Mechanistic Studies on CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-Dehydrase: Identification of His-220 as the Active-Site Base by Chemical Modification and Site-Directed Mutagenesis†

Yenyoung Lei, Olivier Ploux,‡ and Hung-wen Liu*

Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455

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ABSTRACT: CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydrase (E_1) purified from *Yersinia pseudotuberculosis* is a pyridoxamine 5′-phosphate (PMP) dependent iron−sulfur-containing enzyme which catalyzes the C−O bond cleavage at C-3 of its substrate leading to the formation of 3,6-dideoxyhexose. This enzyme is rapidly inactivated by diethyl pyrocarbonate (DEP) at pH 6.0 and 25 °C. The inactivation of E_1 by DEP, which is reversible upon treatment of hydroxylamine, appears to be attributable solely to the modification of histidine residues. The fact that coincubation of E_1 with its substrate gave almost total protection against DEP inactivation and that only one less histidine residue was modified in the presence of substrate strongly suggested that inactivation is due to the modification of only one reactive histidine residue which resides in or near the active site of E_1 and is critical for E_1's activity. Sequence alignment between the translated *asc*C (E_1) gene and several representative pyridoxal 5′-phosphate (PLP)/PMP dependent enzymes revealed that three of the four invariant residues, glycine, aspartate, and arginine found in all other aminotransferases, are conserved in the E_1 sequence (G169, D191, and R403). However, the highly conserved lysine is replaced by a histidine residue (H220) in E_1. In order to test whether H220 plays an essential role in E_1 catalysis, H220N mutant was constructed and the encoding protein was found to exhibit nearly identical physical characteristics as the wild-type E_1. Interestingly, the mutant protein had lost most of its catalytic activity, and one less histidine residue was modified upon treatment of H220N-mutated protein with DEP. Such a single-point mutation also impaired E_1's capability of catalyzing the solvent hydrogen exchange at C-4′ position of the PMP coenzyme. Our findings strongly suggested that H220 is most likely the active-site base which abstracts the C-4′ proton from the PMP−substrate adduct and initiates the catalysis. Furthermore, E_1's preservation of other invariant residues found in many PLP/PMP dependent enzymes allowed a speculation of their roles in E_1 catalysis. Since sequence alignment between E_1 and its homologs believed to participate as PLP/PMP dependent aminotransferases in the biosynthesis of deoxy amino sugars showed that substitution of the active-site lysine with a histidine residue seems to be characteristic for strictly PMP dependent enzymes, it is thus conceivable that nature's simple substitution of an active-site lysine with a histidine residue may have transformed a normal coenzyme B_6 dependent aminotransferase into a unique PMP dependent catalyst that is no longer an aminotransferase but instead a dehydrase.

The reactions mediated by coenzyme B_6 phosphate dependent catalysts are extremely versatile (Adams, 1976; Evangelopoulos, 1984; Dolphin et al., 1986). Although their catalytic functions are predominantly confined to the metabolism of amino acids and amines, with a process involving glycogen phosphorylation as the only exception (Madsen & Withers, 1986; Hajdu et al., 1987; Palm et al., 1990), a study of the biosynthesis of CDP-ascarylose (CDP-3,6-dideoxy-L-*arabino*-hexopyranose, **1**) in *Yersinia pseudotuberculosis* has led to the discovery of another interesting example of aberration in which the C-3 deoxygenation step is catalyzed by a pyridoxamine 5′-phosphate (PMP) dependent enzyme, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydrase (E_1)[1] (Rubenstein & Strominger, 1974a; Gonzalez-Porqué, 1986; Liu & Thorson, 1994). As shown in Scheme 1, the postulated mechanism for E_1 catalysis, based on the well-established coenzyme B_6 chemistry, is initiated by Schiff base formation between PMP and the C-4 keto group of the substrate CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**2**) followed by a C-4′ proton abstraction from the resulting adduct (**3**) that triggers the expulsion of the C-3 hydroxyl group to produce a $\Delta^{3,4}$-glucoseen intermediate (**4**) in the active site of E_1 (Rubenstein & Strominger, 1974a; Weigel et al., 1992a,b). While this glucoseen intermediate **4** has never been isolated or characterized, subsequent reduction by an

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* To whom correspondence and reprint requests should be addressed. Tel: 612-625-5356. Fax: 612-626-7541.

‡ On leave from Centre National de la Recherche Scientifique, Paris, France.

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[1] Abbreviations: CDP, cytidine diphosphate; E_{od}, CDP-D-glucose 4,6-dehydratase (CDP-D-glucose oxidoreductase); E_1, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydrase; E_3, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase; E_{ep}, CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose 5-epimerase; PMP, pyridoxamine 5′-phosphate; PLP, pyridoxal 5′-phosphate; LB, Luria−Bertani broth; DTT, dithiothreitol; DEAE, (diethylamino)-ethyl; FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DEP, diethyl pyrocarbonate; IPTG, isopropyl β-D-thiogalactoside; CD, circular dichroism; U, units

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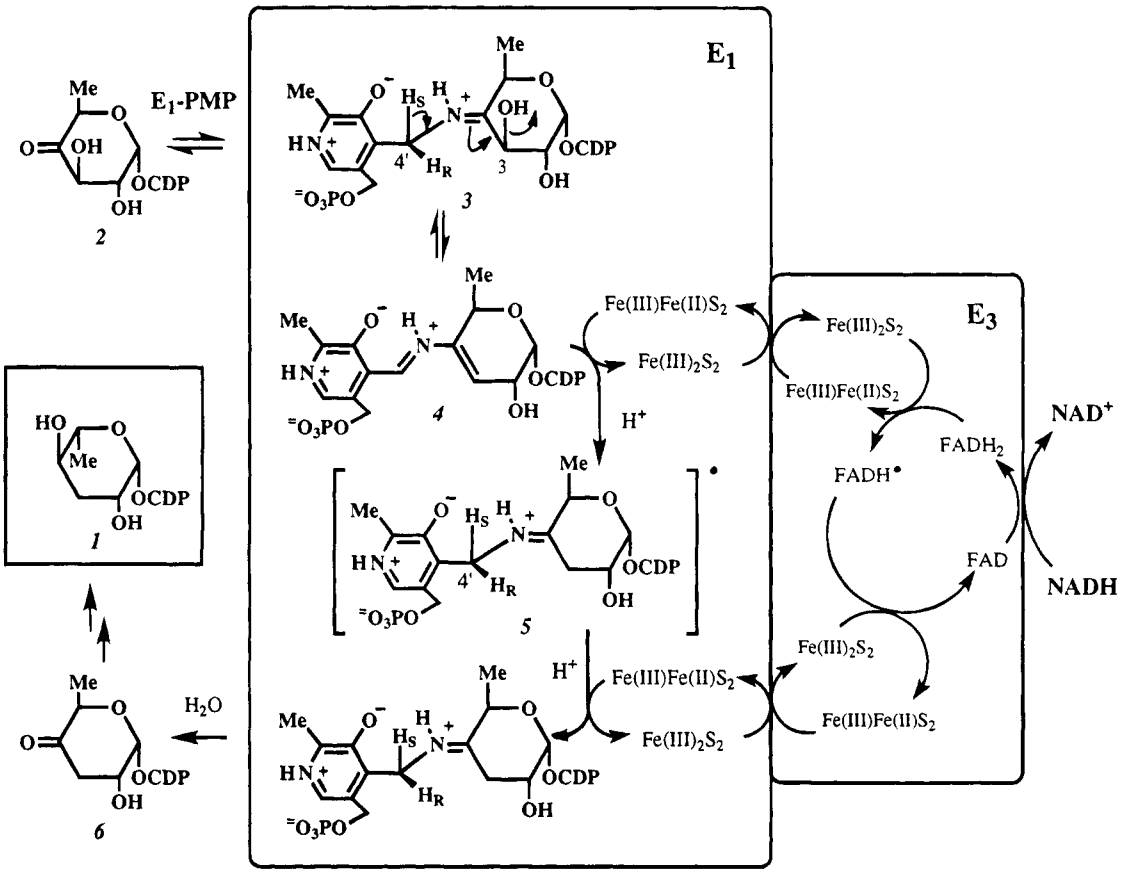
Scheme 1



