Mechanistic Studies on CDP-6-deoxy-$\Delta^{3,4}$-glucoseen Reductase: The Role of Cysteine Residues in Catalysis As Probed by Chemical Modification and Site-Directed Mutagenesis†

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Received August 16, 1994; Revised Manuscript Received November 14, 1994®

ABSTRACT: CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3), which catalyzes the reduction of the C-3 deoxygenation step during the formation of CDP-ascarylose, a 3,6-dideoxyhexose found in the lipopolysaccharide of *Yersinia pseudotuberculosis*, has been expressed at high level in *Escherichia coli* (670 times over the wild-type strain). This flavoenzyme, which also contains one plant ferredoxin type [2Fe-2S] cluster, was inactivated by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide. In both cases the inactivation followed a pseudo first order kinetics. The second order rate constant for the reaction of DTNB with E_3 was 0.25 mM^{-1} min^{-1} at 20 °C, pH 8.0. Detailed characterization of the inactivated enzyme showed that neither the flavin nor the [2Fe-2S] cluster was altered during inactivation. Since this inactivation was reversible by treating the inactivated enzyme with 1 mM D,L-dithiothreitol (DTT), it was concluded that only cysteine residues were modified during inactivation. Analysis of the inactivation using the method developed by Tsou revealed that two cysteines react with DTNB at similar rates and modification of either one is enough to impair E_3's activity. Tryptic digestion of E_3 labeled with *N*-ethyl[2,3-^{14}C]maleimide, followed by fractionation of the digest by high performance liquid chromatography, gave two labeled peptides, both of which were separately isolated as a pair of interconvertible diastereoisomers. Sequence analysis of these labeled peptides allowed the identification of Cys-75 and Cys-296 as the reactive cysteine residues. Interestingly, the C75S and C296S mutant proteins exhibit identical physical and comparable catalytic properties as the wild-type enzyme. Since Cys-296 is a conserved residue in the NAD(P) binding domain of enzymes belonging to the same class, this residue may be involved in stabilizing the charge-transfer complex between E_3 and NADH, thus facilitating hydride transfer from the nicotinamide nucleotide to flavin. A chemically modified Cys-75 which is immediately adjacent to the [2Fe-2S] center in E_3 may prevent the proper juxtaposition of the redox centers and thus impede electron transfer leading to enzyme inactivation. These results may be useful for placing constraints on the peptide folding comprising the active site of E_3 for electron transfer between NADH, FAD, and the [2Fe-2S] center.

Ascarylose (**1**, 3,6-dideoxy-L-*arabino*-hexose), a 3,6-dideoxy sugar found in the *O*-antigen of the lipopolysaccharides of *Yersinia pseudotuberculosis*, is derived via a complex enzymatic series beginning with α-D-glucose 1-phosphate (**2**) (Thorson et al., 1993; Liu & Thorson, 1994). As depicted in Scheme 1, the key transformation of its biosynthesis is the replacement of the C-3 hydroxyl group by a solvent hydrogen (Gonzalez-Porqué & Strominger, 1972; Gonzalez-Porqué, 1986). Early studies have demonstrated the requirement of two enzymes for this deoxygenation: a pyridoxamine 5′-phosphate (PMP) linked CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose 3-dehydrase (E_1)[1] which catalyzes the initial dehydration of **3** (Rubenstein & Strominger, 1974a), and an NADH dependent CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3) which mediates the subsequent reduction of the conjugated enamine intermediate **4** (Rubenstein & Strominger, 1974b). The nascent imine species **5** is then hydrolyzed to give the actual deoxygenated product **6**.

In an effort to elucidate the mechanism by which the sugar deoxygenation is effected, we have purified most of the enzymes involved in this biosynthetic pathway (Yu et al., 1992; Weigel et al., 1992; Lo et al., 1994; Thorson et al., 1994a). The *asc* (ascarylose) gene cluster has also been cloned and sequenced, and the corresponding gene products have been expressed (Thorson et al., 1993, 1994b). Characterization of the recombinant E_1 has unveiled that this enzyme contains, in addition to PMP, an additional cofactor with chemical as well as physical properties consistent with the [2Fe-2S] cluster found in adrenodoxin and/or putidaredoxin (Thorson & Liu, 1993a). Interestingly, examination of the highly purified E_3 showed that this enzyme is also an iron−sulfur protein containing one FAD and one ferredoxin

† This work was supported by National Institutes of Health Grant GM 35906.

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® Abstract published in *Advance ACS Abstracts,* March 1, 1995.

[1] Abbreviations: CDP, cytidine diphosphate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, D,L-dithiothreitol; E_3, CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase; E_1, CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose 3-dehydrase; BSA, bovine serum albumin; FAD, flavin adenine dinucleotide; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; NEM, *N*-ethylmaleimide; PCR, polymerase chain reaction; FAB-MS, fast atom bombardment mass spectroscopy.

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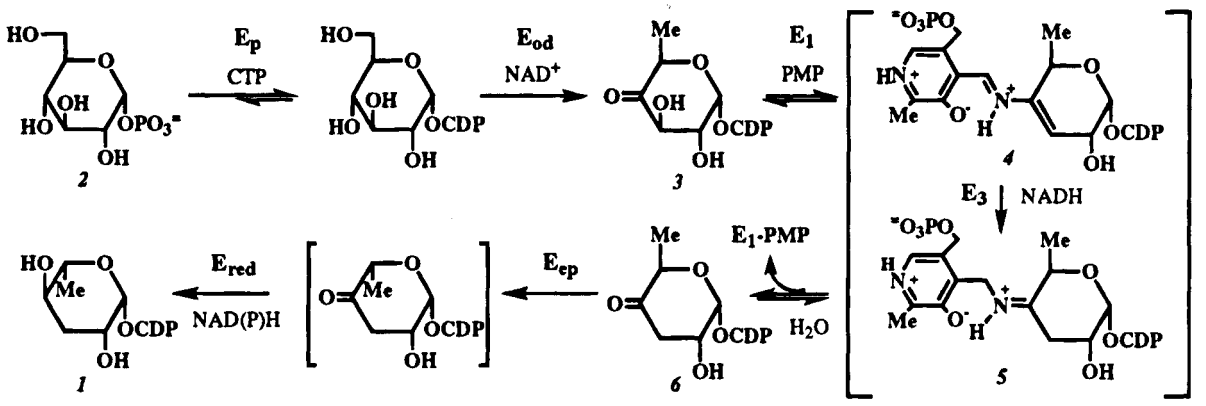
Scheme 1



type [2Fe-2S] center per enzyme molecule (Miller et al., 1993). On the basis of the physical characteristics of E_1 and E_3, the mechanism of C-3 deoxygenation (Scheme 1) has been postulated (Thorson & Liu, 1993b). Although the details of the overall mechanism still remains unresolved, the inherent dependence of E_1 on both PMP and a [2Fe-2S] center and the incorporation of E_3 as an integral part of the reduction conduit clearly make this deoxygenation singular in its own class.

