Probing the Coenzyme and Substrate Binding Events of CDP-D-glucose 4,6-Dehydratase: Mechanistic Implications†

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ABSTRACT: NAD^+-dependent nucleotidyl diphosphohexose 4,6-dehydratases which transform nucleotidyl diphosphohexoses into the corresponding 4-keto-6-deoxy sugar derivatives are essential to the formation of all 6-deoxyhexoses. Studies of the CDP-D-glucose 4,6-dehydratase (E_{od}) from *Yersinia* had shown that this dimeric protein binds only 1 equiv of NAD^+/mol of enzyme and, unlike other enzymes of the same class, displays a unique NAD^+ requirement for full catalytic activity. Analysis of the primary sequence revealed an extended ADP-binding fold (GHTGFKG) which deviates from the common Rossmann consensus (GXGXXG) and thus may have contributed to E_{od}'s limited NAD^+ affinity. In particular, the presence of His17 in the β-turn region and that of Lys21 in a position typically occupied by a small hydrophobic residue may impose electronic or steric perturbations to this essential binding motif. To better understand the correlation between the binding properties and primary sequence, mutants (H17G and K21I) were constructed to provide enzymes containing an ADP binding region which more closely resembles the Rossmann-type fold. Analysis of the cofactor and substrate binding characteristics of the wild-type and mutant enzymes helped define the presence of two binding sites for both CDP-D-glucose and NAD^+ per enzyme molecule. While both mutants displayed enhanced NAD^+ affinity, the H17G mutation resulted in an enzyme with slightly higher k_{cat} and a 3-fold increase in catalytic efficiency (k_{cat}/K_m). The large anticooperativity found for NAD^+ binding ($K_1 = 40.3 \pm 0.4$ nM, $K_2 = 539.8 \pm 4.8$ nM) may explain why the cofactor binding sites of wild-type E_{od} are only half-occupied. Further examination also revealed the purified E_{od} to contain sequestered NADH and that the affinity of E_{od} for NADH ($K_1 = 0.21 \pm 0.01$ nM, $K_2 = 7.46 \pm 0.25$ nM) is much higher than that for NAD^+. Thus, it is possible that E_{od}'s half-site saturation of NAD^+ per enzyme dimer may also be attributed to a significant portion of the cofactor binding sites being occupied by NADH. Interestingly, the sequestered NADH is released upon binding with CDP-D-glucose. These results implicate a new kinetic mechanism for E_{od} catalysis.

The 3,6-dideoxyhexoses, present primarily in the lipopolysaccharide components of Gram-negative cell envelopes (Hanessian, 1966; Butterworth & Hanessian, 1971; Williams & Wander, 1980), have been characterized as important antigenic determinants and are essential to the serological specificity of many immunologically active polysaccharides (Westphal & Lüderitz, 1960; Lüderitz et al., 1966; Bishop & Jennings, 1982; Raetz, 1990). Although there are apparent differences in the biosynthetic precursors of these dideoxy sugars, their formation is consistently initiated with an irreversible intramolecular oxidation−reduction catalyzed by a NAD^+ [1] -dependent nucleotidyl diphosphohexose 4,6-dehydratase, also known as nucleotidyl diphosphohexose oxidoreductase (Glaser & Zarkowsky, 1971; Gabriel, 1973; Grisebach, 1978; Gabriel & VanLenten, 1978; Frey, 1987; Liu & Thorson, 1994). This dehydratase catalyzed reaction, which converts a nucleotidyl diphosphohexose to the corresponding 4-keto-6-deoxyhexose derivative, has been distinguished as the common entry in the formation of all 6-deoxyhexoses (Williams & Wander, 1980).

Enzymes that catalyze this type of reaction have been purified from a wide variety of bacteria (Ginsberg, 1961; Elbein & Heath, 1965; Matsuhashi et al., 1966; Melo et al., 1968; Melo & Glaser, 1968; Zarkowsky & Glaser, 1969; Wang & Gabriel, 1969; Gonzalez-Porqué & Strominger, 1972; Matern et al., 1973; Vara & Hutchinson, 1988; Romana et al., 1991), plants (Liao & Barber, 1972), and mammalian sources (Broschat et al., 1985). As delineated in Scheme 1, the reaction pathway consists of three catalytically discrete steps: oxidation of nucleotidyl diphosphohexose **1** to the corresponding 4-ketohexose **2**, C-5/C-6 dehydration to a 4-keto-$\Delta^{5,6}$-glucoseen intermediate **3**, and reduction at C-6 to give the 4-keto-6-deoxyhexose product **4**. This intramolecular oxidation−reduction has been shown to involve an internal hydrogen transfer from C-4 of the substrate **1** to C-6 of the resulting 4-keto-6-deoxyhexose

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; CDP, cytidine 5′-diphosphate; CI, chemical ionization; CTP, cytidine 5′-triphosphate; dATP, 2′-deoxyadenosine 5′-triphosphate; DEAE, diethylaminoethyl; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EI, electronic ionization; FPLC, fast protein liquid chromatography; GC, gas chromatography; GDP, guanidine 5′-diphosphate; MS, mass spectroscopy; NAD^+, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide, reduced form; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TDP, thymidine 5′-diphosphate; UDP, uridine 5′-diphosphate; UMP, uridine 5′-monophosphate; UV, ultraviolet.

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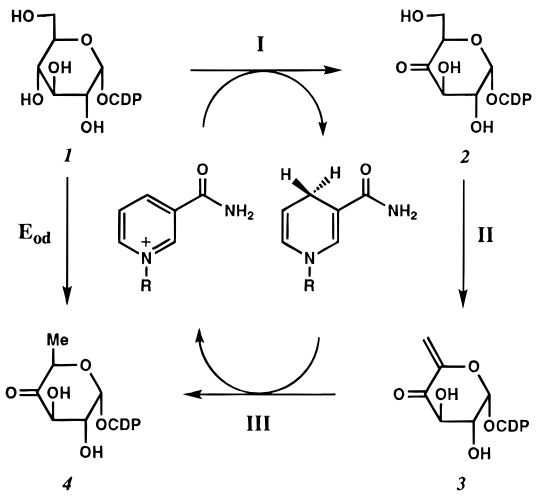
Scheme 1



product **4**, and an enzyme-bound NAD^+ is required for catalysis (Gabriel & Lindquist, 1968; Melo et al., 1968; Snipes et al., 1977). On the basis of the well-established coenzyme chemistry, the NAD^+ apparently functions as a hydride carrier in this catalysis, suggesting these sugar 4,6-dehydratases belong to a small group of enzymes in which the pyridine nucleotide coenzyme is in essence a catalytic prosthetic group, contrary to most other nicotinamide-dependent enzymes in which NAD^+ or $NADP^+$ functions merely as the cosubstrate (Frey, 1987). In addition to the sugar 4,6-dehydratases, this unique group of catalysts also embodies a number of other vital cellular enzymes which include *myo*-inositol-1-phosphate synthase, UDP-D-galactose 4-epimerase, dihydroquinate (DHQ) synthase, UDP-glucuronate decarboxylase, *S*-adenosylhomocysteine hydrolase, ornithine cyclase, urocanase, UDP-apiose synthetase, and TDP-dihydrostreptose synthetase (Frey, 1987).

In an effort to study the mechanisms of 3,6-dideoxyhexose formation (Thorson & Liu, 1993; Thorson et al., 1993, 1994; Liu & Thorson, 1994), we have recently purified a CDP-D-glucose 4,6-dehydratase (E_{od}) from a strain of *Yersinia pseudotuberculosis*. This *Yersinia* dehydratase is a homodimeric protein with a molecular mass of 40.2 kDa per monomer and appears to bind only 1 equiv of NAD^+ per dimer. Similar half-site saturation stoichiometry between cofactor and the functional protein has also been reported for TDP-D-glucose 4,6-dehydrtase from *Escherichia coli* (Wang & Gabriel, 1969) and a number of enzymes using NAD^+ as a catalytic prosthetic group (Frey, 1987). How two identical subunits bind only a single coenzyme molecule in these cases has long been puzzling. Likewise, whether the half-site saturation observed for E_{od}-like dehydratases results from coenzyme sharing between the two subunits or is simply an intrinsic albeit unusual property of these enzymes remains illusive. However, distinct from most other dehydratases which have a tightly bound NAD^+, the purified *Yersinia* dehydratase exhibits an absolute NAD^+ requirement for full activity (Yu et al., 1992). Such NAD^+ dependence on catalysis may be indicative of the inability of E_{od} to hold NAD^+ tightly in its active sites. Interestingly, a close examination of the primary sequence of E_{od} (Thorson et al., 1994) reveals the presence of an extended fold, GHTGFKG (Thorson et al., 1992), that is distinct from the conserved ADP binding $\beta\alpha\beta$ fold (GXGXXG) found in most NAD^+ binding proteins (Wierenga et al., 1985, 1986). The unusual NAD^+ dependence displayed by the *Yersinia* enzyme may thus be ascribed to the displacement of the first invariant glycine with a histidine (His17) and/or the incorporation of a lysine (Lys21) at the locus typically resided by a hydrophobic residue in the cofactor binding domain. To further explore the correlation between the primary structure, cofactor binding characteristics, and the catalysis of this enzyme, we have constructed mutants within this glycine-rich phosphate binding loop designed to better match the conserved sequence. The affinities for NAD^+ of both the wild-type and mutant enzymes were determined and the catalytic properties of these enzymes analyzed. Described in this paper are the results and mechanistic implications of these studies on the catalysis by this interesting enzyme.