NADH dependent flavoenzyme, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3) (Miller et al., 1993; Lo et al., 1994), gives rise to the actual deoxygenation product, CDP-4-keto-3,6-dideoxy-D-glucose (**6**), that had been isolated and identified (Rubenstein & Strominger, 1974b). Our recent characterization of the cloned E_1 expressed in *Escherichia coli* has revealed, in addition to PMP, the presence of a [2Fe-2S] center whose EPR characteristics are akin to those of adrenodoxin and putidaredoxin (Thorson & Liu, 1993a). Interestingly, a plant-type ferridoxin [2Fe-2S] cluster was also found in E_3 (Miller & Liu, 1992). The order of electron flow has been established to be initiated with hydride reduction of FAD in E_3 by NADH. The iron−sulfur cluster of E_3, after receiving electrons one at a time from the reduced flavin, relays the reducing equivalents via the [2Fe-2S] center of E_1 to its acceptor, the glucoseen intermediate **4** (Thorson & Liu, 1993b). Thus, in E_1 catalysis, PMP appears to have a dual function of being responsible for the anion-induced dehydration reaction and also for being an integral part of the subsequent redox process. Although pyridoxal 5′-phosphate (PLP) has been shown to play an essential role in the dehydration mediated by serine dehydrase, there is no precedent that relies on PMP as the cofactor to carry out a deoxygenation in a biological system. The participation of the iron−sulfur cluster of E_1 as part of the electron conduit in the reductive phase of this C−O bond cleavage event further distinguishes this enzyme as singular in its own class. In an attempt to further characterize this unique catalyst, we have undertaken a detailed study to identify particular amino acid residues critical to the catalytic activity of this enzyme. By a multifaceted approach using chemical modification, sequence comparison, and site-directed mutagenesis, we have identified His-220 as the active-site base essential for E_1 catalysis. Reported herein are the results and the mechanistic implications.

EXPERIMENTAL PROCEDURES

General. E. coli JM105 was purchased from Pharmacia (Pistacaway, NJ). DEAE-Sepharose CL6B, Mono-Q 10/10, and Superdex S-200 10/10 were also obtained from Pharmacia. The *asc*C (E_1) gene containing plasmid pJT18 (Thorson et al., 1994) was a gift from Jon S. Thorson. Synthetic oligonucleotides were products of Integrated DNA Technologies (Coralville, IA) and used without further purification. The culture media components were from Difco (Detroit, MI), and the Bradford reagent was from BioRad (Richmond, CA). All restriction enzymes, DNA-modifying enzymes, and their respective buffers were purchased from United States Biochemical (Cleveland, OH). *E. coli* HB101 and plasmids pUC18, pUC118, pUC19, and pUC119 were also from United States Biochemical. [α-^{32}S]dATP and the *in vitro* mutagenesis kit were obtained from Amersham (Arlington Heights, IL). All other biochemicals and chemicals were purchased from either Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) and of the highest purity available. Sequence analysis and comparison were performed using Genetics Computer Group's software (Suite 5.4). CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3) was purified according to a procedure of Ploux et al. (1995). CDP-D-glucose-4,6-dehydratase (E_{od}) (Thorson et al., 1994) was kindly supplied by Xuemei He of this group.

Reconstruction of the ascC Expression Plasmid. The *asc*C gene coding for E_1 was amplified with the GeneAmp PCR kit (Perkin Elmer) using pJT18 plasmid as the template. The construct (pYOE1) was made with a start primer, 5′-

GGCGAATTCATGAGTCAACAT-3′ and a halt primer, 5′-CCGGATCCTCAATTAAAA-3′. The amplified insert was digested with *Eco*RI and *Bam*HI and ligated into the *Eco*RI–*Bam*HI sites of pUC118. Standard recombinant DNA techniques were used for the plasmid construction (Maniatis et al., 1989). After transformation into competent *E. coli* HB101, positive clones were separated, the double-stranded plasmid was isolated and purified, and the sequence of the insert was verified using the Sequenase kit (United States Biochemical, Cleveland, OH).

Growth of Cells. *E. coli* HB101/pYOE1 was grown in Luria–Bertani (LB) broth (Maniatis et al., 1989) supplemented with ampicillin (100 μg/mL) and ferrous ammonium sulfate (final concentration 100 μM). Six 1 L batches were inoculated with an overnight culture (1 mL each), and the cells were grown at 37 °C for 15 h with vigorous agitation. The cells were harvested by centrifugation (15 min, 3000*g*), washed with 50 mM potassium phosphate buffer (pH 7.0) containing 50 mM $MgSO_4$ and 0.4 M NaCl, collected again by centrifugation (15 min, 3000*g*), and kept at −20 °C. A typical yield was 30 g (wet weight) of cells/6 L of culture.

Purification of Wild-Type E_1. The purification was carried out at 4 °C except for the FPLC steps which were run at room temperature. Unless otherwise specified, a 20 mM Tris-HCl buffer (pH 7.6) was used throughout the purification (buffer A). In order to minimize the possibility of enzyme inactivation by molecular oxygen, all buffers were degassed and saturated with nitrogen gas.

Step 1: Crude Extracts. The cell paste was thawed on ice and resuspended in 100 mL of cold buffer A containing 50 μM PMP and 1 mM DTT. The suspension was sonicated for 5 min with a 30 s cooling period every minute. Cellular debris was removed by centrifugation (20 min, 27000*g*), and the supernatant was quickly transferred to a stoppered flask and flushed with nitrogen gas.

Step 2: Ammonium Sulfate Precipitation. Crystals of ammonium sulfate were added portionwise to the crude extract (115 mL) up to 80% saturation, and the solution was stirred for 1 h under nitrogen. The precipitated proteins were collected by centrifugation (15 min, 12000*g*) and redissolved in a minimum amount of buffer A. This solution was dialyzed for 3 h under nitrogen against 1 L of buffer A with three changes of buffer.

Step 3: DEAE-Sepharose CL6B Chromatography. The protein solution from step 2 (35 mL) was loaded onto a DEAE-Sepharose CL6B column (2.5 cm × 40 cm) previously equilibrated with buffer A. After washing with one bed volume of buffer A, the column was eluted with a linear gradient of NaCl from 0 to 0.4 M in buffer A (1 L total) at 50 mL/h. Fractions of 12 mL were collected during the gradient elution, and the active fractions (70–75) were combined. The protein solution was concentrated by ultrafiltration on an Amicon concentrator using a YM10 membrane.

Step 4: FPLC Superdex S-200 Chromatography. The material from step 3 was further purified in batches (250 μL each) by an FPLC equipped with a Superdex S-200 (10/10) column pre-equilibrated with buffer A. Elution monitored at 280 nm was achieved using buffer A at 1 mL/min. The desired fractions were manually collected, concentrated by a Centriprep-10 concentrator (Amicon), and kept at −80 °C.

Protein Assay. Protein concentrations were routinely determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. This assay was calibrated by comparing the results obtained with aliquots of the same sample by both the Bradford method and the quantitative amino acid analysis; the latter was performed by the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota. It was found that the value obtained with quantitative amino acid analysis was only 77% of that deduced by the Bradford method, and thus all results were accordingly corrected.

Enzyme Assay. The E_1 activity was determined by a newly developed continuous assay coupling E_1 with E_3. The assay mixture consists of 25 μM pyridoxamine 5′-phosphate, 200 μM NADH, 100 μM CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**2**; E_1 substrate), and an appropriate amount of E_3, in 800 μL of 50 mM potassium phosphate buffer (pH 7.6). The reaction was initiated by the addition of E_1, and the E_1 activity was determined by measuring the rate of reduction of the absorbance at 340 nm ($\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$) within the initial 1 min. Each reading was calibrated against background activity (ca. 2%, E_3-catalyzed oxidation of NADH in the presence of O_2) recorded prior to the addition of E_1. The E_1-induced NADH oxidation measured separately was also substracted from the observed readings. The E_1 substrate **2** used in the enzyme assay was prepared in large quantities according to a recently reported protocol (Thorson et al., 1994).

Iron and Inorganic Sulfur Analysis. Iron was assayed by the method of Fish (1988), and inorganic sulfur was quantified spectrophotometrically as described by Bienert (1983).

Polyacrylamide Gel Electrophoresis. The SDS–polyacrylamide gel electrophoresis was carried out at 120 V with a 4% stacking gel and a 12% resolving gel in the discontinuous buffer system of Laemmli (1970). Gels were stained with the preparation of Vesterberg (1971) and destained with acetic acid–ethanol–water (15:20:165 by volume).

Molecular Weight Determination. The subunit molecular weight was determined by SDS–polyacrylamide gel electrophoresis as described by Laemmli (1970). The molecular weight of the native enzyme was determined by gel filtration performed on an FPLC Superdex S-200 column eluted with 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl and 1 mM β-mercaptoethanol. The column was calibrated by a separate chromatographic run with protein standards (Sigma): cytochrome *c* (12 300), carbonic anhydrase (28 800), ovalbumin (45 000), bovine serum albumin (66 000), transferrin (81 000), alcohol dehydrogenase (160 000), and β-amylase (200 000). Each standard was dissolved in NaCl (100 mM) containing potassium phosphate buffer (50 mM, pH 7.0) and injected separately to determine the retention time. The molecular weight of the purified protein was estimated by the method of Andrews (1964).

