Identification of an Unusual [2Fe-2S]-Binding Motif in the CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose-3-dehydrase from *Yersinia pseudotuberculosis*: Implication for C-3 Deoxygenation in the Biosynthesis of 3,6-Dideoxyhexoses†

Gautam Agnihotri,‡ Yung-nan Liu, Beth M. Paschal,§ and Hung-wen Liu*

Division of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry and Biochemistry, University of Texas, Austin, Texas 78712

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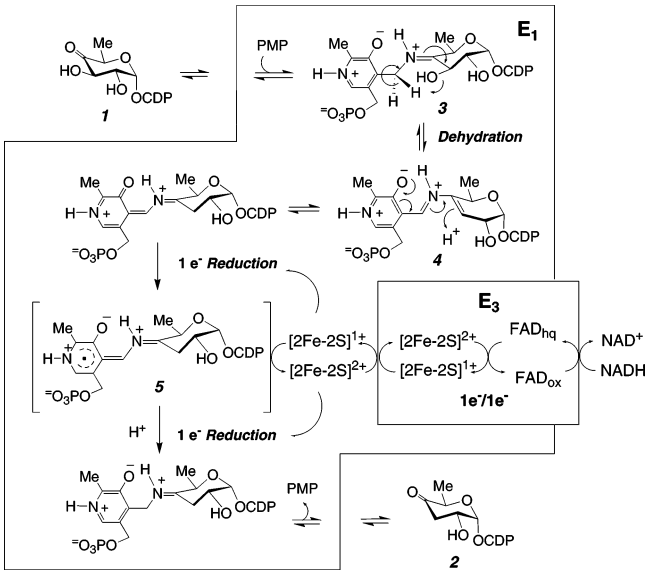
ABSTRACT: CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1) catalyzes the C-3 deoxygenation in the biosynthesis of 3,6-dideoxyhexoses in *Yersinia pseudotuberculosis*. E_1 is a pyridoxamine 5′-phosphate (PMP)-dependent enzyme that also contains a [2Fe-2S] center. This iron−sulfur cluster is catalytically essential, since removal of the [2Fe-2S] center leads to inactive enzyme. To identify the [2Fe-2S] core in E_1 and to study the effect of impairing the iron−sulfur cluster on the activity of E_1, a series of E_1 cysteine mutants were constructed and their catalytic properties were characterized. Our results show that E_1 displays a cluster-binding motif (**C**-X_{57}-**C**-X_1-**C**-X_7-**C**) that has not been observed previously for [2Fe-2S] proteins. The presence of such an unusual iron−sulfur cluster in E_1, along with the replacement of the active site lysine by a histidine residue (H220), reflects a distinct evolutionary path for this enzyme. The cysteine residues (C193, C251, C253, C261) implicated in the binding of the iron−sulfur cluster in E_1 are conserved in the sequences of its homologues. It is likely that E_1 and its homologues constitute a new subclass in the family of iron−sulfur proteins, which are distinguished not only by their cluster ligation patterns but also by the chemistry used in catalyzing a simple, albeit mechanistically challenging, reaction.

The 3,6-dideoxyhexoses are found in lipopolysaccharides of a number of Gram-negative bacteria, where they have been shown to be the dominant antigenic determinants of bacterial pathogenicity and virulence (*1*, *2*). There are seven naturally occurring 3,6-dideoxyhexoses, and with the except of colitose, which is made from GDP-D-mannose, the rest are derived from CDP-D-glucose (*3*−*6*). The most intriguing reaction in the biosynthesis of CDP-3,6-dideoxyhexoses is the C-3 deoxygenation step, in which CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**1**, also known as CDP-4-keto-6-deoxy-D-glucose) is converted to CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**2**, also known as CDP-4-keto-3,6-dideoxy-D-glucose) (*3*−*6*). This C−O bond cleavage event is catalyzed by two enzymes: CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1)[1] and its reductase (E_3) (Scheme 1) (*7*).

E_1 is a dark red-brown protein that exists as a homodimer in its native state, with a subunit molecular mass of approximately 49 kDa (*8*, *9*). Fully reconstituted E_1 contains 1 equiv of PMP and a [2Fe-2S] cluster per subunit (*9*−*11*). E_3 is also a red-brown protein, but it is monomeric with a molecular mass of 36 kDa (*12*). It belongs to the flavodoxin-$NADP^+$ reductase family and contains 1 mol of FAD and a plant-type ferredoxin [2Fe-2S] center per mole of enzyme (*12*, *13*). Recent studies using recombinant E_1 and E_3 obtained by heterologous expression of the corresponding genes (*ddhC/ascC* for E_1 and *ddhD/ascD* for E_3 from *Yersinia pseudotuberculosis*) in *Escherichia coli* (*14*, *15*) have shed light on some of the details of their catalytic mechanisms.

Scheme 1



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* To whom correspondence should be addressed. Fax: (512) 471-2746. E-mail: h.w.liu@mail.utexas.edu.

‡ Current address: Assay Development, NeoGenesis, Inc., 840 Memorial Dr., Cambridge, MA 02139.

§ Current address: New England Biolabs, Inc., 32 Tozer Rd., Beverly, MA 01915.