EXPERIMENTAL PROCEDURES

General. The *E. coli* strains HB101 and JM105 were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and Pharmacia (Pistacaway, NJ), respectively. The pUC19 vector was obtained from Amersham (Arlington Heights, IL), and p*Trc*99A is a product of Pharmacia. The *asc*B (E_{od}) gene containing plasmids pJT7 and pJT8 were generated in an early work of Thorson et al. (1994). The wild-type E_{od} was purified from *E. coli* HB101-pJT8 according to the previously reported procedure (Thorson et al., 1994). Synthetic oligonucleotides were products of Integrated DNA Technologies (Coralville, IA) or National Bioscience Inc. (Plymouth, MN) and were used without further purification. The culture media components were from Difco (Detroit, MI), and the Bradford reagent was from Bio-Rad (Richmond, CA). The *Taq* DNA polymerase and dNTP's as well as buffer used in polymerase chain reaction (PCR) were obtained from Promega (Madison, WI). [α-^{35}S]-dATPαS, all restriction enzymes, Sequenase version 2.0 DNA sequencing kit, DNA modifying enzymes, and their respective buffers were purchased from Amersham. DEAE-cellulose (DE-52) and Cibacron Blue-3GA gel were purchased from Sigma (St. Louis, MO). The FPLC columns, Mono Q 10/10 and Superdex S-200 HR 10/30, were products of Pharmacia. All molecular weight standards, other enzymes used in assays and sample preparation, and most biochemicals and chemicals were purchased from Sigma, Fisher Scientific (Pittsburgh, PA), or Aldrich (Milwaukee, WI) and were of the highest purity available. Methods and protocols for recombinant DNA manipulations are generally referenced (Ausubel et al., 1989; Sambrook et al., 1989).

Construction of H17G Mutant. The H17G mutant was constructed using the overlap extension protocol of Ho et al. (1989) with pJT7 (Thorson et al., 1994) as the template. The oligonucleotides 5′-GGCG*G*TACTGGGTTTAAAGG and 3′-ATTGTCCGC*C*ATGACCCAAA were used as the forward and reverse internal primers, respectively. These internal primers introduce base changes at the indicated positions (*). The flanking primers used included a universal primer 5′-CGGATAACAATTTCACACAGG (start primer) and a designed primer 3′-GACTCGACCTAGGAGTCATG (halt primer) which introduced a *Bam*HI restriction site immediately downstream from the *asc*B gene. The PCR-amplified mutant gene was purified by ethanol precipitation, digested with *Bam*HI and *Pst*I, and inserted into the *Bam*HI/*Pst*I sites of pUC19. The truncated *asc*A gene (encoding α-D-glucose-1-phosphate cytidylyltransferase, E_p) in the resulting construct coamplified during PCR synthesis was deleted by *Acc*I/*Pst*I digestion followed by fill-in religation

(Sambrook et al., 1989) to give mutant plasmid pHE1 which was isolated and transformed into *E. coli* HB101.

Construction of K21I Mutant. The K21I mutant was constructed using the asymmetric PCR protocol of Perrin and Gilliland (1990). The internal primer which introduced a base change at the indicated position (*) and the pair of flanking primers were 3′-ATGACCCAAATA*TCCACC-GACCAACT (internal primer), 5′-CGCGGAGCTCAG-GAGGAAATTTAAAATGATTAATAATAGT (start primer), and 3′-GACTCGACCTAGGAGTCATG (halt primer). The start primer contained a restriction site for *Sac*I, an *E. coli* ribosome binding sequence, a TA-rich region, and the codons for the first five amino acid residues of E_{od}. The halt primer introduced a *Bam*HI restriction site immediately downstream from the *asc*B gene. The PCR-amplified DNA fragment was purified, digested with *Sac*I and *Bam*HI, and ligated between the *Sal*I/*Bam*HI sites of p*Trc*99A to give pHE2. This recombinant plasmid was isolated and transformed into *E. coli* strain JM105.

DNA Sequencing. All desired mutations were confirmed by DNA sequencing. Plasmids were isolated from *E. coli* by Wizard Minipreps DNA purification system (Promega). Nucleotide sequencing was carried out directly on double-stranded templates, using commercially available M13 forward and reverse primers and six synthetic oligonucleotides prepared on the basis of the *asc*B sequence, by the dideoxy chain termination method (Sanger et al., 1977). Sequence analysis and comparison were performed using Genetics Computer Group's software (Suite 5.4).

Purification of H17G Mutant Protein. An overnight culture of *E. coli* HB101-pHE1 was grown in Luria−Bertani (LB) medium supplemented with ampicillin (100 μg/mL) at 37 °C and diluted 10-fold into the same medium. The inoculated culture was incubated with vigorous agitation at 37 °C for 18−20 h and harvested by centrifugation at 4000*g* for 10 min at 4 °C. The collected cells were washed twice with cold 50 mM potassium phosphate buffer (pH 7.5). The yield was 36.7 g of wet cells per 9 L of growth culture. The subsequent operations were carried out at 4 °C with the exception of the FPLC steps which were run at room temperature. Unless otherwise specified, all buffers were supplemented with 1 mM EDTA.

The early steps, which included crude extract preparation (step 1), streptomycin sulfate treatment (step 2), ammonium sulfate precipitation (step 3), and DEAE-cellulose chromatography (step 4), were essentially identical to those used in the purification of wild-type enzyme (Thorson et al., 1994).

Step 5: Cibacron Blue-3GA Affinity Column Chromatography. The material from step 4 was loaded onto a Cibacron Blue-3GA column (2.5 × 17 cm) which had been equilibrated with 5 mM Tris-HCl buffer (pH 7.5). This column was eluted with a 700 mL linear gradient of KCl from 0.10 to 0.35 M in 5 mM Tris-HCl buffer (pH 7.5) at 1.3 mL/min. Active fractions were combined and concentrated (Amicon ultrafiltration, YM10 membrane) to 2.6 mL for subsequent purifications.

Step 6: FPLC Superdex-200 HR 10/30 Gel Filtration Chromatography. Aliquots (200 μL) of the concentrated active fraction from step 4 were further purified by a FPLC Superdex-200 column (25 mL) and eluted with 50 mM potassium phosphate buffer (pH 7.5) at a flow rate of 1 mL/min. Under these conditions, the desired protein was found to have a retention time of about 14 min, which is identical to that of the wild-type enzyme. The combined active fractions were concentrated (Amicon ultrafiltration, YM10 membrane) to 1.4 mL and subjected to the next purification step.

Step 7: FPLC Mono Q (10/10) Ionic Exchange Chromatography. The enzyme solution from the last step was applied to a FPLC Mono Q column and eluted at a flow rate of 3 mL/min using solvent systems A (20 mM Tris-HCl buffer, pH 7.5) and B (A plus 0.5 M NaCl, pH 7.5) with the profile 0% B from 0 to 4 min and a linear gradient of 0−100% B from 4 to 24 min, with a 6 min final wash at 100% B. The active fraction was collected, concentrated, aliquoted (200 μL/aliquot), and stored at −80 °C.

Purification of K21I Mutant Protein. The *E. coli* JM105-pHE2 bacteria were grown in LB medium supplemented with ampicillin (100 μg/mL) at 37 °C overnight and diluted 100-fold into the same medium. The inoculated culture was incubated with vigorous agitation at 37 °C, to which was added isopropyl β-D-thiogalactopyranoside to a final concentration of 62.5 μM when the culture attained an A_{600} = 0.5−0.6. Cells were harvested 3 h after induction, and the yield was 2.9 g (wet weight) from 0.4 L of culture. Except for the omission of the Cibacron Blue-3GA affinity chromatography, the purification sequence and chromatographic conditions were identical to those used to obtain homogeneous H17G mutant protein.

Protein Determination. Protein concentration of the purification fractions was estimated by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. However, the concentration of samples used in the kinetic measurements, NAD^+ and NADH quantitation, and binding studies was determined by quantitative amino acid analysis which was performed by the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota.

Enzyme Assays. Enzyme activity was determined spectrophotometrically by measuring the formation of CDP-4-keto-6-deoxy-D-glucose as previously described (Yu et al., 1992). The typical assay volume was 200 μL in 50 mM potassium phosphate buffer (pH 7.5). Blank was prepared by boiling the enzyme for 5 min prior to addition of other reagents. One unit of enzyme activity corresponds to the formation of 1 μmol of product per hour under the assay condition, and the specific activities are reported as units per milligram of protein.