Quantitation of Bound PMP. The stoichiometry of bound PMP per subunit of E_1 was estimated by fluorometric determination of the quantity of released PMP from a denatured E_1 sample of known concentration. During this measurement, the E_1 solution (1 μM in subunits) in 20 mM Tris-HCl buffer (pH 7.5) was boiled for 5 min and centrifuged, and the supernatant recovered. The solution was diluted and excited at 325 nm and the intensity of fluorescence monitored at 390 nm. The amount of PMP released was deduced by calibrating the reading against a standard

curve established with PMP samples of known concentrations (ranging from 10 to 70 nM).

Inactivation by Diethyl Pyrocarbonate. A typical experiment included the treatment of 15.4 μM of E_1 (in subunits) with varying concentrations of DEP in 0.1 mL of 50 mM potassium phosphate buffer (pH 6.0) at 25 °C. The inactivation was conducted at pH 6.0 to reduce the competing hydrolysis of DEP which may complicate the kinetic analysis. The DEP solution was freshly prepared by diluting the reagent in ice-cold absolute ethanol. Aliquots of 10 μL were removed from the incubation mixture at different time points and the reactions quickly quenched with 1 μL of 0.1 M imidazole buffer (pH 6.0). This aliquot was immediately diluted into the standard coupled assay mixture to initiate the E_1−E_3-catalyzed deoxygenation, and the residual activity was determined. The ethanol content was controlled not to exceed 5% by volume, a concentration found to have no effect on the activity and stability of the enzyme during the incubation times.

Substrate Protection against DEP Inactivation. For the protection experiments, E_1 was incubated with 0.2 mM substrate **2** for 20 min prior to the treatment with DEP. The subsequent incubation and analysis followed an identical protocol as described above.

Spectral Measurement. The extent of *N*-carbethoxylation of histidine residues of E_1 in 50 mM potassium phosphate buffer (pH 6.0, 25 °C) was determined by the time dependent increase in the absorbance at 240 nm (Ovádi et al., 1967). The recorded absorbance was corrected for the blank (buffer, enzyme, and ethanol) over a wavelength range of 235−350 nm to generate difference spectra. The stoichiometry of the formation of modified histidine residues, as a function of time, was calculated from the molar extinction coefficient for *N*-carbethoxyhistidine (ϵ_{240} = 3200 M^{-1} cm^{-1}) (Miles, 1977).

Hydroxylamine Treatment. After 1 h of incubation of E_1 (16.2 μM in subunits) at 25 °C with DEP (1 mM) in 50 mM potassium phosphate buffer (pH 6.0), hydroxylamine (adjusted to pH 7.0 with KOH) was added to a final concentration of 0.2 M. The time course of restoring histidine residues from the *N*-carbethoxyhistidine form was immediately followed by recording continuously the change in absorbance at 240 nm. The effect of hydroxylamine on DEP-inactivated enzyme was determined by incubating the enzyme and DEP at 25 °C for 30 min until the enzyme activity decreased to 3% of its original activity. Unreacted DEP was quenched by the addition of excess imidazole, and hydroxylamine was added to a concentration of 60 mM. The resulting mixture was incubated at 25 °C, and aliquots were removed at different time points to determine the remaining activity. It should be noted that protein started to precipitate after 40 min of incubation when more concentrated NH_2OH (>60 mM) was used.

Site-Directed Mutagenesis and Purification of Mutant Proteins. Mutants H220N and H221N were constructed by the phosphothioate method using an *in vitro* mutagenesis kit purchased from Amersham. The oligonucleotide primers used to introduce the point mutation have a sequence of 5′-CCCGCT*AAT*CATATCACCATGGG-3′ for the H220N mutant and 5′-CCCGCTCAT*AAT*ATCACCATGGG-3′ for the H221N mutant. The resulting plasmids, pYOM1 for H220N and pYOM2 for H221N, were isolated and transformed into *E. coli* TG-1. The sequence of each mutated gene was determined using a Sequenase kit to ensure that only the desired base change was present. These mutant proteins were purified as described for the wild-type E_1. Since the H220N mutant had no activity, the purification was primarily monitored by SDS−PAGE.

Circular Dichroism Studies. The circular dichroism spectra of the wild-type and the mutant enzymes were obtained by scanning the sample solution in 20 mM Tris-HCl buffer (pH 7.5), in a 0.1 mm cell, over the far-UV range (250−185 nm) using a Jasco J-710 spectropolarimeter.

E_1-Catalyzed Deprotonation/Reprotonation at C-4′ of the PMP-$\Delta^{3,4}$-glucoseen Complex. Enzyme E_1 (15.7 μM in subunits) was incubated with its substrate (**2**; 0.5 μmol) and PMP (28 μg, 0.1 μmol) in 50 mM potassium phosphate buffer (pH 7.5) prepared with 600 μL of $[^3H]H_2O$ (20 mCi/mL). The reaction was kept at 25 °C for 4 h and then heated at 100 °C for 5 min to denature the enzyme. The precipitated protein was removed by centrifugation and the supernatant lyophilized. The residue was redissolved in water followed by lyophilization. After this process was repeated three times, the radioactive powder was then redissolved in a minimal amount of water and further purified by HPLC using a Partisil SAX 10 analytical anion-exchange column (4.6 × 250 mm). A linear 20 min gradient from 0.03 to 0.12 M potassium phosphate buffer (pH 6.6) was used with a flow rate of 1 mL/min to elute the compound. Under these conditions, the peak containing the majority of radioactivity was eluted at the same time as standard PMP (12.6 min). This fraction was collected and the radioactivity determined.

RESULTS

Expression and Purification of Wild-Type E_1. In previous reports (Thorson et al., 1994), we have described the cloning and sequencing of *asc*C gene from *Y. pseudotuberculosis* and the expression of the gene product, E_1, in *E. coli* HB101. The expression system used, pJT18, was a pUC18-derived plasmid bearing an *Eco*RI segment of the *asc* cluster which contains the intact *asc*C gene flanked by truncated *asc*B (encoding CDP-D-glucose 4,6-dehydratase, E_{od}) and *asc*E (CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose 5-epimerase, E_{ep}) fragments at the 5′ and 3′ ends, respectively. The expression of E_1 induced by IPTG from this system was extremely efficient in which nearly 50% of the soluble protein in the crude extract was found to be the desired enzyme. Unfortunately such hyperexpression yielded an E_1 typically with a low iron−sulfur content (<0.5 mol [2Fe-2S]/E_1 monomer). Since the metal cluster of E_1 appeared to be relatively inert to molecular oxygen as compared to E_3 (Ploux et al., 1995), and precautions were taken to minimize its exposure to oxygen during enzyme purification, the inferior iron−sulfur content of the expressed enzyme may result from an incomplete *in vivo* reconstitution. That isolated E_1 proved refractory to reconstitution using methods previously described for ferredoxins (Meyer et al., 1992) prompted us to test an approach by lowering the expression level of the *asc*C gene product and to assess such adjustment on the quality of the isolated protein. The initial attempt using uninduced cells of *E. coli* JM105/pJT18 led to an E_1 with a better, yet still low, iron−sulfur stoichiometry. Fortunately, expression in *E. coli* HB101 of a new plasmid, pYOE1, constructed by cloning the PCR-amplified *asc*C gene alone flanked by *Eco*RI and *Bam*HI sites into pUC118, produced the desired E_1 with less abundance but nearly 1 equiv of [2Fe-2S]/E_1 dimer. Whether the approximate 1:1

Table 1: Summary of Enzyme E_I Purification from *E. coli* HB101/pYOE1

purification step	total protein[a]	total activity[b]	specific activity[c]	purification (*x*-fold)	yield (%)
crude extracts[d]	2990	7475	2.5	1	100
ammonium sulfate	616	6283	10.2	4.1	84
DEAE-Sepharose	87.5	4401	50.3	20	59
FPLC S-200	52.5	3780	72	29	51

[a] Protein in milligram. [b] Activity was determined by the coupled assay described in the Experimental Procedures section. U = nmol of NADH consumed/min. [c] Specific activity = U/mg of protein. [d] Crude extracts were obtained from 27.7 g of wet cells (6 L of culture).

stoichiometry of [2Fe-2S] cluster per E_I monomer is an intrinsic property of E_I or simply reflects a continuous imperfect constitution of the metal center requires further investigation. Likewise, the reason for the lower expression of *E. coli* HB101/pYOE1 by deleting sequences flanking the *asc*C open reading frame is not obvious at this point; however, it is clear that a reduced expression has improved the *in vivo* reconstitution of the metal cluster. Table 1 summarizes the purification of wild-type E_I which led to a homogeneous enzyme after a two-step chromatographic procedure. It may be worth mentioning that the addition of ferrous ammonium sulfate and Na_2S to the crude extracts was found to be useful for [Fe-S] reconstitution during the purification of *Anabaena* vegetative ferridoxin mutants (Cheng et al., 1994); however, similar treatment showed little effect on the [Fe-S] stoichiometry of E_I.