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                  211                                                                  280
      DesI  (157) LRKVADEHGLRLYFDAAHALGCAVDGRPAGSLGDAEVFSFHATKAVNAF-EGGAVVTDDADLAARIRALH
    EryCIV  (157) LEKIAAEHQVKLFFDAAHALGCTAGGRPVGAFGNAEVFSFHATKAVTSF-EGGAIVTDDGLLADRIRAMH
     OleN2  (143) LSEVAERHGVRILEDAAQAHGAQAYGRRVGAWS-TTAFSFYPGKNLGGFGDGGAVVTDDAELAERVRLLR
      DesV  (150) LRELADRHGLHIVEDAAQAHGARYRGRRIGAGSSVAAFSFYPGKNLGCFGDGGAVVTGDPELAERLRMLR
      TylB  (159) VGAFAEPHGLAVVEDAAQAT-ARYRGRRIGSGH-RTAFSFYPGKNLGALGDGGAVVTSDPELADRLRLLR
 Gra orf23  (174) VAAIAAEHGLYLVEDN*C*DAVGSYHQGRLTGTFGDLTTTSFYPA*H*HITTG-EGGCVLTRNLALARIVEQLR
      LanQ  (175) IAQLAVDHDLFLIEDN*C*DAVGSTYDGKLTGTFGELTTVSFYPA*H*HLTMG-EGGCVLTADLALARIVESLR
      SpnQ  (203) IAEIAKEHELFLVEDN*C*DAVGSTYRGRLTGTFGDLTTVSFYPA*H*HITSG-EGGCVLTGSLELARIIESLR
      WbdK  (145) INKIIGGRDIILLEDN*C*ESMGATFNNKLAGTFGLMGTFSSFYS*H*HIATM-EGGCIVTDDEEIYHILLCIR
      ColD  (145) ITKITEGKDIFILEDN*C*ESMGARLNGKQAGTYGLMGTFSSFFS*H*HIATM-EGGCVITDDEELYHILLCIR
     Yp-E1  (177) VRRVADKYNLWLIEDC*C*DALGSTYDGKMAGTFGDIGTVSFYPA*H*HITMG-EGGAVFTQSAELKSIIESFR
 Consensus  (211) L  IADEHGLFLVEDACDALGA Y GR  GTFGDLTTFSFYPAKHIT   EGGAVVT D ELA RIRSLR
                              •                    ♦
                  281                                                                  350
      DesI  (226) NFG--------------------------FDLPGGSPAGGTNAKMSEAAAAMGLTSLDAFPEVIDRNRR
    EryCIV  (226) NFG--------------------------IAPDKLVTDVGTNGKMSECAAAMGLTSLDAFAETRVHNRL
     OleN2  (212) NYG--------------------------SREKYRHEVRATNFRLDELQAAVLRVKLAHLDAWTERRAA
      DesV  (220) NYG--------------------------SRQKYSHETKGTNSRLDEMQAAVLRIRLAHLDSWNGRRSA
      TylB  (227) NYG--------------------------AREKYRHEERGTNSRLDELQAAVLSVKLPYLDAWNTRRRE
 Gra orf23  (243) DWGRD*C*W*C*EPGKDNT*C*FKRFDYQLGTLPHGYDHKYIFTHLGYNLKATDIQAALGVSQLSRLDSFGKARRA
      LanQ  (244) DWGRD*C*W*C*EPGESDK*C*LKRFKYQMGTLPAGYDHKYIFSHVGYNLKATDLQAALGLTQLAKLDDFVEARKR
      SpnQ  (272) DWGRD*C*W*C*EPGVDNT*C*RKRFDYHLGTLPPGYDHKYTFSHVGYNLKTTDLQAALALSQLSKISAFGSARRR
      WbdK  (214) AHGWTRNLPKKNKVTGVKS--------DDQFEESFKFVLPGYNVRPLEMSGAIGIEQLKKLPRFISVRRK
      ColD  (214) AHGWTRNLPEFNHITGQKS--------IDPFEESFKFVLPGYNVRPLEMSGAIGIEQLKKLPSFIEMRRK
     Yp-E1  (246) DWGRD*C*Y*C*APGCDNT*C*KKRFGQQLGSLPFGYDHKYTYSHLGYNLKITDMQAACGLAQLEPIEEFVEKRKA
 Consensus  (281) NWG             K            YD KY FS VGYNLKLTELQAALGLTQLA LDAF ERRR
                     • •       •
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FIGURE 1: Sequence alignment of E_1 and its homologue: DesI (the C-4 aminotransferase from the methymycin/pikromycin gene cluster of *Streptomyces venezuelae*; 18% identity with E_1) (*43*), EryCIV (a putative aminotransferase from the erythromycin gene cluster of *Saccharopolyspora erythrea*; 10%) (*44*, *45*), OleN2 (a putative aminotransferase from the oleandomycin gene cluster of *Streptomyces antibioticus*; 23%) (*46*), DesV (the C-3 aminotransferase from the methymycin/pikromycin gene cluster of *Streptomyces venezuelae*; 21%) (*43*), TylB (the C-3 aminotransferase from the tylosin gene cluster of *Streptomyces fradiae*; 20%) (*48*), Gra orf23 (a putative dehydrase from the granaticin biosynthetic gene cluster of *Streptomyces violaceoruber* Tü22; 52%) (*51*), LanQ (a putative dehydrase from the landomycin biosynthetic gene cluster of *Streptomyces cyanogenus*; 53%) (*52*), SpnQ (a putative dehydrase from the spinosyn gene cluster of *Saccharopolyspora spinosa*; 49%) (*53*), WbdK (a putative dehydrase from the colitose gene cluster of *E. coli*; 27%) (*54*), ColD (the C-3 dehydrase from the colitose gene cluster of *Yersinia pseudotuberculosis* VIA; 27%) (*55*), and E_1 (the C-3 dehydrase from the ascarylose gene cluster of *Yersinia pseudotuberculosis* VIA) (*14*). The conserved cysteine residues that bind the [2Fe-2S] cluster and the catalytic histidine residue (H220) in E_1 and its homologues are bold.

It has been established that C-3 deoxygenation catalyzed by E_1 and E_3 is initiated by the formation of a Schiff base between the amino group of PMP and the C-4 keto group of substrate (**1**, Scheme 1). Subsequent abstraction of the *pro-S* 4′-hydrogen of PMP−substrate adduct (**3**) triggers the expulsion of the C-3 hydroxyl group, leading to the formation of the conjugated $\Delta^{3,4}$-glucoseen intermediate (**4**) (*16*). This C−O bond cleavage is a reversible reaction with an equilibrium favoring the reverse direction (**4** → **1**) (*16*, *17*). Hence, a two-electron reduction of intermediate **4** is necessary to drive the reaction to completion (**4** → **2**) and to regenerate the PMP coenzyme. These reducing equivalents are relayed from E_3-bound NADH to the active site of E_1 via a chain of redox-active cofactors, including FAD and the two iron−sulfur centers in E_1 and E_3 (*11*, *18*, *19*).

The involvement of iron−sulfur clusters, which are obligatory one-electron-transfer cofactors, implies that radical intermediates must be formed in the reduction of the $\Delta^{3,4}$-glucoseen intermediate (*20*). Indeed, two transient radicals could be detected by EPR spectroscopy during the E_1−E_3 catalyzed reaction (*11*, *21*). One was identified as the flavin semiquinone radical and the other, which displayed a signal centered at $g = 2.003$ with little g anisotropy, was assigned as a non-flavin organic radical (*11*). The chemical nature of this radical intermediate was elucidated by CW-EPR and pulsed electron nuclear double resonance (pulsed ENDOR) using E_1 reconstituted with isotopically labeled PMP as the catalyst in the incubation mixture (*21*). A radical directly associated with the PMP coenzyme (represented by **5** in Scheme 1) was established on the basis of the EPR results. The participation of PMP in deoxygenation is unusual, (*22*) but the direct involvement of PMP in stabilizing an unpaired electron spin in an electron-transfer reduction makes E_1 unique.

