D-mannoheptose 6-epimerase and NAD with UDP-galactose 4-epimerase

| NAD/NADP atom name | Interaction | Protein residue/atom in AGME | Protein residue/atom in UGE |
|---------------------|------------|------------------------------|-----------------------------|
| Adenosine N7        | H-bond     | Asn\(^{32}\) ND2            | Asn\(^{39}\) OD1            |
| Adenosine N6        | H-bond     | Asn\(^{37}\) O              | Asp\(^{36}\) OD1, Asp\(^{38}\) OD2, Asn\(^{39}\) OD1 |
| Adenosine N1        | H-bond     | None                        | Ile\(^{69}\) N\(^*\)        |
| Adenosine N3        | H-bond     | None                        | Asn\(^{32}\) N, Asn\(^{39}\) OD1 |
| Adenosine-base      | Hydrophobic| Lys\(^{35}\), Gly\(^{76}\), Ala\(^{77}\) | Ala\(^{83}\), Gly\(^{87}\) |
| Adenosine ribose O2 | H-bond     | None                        | Asp\(^{31}\) OD1, Asn\(^{37}\) N |
| Adenosine ribose O3 | H-bond     | Asp\(^{31}\) OD1, Lys\(^{38}\) NZ | Asp\(^{31}\) OD1/OD2, Ser\(^{36}\) OG |
| Adenosine ribose O5 | H-bond     | HOH                         | HOH                         |
| Adenosine 2-phosphate O1 | H-bond     | HOH                         | NA\(^*\)                   |
| Adenosine 2-phosphate O2 | H-bond     | HOH                         | NA\(^*\)                   |
| Adenosine 2-phosphate O3 | H-bond     | Asn\(^{32}\) ND2, Lys\(^{38}\) NZ | NA\(^*\)                   |
| Adenosine phosphate O1 | H-bond   | Ser\(^{39}\) OG             | Asn\(^{35}\) ND2\(^*\)     |
| Adenosine phosphate O2 | H-bond     | Phe\(^{136}\) N, His\(^{177}\) NE2 | Tyr\(^{23}\) N |
| Nicotinamide phosphate O1 | H-bond     | Lys\(^{378}\) NZ            | Lys\(^{378}\) NZ            |
| Nicotinamide phosphate O2 | H-bond     | Ile\(^{12}\) N, HOH         | Ile\(^{12}\) N, HOH          |
| Nicotinamide ring   | Hydrophobic| Tyr\(^{176}\), Val\(^{178}\) | Tyr\(^{177}\), Pro\(^{180}\) |
| Nicotinamide ribose O2 | H-bond     | Tyr\(^{196}\) OH             | Tyr\(^{197}\), Pro\(^{198}\) |
| Nicotinamide ribose O3 | H-bond     | Glu\(^{130}\) O, Lys\(^{144}\) NZ, HOH | Lys\(^{353}\) NZ, Phe\(^{60}\) O, HOH |
| Nicotinamide N7     | H-bond     | Nicotinamide phosphate O1   | Nicotinamide phosphate O1, Lys\(^{64}\) NZ, HOH |
| Nicotinamide O7     | H-bond     | Val\(^{195}\) N             | HOH                         |

\(^{a}\) The significantly different interactions, because of the presence of the 2'-phosphate of NADP are in bold.

\(^{b}\) The 2'-phosphate interactions, which are possible in the NADP·AGME complex, are italicized. NA, not applicable.