Polyacrylamide Gel Electrophoresis. The subunit molecular mass and the purity of enzyme samples were assessed by SDS−PAGE. Electrophoresis was carried out at 25 mA using a discontinuous buffer system (Laemmli, 1970). The separating gel and the stacking gel were 12% and 4% polyacrylamide, respectively. Gels were stained with Coomassie blue (Vesterberg, 1971) and destained with acetic acid−ethanol−water (15:20:165 by volume).

Preparation of Apoenzymes. Apoproteins were prepared by the method of Massey and Curti (1966). The purified enzyme solution (1 mL) obtained from Mono Q chromatography (1−2 mg of protein/mL) was dialyzed against 1 L of 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 2 M potassium bromide. The buffer was changed three times over a period of 2 days, and the progress of NAD^+ washout was monitored by assaying the activity of aliquots of the dialysate taken at different time points. The extent of NAD^+ washout was determined on the basis of the loss of enzyme activity in the absence of exogenous NAD^+. After NAD^+ was completely removed, the dialysis bag containing the apoenzyme was transferred to 1 L of 50

mM potassium phosphate buffer (pH 7.5). Dialysis was continued for 2 days with three changes of buffer to remove potassium bromide. The apoenzyme showed no fluorescence at 443 nm and was catalytically inactive without exogenous NAD^+. However, it regained full activity in the presence of excess NAD^+.

Quantitation of NAD^+ Content of Wild-Type Enzyme. The fluorometric method described by Klingenberg (1984), with minor modification, was used to determine the stoichiometry of NAD^+ per enzyme molecule. The fluorescence spectra were obtained with a Perkin-Elmer LS50B spectrofluorometer (F_{ex} = 365 nm, F_{em} = 454 nm). The excitation and emission slits were 5 and 15 nm, respectively. Purified E_{od} (20 μL, 13.02 mg/mL) in 50 mM potassium phosphate buffer (pH 7.5) was mixed with 100 μL of 0.6 N $HClO_4$ solution and vortexed briefly. The denatured protein was removed by centrifugation at top speed for 5 min using an Eppendorf microcentrifuge. The supernatant was carefully collected without disturbing the protein precipitate, chilled on ice, and diluted with 24 μL of 1 M K_2HPO_4 solution. The pH of this solution was adjusted to 7.4 with 3 N KOH, and the potassium perchlorate precipitate was removed by centrifugation. The supernatant (160 μL) was collected and mixed with 90 μL of 50 mM potassium phosphate buffer (pH 7.4). To this solution was added 250 μL of 100 mM sodium pyrophosphate buffer (pH 8.8) followed by 5 μL of absolute alcohol, and the fluorescence emission at 454 nm was recorded (F_1). An aliquot (1 μL suspension in 3.2 M ammonium sulfate solution) of yeast alcohol dehydrogenase was added (final concentration approximately 6 μg/mL), the mixture was incubated at 23 °C for 6 min, and the fluorescence at 454 nm was measured again (F_2). To this sample was then added 1 μL of a NAD^+ standard solution (1.845 mM, determined using an extinction coefficient of 1.78×10^4 M^{-1} cm^{-1} at 260 nm), and the emission at 454 nm of the resulting solution was taken after 6 min (F_3). The concentration of NAD^+ in this assay solution was calculated from the change of fluorescence emission at 454 nm due to NADH formation: $[F_2 - F_1]$[concentration of NAD^+ standard solution]/$[F_3 - F_2]$.

Quantitation of NADH Content of Wild-Type Enzyme. The NADH quantitation also followed a fluorometric method (F_{ex} = 365 nm, F_{em} = 456 nm). The excitation and emission slits were 5 and 15 nm, respectively. Purified E_{od} (20 μL, 15.13 mg/mL) was diluted with 480 μL of 50 mM potassium phosphate buffer (pH 7.5) which had been degassed and saturated with nitrogen. This sample was denatured by heating at 90 °C for 5 min. The precipitated protein was removed by centrifugation at top speed for 5 min using an Eppendorf microcentrifuge. The supernatant was carefully collected without disturbing the protein precipitate, and the fluorescence emission was recorded. A set of NADH standards were prepared in the same buffer (concentration determined using an extinction coefficient of 6.22 mM^{-1} cm^{-1} at 340 nm) in parallel and subjected to the same heat treatment and centrifugation procedure. The fluorescence data of these standards were recorded to construct a calibration curve. The concentration of NADH extracted from E_{od} was deduced by correlating the reading of the sample solution against the standard curve.

Determination of Kinetic Parameters. Assays were carried out as previously described with varying concentrations of NAD^+ in a total volume of 200 μL of 50 mM potassium phosphate (pH 7.5) buffer. The kinetic parameters of wild-type and mutant enzymes under saturating (CDP-D-glucose) conditions were determined using the corresponding apoproteins and by fitting the experimental data to the Michaelis−Menten equation. The values for K_m and V_{max} were derived from a Lineweaver−Burk plot of the experimental data.

Determination of NAD^+ Binding Affinities (K_d) of Wild-Type and Mutant Enzymes. Binding of NAD^+ to E_{od} and its mutants was monitored by following the quenching of tryptophan fluorescence intensity, induced by the binding of NAD^+ to the apoenzyme (F_{ex} = 295 nm, F_{em} = 339 nm) (Corbier et al., 1990; Clermont et al., 1993). The excitation and emission slits were both 3 nm. The experiment involved successive titration of the apoenzyme solution (in 50 mM potassium phosphate, pH 7.5, total volume 500 μL) with NAD^+. The NAD^+ concentration was determined spectrophotometrically using an extinction coefficient of 1.78×10^4 M^{-1} cm^{-1} at 260 nm, and proper enzyme concentrations (0.245 μM for wild-type E_{od}, 0.264 μM for K21I mutant, and 0.316 μM for H17G mutant) were chosen in order to ensure the free ligand concentrations corresponded to 10−90% of the total concentration of the added NAD^+. All measurements were carried out at 23 °C. From the measured fluorescence intensity I at a given NAD^+ concentration, the average number υ of moles of NAD^+ bound per mole of protein was deduced by using $\upsilon = n(I - I_a)/(I_h - I_a)$, where n represents the number of coenzyme binding sites; I_a, the fluorescence of the apoenzyme; and I_h, the fluorescence of the holoenzyme with its n coenzyme binding sites saturated with NAD^+. The free NAD^+ concentration x (or $[NAD^+]_f$) was estimated using $x = [NAD^+]_t - n[E]_t$ in which $[NAD^+]_t$ and $[E]_t$ represent the total NAD^+ and apoenzyme concentration, respectively. A nonlinear least-squares procedure was used to fit the υ and x to the Adair−Klotz equation (eq 1)

$$\upsilon = \frac{\frac{x}{K_1} + \frac{2x^2}{K_1K_2} + ... + \frac{nx^n}{K_1K_2...K_n}}{1 + \frac{x}{K_1} + \frac{x^2}{K_1K_2} + ... + \frac{x^n}{K_1K_2...K_n}} \quad (1)$$

(Corbier et al., 1990; Clermont et al., 1993) to determine the dissociation constants (K_ds).

Determination of CDP-D-glucose and NADH Binding Affinity with Wild-Type Enzyme. Following the same procedure described above to determine the K_d for NAD^+, quenching of the tryptophan fluorescence (F_{ex} = 297 nm, F_{em} = 334 nm) of apo-E_{od} (60.7 nM, functional dimer) upon binding with substrate (varied from 0 to 1.2 μM) was used to determine the affinity of the wild-type enzyme for CDP-D-glucose. The excitation and emission slits were 3 and 9 nm, respectively. In a separate experiment to obtain the K_d for NADH, the enhancement of the fluorescence emission of NADH upon binding with E_{od} was monitored. It should be noted that the maximum of free NADH fluorescence emission in 50 mM potassium phosphate buffer is at 456 nm, while enzyme-bound NADH undergoes a blue shift to 443 nm with great intensity enhancement. Thus, the excitation wavelength was set at 360 nm, and the emission of fluorescence at 443 nm was monitored. The excitation and emission slits were 5 and 15 nm, respectively. Since the bulk of the added NADH in each titration was not enzyme bound, the fluorescence of those free NADH molecules also contributed to the observed emission at 443 nm. As a result,

each of the measured values at 443 nm was corrected by subtracting the contribution from the unbound NADH, estimated with a NADH solution of respective concentration (in 50 mM potassium phosphate buffer, pH 7.5) used in the titration. The extinction coefficient of 6.22 mM^{-1} cm^{-1} at 340 nm was used to calculate the concentration of NADH solution used for the titration. A total of 101.2 nM (functional dimer) apo-wild-type enzyme was used, while the total NADH concentration varied from 0 to 0.6 μM. All measurements were carried out at 23 °C. Data analyses to determine K_ds in both cases were identical to that used to determine K_d for NAD^+ as described above.