Although formation of a cofactor-based radical is unprecedented in coenzyme B_6-dependent reactions, E_1 shares several characteristics, including a modest sequence identity, with other B_6-dependent enzymes, albeit with two important distinctions (*23*). First, a highly conserved lysine that serves as an anchor for PLP in most B_6-dependent enzymes is absent in E_1. Instead, a histidine residue is present in its place at position 220 in E_1 (see Figure 1). Site-directed mutagenesis studies has shown that His220 is likely the active site base responsible for the 4′-proton abstraction (*10*). Second, E_1, is a rare example of a B_6-dependent enzyme that harbors a [2Fe-

[1] Abbreviations: CDP, cytidine 5′-diphosphate; DEAE, diethylaminoethyl; E_1, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase; E_3, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase; E_{od}, CDP-D-glucose 4,6-dehydratase; DTT, dithiothreitol; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; GABA, γ-aminobutyric acid; GC−MS, gas chromatography−mass spectrometry; NADH, β-nicotinamide adenine dinucleotide, reduced form; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMP, pyridoxamine 5′-phosphate; SDS, sodium dodecyl sulfate; UV, ultraviolet; GCG, Genetics Computer Group, Inc.

2S] cluster (*9*). As shown in Figure 1, E_1 has eight cysteines, out of which four are expected to be involved in the binding of the [2Fe-2S] cluster. However, none of the cysteine pairs in E_1 is compatible with the consensus sequence of **C**-X_2-**C**, which is generally considered to be the minimal structural motif for a [2Fe-2S] cluster (*24*, *25*). Thus, E_1 must have an unusual binding motif for the iron−sulfur center. It is conceivable that the presence of such a unique iron−sulfur cluster in E_1, along with the replacement of the active site lysine by a histidine residue, reflects a different evolutionary path for this enzyme. To identify the [2Fe-2S] binding site in E_1 and to study the effect of the iron−sulfur cluster on the activity of E_1, a series of E_1 cysteine mutants were constructed and their catalytic properties characterized. Reported in this paper are the results of this investigation as well as the implications for assigning the catalytic function and mechanism of other E_1 homologues.

EXPERIMENTAL PROCEDURES

Materials. The construction of the plasmid containing the E_1 gene (pJT18) in pUC119 have been reported elsewhere (*14*). Enzymes E_1, (*10*, *11*), E_3, (*11*, *13*), and CDP-D-glucose 4,6-dehydratase (E_{od}) (*14*) were prepared according to published procedures. Purification and reconstitution of E_1 was carried out under semianaerobic conditions as previously described (*10*, *11*). All culture media were products of Difco (Detroit, MI), and the Bradford reagent for protein quantitation was purchased from Bio-Rad (Hercules, CA). Synthetic oligonucleotides were obtained from Gibco-BRL (Grand Island, NY) and were used without further purification. All restriction endonucleases, DNA modifying enzymes, and their respective buffers were purchased from Amersham (Arlington Heights, IL), Gibco-BRL, Stratagene (La Jolla, CA), or Promega (Madison, WI). The pTrc99A expression vector was obtained from Pharmacia (Piscataway, NJ), while pUC119 DNA and the Sculptor in vitro mutagenesis kit were purchased from Amersham. The QuickChange site-directed mutagenesis kit was a product of Stratagene (La Jolla, CA). [α-^{35}S]dATP for DNA sequencing was obtained either from Amersham or DuPont NEN (Boston, MA). DNA minipreps were performed using the Wizard DNA purification kit from Promega. GeneClean DNA purification kits were purchased from BIO 101, Inc. (La Jolla, CA). DNA ladders (100 bp and 1 kb) were obtained from Gibco-BRL. All other biochemicals and chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were of the highest purity available. Sequence analysis and comparison were performed using the Genetics Computer Group (GCG) or the GeneWorks software. DEAE-Sepharose CL6B, Mono Q H/R 10/10, and Superdex S-200 H/R 10/30 used in enzyme purification are products of Pharmacia. Centricon filters were purchased from Amicon (Danvers, MA).

Site-Directed Mutagenesis and Purification of E_1 Mutants. Point mutations at C193, C251, C253, C257, and C261 in E_1 were achieved using the Sculptor in vitro mutagenesis kit. The mutagenesis primers for the C193A, C251A, C253A, C257A, and C261A mutants were 5′-TGGTTAATTGAAGACTGC**GCT**GATGCGTTGGGTTCCACC-3′; 5′-TGGGGTCGTGAT**GCT**TATTGTGCTCCAGGC-3′; 5′-TGGGGTCGTGATTGTTAT**GCT**GCTCCAGGCTGT-3′; 5′-TGTGCTCCAGGC**GCT**GACAACACATGTAAA-3′; and 5′-GGCTGTGACAACACA**GCT**AAAAGGCGTTTCGG-3′, respectively, where the sequence in bold denotes the codon change for the respective mutants. Mutant genes were cloned into pTrc99A for overexpression, and the produced mutant proteins were purified using the procedure reported for the wild-type enzyme (*10*, *11*). The C192A, C298A, and C327A mutants were prepared using the wild-type gene as the template in the QuickChange site-directed mutagenesis method (Stratagene). The C192A/193A double mutant was prepared by the same method using the C193A gene as the template. The following primers were used to introduce the point mutations, where the sequence in bold denotes the codon change for the respective mutations: C192A (forward), 5′-GGTTAATTGAAGAC**GC**CTGCGATGCGTTGG-3′; C192A (reverse), 5′-CCAACGCATCGCAG**GC**GTCTTCAATTAACC-3′; C298A (forward), 5′-GATATGCAGGCTGCC**GC**TGGTTTGGCGCAACTAG-3′; C298A (reverse), 5′-CTAGTTGCGCCAAACCA**GC**GGCAGCCTGCATATC-3′; C327A (forward), 5′-GACGCACTCCAATCT**GC**CGCTGACTTCATTGAG-3′; C327A (reverse), 5′-CTCAATGAAGTCAGCG**GC**AGATTGGAGTGCGTC-3′; C192A/193A (forward), 5′-GTGGTTAATTGAAGAC**GC**C**GCT**GATGCGTTGGGTTC-3′; and C192A/C193A (reverse), 5′-GAACCCAACGCATC**AGC**G**GC**GTCTTCAATTAACCAC-3′. The mutant proteins were purified by the same procedure described for the wild-type enzyme (*10*, *11*).