**Structural Basis for ADP-hep 6-EPimerase NADP Binding Preference**—The recently determined crystal structure of ADP-hep 6-epimerase (10) (Fig. 5A) closely resembles the structure of UGE (Fig. 5b) (8, 9). The two enzymes, both from *E. coli*, share the same fold and 24% sequence identity. Despite the obvious structural similarity, UGE utilizes only NAD\(^+\) as its cofactor, whereas ADP-hep 6-epimerase prefers NADP\(^+\) in *vitro*. There are several important structural features that define this modified cofactor specificity. It can be seen that the largest structural difference in the N-terminal domain (i.e. the dinucleotide-binding domain) occurs in the region immediately surrounding the 2'-phosphate in the ADP-hep 6-epimerase structure (Fig. 5a; see arrow). There are five additional amino acid residues present in the UGE structure in the polypeptide chain from residues 30 to 74, which correspond to residues 30 to 69 (Fig. 5b) in ADP-hep 6-epimerase. These extra residues in UGE extend the conformation of the interconnecting loop, between the secondary structure elements, away from the dinucleotide and thus prevent any direct interaction with a 2'-phosphate. They also allow significant changes in the orientation of one of the short helices. On closer inspection this region contributes all the specific residues that participate in coordinating the 2'-phosphate in ADP-hep 6-epimerase (Fig. 5C). Although there is a high degree of structural and sequence homology for much of the nicotinamide dinucleotide-binding site, there is little similarity around the 2'-phosphate in ADP-hep 6-epimerase (Table III). Thus, as shown in Fig. 5C, the NAD/NADP binding fingerprint motifs of the two proteins match very closely (Gly\(^9\) and Ile\(^{11}\) in ADP-hep 6-epimerase are shown), as well as the catalytically important residues (Ser\(^{116}\), Tyr\(^{140}\), and Lys\(^{144}\) in ADP-hep 6-epimerase). However, major differences occur where there are positively charged residues (Lys\(^{38}\) and particularly Lys\(^{53}\) in ADP-hep 6-epimerase that play an important role in conferring the additional NADP\(^+\) specificity. Lys\(^{53}\) extends along and forms a large hydrophobic contact with the adenine base, and it also then coordinates the 2'-phosphate through its side chain-terminal side-chain nitrogen atom. The orientation of Lys\(^{53}\) precludes any direct H-bond interactions with the adenosine N1 and N3, which are present in UGE and also serves to allow Asn\(^{32}\) to interact directly with the 2'-phosphate (Table III).

**DISCUSSION**

Many dinucleotide-binding proteins specifically require either NAD\(^+\) or NADP\(^+\) as cofactor, although some of them show a dual specificity (5, 16–20). Our study provides strong evidence that NADP\(^+\) is the natural coenzyme of ADP-hep 6-epimerase, although NAD\(^+\) can substitute for NADP\(^+\) and still allow the epimerization reaction to proceed at a slower rate. The apparent dissociation constant determined for NADP\(^+\) versus that of NAD\(^+\) is consistent with preferential binding of NADP to ADP-hep 6-epimerase.

Since the 1970s, there has been substantial interest in elucidating the fundamental basis for NAD\(^+\)/NADP\(^+\) specificity of many enzymes. The fingerprint sequence Gly-X-Gly-X-X-Gly (20) has been recognized as the stereotypic hallmark of dinucleotide binding, and therefore it is not surprising that the study of NAD\(^+\)/NADP\(^+\) specificity has focused predominately on this sequence.

To investigate the coenzyme preference of ADP-hep 6-epimerase, we have linked the biochemical and molecular modeling studies with information derived from our recent three-dimensional structural analysis. This combined approach has allowed us to (1) determine kinetic parameters that indicated a preference for NADP by ADP-hep 6-epimerase and (2), the detailed analysis of the amino acids and structural attributes of enzyme that confer preference for nicotinamide adenine dinucleotide with or without an additional 2'-phosphate. The structural studies of ADP-L-glycero-D-mannoheptose 6-ADP-hep 6-epimerase indicate that positively charged Lys\(^{38}\) and Lys\(^{63}\) are the major contributors to the electrostatic compensation for the 2'-phosphate group of NADP. The occurrence of these positively charged residues conferred the preference of the enzyme for NADP\(^+\). This is consistent with the results of Scrutton et al. (19), which demonstrated that the introduction of positively residues (Arg\(^{196}\) and Arg\(^{204}\)) in the NAD\(^+\)-binding site of *E. coli* glutathione reductase conferred NADP\(^+\) binding preference to the redesigned enzyme. Rizzi et al. (21) reported in a recent structural study of GDP-4-keto-6-deoxy D-mannoepimerase/reductase that in its NADP\(^+\)-binding site two positively charged residues (Arg\(^{12}\) and Arg\(^{26}\)) play important roles in providing electrostatic compensation for the NADP\(^+\) ribose 2'-phosphate group.