Effects of NAD^+ Structural Mimics on the Catalytic Activity of E_{od}. NADH, ADP, 5′-phosphoribose 1-pyrophosphate (5′-PRPP), β-nicotinamide mononucleotide (β-NMN), nicotinamide, and 3-aminobenzamide were used to test for the possible allosteric effect of these compounds on the activity of E_{od}. The wild-type enzyme (0.12 mg/mL) was incubated separately with each of the above compounds (at 1 μM and 1 mM) at room temperature for 5 min. The activity of the incubation mixture was then subjected to normal E_{od} assay procedure, omitting exogenous NAD^+. A control was prepared without the preincubation with any of the above compounds.

Detection of Enzyme-Bound Substrate and Other Sugar Derivatives. Detection of enzyme-bound sugar derivatives involved the isolation and derivatization of these compounds followed by GC−MS characterization (Weigel et al., 1992; Thorson et al., 1993). A 19.5 mL solution containing 14.8 mg of wild-type E_{od} in potassium phosphate buffer (50 mM, pH 7.5) was boiled for 10 min, and the protein precipitate was removed by centrifugation on an Eppendorf microcentrifuge at top speed for 5 min. The supernatant was concentrated with a Savant Speed Vac SC100 to a final volume of 0.3 mL. This solution was then subjected to sodium borodeuteride reduction, followed by acid hydrolysis and derivatization. The resulting products were submitted to GC−MS analysis.

RESULTS

Properties of CDP-D-glucose 4,6-Dehydratase. When purified to homogeneity, the recombinant wild-type E_{od} is typically only 50−60% active in the absence of exogenous NAD^+. Among other potential cofactor surrogates, only $NADP^+$ could stimulate activity, albeit at 20% of the rate obtained with the NAD^+ under identical conditions. Since incubation of the cofactor-deficient sample with excess NAD^+ followed by dialysis failed to restore full enzyme activity, this dehydratase, akin to the wild-type enzyme isolated directly from *Y. pseudotuberculosis*, shows an absolute requirement of NAD^+ for optimal activity.

Construction of H17G and K21I Mutants and Purification of Mutant Proteins. The H17G mutant was prepared, and the resulting plasmid (pHE1) containing the mutagenic fragment was expressed in *E. coli* HB101. The mutant protein was purified (0.5 mg/36.7 g of wet cells) with a specific activity of 167 units/mg. Construction of the K21I mutant was accomplished by the asymmetric PCR method. The amplified mutagenic gene was cloned into pTrc99A (pHE2) and overexpressed in *E. coli* JM105. The expressed protein (7.6 mg/2.9 g of wet cells) was purified with a specific activity of 96 units/mg. The summary of the purification results for H17G is presented in Table 1 as an example. Interestingly, these mutant proteins were only partially active prior to NAD^+ reconstitution, a phenomenon also noted for the wild-type E_{od} (Yu et al., 1992; Thorson et al., 1992).

Table 1: Summary of H17G Mutant Protein Purification from *E. coli* HB101-pHE1

purification step	total protein (mg)[a]	sp act. (units/mg)[b]	total act. (units)	purification (*x*-fold)	yield (%)
crude extracts[c]	4373	0.15	656	1.0	100
streptomycin sulfate	2545	0.22	560	1.5	85.4
ammonium sulfate	1846	0.28	517	1.9	78.8
DEAE-cellulose	208	2.33	485	15.5	73.9
Cibacron Blue-3GA	15	23.4	351	156	53.5
Superdex-200	1.9	56.4	107	376	16.3
Mono Q	0.5	167	83.5	1113	12.7

[a] Determined by Bradford assay. [b] Activity was determined as detailed in Experimental Procedures; units represent micromoles of product formation per hour. [c] From 36.7 g of wet cells.

Table 2: Kinetic Parameters and Affinity for NAD^+ of Wild-Type E_{od} and Its Mutants

	WT G**H**T**G**FK**G**	K21I GHTGF**I**G	H17G G**G**TGFKG
V_{max} (μmol min^{-1} mg^{-1})[a]	2.75 ± 0.07	1.74 ± 0.08	3.17 ± 0.05
k_{cat} (min^{-1})	221.2 ± 5.6	139.9 ± 6.4	254.6 ± 4.0
K_m (μM)	0.33 ± 0.03	0.24 ± 0.03	0.15 ± 0.01
k_{cat}/K_m (min^{-1} μM^{-1})	670	595	1686
K_{d,NAD^+} (nM)[b]			
K_1	40.3 ± 0.4	8.28 ± 0.16	2.19 ± 0.02
K_2	539.8 ± 4.8	33.7 ± 0.7	27.5 ± 0.3

[a] The kinetic parameters were determined using apoenzyme under substrate (CDP-D-glucose) saturation conditions as described in Experimental Procedures. [b] The experimental details are described in Experimental Procedures

Quantitation of Bound NAD^+ and Determination of K_m for NAD^+. Quantitation of the NAD^+ content of the purified enzyme which displayed 58.5% activity prior to NAD^+ reconstitution revealed a ratio of ∼0.73 ± 0.02:1 (NAD^+ per protein dimer), suggesting the loss of activity may be directly proportional to the deficiency of this cofactor. The kinetic constants for the apoform of both the wild-type and mutant enzymes were determined as described in Experimental Procedures. The dependence of the reaction rate on NAD^+ concentration in each case was found to follow classical Michaelis−Menten saturation kinetics. As listed in Table 2, the wild-type enzyme displayed a K_m for NAD^+ of 0.33 μM, while the K21I and H17G mutant species, as anticipated, showed a lower K_m for NAD^+ of 0.24 and 0.15 μM, respectively. Preparation of the H17G/K21I double mutant whose cofactor binding domain should more closely resemble the typical **G**X**G**XX**G** fold was also attempted. Unfortunately, the desired protein was obtained as inclusion bodies. Efforts to express this double mutant enzyme in soluble form or to renature the insoluble form of the expressed protein proved futile. Interestingly, because the catalytic efficiency (k_{cat}/K_m) of the H17G mutant enzyme is higher than that of the wild-type E_{od}, this can be considered an unusual example of a biocatalyst that has been genetically altered.

NAD^+ Binding Affinities (K_d) of Wild-Type and Mutant Enzymes. Sequence alignment showed that Trp25 of E_{od} is part of the conserved ADP binding $\beta\alpha\beta$ fold found in most NAD^+-binding proteins (Wierenga et al., 1985, 1986). Due to its close proximity to the NAD^+ binding site, this residue

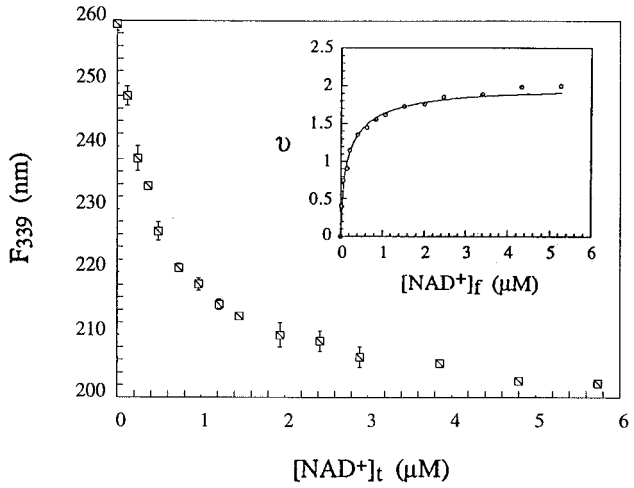


FIGURE 1: Effect of binding of NAD^+ on the fluorescence emission at 339 nm of apo-wild-type CDP-D-glucose 4,6-dehydratase. The apoenzyme concentration was 245 nM in 50 mM potassium phosphate buffer, pH 7.5. The inset displays the plot of $\upsilon = n(I - I_a)/(I_h - I_a)$, where n represents the number of coenzyme binding sites; I_a, the fluorescence of the apoenzyme; and I_h, the fluorescence of the holoenzyme with its n coenzyme binding sites saturated with NAD^+. The free NAD^+ concentration was estimated using $[NAD^+]_f = [NAD^+]_t - n[E]_t$ in which $[NAD^+]_t$ and $[E]_t$ represent the total NAD^+ and enzyme concentration, respectively. A nonlinear least-squares procedure was used to fit the υ and $[NAD^+]_f$ to the Adair−Klotz equation (eq 1) to determine the dissociation constants.