Enzyme Characterization and Activity Assay. The E_1 substrate (**1**) was prepared from CDP-glucose by the action of E_{od} as previously described (*26*). The activity of E_1 and E_1 mutants was determined by a published continuous spectrophotometric assay that monitors the consumption of NADH in the presence of E_1 (or its mutants), E_3, and the E_1 substrate (**1**) (*10*, *15*). The amount of iron associated with wild-type E_1 and the E_1 mutants was quantitated using the method of Fish (*27*). The stoichiometry of bound PMP per E_1 subunit was determined by a fluorimetric measurement of the quantity of released PMP from a denatured enzyme sample of known concentration (*10*).

Reconstitution of the Iron−Sulfur Center. The reconstitution of the iron−sulfur center of E_1 mutants was carried out according to a published procedure (*21*). Briefly, the protein samples were denatured in the presence of DTT by the addition of urea to a final concentration of 3 M. This was followed by the addition of 6-fold excess Fe^{2+} in the form of ferrous ammonium sulfate and S^{2-} in the form of Na_2S. The resulting mixture was incubated at room temperature for 2 h and the reconstituted enzyme was purified through a DEAE-Sepharose column (1 × 10 cm) that had been preequilibrated with 25 mM Tris·HCl buffer, pH 7.5. Elution was conducted using a linear gradient of 0.16−0.36 mM NaCl in the same buffer.

GC−MS Assay To Detect ^{18}O-Label Incorporation. The GC−MS assay was performed as previously described with minor modifications (*8*, *16*). Specifically, the E_1 substrate (**1**, 1.5 μmol) and PMP (0.1 nmol) in 300 μL of 25 mM Tris·HCl buffer (pH 7.5) were lyophilized and redissolved in 300 μL of [^{18}O]H_2O (95% enrichment). To this solution was added E_1 (750 μg), and the resulting mixture was incubated at 37 °C for 1.5 h. Subsequently, $NaBH_4$ (3 mg) was added. The reaction was allowed to incubate for 1 h at room temperature. Dilute HCl was then added to adjust the pH of the solution to ∼2, whereupon the solution was placed

in a boiling water bath for 10 min. The reaction was cooled and the pH was then adjusted to ~7 by the dropwise addition of dilute KOH. The resulting neutral solution was lyophilized and the solid residue was dissolved in 600 μL of 0.5 N NH_4-OH. To this solution was added 1 mL of a 1 mg/mL solution of $NaBH_4$ in DMSO. The reaction mixture was incubated at 40 °C for 1.5 h, cooled to room temperature, and carefully quenched by the dropwise addition of 100 μL of glacial acetic acid. Subsequently, 1-methylimidazole (250 μL) and 6 mL of acetic anhydride were added, and the acetylation reaction was carried out at room temperature for 1 h. The reaction mixture was then cooled in an ice bath and quenched slowly with 6 mL of methanol, followed by 10 mL of water. The resulting mixture was washed with chloroform (3 × 15 mL), and the combined organic layers were washed with water (2 × 20 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness on a rotary evaporator. The residue was dissolved in a small quantity of chloroform and subjected to GC−MS analysis.

RESULTS

Construction and Purification of E_1 Mutants. Since the binding ligands for the iron−sulfur clusters are typically cysteine thiolates, the cysteine residues in the central portion of the primary sequence of E_1, specifically those within the segment from 251 to 261 were targeted. This sequence, **C**^{251}-Y-**C**-A-P-G-**C**-D-N-T-**C**^{261}, contains four cysteines in close proximity (at positions 251, 253, 257, and 261). Cys193, which precedes these cysteines in this central fragment, was also selected. The construction of the corresponding Cys-to-Ala variants at each location was achieved using the Sculptor in vitro mutagenesis kit. The resulting mutant genes were excised from the pUC119 vector and cloned into the *Eco*RI and the *Bam*HI site of the expression vector pTrc99A. The C192A, C298A, C327A, and C192A/193A mutants were prepared at a later stage using the QuickChange site-directed mutagenesis kit. Each mutant protein was purified to homogeneity, as determined by SDS−PAGE, on the basis of the published procedure developed for the wild-type E_1 (*10*, *11*).

Quantitation of Enzyme-Bound PMP. As observed with wild-type E_1, all E_1 mutants displayed a prominent absorption maximum near 330 nm (Figure 2), an indicator of the presence of bound PMP. The amount of PMP bound to each mutant was quantitatively assayed by denaturing the enzyme to release the cofactor in solution. The presence of PMP in solution was detected using a flourimetric method, which relies on the intensity of the emitted radiation at 393 nm when the cofactor is excited using an incident radiation of wavelength 325 nm (*10*). The amount of released PMP was calculated on the basis of a calibration curve constructed using the fluorescence intensities of standard PMP solutions. As shown in Table 1, the ratios of bound PMP to enzyme concentration for these E_1 mutants varied from 0.25 to 0.68 mol/dimmer. It should be noted that PMP is bound to E_1 through noncovalent interactions so that the amount of cofactor bound to the enzyme fluctuates greatly, even among different preparations of wild-type E_1 (*7*). The observed values for the mutants are within the range normally observed for most wild type enzyme preparations. It is only by reconstitution with excess PMP that a stoichometic ratio of one PMP per E_1 monomer can be achieved. Hence, it appears

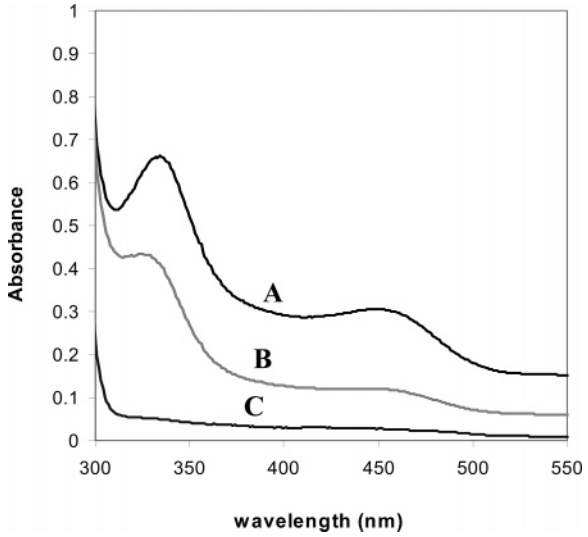


FIGURE 2: Electronic absorption spectra of (A) wild-type E_1, (B) C192A, and (C) C192A/C193A mutants at 4 mg/mL in 50 mM potassium phosphate buffer, pH 7.5.