Molecular Properties. When run on a calibrated FPLC column of Superdex S-200, the isolated E_I exhibits a native molecular weight of 97 kDa. The subunit molecular weight of 49 kDa, based upon SDS−PAGE, is consistent with the predicted molecular weight of 48 371 Da from the deduced amino acid sequence. Therefore, the active enzyme exists as a homodimer. The electronic absorption spectrum of native E_I exhibits the characteristic absorption of an iron−sulfur protein (Figure 1, the absorbance at 278 nm is 1.01 for an E_I solution of 1 mg/mL). Spectrophotometric quantitation of iron and sulfur contents was repeated with samples obtained from different enzyme preparations. Although the iron and sulfur contents varied among protein batches, these analyses revealed 1.2 equiv of Fe and 1.1 equiv of S^{2-} per mole of E_I monomer, consistent with at least one [2Fe-2S] center per enzyme (E_I dimer). These results imply that either the isolated E_I remains only partially constituted with its metal cluster or this unusual iron−sulfur center is shared between E_I's two subunits. Thus, it is possible that E_I may resemble the *Azotobacter* nitrogenase whose dimeric structure is linked via a [4Fe-4S] center (Kim & Rees, 1994) and be a new member of a rare class of enzyme. However, the presence of 1 equiv of bound PMP/E_I subunit established by fluorometric quantitation of PMP released from denatured protein suggests that the unusual Fe/S stoichiometry found for purified E_I is more likely a problem of incomplete reconstitution.

Catalytic Properties. The kinetic parameters of E_I catalysis were determined using a newly developed assay monitoring the consumption of NADH during the coupled reduction catalyzed by E_3. As a sugar dehydrase, E_I displayed normal Michaelis−Menten saturation kinetics. The values for a K_M of 44.3 μM for CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**2**) and a V_{max} of 72 nmol min^{-1} mg^{-1} for its catalysis were determined by plotting the data according to the method of Eisenthal and Cornish-Bowden (1974).

Inactivation of E_I by DEP. Incubation of E_I with excess DEP (0.2−0.8 mM) in 50 mM potassium phosphate buffer (pH 6.0) at 25 °C resulted in a time dependent loss of enzyme activity and a concomitant increase in absorbance at 240 nm (A_{240} for E_I at 1 mg/mL is 1.09). The kinetic analysis of this process showed that the inactivation followed pseudo-first-order kinetics (Figure 2). As expected, when the concentration of DEP was increased, so did k_{obs}. However, a plot of pseudo-first-order rate constants for inactivation (k_{obs}) versus DEP concentration gave a concave line revealing a complex kinetics phenomenon. Since incubation was conducted with excess of inhibitor, the nonlinear plot is not a result of deficiency of DEP over the target enzyme. Thus,

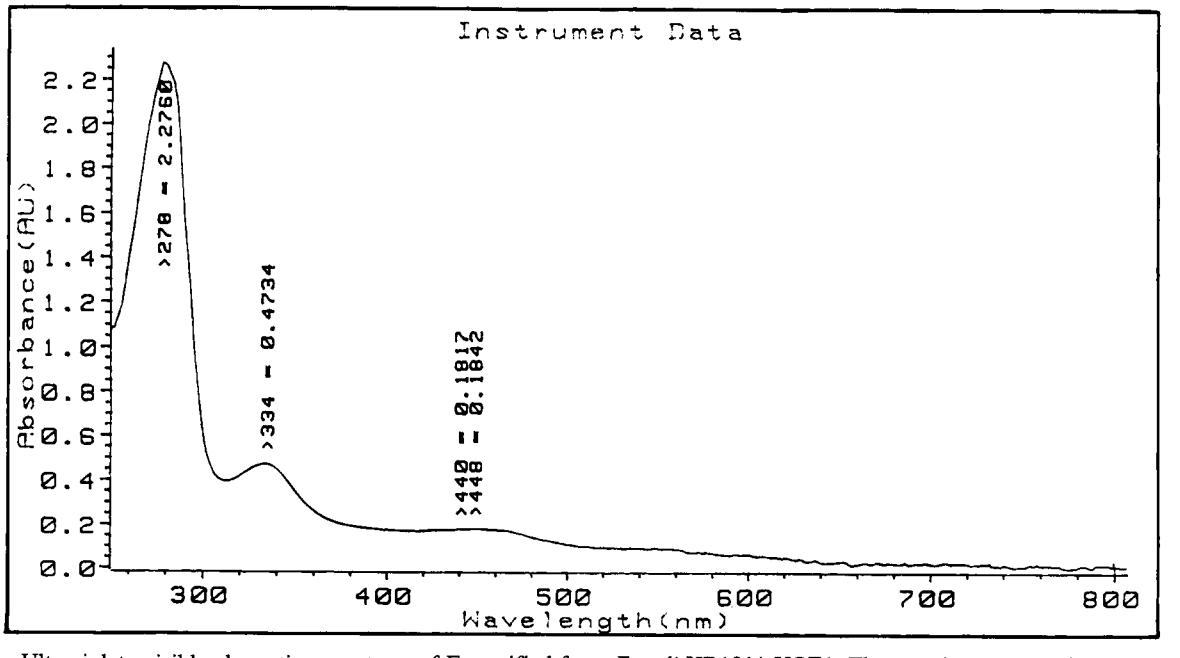


FIGURE 1: Ultraviolet−visible absorption spectrum of E_I purified from *E. coli* HB101/pYOE1. The protein concentration was 46 μM (in subunits) in 100 mM potassium phosphate buffer, pH 7.3.

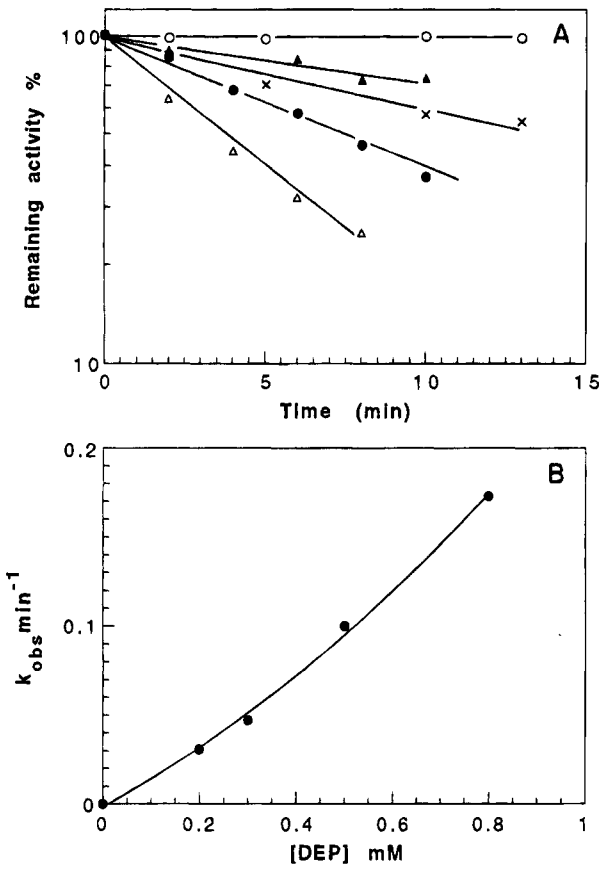


FIGURE 2: Time dependent inactivation of E_I by diethyl pyrocarbonate. (A) The enzyme (15.4 μM in subunits) was incubated at 25 °C in 0.1 mL of 50 mM potassium phosphate buffer, pH 6.0, with (△) 0.8, (●) 0.5, (×) 0.3, (▲) 0.2, and (○) 0 mM DEP. At the indicated incubation times, aliquots (10 μL) were withdrawn to assay the remaining enzyme activity. (B) Plot of apparent first-order rate constants for inactivation (k_{obs}) at various concentrations of DEP.

it may partially be ascribed to the variance of DEP concentration during incubation resulting from competing hydrolysis of DEP. From the plot shown in Figure 2B, a second-order rate constant of $k_{inact} = 0.14$ mM^{-1} min^{-1} at low DEP concentration was estimated from the linear portion of the curve. This rate constant is faster than those found for the modification of lysine, cysteine, or tyrosine residues (Holbrook & Ingram, 1973; Wells, 1973).