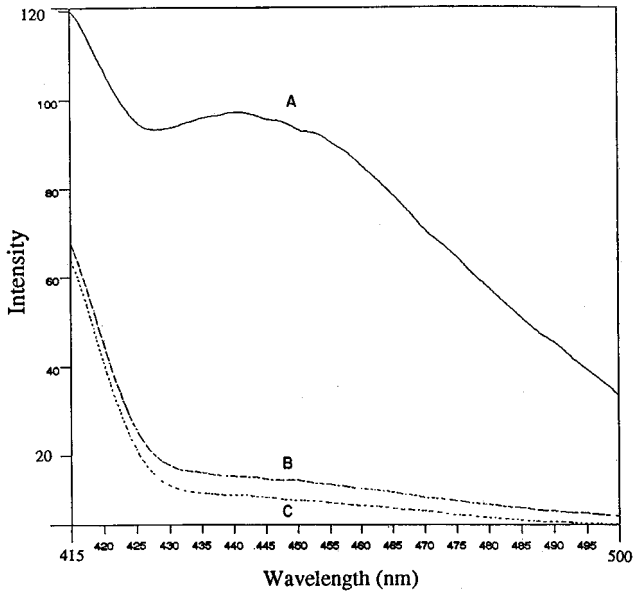


FIGURE 2: Fluorescence spectra of wild-type and apo-CDP-D-glucose 4,6-dehydratase. The enzyme concentration of each sample was 0.21 μM in 50 mM potassium phosphate buffer, pH 7.5. The excitation wavelength was set at 360 nm, and the emission of fluorescence between 415 and 500 nm was recorded. The excitation and emission slits were 5 and 15 nm, respectively. The spectra are (A) wild-type E_{od}, (B) apo-wild-type E_{od}, and (C) buffer.

is expected to be a good probe as its fluorescence characteristics may reflect the conformational changes associated with cofactor binding. Figure 1 shows the effect of NAD^+ binding on the fluorescence emission of apo-E_{od} at 339 nm, from which the dissociation constant K_d is derived by plotting υ versus $[NAD^+]_f$ (see inset). The curve is best fitted with the Adair−Klotz equation (eq 1) assuming $n = 2$, which leads to two constants, $K_1 = 40.3 \pm 0.4$ nM and $K_2 = 539.8 \pm 4.8$ nM. From these data, a 2:1 stoichiometry between bound NAD^+ and the homodimeric enzyme could thus be deduced. It should be noted that additional quenching of fluorescence was observed for both the wild-type and mutant enzymes in the presence of a high molar excess of NAD^+, probably due to a nonspecific binding of NAD^+ to these enzymes. Assuming such an additional quenching prevails equally in all three cases (wild type, K21I, and H17G), where the fluorescence varies linearly with total NAD^+ concentration, the fluorescence intensity I_h associated with the saturation of the specific coenzyme binding site could be easily computed from the measured intensity I by $I_h = I + a[NAD^+]_t$ (Clermont et al., 1993), where a was deduced from the H17G mutant which has the highest affinity for NAD^+. These data along with other kinetic parameters determined with the corresponding apoproteins of the wild-type and mutant enzymes are listed in Table 2. Consistent with the K_m results, the dissociation constant (K_1) of the K21I mutant decreases by approximately 5-fold and those (K_1 and K_2) of H17G by nearly 20-fold as compared to the wild-type enzyme.

Incubation with NAD^+ Structural Mimics. It has been well established that the addition of exogenous NAD^+ is essential to render the wild-type E_{od} fully active. In order to assess whether the added NAD^+ binds at allosteric site(s) and thus enhances the catalytic activity of E_{od}, several NAD^+ structural mimics were tested for their competence as allosteric effectors of E_{od}. Interestingly, all of these compounds exhibited inhibitory effects with varying degrees on the catalysis. It was thus concluded that the E_{od}-catalyzed reaction is unlikely regulated allosterically by exogenous NAD^+.

Detection and Quantitation of Bound NADH. The electronic spectrum of the apoenzyme is that of a simple polypeptide since it is devoid of any chromophoric absorption above 300 nm (data not shown). However, a close examination of the UV−vis spectrum of the wild-type E_{od} as isolated revealed the presence of an absorption around 340 nm whose intensity appeared to be inversely proportional to the activity of the enzyme. Furthermore, upon excitation at 365 nm, the wild-type enzyme gave a strong fluorescence emission around 443 nm, a phenomenon that was not found for the apoenzyme (Figure 2). Both observations insinuated the presence of enzyme-bound NADH. Further analysis indeed revealed a 0.340 ± 0.002:1 stoichiometry between NADH and the dimeric enzyme. It should be noted that NADH is known to be more stable at alkaline pH (Klingenberg, 1984); however, denaturation of E_{od} under alkaline conditions led to a fine suspension which was difficult to separate by centrifugation and thus interfered with fluorometric measurement. In order to avoid such complications, our experiment was performed at pH 7.5. Hence, although the 0.34:1 ratio was deduced by calibrating against a standard curve constructed with standards prepared under identical conditions, it should still be considered only as the low limit of the actual stoichiometry. Furthermore, the less than quantitative release of NADH by heat denaturing of E_{od} may have also contributed to the observed low stoichiometry.

Determination of K_d for NADH. Figure 3 illustrates the relationship of fluorescence intensity to total NADH concentration. A setting of $n = 2$ is again required to better fit the curve with the Adair−Klotz equation (eq 1). The two dissociation constants of K_1 and K_2 derived from these data are 0.21 ± 0.01 nM and 7.46 ± 0.25 nM, respectively. These results point to two cofactor binding sites per functional dimer, and E_{od} has a much higher affinity for

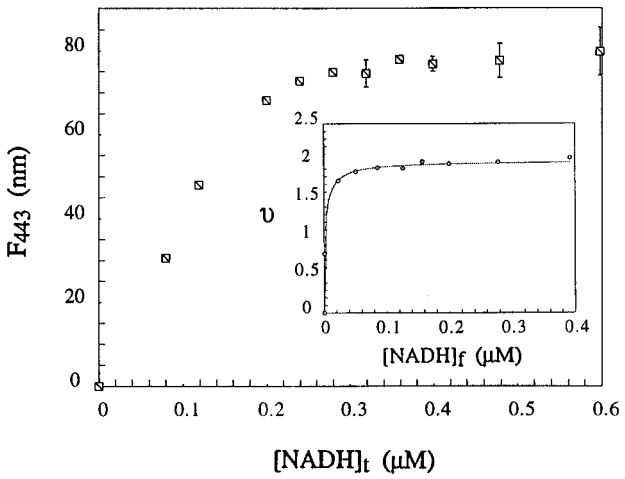


FIGURE 3: Determination of dissociation constants of NADH based on its fluorescence emission at 443 nm upon binding with apo-wild-type CDP-D-glucose 4,6-dehydratase. The apoenzyme concentration was 101.2 nM in 50 mM potassium phosphate buffer, pH 7.5. The inset displays the plot of $v = n(I - I_i)/(I_f - I_i)$, where n represents the number of coenzyme binding sites; I_i, the initial fluorescence of the sample solution prior to NADH titration; and I_f, the final fluorescence of the sample solution in which the n coenzyme binding sites of E_{od} had been saturated with NADH. The free NADH concentration was estimated using $[NADH]_f = [NADH]_t - n[E]_t$ in which $[NADH]_t$ and $[E]_t$ represent the total NADH and enzyme concentration, respectively. A nonlinear least-squares procedure was used to fit the v and $[NADH]_f$ to the Adair−Klotz equation (eq 1) to determine the dissociation constants.

NADH than for NAD^+. Due to the tight binding between NADH and E_{od}, only a trace amount of NADH is needed to saturate the available binding sites.

Release of NADH from E_{od} upon Binding with Substrate. Interestingly, when the wild-type enzyme was treated with CDP-D-glucose, NADH was found to be released. Shown in Figure 4 are the NADH fluorescence spectra of samples of wild-type E_{od} preincubated with excess NAD^+, CDP-D-glucose, or mixture of both for 15 min prior to emission measurement. Since CDP-D-glucose does not absorb at the wavelength used to excite the sample nor at where enzyme-bound NADH emits, the decrease of fluorescence intensity at 443 nm upon substrate binding (Figure 4, curve C) must therefore result from the liberation of NADH from E_{od}. This conclusion is supported by the similar fluorescence emission spectrum found for apo-E_{od} which is known to be NADH free (Figure 2, curve B). Our studies also showed that no significant NADH release was detected by incubation with excess NAD^+ alone (Figure 4, curve B). These findings suggest that NADH and NAD^+ compete for the same site and are consistent with the fact that E_{od} has a higher affinity for NADH than NAD^+. An analogous experiment was also performed with E_{od} samples prepared by reconstituting the apoenzyme with NADH. As illustrated in Figure 5, the decline of enzyme-bound NADH fluorescence at 443 nm induced by binding with CDP-D-glucose occurs with concomitant increase of the tryptophan fluorescence at 334 nm.