Further, residues (Gly\(^{9}\) and Ile\(^{11}\)) present in the Gly-X-Gly-X-Gly motif (21) are positioned near the diphosphate bridge of the ADP-hep 6-epimerase-bound NADP. Residues Ser\(^{116}\), Tyr\(^{140}\), and Lys\(^{144}\), thought to be involved in the catalytic mechanism of ADP-hep 6-epimerase, are located near the nicotinamide ring and its attached ribose. Thus, there exists a
significant degree of structural and sequence similarity for the NAD(P)⁺-binding sites of ADP-hep 6-epimerase and other NAD(P)-binding proteins. Further mutagenesis studies will be used to provide additional insight into the cofactor specificity of ADP-hep 6-epimerase and to determine in more detail the significance of the residues involved.

REFERENCES

1. Coleman, W. G., Jr. (1983) J. Biol. Chem. 258, 1985–1990
2. Coleman, W. G., Jr., Chen, L., and Ding, L. (1992) in Pseudomonas: Molecular Biology and Biotechnology (Galli, E., Silver, S., and Witholt, B., eds) pp. 161–169, American Society for Microbiology, Washington, D. C.
3. Adams, G. A., Quading, C., and Perry, M. B. (1987) Can. J. Microbiol. 13, 1605–1613
4. Pegues, J. C., Chen, L., Gordon, A. W., Ding, L., and Coleman, W. G., Jr. (1990) J. Bacteriol. 172, 4652–4660
5. Bellamacina, C. R. (1996) FASEB J. 10, 1257–1269
6. Ding, L., Seto, B. L., Ahmed, S. A., and Coleman, W. G., Jr. (1994) J. Biol. Chem. 269, 24384–24390
7. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402.
8. Bauer, A. J., Rayment, I., Frey, P. A., and Holden, H. M. (1992) Proteins Struct. Funct. Genet. 9, 135–142
9. Thoden, J. B., Frey, P. A., and Holden, H. M. (1996) Protein Sci. 5, 2149–2161
10. Deacon, A. M., Ni, Y. S., Coleman, W. G., Jr., and Ealick, S. E. (2000) Structure 5, 453–462
11. Ding, L., Zhang, Y., Deacon, A. M., Ealick, S. E., Ni, Y. S., Sun, P., and Coleman, W. G., Jr. (1999) Acta Crystallogr. Sec. D 55, 685–688
12. Gomi, T., Takata, Y., and Fujikawa, M. (1989) Biochim. Biophys. Acta 994, 172–179
13. Daussmann, T., Aivasidis, A., and Wandrey, C. (1997) Eur. J. Biochem. 248, 889–896
14. Provencher, S. W., and Glockner, J. (1981) Biochemistry 20, 33–37
15. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991). Acta Crystallogr. Sect. A 47, 110–119
16. Lilley, K. S., Baker, P. J., Britton, K. L., Stillman, T. J., Brown, P. E., Moir, A. J. G., Engel, P. C., Rice, D. W., Bell, J. E., and Bell, E. (1991) Biochim. Biophys. Acta 1080, 191–197
17. McPherson, M. J., and Wootton, J. C. (1983) Nucleic Acid Res. 11, 5257–5266
18. Moyer, W. S., Amura, N., Ras, J. K. M., and Zalkin, H. (1985) J. Biol. Chem. 260, 8502–8508
19. Scrutton, N. S., Berry, A., and Perham, R. N. (1990) Nature 343, 38–43
20. Wierenga, R. K., Terpstra, P., and Hol, W. G. J. (1986) J. Mol. Biol. 187, 101–107
21. Rizzi, M., Tonetti, M., Vigevani, P., Sturla, L., Bisso, A., De Flora, A., Borda, D., and Bolognesi, M. (1998) Structure 1453–1465
Evidence that NADP⁺ Is the Physiological Cofactor of ADP-1-glycero-d-mannoheptose 6-Epimerase
YiSheng Ni, Peter McPhie, Ashley Deacon, Steve Ealick and William G. Coleman, Jr.

J. Biol. Chem. 2001, 276:27329-27334.
doi: 10.1074/jbc.M102258200 originally published online April 19, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102258200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 19 references, 4 of which can be accessed free at
http://www.jbc.org/content/276/29/27329.full.html#ref-list-1