Table 1: Biochemical and Catalytic Properties of Wild-Type E_1 and Its Mutants

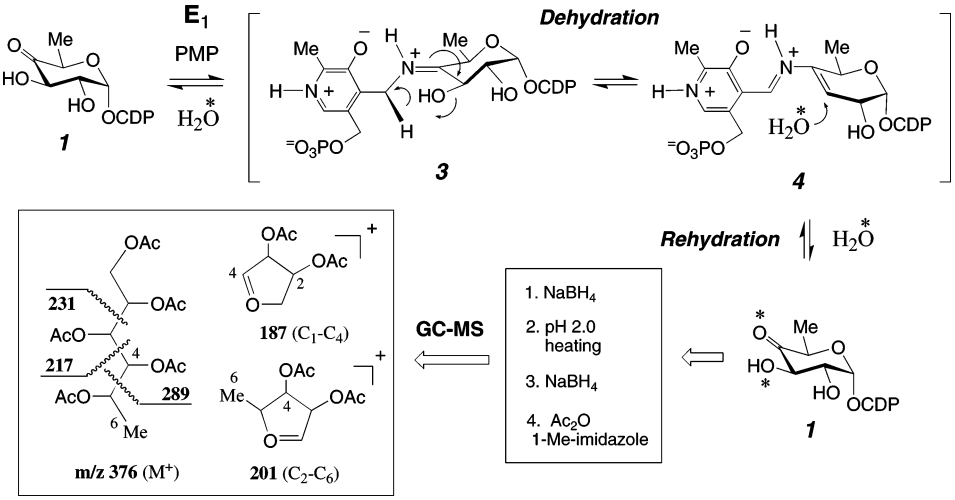
protein	relative Fe content[a]	relative PMP content[b]	relative activity[c]
wild-type	1	1	1
C192A	0.57	0.60	0.71
C193A	0.50	0.57	0.77
C251A	0.05	0.58	0.07
C253A	0.04	0.45	0.06
C257A	0.87	0.25	0.85
C261A	0.07	0.68	0.09
C298A	0.80	0.60	0.86
C327A	0.87	0.66	0.95
C192A/C193A	0.05	ND[d]	0.02

[a] The iron content was determined according to the method of Fish (*27*) as described in the Experimental Procedures. [b] The PMP content was determined by a flourometric assay as described in the Experimental Procedures. [c] The enzyme activity was determined by a coupled assay as described in the Experimental Procedures. [d] Not determined.

that the presence of the iron−sulfur cluster, or the lack thereof, does not significantly alter the ability of E_1 and E_1 mutants to bind PMP.

Quantitation of Enzyme-Bound Iron. To elucidate which cysteine residues are crucial for coordinating the iron−sulfur cluster, the iron content of each mutant was determined (*27*). This assay relies on the formation of a colored complex when the iron released from the enzyme is complexed with the water-soluble chelators ferrozine and neocuprine. The amount of released iron was quantitated using a calibration curve. As shown in Table 1, the C251A, C253A, and C261A mutants clearly lose their ability to bind iron, because the amount of iron associated with these three mutants corresponds to background levels. The C257A, C298A, and C327A mutants, on the other hand, bind iron comparably to the wild-type enzyme. Thus, Cys251, Cys253, and Cys261, but not Cys257, Cys298 and Cys327, can be assigned as ligands for the iron−sulfur cluster. This leaves Cys192 and Cys193 as the two remaining candidates for the fourth ligand of the iron−sulfur cluster. However, to our surprise, both C192A and C193A mutants retained significant ability to bind iron (Table 1). A possible explanation for this observa-

Scheme 2



tion is that while one of the two cysteines (such as Cys193) is involved in cluster coordination, the other (such as Cys192) which is immediately adjacent, can "rescue" the assembly of the cluster when the original ligand (Cys193) is not present. In accord with this hypothesis, mutation of both Cys192 and Cys193 results in a total depletion of iron in E_1. An analogous rescue has been observed earlier, where the lack of a catalytic histidine at position 220 in the H220N mutant of E_1 can be partially compensated by the adjacent histidine residue at position 221 (*10*). As discussed later, the fact that Cys193 is conserved among E_1 homologues (see Figure 1) leads us to assign Cys193 as the more likely candidate for the fourth ligand.

Reconstitution of E_1 Mutants with Iron. To conclusively establish that the cysteine side-chain thiolate at positions 193 (or 192), 251, 253, and 261 are necessary for the formation of the [2Fe-2S] cluster in E_1, the reconstitution of the corresponding E_1 mutants with iron and sulfur was attempted under denaturing conditions. As expected, the reconstitution effort did not increase the iron content of the C192A, C193A, C251A, C253A, C261A, and C192A/C193A mutants (data not shown). Evidently, the inability of these mutants to form or bind a [2Fe-2S] cluster is not due to variations in the quality of the respective preparations but an inherent property of the respective proteins that is due to the lack of a cysteinyl thiolate in each mutant.

Activity Assay of the E_1 Mutants. The catalytic activity of E_1 can be determined by a coupled assay that utilizes its partner reductase, E_3. Excess PMP is added to the assay cocktail to compensate for variations in the amount of enzyme-bound PMP. The assay to monitor the catalytic activity of E_1 can be performed in a continuous, time-dependent manner by monitoring the decrease in absorbance at 340 nm due to the consumption of NADH by E_3 during the E_3-catalyzed reduction of the $\Delta^{3,4}$-glucoseen intermediate (**4**) (*10*, *15*). As shown in Table 1, the C192A/C193A, C251A, C253A and the C261 mutants show a very low level basal activity, after correcting for background readings. In contrast, the C192A and C193A mutants exhibited much higher activities of similar magnitude. As anticipated, the activities of the C257A, C298A and C327A mutants are comparable to that of the wild-type E_1. The level of activity displayed by each mutant appears to be directly related to its iron content. These observations are consistent with the assigned roles of these cysteine residues and highlight the importance of the iron−sulfur cluster in the catalytic mechanism of E_1.