Determination of K_d for CDP-D-glucose. The above experiment demonstrated that substrate binding is not only a prerequisite for turnover but is also important to transform the enzyme from an inactive form (NADH bound) to the catalytically active form (NAD^+ bound). In order to gain more insights into this regulation, the affinity of wild-type apo-E_{od} for CDP-D-glucose was determined by monitoring the quenching of the tryptophan fluorescence of E_{od} upon substrate binding at 334 nm. As shown in Figure 6, the v versus $[CDP\text{-glucose}]_f$ plot is best fitted to the Adair−Klotz equation (eq 1) with $n = 2$ to give two dissociation constants, $K_1 = 216.7 \pm 0.9$ nM and $K_2 = 138.9 \pm 0.6$ nM. Thus, the binding of substrate to the wild-type enzyme under our experimental conditions exhibited a 2:1 stoichiometry (CDP-D-glucose:dimeric enzyme) with comparable binding affinities for both sites.

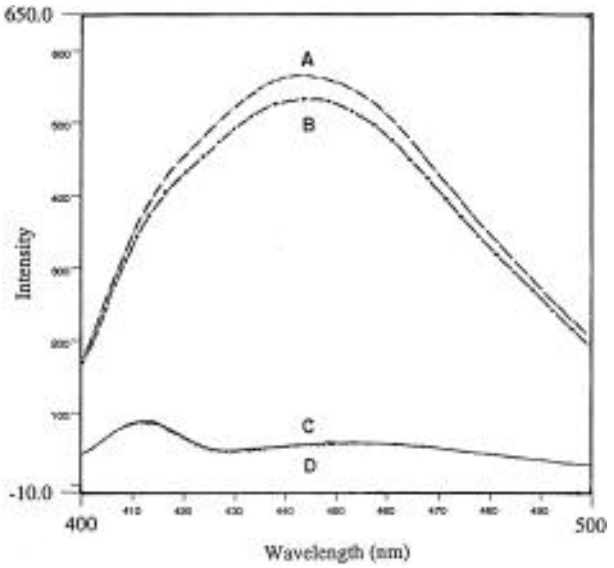


FIGURE 4: Effect of addition of NAD^+ and CDP-D-glucose on the fluorescence spectra of wild-type CDP-D-glucose 4,6-dehydratase. The enzyme concentration in each sample was 1.2 μM in 50 mM potassium phosphate buffer, pH 7.5. All samples were incubated at 37 °C for 15 min prior to fluorescence measurement. The excitation wavelength was set at 360 nm, and the emission of fluorescence between 400 and 500 nm was recorded. The excitation and emission slits were 5 and 15 nm, respectively. The spectra are (A) wild-type E_{od}, (B) wild-type E_{od} plus NAD^+ (29.9 μM), (C) wild-type E_{od} plus CDP-D-glucose (35.0 μM), and (D) wild-type E_{od} plus NAD^+ (29.9 μM) and CDP-D-glucose (35.0 μM).

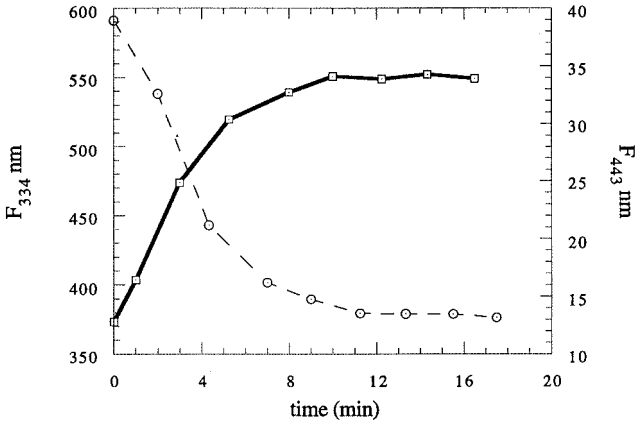


FIGURE 5: Time-dependent release of NADH from wild-type CDP-D-glucose 4,6-dehydratase upon binding with CDP-D-glucose. The sample solution containing apoenzyme (60.7 nM) and NADH (266.4 nM) in 0.5 mL of 50 mM potassium phosphate buffer (pH 7.5) was incubated for 3 min at 23 °C and then mixed with 2 μL of CDP-D-glucose (final concentration 344 nM). At the indicated times the fluorescence was measured. To follow the release of NADH, the excitation wavelength was set at 360 nm, and the emission of fluorescence at 443 nm due to the bound NADH was recorded. The excitation and emission slits were 5 and 15 nm, respectively. To monitor the change at the coenzyme binding site due to the release of NADH, the excitation wavelength was set at 297 nm, and the emission of fluorescence at 334 nm was recorded. The excitation and emission slits were 3 and 9 nm, respectively. The thick line represents the fluorescence emission at 334 nm, and the dash line traces the change of fluorescence at 443 nm.

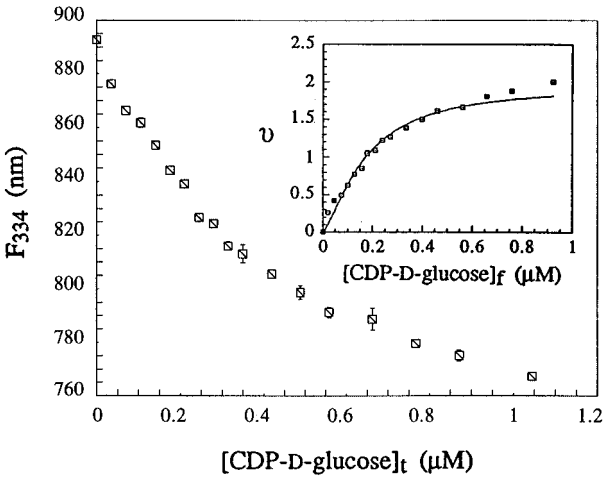
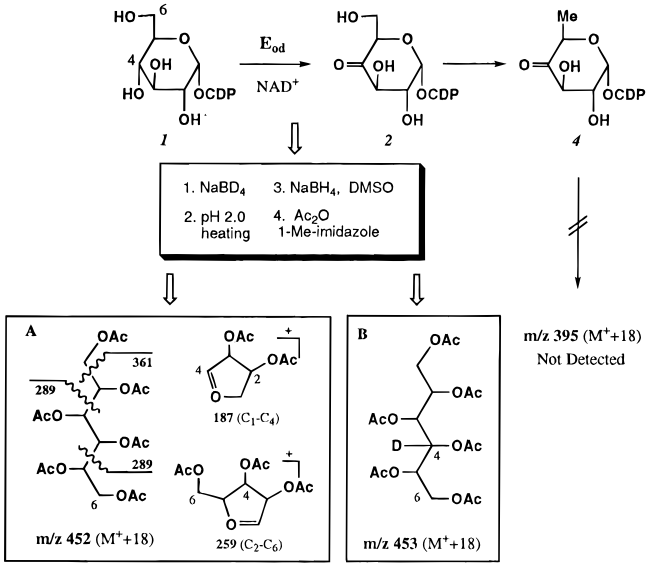


FIGURE 6: Effect of binding of CDP-D-glucose on the fluorescence emission at 334 nm of apo-wild-type CDP-D-glucose 4,6-dehydratase. The apoenzyme concentration was 60.7 nM in 50 mM potassium phosphate buffer, pH 7.5. The inset displays the plot of $\upsilon = n(I - I_a)/(I_h - I_a)$, where n represents the number of coenzyme binding sites; I_a, the fluorescence of the apoenzyme; and I_h, the fluorescence of the holoenzyme with its n coenzyme binding sites saturated with CDP-D-glucose. The free substrate concentration was estimated using $[\text{CDP-glucose}]_f = [\text{CDP-glucose}]_t - n[\text{E}]_t$ in which $[\text{CDP-glucose}]_t$ and $[\text{E}]_t$ represent the total CDP-D-glucose and enzyme concentration, respectively. A nonlinear least-squares procedure was used to fit the υ and $[\text{CDP-glucose}]_f$ to the Adair−Klotz equation (eq 1) to determine the dissociation constants.

Scheme 2



Detection of Bound Sugar Derivatives. As shown above, the complex formation between substrate and E_{od} is reasonably tight. Since the apoenzyme can bind substrate in the absence of NAD^+, we decided to investigate whether the isolated wild-type E_{od} contains sequestered substrate or its analogs. As described in Experimental Procedures, the purified enzyme was heat denatured, and the liberated sugar derivatives were subjected to sodium borodeuteride reduction, acid hydrolysis, further reduction, and then acetylation to yield glycidol peracetates whose MS fragmentation patterns had been well characterized (Rubenstein & Strominger, 1974; Lönngren & Svensson, 1974). On the basis of the GC/MS (both EI and CI) results, it was concluded that this mixture contains glucitol hexaacetate (Scheme 2, panel A) which has a molecular ion peak at *m/z* 452 ($M^+ + 18$), and a distinct GC retention time identical to that of an authentic sample prepared from CDP-D-glucose. A 4-keto-hexose species was also detected as a minor component (*m/z* 453) (Scheme 2, panel B). The assignment was based on the observation that the MS fragments containing the C-4 oxygen linkage (*m/e* 187, 259, 289, and 361) are all uniformly shifted by one mass unit. The increment of one mass unit of these fragments bearing the C-4 ester linkage is indicative of a deuterium incorporation through reduction into the 4-keto group of a hexose which is likely a 4-ketoglucose derivative. Since early studies on substrate specificity indicated no turnover could be detected with nucleotidyl diphosphoglucoses other than CDP-D-glucose (Yu et al., 1992), the sugar molecules found in the wild-type enzyme are most likely CDP-glucose and/or its derivatives. Interestingly, no trace of E_{od} product (**4**) was found in this analysis.

