Articles

Biosynthesis of 3,6-Dideoxyhexoses: *In Vivo* and *in Vitro* Evidence for Protein−Protein Interaction between CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-Dehydrase (E_1) and Its Reductase (E_3)†

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ABSTRACT: CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydrase (E_1), together with its reductase (E_3), catalyzes a novel deoxygenation reaction essential for the biosynthesis of 3,6-dideoxyhexoses. In an attempt to gain evidence substantiating the $E_1 \cdot E_3$ complex formation as a prerequisite for the C-3 deoxygenation activity, we have carried out experiments to study the interaction between these two proteins. The detection of a new species when a mixture of E_1 and E_3 was analyzed by size-exclusion chromatography was the initial indication supporting the proposed complex formation. Additional evidence for the expected complex formation was provided by the change of the CD spectrum of E_1 upon its coupling with E_3. The fact that the catalytic efficiency of this system is limited by the quantity of one enzyme, which becomes catalytically competent only after coupling with the second enzyme, further illustrated the importance of such a complex formation to the deoxygenation activity. By using the two-hybrid system which scores for interactions between two proteins coexpressed in yeast, the $E_1 \cdot E_3$ complex formation *in vivo* was also firmly established. These results, when considered with the incompatibility of other electron transfer proteins as replacements for E_3 in this electron relay, nicely demonstrated the specificity of the E_1-E_3 recognition. The apparent dissociation constant of the $E_1 \cdot E_3$ complex formed in rapid equilibrium was estimated to be 288 ± 22 nM from the correlation between the initial rate of the overall reaction and the concentration of one protein component, and the stoichiometry between E_3 and E_1 of this complex was deduced as 1.7. Interestingly, while the conformation of the $E_1 \cdot E_3$ complex was sensitive to the salt concentration in the buffer, the decrease in the catalytic activity at high ionic strength was most likely due to the retardation of the electron transfer mediated by E_3. In conjunction with early mechanistic studies, the present data establish the significance of the $E_1 \cdot E_3$ complex formation for catalysis and, consequently, corroborate the mechanism proposed for the overall deoxygenation process.

Studies of the biosynthesis of ascarylose (**1**), a 3,6-dideoxy-L-*arabino*-hexopyranose found in the lipopolysaccharide of *Yersinia pseudotuberculosis* V (Ovodov et al., 1992), have shown that the C-3 deoxygenation is a process consisting of two enzymatic steps (Liu & Thorson, 1994). The first half of this transformation is a dehydration catalyzed by CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydrase (E_1)[1] (Rubenstein & Strominger, 1974a; Weigel et al., 1992a,b). This enzyme is unique since it is the only pyridoxamine 5′-phosphate (PMP) dependent protein that also contains an adrenodoxin/putidaredoxin-like [2Fe-2S] center (Thorson & Liu, 1993a). The second half of the reaction is a NADH-dependent reduction initiated by CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose 3-dehydrase reductase (E_3) (Rubenstein & Strominger, 1974b; Miller et al., 1993). E_3, formerly known as CDP-$\Delta^{3,4}$-glucoseen reductase, contains a plant-type ferredoxin [2Fe-2S] cluster and a FAD in its active site (Miller & Liu, 1992). The proposed mechanism of this deoxygenation is shown in Scheme 1. The PMP coenzyme of E_1 is responsible for the reversible dehydration of **2**. The conversion of the nascent intermediate **3** to CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**4**) is initiated upon reduction of the E_3 FAD by NADH, followed by electron transfer from the reduced flavin to the E_3 [2Fe-2S]