Determination of Numbers of DEP-Sensitive Histidine Residues in E_I. The difference absorbance spectra of E_I after incubation with DEP for varying lengths of time are shown in Figure 3. The absorbance difference reached a maximum at 240 nm after 60 min which is characteristic for *N*-carbethoxylation of histidine residues (Miles, 1977; Avaeva & Krasnova, 1975). Since a large decrease in absorbance at 280 nm ($\epsilon_{278} = 13\,000$ M^{-1} cm^{-1}) (Muhlrad et al., 1967; Burnstein et al., 1974) is expected for the formation of *O*-carbethoxytyrosine, the absence of significant spectral change around 280 nm during the incubation disfavored the modification of tyrosine residues by DEP as a possible cause for inactivation. On the basis of $\epsilon_{240} = 3200$ M^{-1} cm^{-1}, the concentration of *N*-carbethoxyhistidine formed at the final stage of modification was calculated, from which the number of histidinyl residues being modified per mole of E_I monomer was determined to be five (Figure 4). It should be pointed out that each E_I monomer contains eight histidine residues.

Substrate Protection against DEP Inactivation of E_I. As illustrated in Figure 5, coincubation of E_I with **2** at 0.2 mM concentration (10 × K_M) afforded nearly 95% protection against DEP-induced inactivation. Interestingly, the number of modified histidine residues per E_I monomer, calculated from the difference spectra of the wild-type enzyme incubated with substrate and DEP, was found to be only four (Figure 4). The fact that one less histidine was modified and the modified enzyme was still active strongly suggested the presence of a single histidine residue in or proximal to the active site of E_I. This histidine must be immune to DEP attack under substrate protection, and modification of which by DEP leads to total inactivation of E_I.

Specificity of Modification and Effect of Hydroxylamine Treatment on DEP-Inactivated E_I. Treatment of DEP-inactivated E_I with NH_2OH led to a rapid quenching of the absorption at 240 nm and restored a great portion of E_I's original activity. As shown in Figure 6B, the activity of E_I was regenerated from almost nil to more than 80% within 40 min. Since decarbethoxylation of *O*-(ethoxyformyl)-tyrosyl derivatives is known to occur at a much slower rate (Melchoir & Fahrney, 1970), such facile restoration is irreconcilable with the modification of tyrosine residues in E_I by DEP and thus further substantiates our early conclusion. Moreover, modification of cysteines and primary

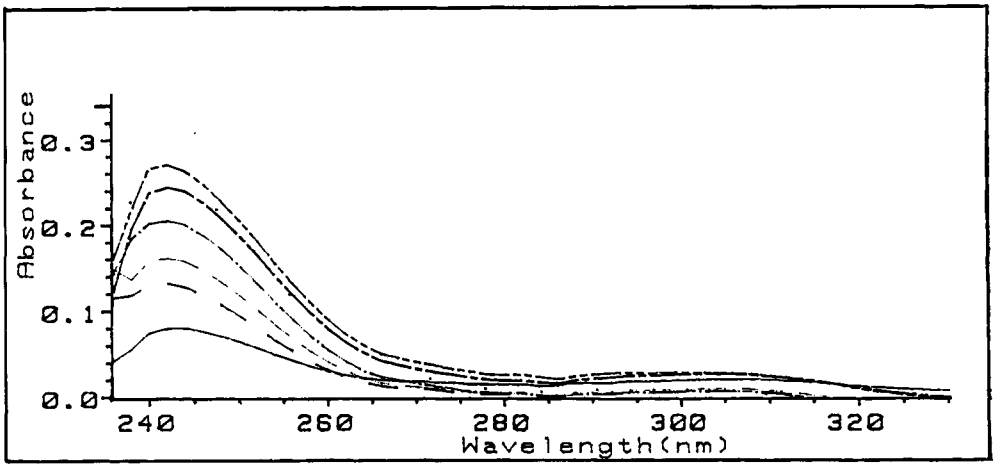


FIGURE 3: UV difference spectra showing the effect of time of incubation of E_I with diethyl pyrocarbonate. In this experiment, E_I (16.2 μM in subunits) was incubated with DEP (1 mM) in 50 mM potassium phosphate buffer (pH 6.0) at 25 °C. The reaction was followed spectrophotometrically at 240 nm. The recording times are 1, 3, 5, 10, 20, and 60 min.

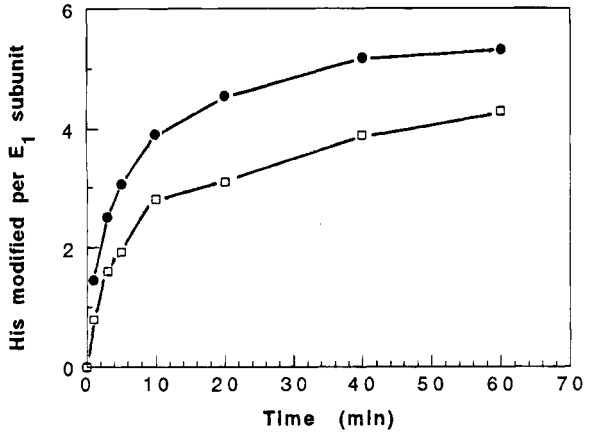


FIGURE 4: Effect of preincubation of substrate **2** on the modification of E_I by DEP. E_I (15.4 μM in subunits) was incubated at 25 °C in 50 mM potassium phosphate buffer, pH 6.0, in the presence (□) and/or absence (●) of substrate **2** (0.2 mM) for 20 min followed by treatment with 1 mM DEP. The change of absorption at 240 nm was monitored. The concentration of *N*-carbethoxyhistidine formed was determined on the basis of the absorbance at 240 nm (ϵ_{240} = 3200 M^{-1} cm^{-1}).

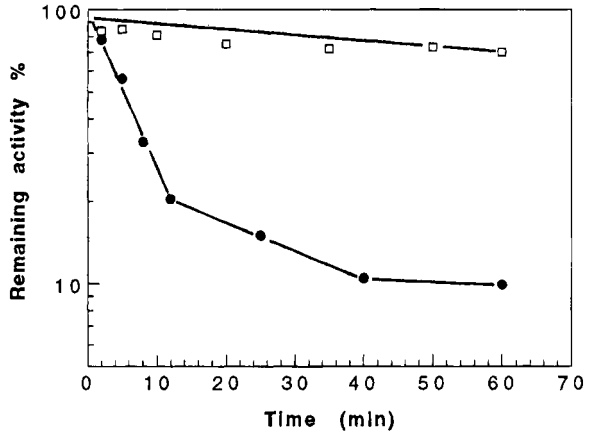


FIGURE 5: Effect of coincubation of substrate **2** on the inactivation of E_I by DEP. E_I (15.4 μM in subunits) was incubated at 25 °C in 0.1 mL of 50 mM potassium phosphate buffer, pH 6.0, with substrate **2** (0.2 mM) for 20 min followed by treatment with 0.5 mM DEP. At different time intervals, an aliquot (10 μL) was withdrawn to assay the remaining enzyme activity. An identical repeat, omitting the coincubation with substrate, was performed as the control [(●) no substrate and (□) with substrate].

amines by DEP and di-*N*-carbethoxylation of histidines can not be reversed by hydroxylamine treatment (Miles, 1977). Thus, the fact that the catalytic capability of DEP inactivated E_I can be restored by hydroxylamine provided strong evidence establishing that DEP-modification of E_I is indeed histidine specific. The incomplete recovery of activity may be due to the irreversible denaturation of the enzyme by hydroxylamine and is consistent with similar difficulties in restoring enzyme activities reported by other workers (Gao & Fonda, 1994; Deka et al., 1992).