While E_1 is a unique catalyst, E_3, which is formally an electron transport protein, is very similar to a number of iron−sulfur flavoprotein reductases found in multicomponent hydroxylating systems (Mason & Cammack, 1992), such as methane monooxygenase reductase (Stainthorpe et al., 1990), phthalate dioxygenase reductase (Batie et al., 1991), benzoate 1,2-dioxygenase reductase (Neidle et al., 1991), vanillate demethylase oxidoreductase (Brunel & Davison, 1988), xylene monooxygenase reductase (Shaw & Harayama, 1992), NADH-ferrodoxin$_{NAP}$ reductase (Haigler & Gibson, 1990), putidaredoxin reductase (Roome et al., 1983), trimethylamine dehydrogenase (Lim et al., 1988), and a few others. Sequence comparison also reveals excellent homology of E_3 and many other flavin containing reductases whose function is to shuttle electrons between NAD(P)H and a one electron carrier, typically a metal center in another protein (Mason & Cammack, 1992). Several well-known examples include ferredoxin-NADP$^+$ reductase, cytochrome P-450 reductase, cytochrome b_5 reductase, nitric oxide synthase, and nitrate and sulfite reductases (Mason & Cammack, 1992). Like most of these reductases, E_3 is sensitive to thiol-directing agents (Miller et al., 1993). However, this inactivation and in particular the role of cysteine residues of this important class of iron−sulfur flavoprotein reductases have never been fully characterized. In order to further elucidate the intimate mechanism of the C−O bond cleavage catalyzed by E_1 and E_3, we have undertaken a detailed study based on an approach combining chemical modification and site-directed mutagenesis to probe the possible involvement of reactive cysteine residues in E_3 catalysis. The availability of the complete primary sequence of E_3 and the three-dimensional structure of mutant proteins enabled us to locate these residues within the amino acid sequence. Although mutant proteins with each of these reactive cysteines replaced by serine showed little effect on their catalytic activity, the insights gained from this study may be useful for placing constraints on the peptide folding comprising the active site of E_3 for electron transfer between NADH, FAD, [2Fe-2S], and the metal center of E_1.

EXPERIMENTAL PROCEDURES

General. Escherichia coli JM105 and plasmid pKK223-3 were purchased from Pharmacia (Pistacaway, NJ). DEAE-Sepharose CL6B, phenyl-Sepharose CL4B, and MonoQ 10/10 were also obtained from Pharmacia. The *asc*D (E_3) gene containing plasmid pSFL28 prepared earlier (Lo et al., 1994) was a gift from Stanley F. Lo of this group. Synthetic oligonucleotides were products of Integrated DNA Technologies (Coralville, IA) and were used without any further purification. The culture media components were from Difco (Detroit, MI), and the Bradford reagent was from Bio-Rad (Richmond, CA). All restriction enzymes, DNA modifying enzymes, and their respective buffers were purchased from United States Biochemicals (Cleveland, OH). [α-^{32}S]dATP and *N*-ethyl[2,3-^{14}C]maleimide were obtained from Amersham (Arlington Heights, IL). All other biochemicals and chemicals were purchased either from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) and were of the highest purity available. Sequence analysis and comparison were performed using IntelliGenetics software (Suite 5.4). CDP-6-deoxy-D-*glycero*-L-*threo*-4-hexulose 3-dehydrase (E_1) and CDP-D-glucose 4,6-dehydratase (E_{od}) were kindly supplied by Jon S. Thorson and Xuemei He of this group.

Construction of the Overproducing Strain. The *asc*D gene coding for E_3 was amplified with the GeneAmp PCR kit (Perkin Elmer) using pSFL28 plasmid as the template. Two constructs were made (OP1 and OP2) with the following primers (sequences are written 5′ to 3′): start primer (primer 1): CCCGGGAATTCATGTCATTAAATGTTAAGCTGCAT and halt primer (primer 2): GCGCAAGCTTTTATTTTGATGGCACAAA for construct OP1; start primer (primer 3): CGCGCGAATTCAGGAGGAAATTTAAAATGTCATTAAAT and halt primer (primer 4): GCGCAAGCTTTTATTTTGATGGCACAAA for construct OP2. The amplified inserts were digested with *Eco*RI and *Hin*dIII and inserted into the *Eco*RI-*Hin*dIII sites of pKK223-3. Standard recombinant DNA techniques were used for all plasmid constructions (Maniatis et al., 1989). After transformation into competent *E. coli* JM105, positive clones were isolated, the double stranded plasmids were isolated and purified, and the sequences of the inserts were verified using the Sequenase kit (United States Biochemicals, Cleveland, OH).

Growth of Cells. Six 1-L batches of Terrific Broth medium (Maniatis et al., 1989) supplemented with ampicillin (100 μg/mL) and ferrous ammonium sulfate (final concentration 100 μM) were inoculated with an overnight culture (1 mL each), and the cells were grown at 37 °C with vigorous agitation. Isopropyl β-D-thiogalactopyranoside was added

to a final concentration of 1 mM when the cultures attained an $A_{660} = 0.6$, and the agitation was continued overnight (ca. 10−15 h). The cells were then 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 approximately 12 g (wet weight) of cells per 1 L of culture.

Purification of E_3. The purification was carried out at 4 °C except for the FPLC steps which were run at room temperature. In order to minimize the possibility of enzyme inactivation by molecular oxygen, all buffers were degassed and saturated with nitrogen gas.

(i) Step 1. Crude Extracts. The cell paste was thawed on ice and resuspended in 100 mL of cold buffer A (20 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.1 mM EDTA) which contained 1 mM phenylmethanesulfonyl fluoride. The suspension was sonicated for 5 min with a 30 s cooling period every minute. Cellular debris was removed by centrifugation (15 min, 12000*g*), and the supernatant was quickly transferred to a stoppered flask and flushed with nitrogen gas.

(ii) Step 2. Ammonium Sulfate Precipitation. Crystals of ammonium sulfate were added portionwise to the crude extract up to 25% saturation, and the solution was stirred for 1 h under nitrogen. After centrifugation (10 min, 12000*g*), more ammonium sulfate was added to the supernatant, under nitrogen and with stirring, to give a final concentration of 70% saturation. The precipitated proteins were collected 1 h later by centrifugation (10 min, 12000*g*) and redissolved in a minimum amount of buffer A. This solution was dialyzed for 2 h under nitrogen against 1 L of buffer A with three changes of buffer.

(iii) Step 3. DEAE-Sepharose CL6B Chromatography. The protein solution from step 2 (50 mL) was loaded onto a DEAE-Sepharose column (2.5 cm × 40 cm) equilibrated with buffer A. After washing with 1 bed volume of buffer A, the column was eluted with a linear gradient of NaCl from 0 to 0.3 M in buffer A (1 L total) at 50 mL/h. Fractions of 15 mL were collected during the gradient elution, and the active fractions were combined. The proteins were precipitated by the addition of solid ammonium sulfate up to 70% saturation. After centrifugation (10 min, 12000*g*), the pellet was dissolved in a minimum amount (40 mL) of cold buffer B (20 mM Tris-HCl, pH 8.0, 1 mM DTT, 0.1 mM EDTA, 3 M NaCl).

(iv) Step 4. Phenyl-Sepharose CL4B Chromatography. The protein solution from step 3 was chromatographed on a phenyl-Sepharose column (2.5 cm × 20 cm) pre-equilibrated with buffer B. The column was first washed with buffer B (1 column volume) followed by an elution with a linear descending gradient of NaCl from 3 M (250 mL of buffer B) to 0 M (250 mL of buffer A) at 50 mL/h. The active fractions were pooled and treated with ammonium sulfate up to 70% saturation. The orange-brown protein precipitates were collected by centrifugation (10 min, 12000*g*) and dissolved in 10 mL of cold buffer C (20 mM Tris-HCl, pH 8.0). The resulting solution was dialyzed against 2 L of buffer C overnight under nitrogen.

(v) Step 5. MonoQ Chromatography. The material from step 4 was further purified by an FPLC equipped with a MonoQ 10/10 column pre-equilibrated with buffer C. Elution monitored at 280 nm was achieved using a linear gradient from 0 to 0.5 M NaCl in buffer C at 4 mL/min in 20 min. The desired peak was manually collected, and the solution was immediately frozen in liquid nitrogen. The purified enzyme was kept at −80 °C for months without loss of activity.

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 by both the Bradford method and the quantitative amino acid analysis (Tarr, 1986) performed by the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota using aliquots of the same sample. It was found that the Bradford method gave a value of 10% higher than that obtained by quantitative amino acid analysis, and thus all results were accordingly corrected.