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[1] Abbreviations: CDP, cytidine 5′-diphosphate; CD, circular dichroism; CTP, cytidine 5′-triphosphate; DCPIP, dichlorophenolindophenol; DEAE, diethylaminoethyl; DMS, dimethyl suberimidate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 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; E_p, α-D-glucose-1-phosphate cytidylyltransferase; EDC, [1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide]; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; FPLC, fast protein liquid chromatography; MMO, methane monooxygenase; NAD^+, β-nicotinamide adenine dinucleotide; NADH, β-nicotinamide adenine dinucleotide, reduced form; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; UV, ultraviolet; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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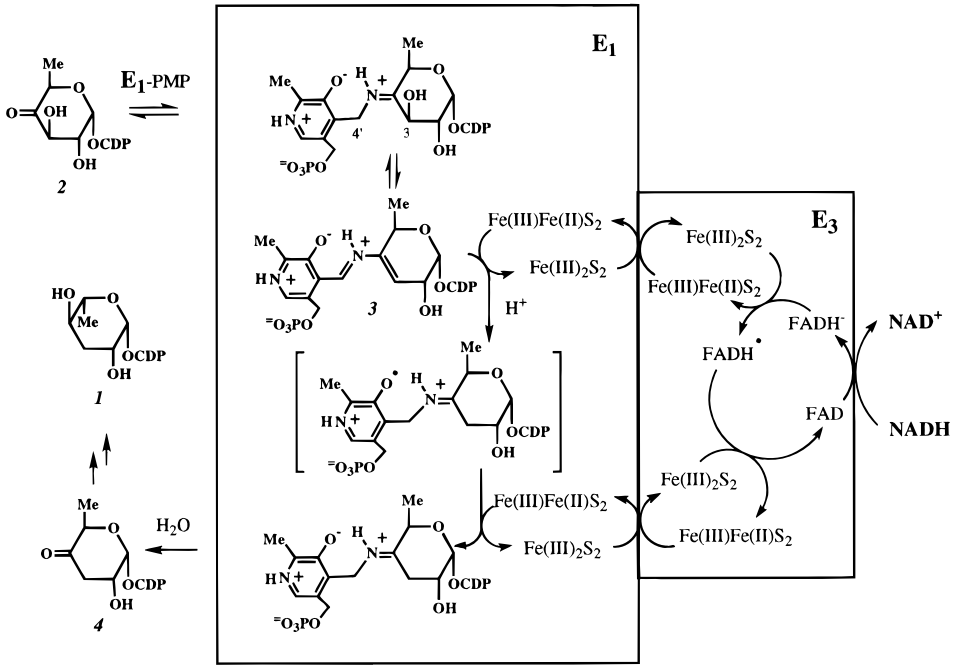
Scheme 1



center, which then relays the reducing equivalents via the [2Fe-2S] center in E_1 to **3** (Miller et al., 1993). Since these [2Fe-2S] centers are well-known one-electron carriers, the reduction of the PMP-glucoseen intermediate **3** must occur via a radical process. The formation of an E_1·E_3 complex is proposed to be a prerequisite for the overall catalysis, especially since these two proteins must have an intimate interaction for the electron-relay process to proceed from the iron−sulfur center of E_3 to that of E_1. This catalytic model was supported by the detection of a phenoxyl radical intermediate in an EPR study (Thorson & Liu, 1993b) and was also implicated in a recent kinetic analysis of the reduction catalyzed by E_1 and E_3 in which electron transfer from E_3 to E_1 was clearly noted (Gassner et al., 1996; Johnson et al., 1996). However, the more fundamental issues related to the occurrence and significance of such an enzyme complex formation have never been addressed. In order to further substantiate the proposed catalytic model shown in Scheme 1, we have carried out experiments to test the formation of an E_1·E_3 complex and to estimate the affinity of these proteins. Reported herein are the results of these studies which provide both *in vitro* and *in vivo* evidence for the E_1·E_3 complex formation.

MATERIALS AND METHODS

General. *Escherichia coli* strain HB101 was purchased from Bethesda Research Laboratories (Gaithersburg, MD) and used for general DNA manipulation. Yeast strain *Saccharomyces cerevisiae* L40 (Vojtek et al., 1993), plasmid vector pBTM116 for the DNA-binding domain LexA hybrid, and pVP16 for the activation domain VP16 hybrid, as well as control plasmids pLexA-RAS, pVP-CYR, pLexA-VP, and pLexA-lamin, were kindly provided by Drs. Stanley Hollenberg (Fred Hutchinson Cancer Research Center, Seattle, WA) and Timothy Behrens (Department of Medicine, University of Minnesota, Minneapolis, MN). DEAE-Sepharose CL-6B, Phenyl Sepharose CL-4B, and the FPLC columns Mono-Q 10/10 and Superdex 200 HR 10/30 were products of Pharmacia (Piscataway, NJ). The general culture media components were from Difco (Detroit, MI), and the amino acids for preparation of synthetic media were from Sigma (St. Louis, MO). Synthetic oligonucleotides were prepared by Integrated DNA Technologies (Coraville, IA). *Taq* DNA polymerase, dNTPs, and buffer used in PCR were obtained from Promega (Madison, WI). All restriction enzymes, DNA modification enzymes, and their respective buffers were purchased from Amersham (Arlington Heights, IL). The molecular weight standards, other biochemicals, and chemicals were purchased from Sigma, Fisher Scientific (Pittsburgh, PA), or Aldrich (Milwaukee, WI) and were of the highest purity available. Methods and protocols for recombinant DNA manipulations are generally referenced (Ausubel et al., 1989; Sambrook et al., 1989).