DISCUSSION

Extensive structural work has established a consensus for the typical ADP binding fold of nicotinamide-dependent enzymes known as the Rossmann fold domain (Rossmann et al., 1975; Ohlsson et al., 1974; Matthews et al., 1979; Wierenga et al., 1983, 1985; McKie & Douglas, 1991). To help define the absolute requirement of NAD^+ for full activity of E_{od} from *Y. pseudotuberculosis*, a comparison of the Rossmann fold was made with other members of the same class as E_{od} using NAD^+ as a prosthetic group. As shown in Table 3, the typical $\beta\alpha\beta$ fold is found in most cases (entries 1−13), among which *E. coli* DHQ synthase (Bender et al., 1989), *E. coli* and yeast UDP-galactose 4-epimerases (Frey, 1987), and the *S. typhimurium* TDP-D-glucose 4,6-dehydratase (Romana et al., 1991) are known to bind NAD^+ tightly. However, the primary sequence of E_{od} (entry 17) along with that of three other enzymes (entries 14−16) shows a clear divergence from the **G**X**G**XX**G** consensus pattern. The apparent deviation from the normal NAD^+ binding consensus suggests the presence of an alternative cofactor binding motif for this group of enzymes, contributing to the difference in NAD^+ binding characteristics displayed by the *Yersinia* dehydratase (Thorson et al., 1992). The preferred alignment with the typical **G**X**G**XX**G** consensus, G**H**T**G**FK**G** (alignment I), results in the placement of a histidine near the N-terminal end of the α-helix which may decrease the α-helix dipole often thought to be important in pyrophosphate ligand binding (Sancho et al., 1992). The alternative alignment, **G**H**T**GF**K**G (alignment II), substitutes a relatively more bulky threonine for the universally conserved second glycine believed to be important in minimizing steric interaction with the coenzyme. Furthermore, the presence of a positively charged lysine at the position typically occupied by a hydrophobic residue may also impose a severe perturbation on the NAD^+ binding domain with this alignment.

To help better understand the NAD^+ dependence of the wild-type enzyme and the structure−cofactor binding correlation, H17G and K21I mutants were prepared and characterized. It is evident from Table 2 that the K_1 and K_2 constants for NAD^+ binding of the K21I mutant decreased by factors up to 5 and 16, respectively. Since the core of the conserved dinucleotide binding $\beta\alpha\beta$ fold is essentially hydrophobic, replacing the hydrophilic lysine with isoleucine may have alleviated the adverse interaction between lysine and the $\beta\alpha\beta$ core in the wild-type E_{od}. The H17G mutant exhibits a more significant improved NAD^+ affinity in which both the K_1 and K_2 constants decreased by nearly 20-fold.

Table 3: ADP Binding $\beta\alpha\beta$ Folds of Proteins Containing Active Site Bound NAD^+

	β_A	α_B	β_B
(1)[a]	C L V T G **G A G** Y I **G** S H T V V E L C E A G - - Y K C I V V D N		
(2)[b]	I L V L G **G A G** Y I **G** S H M V D R L V E K G - Q E K V V V V D S		
(3)[c]	V L V T G **G S G** Y I **G** S H T C V Q L L Q N G - - H D V I I L D N		
(4)[d]	V L V T G **G A G** Y I **G** S H T V V E L I E N G - - Y D C V V A D N		
(5)[e]	V L V T G **G A G** Y I **G** S H T V L E L L E A G - - Y S P V V I D N		
(6)[f]	Y L V T G **G A G** Y V **G** S V V A Q H L V E A G - - N E V V V L H N		
(7)[g]	V L V T G **G S G** Y I **G** S H T C V X L L X N G - - H D V V I L D N		
(8)[h]	L L I T G **G** C **G** F L **G** S N L A S F A L S Q G - - I D L I V F D N		
(9)[i]	I L I T G **G A G** F I **G** S A V V R H I I K N T - Q D T V V N I D K		
(10)[j]	V L V T G **G A G** F I **G** S H Y V R Q L L G G A - Y P A F A G A D V		
(11)[k]	T L V A L **G G G** V V **G** D L T G F A A A S - - - - - Y Q R G V R F		
(12)[l]	V V I A L **G G G** V I **G** D L T G F V A S T - - - - - Y M R G V R Y		
(13)[m]	V M V A I **G G G** V I **G** D M I G F V A S T - - - - - F M R G V R V		
(14)[n]	W L V T **G** A S **G** M L **G** R E L T P L L D R R G - - - - - A A V T A		
(15)[o]	L L V T **G** A A **G** F I **G** S Q Y V R T L L G P G - G P P D V V V T A		
(16)[p]	V F V T **G** H T **G** F K **G** S W L S L W L T E M G A I V K G Y A L D A		
(17)[q]	V F V T **G** H T **G** F K **G** G W L S L W L Q T M G A T V K G Y S L T X		
Align I		**G X G X X G**	
Align II		**G X G X X G**	

[a] *Kluyveromyces lactis* UDP-galactose 4-epimerase (Webster & Dickson, 1988). [b] *Streptococcus thermophilus* UDP-galactose 4-epimerase (Poolman et al., 1990). [c] *E. coli* UDP-galactose 4-epimerase (Lemaire & Muller-Hill, 1986). [d] *Saccharomyces cerevisiae* UDP-galactose 4-epimerase (Citron & Donelson, 1984). [e] Rat myoblast UDP-galactose 4-epimerase (Zeschnigk et al., 1990). [f] *Streptomyces lividans* UDP-galactose 4-epimerase (Adams et al., 1988). [g] *Salmonella typhimurium* UDP-galactose 4-epimerase (Houng et al., 1990). [h] *S. typhimurium* CDP-tyvelose 2-epimerase (Verma & Reeves, 1989). [i] *S. typhimurium* TDP-D-glucose 4,6-dehydratase (Jiang et al., 1991). [j] *S. erythrea* TDP-D-glucose 4,6-dehydratase (Pissowotzki et al., 1991).[k] *E. coli* dehydroquinate synthase (Millar & Coggins, 1986). [l] *Aspergillus nidulans* dehydroquinate synthase (Charles et al., 1986). [m] *S. cerevisiae* dehydroquinate synthase (Duncan et al., 1987). [n] *Streptomyces griseus* TDP-dihydrostreptose synthase (Pissowotzki et al., 1991). [o] *S. griseus* TDP-D-glucose 4,6-dehydratase (Pissowotzki et al., 1991). [p] *S. typhimurium* CDP-D-glucose 4,6-dehydratase (Jiang et al., 1991). [q] *Yersinia pseudotuberculosis* CDP-D-glucose 4,6-dehydratase (Thorson et al., 1994).

Thus, removal of the interference with the α-helix dipole by replacing the histidine near the N-terminal end of the α-helix in the Rossmann fold with glycine has clearly improved the cofactor binding ability of this enzyme. Interestingly, both the wild-type and H17G mutant enzymes showed strong anticooperativity while the K21I mutation greatly reduced the effect. Since a tightly bound NAD^+ is pivotal for initiating an effective intramolecular redox turnover, the catalytic efficiency (k_{cat}/K_m) of this important biosynthetic enzyme, as expected, has also benefited from remodeling of its coenzyme binding fold to enhance the NAD^+ affinity. Our successful construction of a mutant protein having better catalytic efficiency than the wild-type enzyme nicely demonstrates the feasibility of predicting and rationally modifying a cofactor binding site. It also holds promise toward the refinement of the catalytic activity of this class of enzymes and perhaps of other nicotinamide-dependent proteins as well.

Such quantitative analysis of NAD^+ binding behavior has only been studied for a small number of proteins that utilize nicotinamide cofactor as a prosthetic group. The best defined nicotinamide binding characteristics within this class have been done with DHQ synthase from *E. coli* (Bender et al., 1989) which has a $K_m(NAD^+)$ of 80 nM under turnover conditions. From the reported k_{on} and k_{off} values for DHQ synthase without substrate, a dissociation constant for NAD^+ of 2 nM can be deduced. This number as compared to those derived from Figure 1 for E_{od} denotes at least a 20-fold difference of NAD^+ affinity. Qualitatively consistent with the variance of nicotinamide binding displayed by these two enzymes is the observation that E_{od} preincubated with excess cofactor followed by dialysis, or gel filtration, failed to restore full enzyme activity. However, tight binding of NAD^+ to the apo-DHQ synthase under similar conditions was evident (Bender et al., 1989). It is worth mentioning that the K_d for NADPH of human aldose reductase (Kubiseski & Flynn, 1995) was found to be <1 μM and that for NAD^+ of *Bacillus stearothermophilus* glyceraldehyde-3-phosphate dehydrogenase is 0.26 μM (Clermont et al., 1993). Clearly, the K_ds for cofactor in the latter two cases that use NAD(P)$^+$/NAD(P)H as a cosubstrate are higher than those determined for DHQ synthase and E_{od} in which NAD^+ participates in the catalysis as a coenzyme.