Enzyme Assays. The activity of E_3 was assayed by following E_3 catalyzed NADH reduction of 2,6-dichlorophenolindophenol ($\epsilon_{600} = 22\,000\ M^{-1}\ cm^{-1}$), as had been previously described (Lo 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).

Thiol Titrations. All experiments were run in buffers saturated with nitrogen gas. A typical experiment involves the addition of the denaturing solution consisting of 1 mM EDTA and 6.0 M guanidine hydrochloride in 800 μL of 50 mM potassium phosphate buffer (pH 7.4) to a solution of 1 mg of E_3 in 200 μL of 20 mM Tris-HCl buffer (pH 8.0). For these experiments in which reduced E_3 was used, the enzyme sample was further treated, in addition to the above denaturing solution, with DTT (final concentration 5 mM) for 30 min. After thorough mixing, 50 μL of 12 N HCl was added to the incubation mixture and the resultant solution was kept under a stream of nitrogen at room temperature for 1 h to evaporate the nascent H_2S. This mixture was then loaded onto a 10DG desalting column (Bio-Rad) pre-equilibrated with EDTA (1 mM) and guanidine hydrochloride (6.0 M) containing potassium phosphate buffer (50 mM, pH 7.4), and the protein was eluted using the same buffer. The protein fractions were pooled, and the exposed thiols in the denatured E_3 were titrated by adding 5 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to the solution and measuring the change in absorbance at 412 nm ($\epsilon_{412} = 13\,700\ M^{-1}\ cm^{-1}$) (Riddles et al., 1983). The protein concentration was determined by the Bradford method.

Inactivation by Thiol-Directing Agents. Typically, 1.75 nmol of E_3 was treated with excess reagent (DTNB, NEM, iodoacetic acid, or iodoacetamide) in 0.1 mL of buffer (0.1 M potassium phosphate or 0.1 M Tris-HCl at various pH). Aliquots of 10 μL were removed at different times, diluted with 90 μL of cold 0.1 M potassium phosphate buffer (pH 7.0), and immediately subjected to activity assay. When DTNB was used as the thiol modifying reagent to determine the number of critical cysteines for E_3 activity, the incubation was conducted with 10.5 nmol of E_3 and 0.5 mM DTNB in 600 μL of 0.1 M Tris-HCl buffer (pH 8.0). The correlation between the number of modified cysteines and the extent of inactivation was established by measuring the change in absorbance at 412 nm ($\epsilon_{412} = 14\,150\ M^{-1}\ cm^{-1}$) (Riddles et al., 1983) and the residual activity at the same time.

Characterization of DTNB Inactivated E_3. E_3 (52.5 nmol) was treated with 0.5 mM DTNB in 0.1 M Tris-HCl buffer (pH 8.0, total volume 3 mL) for 60 min at room temperature.

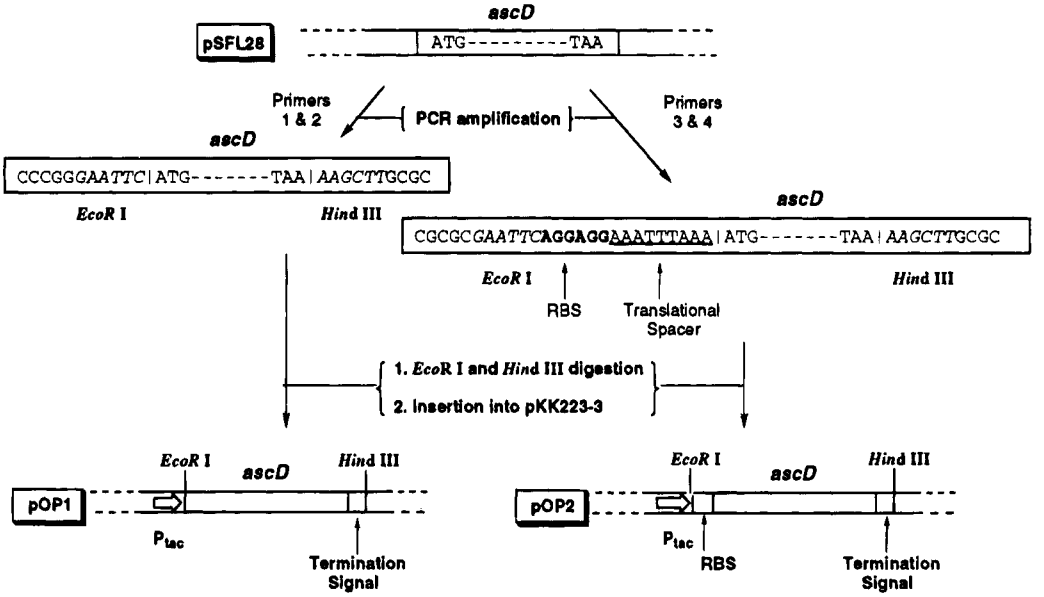


FIGURE 1: Construction of an efficient expression vector for E_3. The *asc*D gene was amplified from pSFL28 by PCR using two different sets of primers. The inserts were then ligated into pKK223-3 by standard recombinant DNA technology. The details are described under Experimental Procedures.

The inactivated enzyme was desalted on a Bio-Rad 10DG column using 20 mM Tris-HCl buffer (pH 8.0) as eluent. A control was run in parallel except that DTNB was omitted. The two preparations were characterized by UV−vis spectroscopy and assayed for activity as well as iron−sulfur content. The inactive E_3 was reactivated by treatment with 5 mM DTT in 20 mM Tris-HCl buffer, pH 8.0. The amount of released 5-mercapto-2-nitrobenzoate during reactivation was deduced from its absorbance at 412 nm (ϵ_{412} = 14 150 M^{-1} cm^{-1}) (Riddles et al., 1983). The solution was then desalted by passage through a 10DG column (Bio-Rad), and the protein fraction was collected and its physical and catalytic properties were fully characterized.

Protection of Inactivation of E_3 by DTNB. E_3 (1.75 nmol) was incubated with 0.5 mM DTNB, in the presence of NADH (0, 0.2, or 0.5 mM) and/or E_1 (0 or 1.75 nmol) in 0.1 M Tris-HCl buffer (pH 8.0) at room temperature. Aliquots of 10 μL were withdrawn at different times and diluted 10 times in cold 0.1 M potassium phosphate buffer (pH 7.0). The residual activity was measured as described above.

Identification of Modified Cysteines. Seven milligrams of E_3 was treated with 5 mM *N*-ethyl[2,3-^{14}C]maleimide (7.4 mCi/mmol) in 0.1 M Tris-HCl buffer (pH 8.0, total volume 0.5 mL) at room temperature for 25 min. Excess NEM was removed by a 10DG desalting column (Bio-Rad) using 50 mM ammonium acetate buffer (pH 8.5) as eluent. The radioactivity of the labeled protein was measured and its concentration determined. After being concentrated on Centricon 10 (Amicon), the labeled protein was digested using an equal volume of 1 mg/mL trypsin (porcin pancreas type IX, Sigma) in 50 mM ammonium formate buffer (pH 8.5). The tryptic fragments, after treatment with 1 mM DTT, were separated by HPLC on a Vydac C_{18} (4.6 mm × 25 cm) column using a 0−25% linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid/water over a period of 70 min followed by a 100% acetonitrile wash. The elution was monitored at 220 nm, and the flow rate was 1 mL/min. After further purification by the same HPLC conditions, four peptides, A, B, C, and D, which carried the majority of the radioactivity, were sequenced by automated Edman degradation at the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota. The effluents obtained after each cycle were also counted for radioactivity.