Table 2: GC−MS Analysis of PMP-Mediated Reversible Dehydration Reactions with $[^{18}O]H_2O$ (also see Scheme 2)

fragment	m/e	*m/e* (in $[^{18}O]H_2O$ + PMP + E_1)
C_1-C_4	289	291, 293
C_3-C_6	231	233, 235
C_1-C_3	217	217, 219
C_2-C_6	201	203, 205
C_1-C_4	187	189, 191

Catalysis of the First Half-Reaction by E_1 Mutants. As described above, mutants of E_1 that are deficient in the iron−sulfur cluster failed to catalyze the C-3 deoxygenation reaction in the presence of E_3. This is undoubtedly due to the loss of a key component of the electron shuttle network that transports reducing equivalents from NADH via E_3 to reduce the dehydration product **4** in the active site of E_1. However, these mutants should retain some ability to catalyze the first half-reaction because the formation of the Schiff base and the subsequent elimination of water do not require the participation of the iron−sulfur cluster. To investigate whether the E_1 mutants, including C192A, C193A, C251A, C253, and C261A, are competent in catalyzing the reversible dehydration reaction, each mutant protein was incubated in the presence of $[^{18}O]H_2O$ and substrate, and the recovered substrate was subjected to reduction and acetylation. Since the MS fragmentation patterns of the glycitol pentaacetate product are well-characterized (*28*, *29*), any ^{18}O incorporation is readily detected using a GC−MS assay (see Scheme 2) (*8*, *16*). Our results showed that the observed mass-to-charge ratio of the C_3-C_6 fragment (*m*/*e* 231) in all samples is shifted by four mass units (Table 2), an outcome resulting from the incorporation of two ^{18}O labels. One of these is found at C-4 and is incorporated by solvent oxygen exchange into the 4-keto group of the recovered substrate. The other is found at C-3 and is incorporated by reversible dehydration/rehydration due to enzyme catalysis. Similar labeling patterns were also noted for fragments C_1-C_4 (*m*/*e* 187, 289) and C_2-C_6 (*m*/*e* 201). These observations demonstrate that the

Table 3: Cluster Ligation Patterns for Selected [2Fe-2S] Clusters

Protein	Ligation Pattern
Pseudomonas putidaredoxin[32]	^{39}**C**—X_5—^{45}**C**—X_2—^{48}**C**—X_{37}—^{86}**C**
Bovine adrenodoxin[33]	^{46}**C**—X_5—^{52}**C**—X_2—^{55}**C**—X_{36}—^{92}**C**
Anabaena 7120 ferredoxin[34]	^{41}**C**—X_4—^{46}**C**—X_2—^{49}**C**—X_{29}—^{79}**C**
P. cepacia phthalate dioxygenase reductase[35]	^{272}**C**—X_4—^{277}**C**—X_2—^{280}**C**—X_{27}—^{308}**C**
Escherichia coli FNR[36]	^{20}**C**—X_2—^{23}**C**—X_5—^{29}**C**—X_{92}—^{122}**C**
Paracoccus denitroficans FnrP[37]	^{14}**C**—X_2—^{17}**C**—X_7—^{25}**C**—X_{87}—^{113}**C**
Clostridium ferredoxin[38]	^{11}**C**—X_{12}—^{24}**C**—X_{31}—^{56}**C**—X_3—^{60}**C**
Mouse liver xanthine dehydrogenase[39]	^{115}**C**—X_2—^{118}**C**—X_{32}—^{151}**C**—X_1—^{153}**C**
O. carboxidovorans CO dehydrogenase CoxS[40]	^{102}**C**—X_2—^{105}**C**—X_{32}—^{138}**C**—X_1—^{140}**C**
Desulfovibrio gigas aldehyde oxidoreductase[41]	^{100}**C**—X_2—^{103}**C**—X_{33}—^{137}**C**—X_1—^{139}**C**
Escherichia coli SoxR[42]	^{119}**C**—X_2—^{122}**C**—X_1—^{124}**C**—X_5—^{130}**C**
Yersinia pseudotuberculosis E_1[14]	^{193}**C**—X_{57}—^{251}**C**—X_1—^{253}**C**—X_7—^{261}**C**

absence of the cysteine side-chain thiolate at positions 192, 193, 251, 253, and 261 does not interfere with the ability of the respective mutants to bind substrate and initiate the events leading to the formation of the $\Delta^{3,4}$-glucoseen intermediate (**4**). Clearly, the active site of E_1 remains functional, despite the lack of the iron−sulfur cluster.

DISCUSSION

Iron−sulfur proteins are found in all organisms, from "primitive" archea and bacteria to higher plants and animals. Dubbed as "nature's modular, multipurpose structures" (*30*), the iron−sulfur centers in these proteins function as components of electron-transfer pathways, catalytic centers, and sulfur donors and as sensors of iron and oxygen. They participate in a variety of processes, such as hydrogen metabolism, fixation of nitrogen or carbon monoxide, oxidative phosphorylation, dehydration, mitochondrial hydroxylation, and the reduction of nitrite and sulfide (*24*, *30*, *31*). The ubiquitous occurrence of iron−sulfur proteins and their presence in ancient organisms has led to the hypothesis that these proteins were among the earliest proteins in existence.

Four types of iron−sulfur clusters have been structurally characterized: [1Fe], [2Fe-2S], [3Fe-4S], and [4Fe-4S]. While the thiolates of cysteine residues are the most common ligands for coordinating the iron−sulfur center, variations from cysteine ligation are known such as the oxygen ligation in aconitase and the histidine ligation in Rieske centers (*25*). Sequence comparison of [2Fe-2S] proteins have revealed only a handful of cluster binding motifs, as shown in Table 3 (*32*−*42*). While the spacing between the cysteine ligands has been used as a "signature" to distinguish the class to which a given iron−sulfur protein belongs, the **C**-X_n-**C**-X_1-**C**-X_7-**C** ligation pattern displayed by E_1 does not fall into any of the common categories.