Construction and Purification of H220N Mutant. Alignment of the E_I sequence with several representative PLP/PMP dependent enzymes revealed the substitution of the highly conserved active-site lysine of the latter enzymes by a histidine at position 220 in E_I (Figure 7). It was then speculated that this H220 could be the essential histidine residue and site-directed mutagenesis converting this histidine to an amino acid residue which is incapable of abstracting protons would impair E_I. Since asparagine which is sterically less bulky than histidine can not function as a general

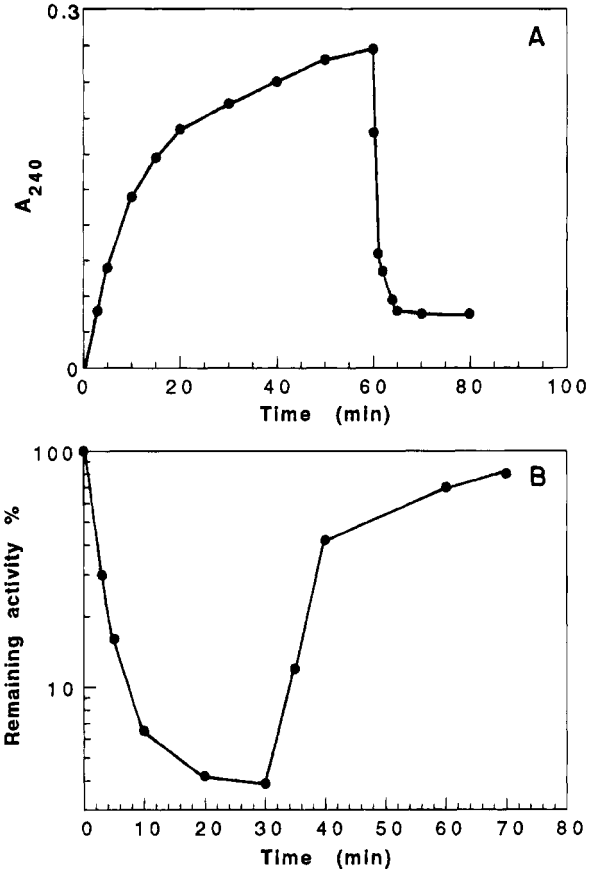


FIGURE 6: Effect of hydroxylamine on DEP-inactivated E_I. (A) After 60 min of incubation of the enzyme (16.2 μM in subunits) with DEP (1 mM) in potassium phosphate buffer (pH 6.0) at 25 °C, NH_2OH was added to a final concentration of 0.2 M. The change of absorption at 240 nm was monitored. (B) Restoration of activity of DEP-treated E_I with NH_2OH. E_I (16.2 μM in subunits) was incubated with DEP (1 mM) in potassium phosphate buffer (pH 6.0) for 30 min at 25 °C. The unreacted DEP was quenched with imidazole (10 mM), after which the sample was reacted with 60 mM NH_2OH for 40 min. An aliquot (10 μL) of the reaction mixture was withdrawn at different time intervals and assayed for E_I reactivity.

base, and its codon differs by only one base from that of histidine, it is a conservative substituent for histidine. Thus, the mutant H220N was constructed using the well-established phosphothioate method, and the mutated gene was sequenced to confirm the presence of the desired mutation. Expression and purification of E_I H220N was achieved as for the wild-type enzyme.

Characterization of H220N Mutant Protein. The electronic absorption spectrum of the mutant protein is indistinguishable from that of the wild-type E_I (data not shown). In conjunction with the results of Fe and S^{2-} quantitation which revealed 0.9 equiv of Fe and 1.2 equiv of S^{2-} per protein subunit, these data confirmed the presence of an intact metal cluster in the mutant enzyme. The conformational integrity of the mutant protein was also probed by circular dichroism spectroscopy. Since the CD spectra of the wild-type E_I and of the H220N mutant protein were almost superimposable (data not shown), the H220N mutation evidently did not induce any gross conformational change of the enzyme. It should be noted that H220N mutant protein still binds 1 equiv of PMP/monomer. This result is important since it nicely demonstrated that the point mutation of an active-site residue in E_I did not impair its PMP binding. However, the activity of the mutant protein determined by

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                145
      aspAtec  DFDALINSLN EAQAGDVVLF HGCCHNPTGI DPTLEQWQTL AQLSVEKGWL
      AcornAT  HAVKAVMDDH TC....AVVV EPIQGEGGVT AATPEFLQGL RELCDQHQAL
          dgd  DYAFDLIDRQ SSGNLAAFIA EPILSSGGII ELPDGYMAAL KRKCEARGML
         ascC  NVNASLIEAA VSDKTKAIMI AHTLGNLF.. ..D...LAEV RRVADKYNLW
     yrf7salty NIDASLIEAA VTEKSKAIMI AHTLGNAF.. ..N...LSEV RRIADKYNLW
         dnrj  LMDTGRLRSV IGPRTRCLLP VHLYGQSV.. ..D...MTPV LELAAEHDLK
        erycl  .LDPALVEQA ITPRTAAILP VHLYGHPA.. ..D...LDAL RAIADRHGLA
         tylb  LLDPDRLEAA LTPRTRAVMP VHLYGHPV.. ..D...LDPV GAFAEPHGLA
         degt  NIDPAQVEAA VTEKTKAIIP VHLYGQMA.. ..D...MEAI AAIAKRHGLV
       ipq65d  NIDPVKLEAA VTPRTKAVVP VHFGGQSC.. ..D...MDAI LAVAQNHGLF
         lmbs  TFDPDQVKSL ITERTKAVVP VHLFGRMA.. ..A...MEPL RELCDSHGLT
         prg1  GLDPKALEAA ITENTRVVTV VHQWGHPC.. ..D...MDAI LGVAERYGLR
   perosamine  QVSVEDVKRK INKKTKAVMA VHIYGQAC.. ..D...IQSL RDLCDEHGLY
         mosb  NISPTALAAA ITPDVKAIIV VHMHGISC.. ..D...MDEI ICHAREQGIA
         yifi  NIDETLIEAA ITDKTRVIVP VHYAGVAC.. ..E...MDTI MALAKKHNLF
        serATh TLQE..VEEG LAQHKPVLLF LTHGESSTGV LQP...LDGF GELCHRYKCL
         shmt  DYAD..LEKQ AKEHKPKMII GGFSAYSGVV ..D...WAKM REIADSIGAY

                188 •
      aspAtec  PLFDFAYQGF ARGLEE.DAE GLRAFAAMHK ELIVASSYSK NFGLYNERVG
      AcornAT  LVFDEVQCGM GRTGD..... ..LFAYMHYA LAPDILTSAK ALGGGFP...
          dgd  LILDEAQTGV GRTGT..... ..MFACQRDG VTPDILTLSK TLGAGLP...
         ascC  LIEDCCDALG STYDG..... ..KMAGTFGD IGTVSFYPAH HIT.MGE...
     yrf7salty LIEDCCDALG TTYEG..... ..QMVGTFGD IGTVSFYPAH HIT.MGE...
         dnrj  VLEDCAQAHG ARRHG..... ..RLVGTQGH AAAFSFYPTK VLGAYGD...
        erycl  LVEDVAQAVG ARHRG..... ..HRVGAGSN AAAFSFYPGK NLGALGD...
         tylb  VVEDAAQA.T ARYRG..... ..RRIGSG.H RTAFSFYPGK NLGALGD...
         degt  VIEDAAQAIG AKYNG..... ..KCVGELGT AATYSFFPTK NLGAYGD...
       ipq65d  VLEDAAHAVY TTYKQ..... ..RMIGSIGD ATAFSFYATK NLAT.GE...
         lmbs  LLEDAAHTLP ARDGD..... ..AVAGRAGD ASAFSFFATK PITT.AE...
         prg1  VLEDCSHAHG SRYKG..... ..KPVGTFGD AAVFSLQANK AVYA.GE...
   perosamine  LIEDCAEAIG TAVNG..... ..KKVGTFGD VSTFSFFGNK TITS.GE...
         mosb  VIEDCAQAHG ALYKG..... ..QHVGLLSD IGCFSMQKSK HLSA.GD...
         yifi  VVEDAAQGVM STYKG..... ..RALGTIGH IGCFSFHETK NYTAGGE...
        serATh LLVD..SVAS LGGTP..... ..LYMDR.QG IDILYSGSQK ALNAPPG...
         shmt  LFVDMAHVAG LVAAG..... ..VYPNPVPH AHVVTTTTHK TLAGPRG...