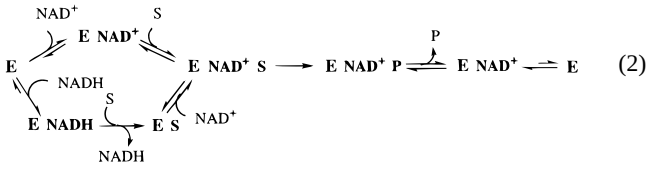
A more significant result from this affinity investigation is that two NAD^+ binding sites per E_{od} dimer were deduced from the K_d determination. A similar conclusion was previously reached for *E. coli* UDP-D-galactose 4-epimerase on the basis of its X-ray crystal structure which had been solved to a resolution of 2.5 Å (Bauer et al., 1992). In contrast to extensive biochemical literature suggesting that this epimerase binds only one NAD^+ per dimer, the appearance of electron density for two pyridine nucleotides in symmetry-related positions in the dimer was found. It was also noted that each subunit contains one substrate binding site. Unfortunately, NAD^+ is essentially irreversibly bound to this epimerase which precipitates when NAD^+ is removed *in vitro* (Bauer et al., 1992). The obstinate nature of this epimerase to form the apoenzyme has hampered the quantitative analysis of its cofactor binding characteristics. Thus, despite the fact that *E. coli* UDP-galactose 4-epimerase is perhaps the best studied enzyme of this class, a direct comparison of its affinity for NAD^+ binding with E_{od} is not possible.

While the affinity for NAD^+ has been enhanced by altering the coenzyme binding motif of E_{od}, it was surprising to note that these mutant proteins were still only partially active in the absence of exogenous NAD^+. Although it is conceivable that E_{od} and its mutants could each be isolated as a mixture of apo- and holoenzyme as previously surmised, the fact that both the wild-type and H17G mutant displayed little increase of their activities after reconstitution with excess NAD^+ and then dialysis is incompatible with the enhanced affinity for coenzyme binding shown for the H17G mutant. It was the unusually high background NADH fluorescence observed in our early quantitation of NAD^+ under heat denaturation conditions (data not shown) that provided the initial hint indicating the coexistence of NADH in the wild-type enzyme. A discernible absorption near 340 nm of the concentrated E_{od} sample, which became readily available after its gene (*asc*B) was overexpressed, also suggested the presence of NADH-bound enzyme (data not shown). In fact, it is well documented that the highly purified *E. coli* UDP-galactose 4-epimerase contains a significant amount of NADH (Wong et al., 1978; Swanson & Frey, 1993), and an early study of TDP-D-glucose 4,6-dehydratase from *E. coli* also reported that some of the nicotinamide coenzyme bound to the enzyme

is present in reduced form (Wang & Gabriel, 1969). In view of such precedence, the occupation of a portion of the NAD^+ cofactor binding sites by NADH seems to be a more appealing explanation of the low NAD^+ content and ineffective reconstitution. Indeed, quantitative analysis of the NAD^+ and NADH content confirmed that E_{od} as isolated is comprised of at least two forms distinct in the oxidation states of the active site bound pyridine nucleotide coenzyme. Identical to the NAD^+ titration results, two coenzyme binding sites per E_{od} dimer could also be deduced from titration of the apoenzyme with NADH (Figure 3). Since the dissociation constants for the reduced nicotinamide coenzyme are smaller than those of NAD^+, it is not too surprising that a significant portion of the wild-type enzyme exists in the NADH-bound form. Although the total amount of cofactor (both NAD^+ and NADH) extracted from E_{od} is less than that expected for a fully constituted sample, it should be stressed that the quantity of coenzymes, especially NADH, associated with E_{od} may be underestimated in our experimental conditions. However, in light of the large anticooperativity found for NAD^+ binding, it is also possible that the NAD^+-bound enzyme is only half-site saturated. Hence, whether all of the cofactor binding sites of purified E_{od} are fully constituted with NAD^+ and/or NADH must await a detailed structure elucidation of the native protein.

Fluorescence quenching experiments, carried out by titrating the apoenzyme with CDP-D-glucose (Figure 6), nicely demonstrated that a tight binary complex was formed in the absence of coenzyme. Again, a 1:1 stoichiometry between the substrate binding site and each subunit of E_{od} was deduced from our data analysis. The comparable magnitude of the two dissociation constants derived from this study is consistent with E_{od} being composed of two catalytically equivalent subunits. These results also provide direct evidence suggesting that cofactor and substrate binding in the E_{od} reaction can be treated as two independent events. While the fact that both substrate and NAD^+ can bind to E_{od} alone is a necessary but not a sufficient condition for a random sequential mechanism, this observation at least indicates that the catalysis is unlikely an ordered mechanism as previously proposed (Thorson et al., 1992). Considering the apo-wild-type enzyme after reconstitution with NAD^+ followed by dialysis still exhibits partial NAD^+ dependence on its activity, the association of the E−NAD^+ complex is most likely a reversible process.

Since NADH associated with *E. coli* UDP-D-glucose 4-epimerase was found to be a part of a ternary complex containing tightly bound uridine nucleotide such as UMP, UDP, or UDP sugars (Vanhooke & Frey, 1994), it was interesting to determine whether a similar situation is also preserved by the *Yersinia* dehydratase. As anticipated, our GC−MS results clearly showed the presence of hexose derivatives in the highly purified E_{od}. These sugar components are most likely derivatives of cytidine nucleotide since glucose itself cannot bind with E_{od} (data not shown) and other nucleotidyl diphosphoglucoses are not substrates of this enzyme (Yu et al., 1992). In view of the fact that both the NADH- and 4-ketohexose-containing enzyme species are present, it is tempting to hypothesize that their formation may be ascribed to the untimely release of either the sugar intermediate or the reduced nicotinamide coenzyme during turnover. However, this conjecture is inconsistent with an early report in which the thymidine diphosphate sugars were found to be tightly bound to the reduced TDP-D-glucose 4,6-dehydratase whose nicotinamide coenzyme had been converted to the NADH form (Zarkowsky et al., 1970). Thus, a definitive answer for the CDP counterpart must await further experiments. Interestingly, incubation of CDP-D-glucose with the apoenzyme pre-reconstituted with NADH led to the release of enzyme-bound NADH (Figure 5). These results indicate that the NADH-containing enzyme cannot be a ternary complex since NADH and substrate are mutually exclusive. This intriguing difference in substrate−coenzyme association clearly distinguishes E_{od} from UDP-galactose 4-epimerase from *E. coli*. In a recent report, Burke and Frey (1993) elegantly demonstrated that binding of uridine nucleotides by UDP-galactose 4-epimerase from *E. coli* induces a protein conformational change which perturbs the nicotinamide ring and increases the intrinsic reactivity of NAD^+. It is thus conceivable that, when E_{od} binds with substrate, it may also undergo a conformational change establishing a preference for NAD^+ over NADH and converting an abortive complex to an active catalyst. Clearly, the possible ability of E_{od} to adjust its conformation by substrate binding to discriminate between these two coenzymes of distinct oxidation states is novel. The observed cofactor selection regulated via substrate binding is an intriguing mechanism worthy of further investigation. Since the *Yersinia* dehydratase contains a significant amount of NADH, eq 2 may



(2)

be a more reasonable description of the E_{od} reaction *in vivo*.

CONCLUSION

Studies of the cofactor and substrate binding characteristics of the wild-type and mutant E_{od} revealed the presence of two binding sites for both CDP-D-glucose and NAD^+ per enzyme molecule. Further analysis also showed that the purified E_{od} contains sequestered NADH. The fact that mutant with enhanced NAD^+ affinity still exhibits NAD^+ dependence for full activity suggested that E_{od}'s half-site saturation of NAD^+ per enzyme dimer may not be attributed solely to weak binding as previously surmised. The large anticooperativity found for NAD^+ binding may explain why the cofactor binding site of wild-type E_{od} is only half-occupied. Since the affinity of E_{od} for NADH is much higher than that of NAD^+, it is also conceivable that a significant portion of the cofactor binding sites is occupied by NADH. The capability of E_{od} to regulate the release of the sequestered NADH upon binding with CDP-D-glucose brought to light a new possible kinetic mechanism for E_{od} catalysis. Interestingly, the H17G mutation resulted in an enzyme with slightly higher k_{cat} and a 3-fold increase in catalytic efficiency (k_{cat}/K_m), providing an interesting example in which site-directed mutagenesis enhances catalytic efficiency.

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