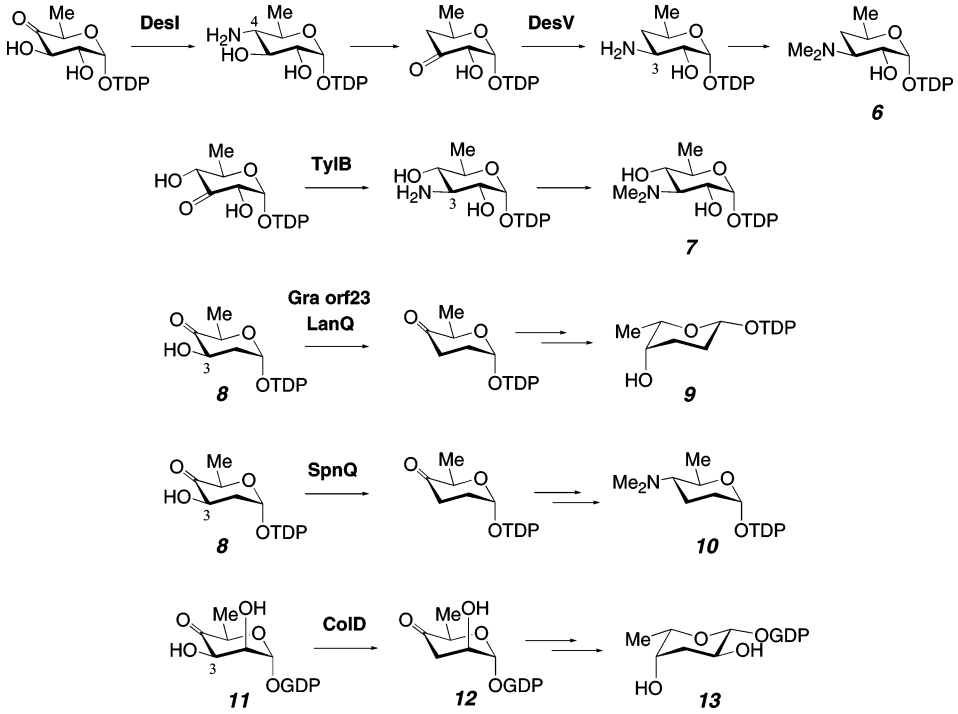
As shown in Table 3, the metal cluster of E_1 is composed of three proximal cysteines and a fourth ligand that is relatively distant in the primary sequence. However, unlike many [2Fe-2S]-containing proteins of this class in which the distal ligand is located downstream to the clustered cysteines (such as Cys86 in *Pseudomonas* putidaredoxin), the distal ligand (Cys193) in the E_1 metal-binding motif is located upstream to the three proximal cysteine residues with a gap between the first and the second cysteine of 57 amino acids. It is likely that such an unusual coordination environment around the iron−sulfur cluster plays an important role in conferring many unusual attributes to the catalytic properties of E_1.

Another unusual feature is the **C**-X_1-**C** configuration, which has only been observed in xanthine dehydrogenases (*39*) and its related proteins, such as the small subunit (CoxS) of carbon monoxide dehydrogenase from *Oligotropha carboxidovorans* (*40*) and a molybdenum-containing aldehyde oxidoreductase from *Desulfovibrio gigas* (*41*). A 12 amino acid sequence containing a **C**-X_1-**C** motif has also been identified as part of the [2Fe-2S] core in SoxR (Table 3) (*42*), which governs an oxidative response regulon in *E. coli*. The crystal structure of *D. gigas* aldehyde oxidoreductase, solved to 2.25 Å resolution, revealed that the domain of its iron−sulfur cluster, as part of a 2-fold symmetric four-helix bundle where Cys100 and 139 are linked to one iron and Cys103 and 137 are linked to another iron atom, is unique among the [2Fe-2S] proteins (*41*). Although both E_1 and aldehyde oxidoreductase contain the rare **C**-X_1-**C** motif, the spacing between this motif and two other cysteine residues diverges for the E_1 and aldehyde oxidoreductase cluster. Hence, the protein fold in the region around the iron−sulfur center is expected to be different for these two enzymes.

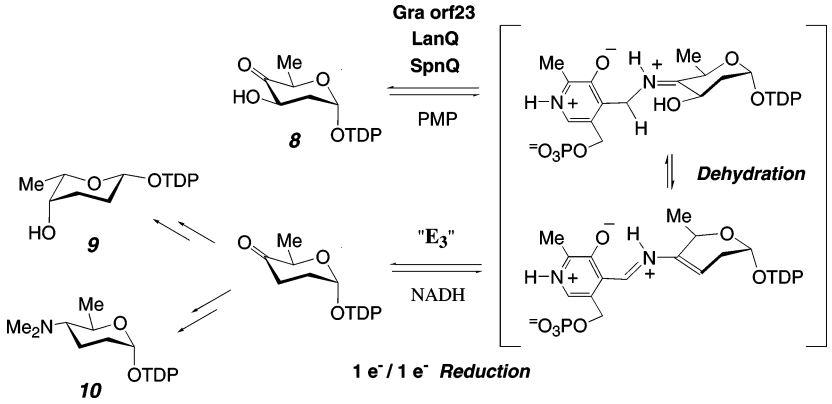
The redox potentials of [2Fe-2S] ferredoxins usually fall within a narrow range from −380 to −440 mV (*24*, *30*, *31*), although the corresponding value for *D. gigas* aldehyde oxidoreductase lies between −260 and −285 mV. However, the redox potential of the E_1 [2Fe-2S] cluster was found to be even higher (−209 mV) and is therefore significantly different as compared to the values for most [2Fe-2S] proteins (*19*). In view of the broader context of evolution, the singular catalytic nature of E_1 may have evolved concomitant with modifications in its cluster ligation patterns that enhanced redox communication of the E_1 [2Fe-2S] cluster with its partners, the [2Fe-2S] cluster of E_3 and the PMP-$\Delta^{3,4}$-glucoseen intermediate (**4**).

The sequence alignment of E_1 with a selection of known or putative transaminases and dehydrases involved in the biosynthesis of unusual sugars is shown in Figure 1. E_1 exhibits low to modest sequence identity with DesI (18%) (*43*), EryCIV (10%) (*44*, *45*), and OleN2 (23%) (*46*). Among these enzymes, DesI has been identified as a C-4 aminotransferase that catalyzes a key step in the biosynthesis of desosamine (**6**, Scheme 3) (*47*). E_1 also shows moderate sequence identity with DesV (21%) (*43*) and TylB (20%), (*48*) both of which have been identified as C-3 aminotransferases involved in the biosynthesis of desosamine (**6**) (*49*) and mycaminose (**7**), (*50*) respectively (Scheme 3). Interestingly, the translated sequences of *gra* orf23 from the granaticin gene cluster of *Streptomyces violaceoruber* T22 (*51*), *lanQ* from the landomycin gene cluster of *Streptomyces cyanogenus* (*52*), and *spnQ* of the spinosyn gene cluster of *Saccharopolyspora spinosa* (*53*) show much higher identity with that of the E_1 gene (52% for Gra orf23, 53% for LanQ, and 49% for SpnQ). The proteins encoded by these genes are proposed to be dehydrases, with Gra orf23 and LanQ catalyzing the C-3 deoxygenation of **8** in the biosynthesis of l-rhodinose (**9**) (*51*, *52*) and SpnQ catalyzing the C-3