Site-Directed Mutagenesis and Purification of Mutant Protein. The mutants C75S and C296S were constructed by the overlap extension PCR methodology developed by Higuchi et al. (1988) using primers 5′-CATTGTAG-CTCTAAGGC for the C75S and 5′-GTTTATGCCAGTG-GTTCATTA for the C296S mutations. After amplification and cloning in pKK223-3 vector, positive clones were isolated and the sequence of the mutated gene was verified. Purification of C75S and C296S mutant proteins was performed as described for the wild-type E_3.

Kinetic Characterization of Mutant Proteins. The catalytic competence of C75S and C296S mutant proteins was determined by following their NADH:DCPIP oxidoreductase activity (Lo et al., 1994).

RESULTS

Overexpression of E_3. In a previous report we had described the cloning and sequencing of *asc*D, the gene coding for E_3, from *Y. pseudotuberculosis* and the expression of this gene product in *E. coli* (Lo et al., 1994). Although an 80-fold overexpression was achieved with this pUC19 derived plasmid (pSFL28) in *E. coli* HB101, the yield of E_3 from a 6 L preparation was still less than desirable for our spectroscopic and mechanistic studies. In an attempt to further improve its expression, we have designed and prepared two constructs (pOP1 and pOP2) using PCR. As illustrated in Figure 1, the *asc*D gene was amplified using primers designed to construct two restriction sites flanking the *asc*D gene to facilitate the cloning in pKK223-3. Indeed, the expression of E_3 using the pOP2 construct in *E. coli* JM105 was greatly improved by substituting the *lac* promoter (in pUC19) for the stronger *tac* promotor (in pKK223-3) and by adding a ribosome binding site (MacFerrin et al., 1990; Schreiber & Verdine, 1991) as well as a transcription termination signal (pKK223-3) (Table 1).

Purification of Recombinant E_3. Like many iron−sulfur proteins, E_3 is, albeit not extremely, oxygen sensitive.

Table 1: Comparison of the Expression of E_3 Using Different Expression Systems

expression system	sp act. (μmol/(min·mg))[b]	over-expression fold[c]	pure E_3/6 L of culture (mg)[d]
Y. pseudotuberculosis[a]	<0.003	1	0.2
E. coli HB101/pSFL28[a]	0.24	80	10
E. coli JM105/pOP1	1.0	333	ND[e]
E. coli JM105/pOP2	2.0	666	>70

[a] Lo et al., 1994. [b] Specific activities were determined as described under the Experimental Procedures using the crude extract without further purification. [c] The fold of overexpression is the ratio of the specific activity of the crude extract of each construct over that of wild-type (*Y. pseudotuberculosis*). [d] E_3 was purified according to the protocol described under the Experimental Procedures. [e] Not determined.

However, in order to prevent any unnecessary deactivation by air oxidation, the purification was carried out in O_2 free buffer in an expeditious manner. As described under Experimental Procedures, using the newly developed expression system (*E. coli* JM105/pOP2) and the modified purification protocol, we can now readily obtain nearly 70 mg of pure E_3 (from 6 L of culture) in a routine preparation.

Thiol Titration by DTNB. The translated amino acid sequence of *asc*D predicts 7 cysteines per E_3 which is a monomeric enzyme (Lo et al., 1994). Since four of these cysteines are ligands of the [2Fe-2S] center, this metal cluster was removed by denaturation of the native E_3 under acidic conditions to free the cysteine ligands (Meyer et al., 1992). The total thiol content of the apo-[Fe-S]-E_3 was spectrophotometrically titrated with DTNB. A ratio of 6.4−6.9 mol of thiol/mol of E_3 was found (data not shown), depending on the enzyme batches (data not shown). Since extensive reduction of E_3 by DTT prior to thiol titration gave the same results, the native E_3 therefore does not contain any disulfide bridge.

E_3 Inactivation by Thiol-Directed Reagents. As we already noted, E_3 can be inactivated by thiol-directed reagents such as DTNB, NEM, and various mercurial compounds (Miller et al., 1993). Further characterization of the inactivation of E_3 in the presence of excess DTNB revealed a pseudo first order kinetic for this process (Figure 2A). However, the rate accelerated in a quasilinear fashion, suggesting a very low affinity (much higher than 1 mM) of E_3 for DTNB. From the plot shown in Figure 2B, a rate constant of 0.25 mM^{-1} min^{-1} for the inactivation of E_3 at low DTNB concentration was calculated. It was also discovered that the inactivation was pH dependent with higher rate at high pH. In the presence of 0.1 mM DTNB, the k_{obs} was found to be 0.06 min^{-1} and 0.17 min^{-1} at pH 7.0 and 8.0, respectively. It should be noted that the inactivation follows the same kinetics in potassium phosphate buffer as in Tris-HCl buffer. Surprisingly, neither iodoacetic acid nor iodoacetamide inactivated E_3 at a concentration of 25 mM in 0.1 M Tris-HCl buffer (pH 7.0−9.0) within 1 h. Furthermore, the addition of 25 mM iodoacetic acid to a mixture of E_3 and 0.5 mM DTNB in 0.1 M Tris-HCl buffer (pH 8.0) did not affect the rate of inactivation. Both observations support the immunity of E_3 toward iodoacetic acid. On the contrary, *N*-ethylmaleimide can also inactivate E_3, albeit at a lower rate than DTNB. The inactivation rate constant k_{obs} at pH 8.0 was determined to be 0.29 min^{-1} with 5 mM NEM, and 0.05 min^{-1} with 1 mM NEM.

Characterization of the DTNB Inactivated E_3. Figure 3 shows the electronic absorption spectra of the native and

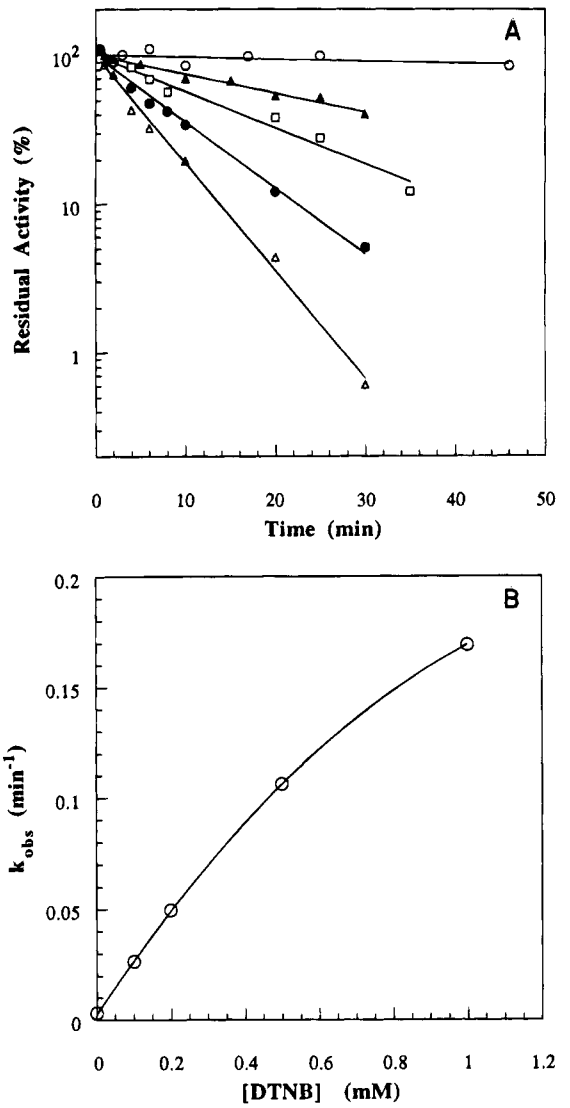


FIGURE 2: (A) Kinetics of the inactivation of E_3 with DTNB. E_3 (1.75 nmol) was incubated with 0 mM (○), 0.1 mM (▲), 0.2 mM (□), 0.5 mM (●), and 1.0 mM (△) DTNB in 0.1 M Tris-HCl buffer (pH 8.0) at room temperature. Aliquots were withdrawn at indicated time points, diluted 10-fold in cold 0.1 M potassium phosphate buffer (pH 7.0), and subjected to activity assay to determine the remaining activity (described under Experimental Procedures). The observed first order rate constants, k_{obs}, were calculated by fitting the data points to simple exponential decays. (B) Effect of varying DTNB concentration on the rate of inactivation of E_3. Rate constants derived from panel A were plotted against DTNB concentration. The second order rate constant was estimated from the linear portion of the curve, from 0 to 0.3 mM DTNB.