                371                                          •
      aspAtec  MRQLFVNTLQ EKGANRDFSF IIKQNGMFSF SGLTKEQVLR LREEFGVYAV
      AcornAT  LKPQYKGR.. ........AR DFLYAGAEAG VMVLNAGPDV MRFAPSLVVE
          dgd  IVKDRRTKEP ADGLGAKITR ECMNLGLSMN IVQLPGMGGV FRIAPPLTVS
         ascC  CADF.IELPE ATENSDPSWF GF........ PITLKEDSGV SRIDLVKFLD
     yrf7salty CTEF.LELPE ATEKSDPSWF GF........ PITLKETSGV NRVELVKFLD
         dnrj  LDGLVLPT.. IAEGNDHVYY VY........ VV.....RHP ERDRILEALT
        erycl  VPGITLPETH PWADS..AWH LF........ VL.....RCE NRDHLQRHLT
         tylb  LPGVTVPEGR V.AEP..VWH QY........ VL.....RSP YRDRLRRRLA
         degt  AVGDLVVTPK EVDGRYHVFH QY........ TI.....RAP KRDELQAFLK
       ipq65d  IPGLITPFVH .DDGRHAWHL YV........ LQVDEKKAGV TRSEMITALK
         lmbs  LDRLELPAAD TATNRSSWYL FP........ VRVHGHRRDA FRQRL.....
         prg1  .......... .......... .......... .......... ..........
   perosamine  LP...MQVHK ESNGTFHSYW LT........ SIILDQEFEV HRDGLMTFLE
         mosb  IP..FFKITR SHVDRTHVWH KIRVGIDYAA VDYFGRSMAE IRRGLRNSLA
         yifi  ......AADR INQQRLALWQ NY.....YDA LRLWRKPGVS SCRRFPMAAC
        serATh LGLQLFVKDP ALRL...... .PTVTTVAVP AGYDWRDIV. ...SYVIDHF
         shmt  RGYKVVSGGT DNHLFLVDLV DKNLTGKEAD AALGRANITV NKNSVPNDPK
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FIGURE 7: Segments of aligned amino acid sequences of E_1 and several representative PLP/PMP dependent enzymes. The abbreviations of the sources of the enzymes are aspATec, aspartate aminotransferase from *E. coli* (Kuramitsu et al., 1985); AcornAT, acetylornithine aminotransferase (Heimberg et al., 1990); dgd, dialkylglycine decarboxylase (Keller et al., 1990); *asc*C, E_1 from *Y. pseudotuberculosis* (Thorson et al., 1994); yrf7salty (*rfb*H), E_1 equivalent from *Salmonella typhimurium* (Jiang et al., 1991); dnrj, *asc*C homolog from daunorubicin cluster (*Streptomyces peucetius*) (Stutzman-Engwall et al., 1992); eryc1, *asc*C homolog from erythromycin cluster (*Saccharopolyspora erythraea*) (Dhillon et al., 1989); tylb, *asc*C homolog from tylosin cluster (*Streptomyces fradiae*) (Merson-Davies & Cundliffe, 1994); degt, *asc*C homolog from *Bacillus stearothermophilus* (Takagi et al., 1990); ipq65d, *asc*C homolog from *Bacillus subtilis* genomic region 325–333 (Glaser et al., 1993); lmbs, *asc*C homolog from lincomycin cluster (*Streptomyces lincolnensis*) (Peschke, U., Schmidt, H., Zhang, H. Z., & Piepersberg, W., unpublished results); prg1, *asc*C homolog from puromycin cluster (*Streptomyces anylatus*) (Lacalle et al., 1992); perosamine, perosamine synthetase from *Vibrio cholerae* (Manning, P. A., unpublished results); mosb, rhizopine biosynthetic gene from *Rhizobium meliloti* (Murphy et al., 1993); yifi, *asc*C homolog from *E. coli* (Daniels et al., 1992); serATh, serine aminotransferase from human (Takada et al., 1990); and shmt, serine hydroxymethyltransferase (Chan & Bingham, 1991). The numbering of residues that corresponds to the sequence of *asc*C (E_1) is given at the top. The two segments shown contain residues from 145 to 227 and 371 to 411. The four highly conserved residues are marked by dots.

the coupled assay was found to be almost completely abolished (2.5 U/mg, <4% of the wild-type activity). Owing to the intricacy and relatively high background activity of the coupled assay, it was difficult to determine the residual activity of the mutant protein more accurately. Nevertheless, following an analogous approach described earlier for the wild-type enzyme, four instead of five histidine residues per mole of E_I subunit were found to be *N*-carbethoxylated at the final stage of modification by DEP (data not shown). This finding provided additional evidence confirming the invariance of the conformation of the mutant protein. Furthermore, the extent of enzyme-catalyzed solvent−hydrogen exchange at C′-4 of PMP was 3.5−4 times lower for H220N mutant protein as compared to the wild-type E_I.

Construction, Purification, and Characterization of H221N Mutant Protein. As shown in Figure 7, immediately adjacent to His-220 is another histidine residue (H221) which is also able to serve as an active-site base. In order to test the possible involvement of His-221 in E_I catalysis, the H221N mutant was constructed and the mutant protein purified. It was found that the mutant protein possessed the same characteristic features as the wild-type enzyme and, more importantly, retained nearly 85% of the wild-type activity.

DISCUSSION

The use of DEP to modify histidine residues is well-known and has been applied to our study to further elucidate the mechanism of E_I catalysis. Although DEP is generally considered to be a histidine specific reagent, other nucleophilic amino acid residues may also be modified under the incubation conditions. However, *O*-carbethoxylation of tyrosyl residues leads to the reduction of absorbance at 278 nm (Muhlrad et al., 1967; Burnstein et al., 1974), while modification of serine residues is reversible in neutral aqueous solution (Miles, 1977). Since both effects were not detected in the DEP-inactivated E_I, inactivation of E_I by DEP appeared to be attributable solely to the modification of histidine residues. The nearly complete restoration of E_I activity, upon treatment of inactivated enzyme with hydroxylamine, further ruled out modification of cysteines and di-*N*-carbethoxylation of histidines as possible causes of inactivation (Miles, 1977). The fact that coincubation of E_I with its substrate, **2**, gave almost total protection against DEP inactivation and that one less histidine residue was modified in the presence of substrate **2** strongly suggested that inactivation is due to the modification of only one reactive histidine residue which resides in or near the active site of E_I and is critical for E_I's activity. Although differential peptide mapping and incubation of [^{14}C]DEP (Igarashi et al., 1985) with E_I, in the presence and absence of substrate, followed by tryptic digestion and sequence analysis should allow the identification of this essential histidine residue, the practical concern involving the maximal half-life of 55 h of *N*-carbethoxyhistidine derivatives (Badet-Denisot & Badet, 1992) had prompted us to consider site-directed mutagenesis as an alternative approach to pinpoint the identity of this histidine residue. Since it is possible that, upon binding with substrate **2**, E_I may have undergone a conformational change which consequently reduces the reactivity of a reactive histidine, such ambiguity associated with substrate protection could also be clarified by performing the site-directed mutation of this histidine and examining the effect on E_I catalysis.

Inspection of the translated *asc*C gene sequence revealed the presence of eight histidine residues per E_I monomer. Since more than 150 amino acid sequences of vitamin B_6 dependent enzymes are known, the wealth of sequence information has made comprehensive alignment on the basis of sequence homology, hydropathy patterns, and predicted secondary structures an appealing means to gain insights into the active-site segments of this class of enzymes. Recently, Mehta et al. (1993) have compared 51 sequences of 14 different PLP/PMP dependent aminotransferases and found that they can be divided into four subgroups and, more importantly, only four amino acid residues are invariant in all 51 sequences. These four conserved residues, numbered according to the numbering of aspartate aminotransferase, are Gly-197, Asp/Glu-222, Lys-258, and Arg-386. On the basis of the crystal structure of aspartate aminotransferase (Kirsch et al., 1984), the specific roles of these residues have been assigned as Gly-197 participating in turn 194−197 located at domain interface, Asp/Glu-222 interacting with N-1 of pyridoxal ring, Lys-258 forming a Schiff base with PLP, and Arg-386 constituting a salt bridge with α-carboxylate group of substrate. Since these residues may be considered the fingerprints of this class of enzymes, searching for such characteristics via sequence alignment holds promise for predicting the regions encompassing the active site of a PLP/PMP dependent enzyme.