Scheme 3



Scheme 4

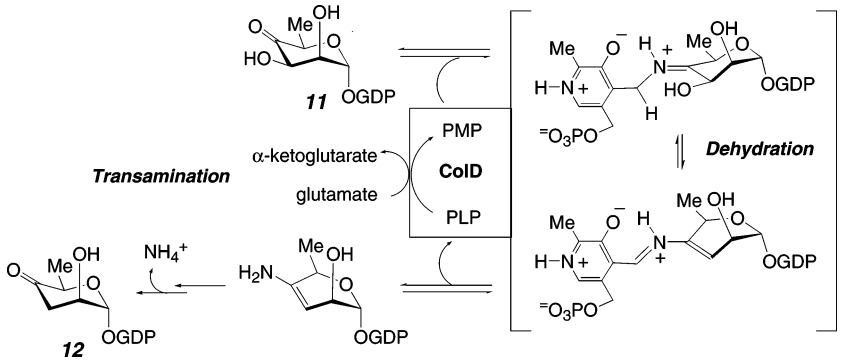


deoxygenation of **8** in the biosynthesis of D-forosamine (**10**) (*53*) (Scheme 3).

The sequence alignment in Figure 1 also shows a distinct divergence between the two subgroups of B_6-dependent catalysts involved in the biosynthesis of unusual sugars. E_1 and those believed to function as dehydrases (such as Gra orf23, LanQ, SpnQ) have a catalytic histidine at position 220 (using the E_1 numbering system) and display an unusual, albeit well-defined, iron−sulfur binding motif. In fact, among the eight cysteine residues in E_1, only the four implicated in this study (Cys193, 251, 253, and 261) are absolutely conserved in this subgroup of B_6-dependent enzymes. Since Cys193 (using E_1 numbering system), but not Cys192, is conserved among these genes, it is more likely to be the fourth residue serving as the ligand for coordinating the iron−sulfur cluster. In contrast, those enzymes believed to function as transaminases (such as DesI, EryCIV, OleN2, DesV, TylB) have a lysine at position 220 and lack a motif for binding the iron−sulfur cluster. Having confirmed the in vitro catalytic functions of E_1, DesI, DesV, and TylB, the sequence divergence mentioned above may be used to distinguish a dehydrase from other B_6-dependent enzymes. In addition, reactions catalyzed by the E_1 homologues Gra orf23, LanQ, and SpnQ are expected to share a catalytic mechanism similar to that of E_1, because they share cofactor-binding and catalytic residues, including the active site histidine and an iron−sulfur cluster (see Scheme 4).

The translated sequence of *colD* from the colitose gene cluster of *Y. pseudotuberculosis* VIA and the corresponding gene (*wbdK*) from *E. coli* also show modest sequence identity with that of the E_1 gene (27% for ColD and 27% for WbdK) (*54*, *55*). Of particular interest is the fact that ColD and WbdK have an equivalent of His220 (using the E_1 numbering system) but lack the cluster of cysteines that provide three of the four ligands to the iron−sulfur cluster. Thus, whether ColD and/or WbdK functions as a dehydrase or a transaminase is not immediately obvious. To determine the catalytic mechanism, the ColD protein was recently purified and was shown to be a dehydrase, catalyzing the C-3 deoxygenation of **11** in the biosynthesis of L-colitose (**13**) (*55*). It was found that the cofactor (PMP) in the ColD-catalyzed reaction is regenerated by a second function of ColD, an aminotransferase activity (see Scheme 5), instead of by the direct regeneration by a two-electron reduction, as seen in the E_1/

Scheme 5



E_3 catalyzed deoxygenation (see Scheme 1). Thus, ColD can be considered a de facto but unusual transaminase that catalyzes a dehydration reaction. This study suggests that mechanistic variations exist even among putative dehydrases. Further studies in this area are ongoing with an examination of the function of SpnQ. Clearly, many more interesting discoveries lie ahead in the area of C−O bond cleavage reactions of sugars.

In summary, sequence alignment showed that E_1 belongs to a group of structurally well characterized PLP-dependent enzymes, such as 3-amino-5-hydroxybenzoic acid synthase (*56*), arnb aminotransferase (*57*), and *Thermus thermophilus* aspartate aminotransferase (*58*). However, the loop containing three of the four cysteine residues implicated in [2Fe-2S] cluster ligation in E_1 is missing in these enzymes. Taken together, the results from this investigation suggest that E_1 is a unique iron−sulfur-containing enzyme. First, due to the inherent differences in the chemistry of B_6-dependent reactions and Fe−S cluster-mediated electron transfer reactions, there are only a few B_6-dependent enzymes that contain Fe−S clusters. Apart from E_1, other notable examples are lysine 2,3-aminomutase (*59*) and GABA-aminotransferase (*60*). Among these enzymes, E_1 is the only PMP-dependent enzyme, whereas the other enzymes are PLP-dependent. Also, while lysine 2,3-aminomutase contains a [4Fe-4S] cluster, E_1 and GABA-aminotransferase contain a [2Fe-2S] cluster. Since the catalytic mechanism of the Fe−S cluster in GABA-aminotransferase remains elusive, E_1 is the only example of a PMP-dependent enzyme that contains a mechanistically defined and relevant [2Fe-2S] cluster. Second, E_1 displays a cluster-binding motif (**C**-X_{57}-**C**-X_1-**C**-X_7-**C**) that has not been observed for [2Fe-2S] proteins and includes the rare **C**-X_1-**C** feature, of which there are only a handful examples. Since structural information for E_1 itself is lacking, a more thorough comparison between E_1 and other [2Fe-2S] proteins will have to wait until such information is available. However, the cysteines that have been implicated in the binding of the iron−sulfur cluster in E_1 are conserved in the sequences of its homologues. Therefore, E_1 and its homologues may constitute a new subclass in the family of iron−sulfur proteins that are distinguished not only by their cluster ligation patterns but also by the chemistry used in catalyzing a simple, albeit mechanistically challenging, reaction. While a number of putative ORFs believed to be dehydrases or transaminases have been identified, the mechanisms of some of these enzymes have yet to be confirmed. The results of this investigation can be used to assign functions and mechanisms for these and new related enzymes.

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