modified E_3. Although the spectral features at 270 nm and around 350 nm of the inactivated E_3 were altered due to the presence of 5-mercapto-2-nitrobenzoate moieties in the thiol modified protein, the flavin and [2Fe-2S] chromophores at 455 and 550 nm (shoulder), respectively, were primarily unchanged. Furthermore, chemical quantification of the iron−sulfur content of the inactivated E_3 gave 1.7 ± 0.2 mol of [Fe-S]/mol of enzyme, a result comparable to that obtained from a control experiment in which E_3 was similarly treated but without DTNB. These data clearly indicated that only cysteines were modified during inactivation and the [2Fe-2S] cluster as well as the flavin cofactor remained intact in the inactivated enzyme. To test if the inactivation was reversible, the inactivated E_3 was incubated with excess DTT to reverse the modification by thiol−disulfide interchange. Indeed, treatment of inactive E_3 (having 1.7 cysteines

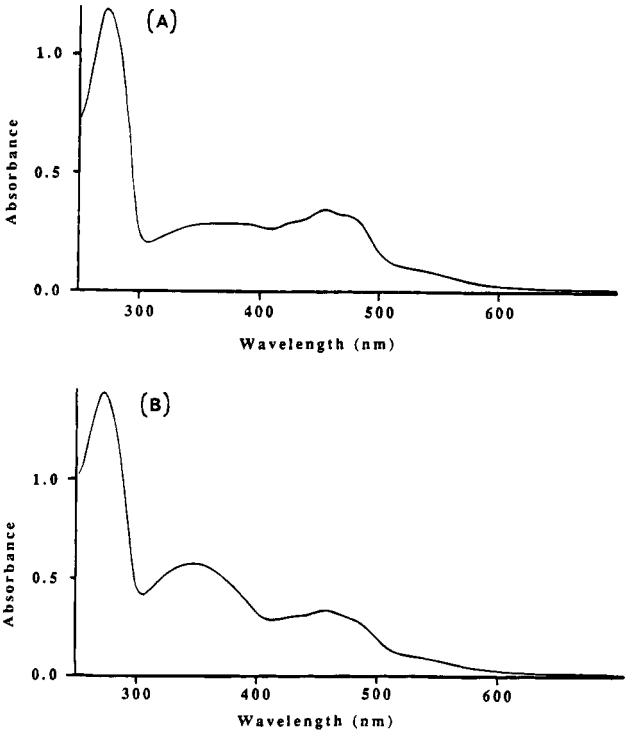


FIGURE 3: Electronic absorption spectra of native and inactivated E_3. Spectra were recorded at room temperature with (A) native E_3 (20 μM) and (B) purified inactivated E_3 (20 μM), both in 20 mM Tris-HCl buffer, pH 8.0.

modified and 2% residual activity) with 5 mM DTT at pH 8.0 led to the release of 1.4 mol of 5-mercapto-2-nitrobenzoate/mol of E_3 which was quantified based on the change of absorbance at 412 nm (ϵ_{412} = 13 700 M^{-1} cm^{-1}) (Riddles et al., 1983). These results clearly excluded the possibility that DTNB modification of E_3 involved the formation of intramolecular disulfide linkages. The enzyme thus obtained fully recovered (greater than 90%) its activity.

Protection of the Inactivation of E_3 by DTNB. Protection of the inactivation by DTNB was tested using NADH, which is formally a substrate for E_3. Since E_1 is believed to form a complex with E_3 during catalysis, protection by E_1 was also attempted. It was found that the protection by NADH was weak, decreasing the inactivation rate by only 30% at high concentration (20 times the K_m). Interestingly, E_1 did not protect against inactivation. Since formation of such complex is expected prior to E_3 inactivation, the above result implied that these reactive thiols are not exterior residues at or near the E_1–E_3 recognition–interaction domain.

Graphical Analysis of the Inactivation by Tsou Plot. E_3 was treated with excess DTNB at pH 8.0, and the residual activity together with the number of modified sulfhydryls per E_3 were determined for each aliquot drawn from the incubation mixture at various time points. The data were plotted according to:

$$\sqrt[i]{a} = 1 - x/N \qquad (1)$$

where N is the total number of reactive cysteines per E_3, x is the number of modified cysteines per E_3 at the given point, a is the fraction of remaining activity when x residues have been modified, and i (a positive integer) is the number of essential residues for catalytic activity as definded by Tsou (1962). The number of essential residues is the value of i that gives a straight line when $a^{1/i}$ (a, $a^{1/2}$, $a^{1/3}$, etc.) is plotted against x. As shown in Figure 4, our data fit to a straight

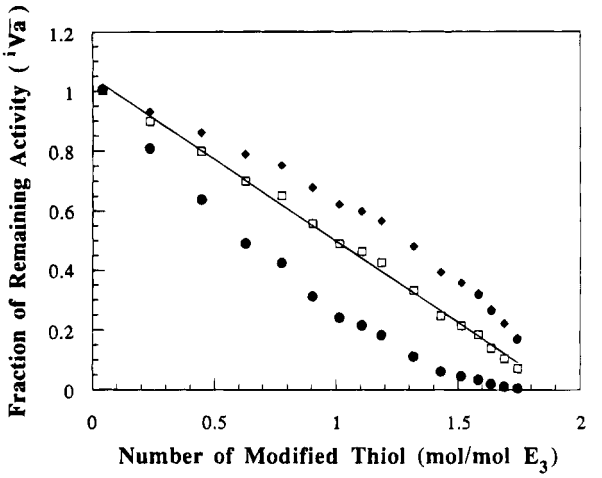


FIGURE 4: Tsou plot for the inactivation of E_3 with DTNB. E_3 (10.5 nmol) was incubated with 0.5 mM DTNB in 0.1 M Tris-HCl buffer (pH 8.0) at room temperature for 60 min. The absorbance at 412 nm (ϵ_{412} = 14 150 M^{-1} cm^{-1}) together with the activity were monitored at the same time (see Experimental Procedures). The fraction of residual activity, $a^{1/i}$, was plotted against the number of modified thiols, (●) i = 1, (□) i = 2, (♦) i = 3, per E_3 monomer. The data points for i = 2 were fitted to a straight line using a least squares regression analysis: $a^{1/2} = 1 - x/1.9$, r = 0.99.

line when i = 2. The abscissa intercept, N = 1.9, indicates that only two cysteine residues are modified by DTNB during inactivation. The other points shown in Figure 4 are a plotting of the same data but assuming that one or three essential residues are responsible for the inactivation. Clearly, when i = 1 or i = 3, the data deviate from a linear relationship. On the basis of these analyses, we can now conclude that E_3 has two cysteines reacting with DTNB at a similar rate and both seem to be critical for E_3's catalytic activity.