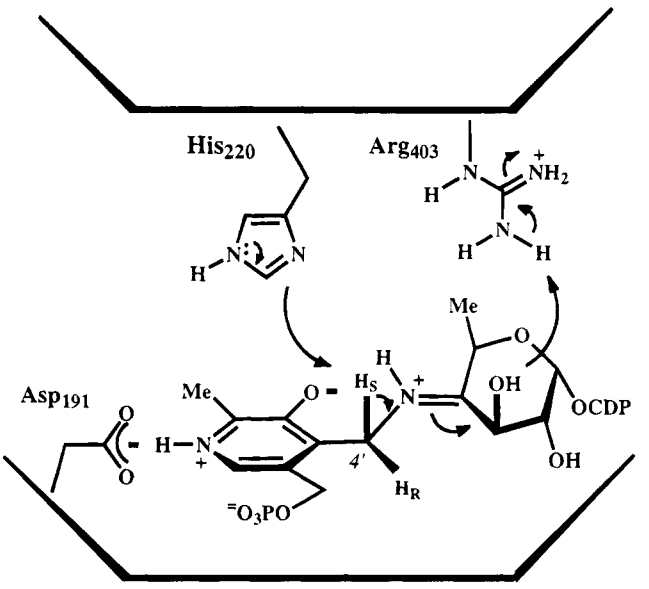
Adopting a similar approach, we had aligned the translated amino acid sequence of E_I with several representative aminotransferases chosen from different subgroups assigned by Mehta et al. (1993). Despite low homology among these sequences, it was noted that the invariant glycine, aspartate, and arginine are conserved in the translated *asc*C sequence (G169, D191, and R403). While not all of the sequences listed in Figure 7 showed perfect alignment, in most cases, only a minor adjustment would allow the anticipated conserved residues to be aligned with those present in the sequence of other aminotransferases. Interestingly, a histidine residue (H220) in E_I was found at a locus equivalent to the active-site lysine (K258) of aspartate aminotransferase. This finding is intriguing and insinuates a pivotal role of His-220. It should be noted that the same conclusion was also reached in a recent report by Pascarella and Bossa (1994) in which a multiple sequence alignment among aspartate aminotransferase, dialkylglycine decarboxylase, and serine hydroxymethyltransferase was shown to be useful to detect distantly related PLP dependent enzyme. When this alignment was applied to analyze the translated *asc*C sequence and its homologs (Figure 7), it was found that histidine substitution of the active-site lysine occurs in E_I and appears to be a phenomenon only in the PMP dependent enzymes (Pascarella & Bossa, 1994). It is worth mentioning that unlike other vitamin B_6 dependent enzymes in which the coenzyme, existing in the aldehyde form, forms a Schiff base with a lysine residue in the active sites, the PMP coenzyme of E_I is not covalently bound and thus can be partially depleted if the purification is not conducted expeditiously. The substitution of this conserved active-site lysine with a histidine residue in E_I which is incapable of covalently anchoring the PMP coenzyme in E_I's active site correlated nicely with the observed properties of E_I.

It is well known that the active-site lysine residue not only participates in Schiff base formation with the PLP coenzyme in the active site, the ϵ-amino group of the same lysine residue, with few exceptions (Schirch et al., 1993), also

serves as a base catalyzing the deprotonation from the substrate–coenzyme aldimine adduct to initiate the catalysis. This was clearly demonstrated in aspartate aminotransferase by site-directed mutagenesis substituting the active-site lysine with a histidine. While the mutated protein retains the proper fold and the capacity of binding PLP/PMP, the catalytic activity was significantly reduced compared to that of the wild-type enzyme (Fukaki et al., 1990; Ziak et al., 1990). Since the activity of K258H mutant of aspartate aminotransferase is still measurable, the substituted histidine residue is evidently an acceptable and competent replacement. It is thus not too surprising that a histidine residue, in lieu of the active-site lysine of a PLP/PMP-linked enzyme, may be nature's choice as the active-site base in a PMP dependent catalyst. In order to test whether His-220 plays an essential role in E_1 catalysis, H220N mutant was constructed and the encoding protein overexpressed. Spectral analysis and iron–sulfur quantitation revealed that the highly purified mutant protein exhibited nearly identical physical characteristics as the wild-type E_1. The result that only one less histidine residue was modified upon treatment of H220N-mutated protein with DEP was compatible with that found with substrate-protected wild-type E_1. This observation not only confirmed that His-220 is indeed an active-site residue but also substantiated that no gross conformational change was induced by such a single-point mutation. Although it was technically difficult to obtain a more precise measurement of the residual activity and acquire kinetic parameters of this mutant protein, there was no doubt that the H220N mutant protein had lost most of its catalytic activity.

Whether this histidine residue truly functions as an active-site base in E_1 was corroborated by examining its capability of catalyzing the solvent hydrogen exchange at the C-4′ position of the PMP coenzyme. Since E_1-mediated dehydration–rehydration is a reversible process and initiated by a C-4′ deprotonation of the PMP–substrate complex (Weigel et al., 1992b), if the reaction is run in tritiated buffer, tritium is thus expected to be incorporated into C-4′ of the PMP–glucoseen complex **4** during rehydration. As predicted, the PMP coenzyme recovered from the incubation of wild-type E_1 with its substrate was tritium labeled. More importantly, the level of tritium incorporation was significantly lower when the incubation was performed with the H220N mutant protein. Clearly, mutation of the active-site histidine has impaired E_1's capability to abstract the C-4′ proton and consequently diminished the extent of tritium exchange at that locus. The magnitude of reduction was significant; however, the extent of exchange was higher than that expected for an inactive protein. It is possible that the juxtaposition of the PMP–substrate complex in the active site of mutant protein may still permit the C-4′ deprotonation mediated by an adjacent base (e.g., His-221), but the improper alignment may have prevented the hydrogen bonding with a general acid (Arg-403) and thus impeded the elimination of the C-3 hydroxyl group. It should be pointed out that the H221N mutant retains nearly 85% of the wild-type activity, and thus His-221 can not be the base essential for normal catalysis. However, its immediate proximity to His-220 may allow it a limited role under demanding conditions. Nevertheless, the above results furnished compelling evidence important for establishing the role of His-220 as the active-site base pivotal for E_1 catalysis. Since a single base located on one face of the π-system catalyzing reactions in a suprafacial mode can benefit

Scheme 2



catalysis by limiting the number of potentially rate-limiting diffusion-controlled steps (Palcic & Floss, 1986), the deprotonation–reprotonation of E_1 catalysis is likely to be mediated by the same histidine residue. In light of the fact that the stereochemistry of E_1-mediated deprotonation–reprotonation is *pro-S* specific (Weigel et al., 1992b), His-220 acting as the active-site base must be situated on the *si* face of the cofactor–substrate complex (Scheme 2).

In summary, it is clear that the E_1-catalyzed reaction represents a unique PMP dependent catalysis involving a glucoseen radical intermediate in the active site of E_1 proposed during the coupled reduction by E_3 (Scheme 1). The possible intermediacy of a $\Delta^{3,4}$-glucoseen-PMP radical in this deoxygenation process has precedent since a pyridoxal phosphate-stabilized aziridine radical has been suggested as the central intermediate in the reaction catalyzed by lysine 2,3-aminomutase (Petrovich et al., 1991; Ballinger et al., 1992). However, early mechanistic and stereochemical analysis had demonstrated that E_1 still behaves as a normal coenzyme B_6 dependent catalyst. This conclusion is consistent with the results gathered from the sequence alignment described above. The preservation of several fingerprint residues characteristic for PLP/PMP dependent enzymes in E_1 not only provided evidence necessary to identify elementary segments constituting the active site but also lent credence to Dunathan's hypothesis that this class of enzymes, regardless of its catalytic diversity, evolved from a common progenitor (Dunathan, 1971). As shown in Scheme 2, Asp-191 is expected to form a salt bridge/hydrogen bond with N-1 of the PMP cofactor stabilizing the required positive charge during catalysis. Arg-403 of E_1, whose equivalent (R386) in aspartate aminotransferase binds with α-carboxylate of the substrate, may be involved in hydrogen bonding with the C-3 hydroxyl group of **3** and play a more direct role in E_1-catalyzed C–O bond cleavage. Since the displacement of 3-OH by a solvent hydrogen proceeds with retention of configuration (Pieper et al., 1995), Arg-403 is thus expected to be situated on the *si* face of the cofactor–substrate complex (Scheme 2). The His-220 is most likely the active-site base which abstracts the C-4′ proton from the PMP–substrate adduct **3** and initiates the catalysis. Although there are many reports of the use of DEP to assess

the presence of reactive histidine residues in enzymes, very few have the identity of the DEP-sensitive histidine residues elucidated (Hegyi et al., 1977; Igarash et al., 1985; Ko et al., 1991). Thus, our results represent an interesting example in which the active-site histidine residue modified by DEP was deduced from sequence alignment and site-directed mutagenesis. It is worth mentioning that we have recently discovered an evolutionary link between E_1 (*asc*C) and several of the genes believed to participate as PLP/PMP dependent aminotransferases in the biosynthesis of deoxy amino sugars (Thorson et al., 1993). Sequence alignment revealed that they all retain the active-site lysine preserved in other PLP/PMP dependent enzymes (Pascarella & Bossa, 1994), with E_1 and its *Salmonella* counterpart (*rfb*H) (Jiang et al., 1991) as the two only exceptions. Thus, it is likely that nature's seemingly simple substitution of an active-site lysine with a histidine residue may have allowed a normal coenzyme B_6 dependent aminotransferase to be transformed into a unique PMP dependent catalyst that is no longer an aminotransferase but instead a dehydrase.

ACKNOWLEDGMENT

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