Identification of the Modified Cysteines. Because the inactivation of DTNB with E_3 is reversible, *N*-ethylmaleimide (NEM), which exhibits similar inhibition behavior as DTNB, albeit irreversibly, was used to label the two reactive cysteines. As expected, incubation of E_3 with [2,3-^{14}C]NEM, as described under Experimental Procedures, yielded an inactive protein containing 1.9 equiv of NEM per monomer, which is consistent with two cysteines being labeled. Tryptic digestion of the labeled protein gave a mixture of peptides that were fractionated by reversed phase HPLC. Although inspection of the translated amino acid sequence predicts the three free cysteines (not ligands to the metal center) in E_3 should be on separate tryptic fragments, surprisingly, four (A, B, C, and D) instead of the anticipated two radioactive peptides were obtained (Figure 5). It was also intriguing to find that, when pure peptide A was incubated in 50 mM ammonium formate buffer (pH 8.5, 1 mM DTT) at room temperature for 1 h and then reinjected into reversed phase HPLC, two radioactive peaks were detected with retention times corresponding to peptides A and B. Likewise, treating pure peptide B under the same conditions yielded a mixture of peptides A and B. These observations clearly demonstrated that A and B are interconvertible. More importantly, sequencing of these two fragments by standard Edman degradation showed their amino acid sequences to be identical. The exact sequence cognition of peptides A and B strongly suggested that the sole distinction of these two peptides must reside on the appended NEM moiety. Interestingly, the PTH derivatives generated during sequencing of each fragment in its pure form gave a double peak upon

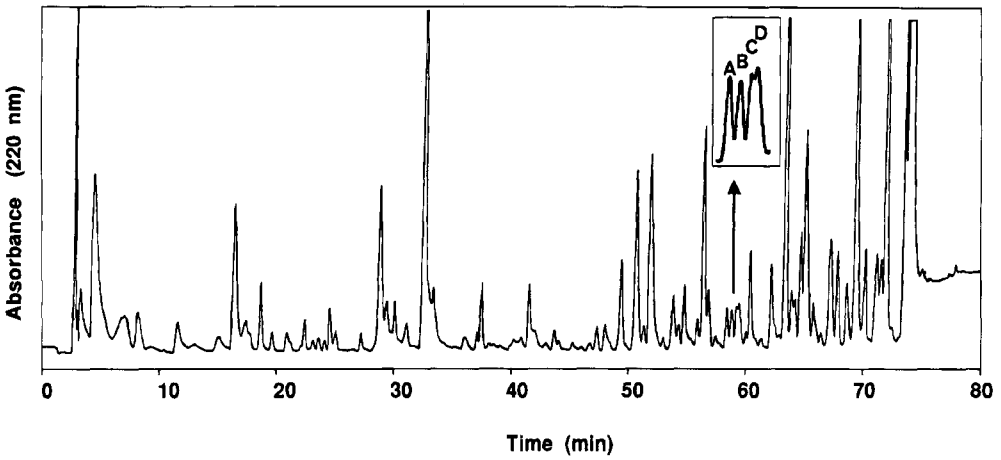


FIGURE 5: Fractionation of the tryptic digest of [2,3-^{14}C]NEM-labeled E_3 by HPLC. The tryptic digest of labeled E_3 (10 nmol), containing 1 mM DTT, was applied to a Vydac C_{18} (4.6 mm × 25 cm) column and eluted at a flow rate of 1 mL/min with a linear gradient of acetonitrile (from 0% to 25% in 70 min) in 0.1% (v/v) trifluoroacetic acid/water (see Experimental Procedures). The elution was monitored at 220 nm and the eluate counted for radioactivity. Inset is the enlargement of the HPLC trace showing the four radioactive peaks A, B, C, and D. Fragments C and D are separable upon further purification under identical conditions.

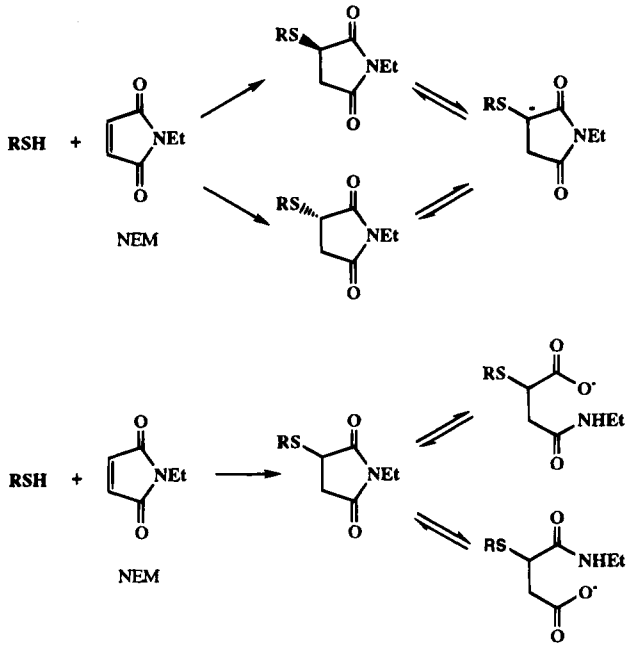


FIGURE 6: Reversible interconversion between two diastereoisomers and regioisomers of *N*-ethylsuccinamic acid.

HPLC elution, only in the cycle corresponding to Cys, substantiating the interconvertibility of the cysteine−NEM derivatives even under the Edman degradation conditions.

Since coupling of NEM and cysteine generates a new chiral center in the S-(*N*-ethylsuccinimido)cysteinyl thioether adduct, fragments A and B could be a pair of diastereomeric cysteinyl peptides arising from a nonstereoselective addition of the SH to the double bond of NEM (Figure 6) or an epimerization of the resulting adduct. However, in view of the fact that the imide ring of the NEM−cysteine product is reactive toward nucleophiles and labile under alkaline pH (Gehring & Christen, 1983), the reversible nonregioselective hydrolysis of the S-(*N*-ethylsuccinimido)cysteinyl thioether adduct to the corresponding *N*-ethylsuccinamic acid during tryptic digestion (trypsin, pH 8.5, overnight) and also during peptide sequencing is another appealing alternative. The ready separation of peptides containing aspartyl and β-aspartyl residues by HPLC nicely demonstrated the feasibility of detecting such regioisomers (Barany & Merrifield, 1980). Furthermore, it has been noted that *S*-(*N*-ethylsuccinimido)-cysteine could undergo partial hydrolysis to give *S*-(1-carboxy-2-(*N*-ethylcarbamoyl)ethyl)cysteine, which is believed to recyclize swiftly under appropriate conditions (Smyth et al., 1964). Thus, as illustrated in Figure 6, formation of such regioisomers could also account for the two peaks observed both upon HPLC of NEM labeled tryptic peptides (A and B) and upon HPLC of the PTH-Cys derivative resulting from Edman degradation. However, a molecular mass consistent with an intact adduct was observed by FAB-MS analysis. This result strongly supports the conclusion that peptides A and B are diastereomeric isomers derived from nonstereoselective addition of cysteinyl-SH to the double bond of NEM or post-addition epimerization of the adduct. The fact that the structure of succinimidocysteinyl thioether adduct remains intact suggests a reversible deprotonation−reprotonation route as the cause for the interconversion between peptides A and B. Interestingly, when a similar situation was previously encountered during inactivation studies of pig heart $NADP^+$-dependent isocitrate dehydrogenase by NEM (Smyth & Colman, 1991), the formation of such diastereomeric products has been invoked. The relatively facile interconversion between these two diastereoisomers at pH 8.5 and during the Edman degradation demands the hydrogen at the newly generated asymmetric center to be readily extractable and exchangeable, a prerequisite which has been substantiated by a NMR study of the *N*-ethylsuccinimide cysteinyl thioether structure derived from glutathione (Kuninori & Nishiyama, 1985). Since epimerization of the *N*-ethylsuccinimide cysteinyl thioester model was found to occur at pH 7.0 (Kuninori & Nishiyama, 1985), the appearance of two epimers for each tryptic fragment is expected even if the incubation is conducted at pH 7.

Because only two labeled peptides are predicted after typtic digestion from which A and B are derivatives of one of them, it is expected that peptides C and D are also a pair of interconvertible isomers. Therefore, only peptide C was sequenced. While the sequence obtained for peptide C is GAILTCCSK (Figure 7) which corresponds to residues 69−77 of E_3, peptides A and B gave a sequence of ACGSLAM (Figure 7) which matches residues 295−301 in the translated sequence of *asc*D. Interestingly, the latter sequence indicates that peptides A and B resulting from cleavage between Tyr_{294}-Ala_{295} and Met_{301}-Ile_{302} are not products of tryptic

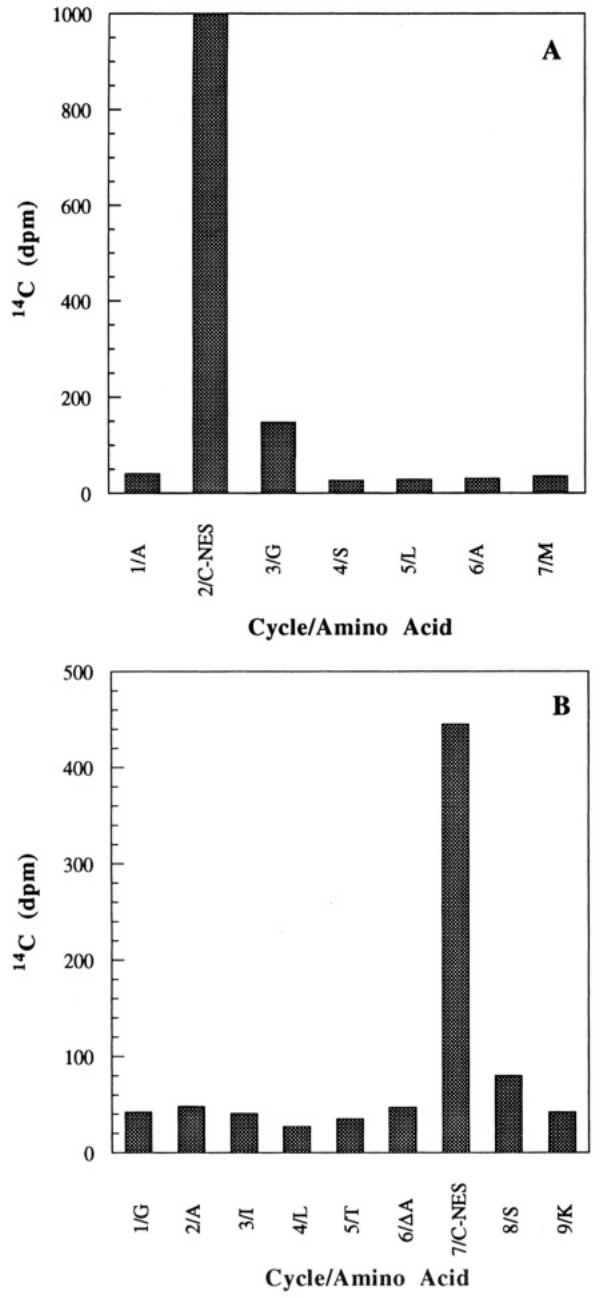


FIGURE 7: Determination of amino acid sequence for peptides B and C. Peptides B (200 pmol) and C (100 pmol) were subjected to automated Edman degradation. After each cycle the effluents were collected and an aliquot of each was counted for radioactivity. Plots A and B show the data obtained for peptides B and C, respectively. ΔA: dehydroalanine, the degradation product of cysteine. C-NES: the adduct of cysteine and NEM.

digestion, but may derive from contaminated chymotrypsin activity. It should be noted that all the radioactivity was released during one cycle of the Edman degradation of each tryptic fragment and was found to be associated with a cysteine residue (Figure 7), confirming the specificity of NEM toward sulfhydryl groups in E_3 inactivation. The two reactive cysteines are thus identified as Cys-75 and Cys-296.

Characterization of C75S and C296S Mutant Proteins. Each of the mutant genes constructed by the overlap extension PCR method was sequenced, and the desired mutation was confirmed. The mutant proteins were expressed, purified, and characterized. The iron−sulfur contents (1.8 ± 0.1 mol of [Fe-S]/mol of enzyme and the electronic absorption spectra of the mutant proteins are identical to those of the wild-type E_3, indicating that both coenzymes, FAD and [2Fe-2S] cluster, are intact in the mutant proteins. While the mutant proteins exhibit slightly different kinetics (Table 2), both mutants are still catalytically active.

Table 2: Kinetic Parameters of Wild-Type E_3 and the C75S and C296S Mutants

source of enzyme	$K_{m,NADH}$ (μM)	V_{max} (U/mg)[a]	V_{max}/K_m
wild-type	23.8	62.5	2.63
C75S	26.3	49.1	1.87
C296S	30.3	87.0	2.87

[a] 1 U = 1 μmol of DCPIP·min^{-1}.

DISCUSSION

There are many flavoprotein reductases that catalyze the transfer of two electrons as a hydride ion from NAD(P)H to a one-electron acceptor, typically an iron-sulfur center or a cytochrome. However, E_3 belongs to a subgroup of reductases that contain not only a flavin prosthetic group but also a fused [2Fe-2S] domain (Mason & Cammack, 1992). Early spectroscopic analyses had established the characteristics of E_3's iron−sulfur cluster as a plant ferredoxin type [2Fe−2S] center (Miller et al., 1993). True to expectations, significant residue congruence was found between E_3 and proteins carrying a plant ferredoxin type center. Since the four cysteine residues serving as ligands in the [2Fe-2S] cluster are highly conserved among proteins of this class, an array of cysteines including Cys-36, Cys-41, Cys-44, and Cys-74 can be assigned as the binding fold for the metal cluster. The spacing between the cysteines in **C**XXXX-**CXXC**, where X is any amino acid, with a fourth cysteine approximately 30 residues downstream toward the carboxy-terminus was characteristic for [2Fe-2S] ferredoxins (Mason & Cammack, 1992).

Incubation of E_3 with thiol-directing agents resulted in a time-dependent inactivation of the enzyme with a rate constant of 0.25 mM^{-1} min^{-1} at low DTNB concentration (less than 0.3 mM). Since the absorption of the iron−sulfur chromophore was unchanged in the inactivated enzyme and the iron−sulfur quantitation gave comparable results as those of the native protein, the observed inactivation must result from modification of free cysteines in E_3, not the ligands of the metal cluster. That the bulk of the enzyme activity can be regenerated under reducing conditions further substantiated that the iron−sulfur cluster was intact. It is worth mentioning that the flavin absorption at 446 nm also remained virtually unchanged, indicating the preservation of the surroundings of the isoalloxazine ring in the inactivated protein.

Sequence analysis has shown that E_3 contains seven cysteine residues per molecule. Because only four are required to constitute the plant ferredoxin type [2Fe-2S] center and no cysteines are coupled to form disulfide linkage based on thiol titration, it is thus conceivable that any one of the remaining three free cysteines could be modified by DTNB or NEM. Further investigation showed that complete inactivation of E_3 is accompanied by the modification of two cysteine residues per monomer. However, it was not obvious whether the loss of enzyme activity resulted from alteration of one or both cysteines. In order to establish the number of critical residues, the method of Tsou was employed. Our

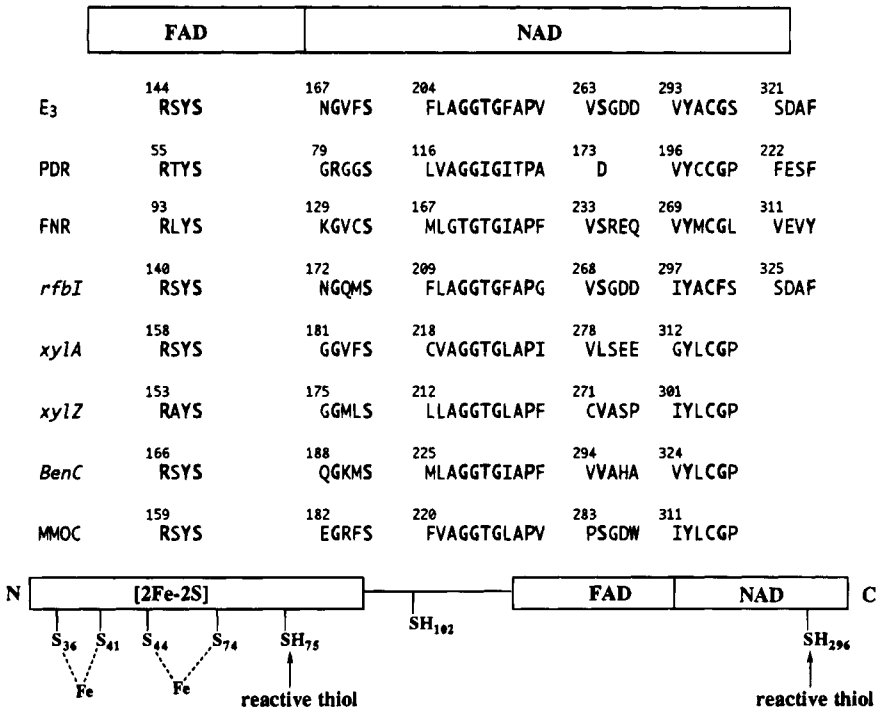


FIGURE 8: Alignment of the adenosine dinucleotide domains of E_3 with analogous domains conserved throughout the FNR family and the domain arrangement of E_3. The postulated residues that interact with the substrate and prosthetic groups are in boldface. Abbreviations: PDR, phthalate dioxygenase reductase; FNR, ferredoxin-NADP$^+$ reductase; *rfb*I, the E_3 equivalent from *Salmonella typhimurium* (Jiang et al., 1991); *xyl*A, xylene monooxygenase reductase (Neidle et al., 1991); *xyl*Z, toluene 1,2-dioxygenase reductase (Neidle et al., 1991); *ben*C, benzoate 1,2-dioxygenase reductase (Neidle et al., 1991); MMOC, methane monooxygenase reductase component C (Stainthorpe et al., 1990).

result suggests that modification of either cysteine is sufficient to impair E_3.

The identity of the reactive cysteines was determined by radiolabeling of the enzyme with [2,3-^{14}C]NEM and sequencing the radiolabeled peptides obtained after tryptic digestion. The detection of a double peak for each labeled tryptic fragment may be ascribed to the presence of two diastereomeric isomers derived from nonstereoselective coupling of SH and NEM, and/or the reversible epimerization at the newly generated chiral center on the succinimide ring. This observation represents a rare example in which the isomers of NEM modified peptide are resolvable and separable and, more importantly, are interconvertible at basic pH and during Edman degradation. Our sequencing data revealed that both Cys-75 and Cys-296 are modified in the inactivated E_3. This outcome suggested that Cys-102, which is not conserved among iron−sulfur flavoproteins and is an unlikely ligand to the metal cluster, may be a buried residue.

As mentioned earlier, E_3 is closely related to phthalate dioxygenase reductase (PDR) and a few other iron−sulfur flavoprotein reductases. On the basis of the structural and sequence similarities, PDR, whose three-dimensional crystal structure was recently determined (Correll et al., 1992), has been assigned to a distinct family of flavoprotein reductases represented by ferredoxin-NADP$^+$ reductase (FNR) (Karplus et al., 1991). Since the six peptide fragments involved in recognition of flavin nucleotides and pyridine nucleotides that have been identified as a fingerprint for the extended FNR family of proteins were also found to be conserved in E_3 (Figure 8), E_3 must be a new member of this protein class (Correll et al., 1993). The difference among E_3, FNR, and PDR lies in their association with the [2Fe-2S] cluster. While the metal cluster in FNR is located on a completely separate protein, ferredoxin, it is an intergral part of the enzyme in PDR and E_3. The iron−sulfur cluster appears to be linked to the amino-terminus of the flavin binding domain in E_3; however, in PDR the [2Fe-2S] cluster is located at the carboxy-terminus of the nicotinamide cofactor binding domain.

According to this deduced domain alignment, it is now possible to assign the two reactive cysteine residues Cys-75 and Cys-296 to be located within the metal cluster domain and the NADH binding site, respectively (Figure 8). The fact that modification of Cys-296 afforded inactive E_3 is not too surprising, since this residue is highly conserved in the class of iron−sulfur flavoproteins and also among NAD(P)H flavoproteins such as FNR and cytochrome b_5 reductase. The catalytic role of this invariant cysteine in the latter two enzymes had been probed by site-directed mutagenesis. While a C277S mutation of FNR led to a 7-fold decrease in its catalytic efficiency (Aliverti et al., 1993), the corresponding C273S mutant of cytochrome b_5 reductase retained essentially all the wild-type activity (Hackett et al., 1986). Although such a disparity may be attributed to a different topology of the NAD(P)H binding site of these two enzymes, the fact that the mutant proteins remain active suggests a nonessential role of this conserved cysteine in the catalysis. The same conclusion can also be applied to E_3, since its C296S mutant is still catalytically active. It was proposed that this cysteine may assist in stabilizing the charge-transfer complex (FAD·NAD[P]H) between the reductase and NAD(P)H, facilitating hydride transfer from nicotinamide nucleotide to flavin (Aliverti et al., 1993). In view of the fact that this cysteine is a conserved residue in the NAD(P) binding domain of all of the enzymes belonging to the FNR family (Aliverti et al., 1993), Cys-296 of E_3 may assume an analogous role in catalysis.

Unlike Cys-296, Cys-75 is not an invariant residue among iron−sulfur flavoproteins, but it is conserved in E_3 (from *Y. pseudotuberculosis*) and its equivalent (*rfb*I product) from *Salmonella typhimurium* (Jiang et al., 1991). The apparent inability of NADH to protect E_3 from being inactivated by DTNB is consistent with the fact that an unmodified Cys-75 is essential for E_3's activity. Protection of Cys-296 alone by NADH could not stop the modification of Cys-75; however, the rate of inactivation appeared to be retarded. Although Cys-75 is immediately adjacent to the iron−sulfur cluster in E_3, chemical modification of this residue did not distort the metal center whose vicinity must be relatively unhindered. However, a minor local conformational change resulting from Cys-75 modification could significantly impair the electron transfer between the two redox centers, FAD and [2Fe-2S]. Indeed, the three-dimensional structure of PDR revealed a close contact (4.7 Å) between the 8-Me of flavin and one of the Cys-S ligand of its iron−sulfur cluster (Correll et al., 1992). The cysteine ligand (Cys-353) of the [4Fe-4S] center and the 8-methyl group of the covalently bound FMN was also reported to be within close proximity (4 Å) in trimethylamine dehydrogenase (Lim et al., 1986). In light of the high sequence, and perhaps structural, homology among these proteins, a chemically modified Cys-75 in E_3 may prevent the proper juxtaposition of the redox centers along the electron conduit and thus impede electron transfer leading to enzyme inactivation. Although thiol and disulfide moieties have been implicated as essential residues involved in several biological electron transfer processes and enzymatic radical mechanisms (Baldwin et al., 1991; Mao et al., 1992; Silverman & Zieske, 1985), the possibility that Cys-75 may directly partake in shuttling electrons from FAD to [2Fe-2S] center in E_3 was ruled out by the finding that the C75S mutant is still catalytically competent. Interestingly, previous doubt as to which cysteine, Cys-74 or the adjacent Cys-75, is a part of the metal cluster was also clarified by these studies. The labeling pattern revealed in the NEM inactivation experiment and the retention of the iron−sulfur center in the C75S mutant are clearly consistent with the assignment of a metal cluster motif deduced from amino acid sequence comparison.

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BI941890W