Enzyme Preparation. Enzyme E_1 was prepared from *E. coli* strain HB101-pJT18 as previously described (Lei et al., 1995). The purified protein contained 2.45 equivalents of iron per E_1 dimer whose absorbance ratios A_{448}/A_{280} and A_{330}/A_{280} were 0.097 and 0.227, respectively. The native E_1 was partially denatured and reconstituted by a newly developed procedure to give 3.95 Fe per homodimer (Johnson and Liu, unpublished results). Enzyme E_3 was prepared from *E. coli* strain JM105-pOP1 according to a published procedure (Ploux et al., 1995). The purified E_3 displayed an A_{454}/A_{271} ratio of 0.323 and was found to have 1.90 Fe per molecule. All buffers used in the protein purifications and *in vitro* studies were degassed and saturated with nitrogen. The concentration of the protein samples used in the kinetic measurements was determined by quantitative amino acid analysis performed by the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota. Unless otherwise specified, the concentration of E_1 cited throughout this paper is per functional dimer and that of E_3 is per monomer. The exact amount of the enzyme-contained iron was determined by the method of Fish (1988).

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out at 25 mA using a discontinuous buffer system

(Laemmli, 1970). The separating gel and the stacking gel were 12% and 4% polyacrylamide, respectively. Gels were stained with Coomassie blue (Vesterberg, 1971) and destained with acetic acid−ethanol−water (15:20:165 by volume).

Enzyme Assays. The catalytic activity of E_1 was determined by a recently published E_1-E_3 coupled assay (Lei et al., 1995). The E_3 activity was measured by following its capability to catalyze NADH reduction of 2,6-dichlorophenolindophenol (DCPIP) as previously described (Lo et al., 1994). The specific activities of the reconstituted E_1 and the purified E_3 used in this work were 188.7 and 47.5 units/mg, respectively.

Synthesis of E_1 Substrate. Preparation of E_1 substrate, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**2**), was achieved by a newly developed method using α-D-glucose 1-phosphate as the starting material. In this preparation, α-D-glucose 1-phosphate (16 mg, 44.6 μmol), CTP (22 mg, 44.6 μmol), and NAD^+ (5 mg, 7.5 μmol) were dissolved in 50 mM potassium phosphate buffer, which also contained 10 mM $MgCl_2$ (pH 7.5). To this solution were added α-D-glucose-1-phosphate cytidylyltransferase (E_p, 0.32 mg) and CDP-D-glucose 4,6-dehydratase (E_{od}, 0.90 mg) to a final volume of 3 mL. The mixture was incubated at 37 °C for 1 h. The desired product was isolated by FPLC with a MonoQ 10/10 column using the published protocol (Thorson et al., 1994a). The concentration of this substrate was determined spectrophotometrically (Yu et al., 1992). The E_p and E_{od} were purified from *E. coli* HB101-pJT12 (Thorson et al., 1994b) and HB101-pJT8 (Thorson et al., 1994a), respectively, as previously reported.

Size-Exclusion Chromatography and Molecular Weight Determination of the E_1·E_3 Complex. Samples of native E_1 and E_3 (20 μM each in 50 μL of 50 mM potassium phosphate buffer, pH 7.5) were loaded separately onto a FPLC Superdex 200 HR 10/30 column which was preequilibrated with the same buffer. Also, a mixture of these two proteins preincubated at room temperature for 1 h was subjected to the same analysis. The respective retention times for E_1, E_3, and the E_1·E_3 complex collected in several runs were averaged. A calibration curve was constructed according to the method of Andrews (1964) using a series of protein standards which included β-amylase (200.0 kDa), yeast alcohol dehydrogenase (150.0 kDa), apotransferrin (81.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), cytochrome *c* (12.3 kDa), and blue dextran 2000 (void volume determination). Each standard was dissolved in 50 μL of 50 mM phosphate buffer (pH 7.5) and injected separately to obtain their retention times. The apparent molecular masses of E_1, E_3, and the E_1·E_3 complex were estimated by calibration against the standard curve.

Effect of E_1·E_3 Complex Formation on the Rate of Catalysis and Estimation of the Binding Affinity of These Proteins. The E_1−E_3 coupled assay (Lei et al., 1995) was used to assess the effect of the E_1·E_3 complex formation on the rate of catalysis under turnover conditions. Included in the assay mixture were 100 μM CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**2**), 25 μM PMP, 200 μM NADH, and 1.18 μM E_3 in 800 μL of 50 mM potassium phosphate buffer (pH 7.5). The reaction was initiated by the addition of E_1 (0.12−1.78 μM) and monitored spectrophotometrically at 340 nm (ε 6.22 mM^{-1} cm^{-1}) within the first 30 s after mixing. The affinity and stoichiometry between E_1 and E_3 of this complex were determined by plotting the rates of NADH consumption versus the corresponding E_1 concentrations. Since the E_1·E_3 complex is formed in rapid equilibrium with free E_1 and E_3 molecules ($E_1 + nE_3 \rightleftharpoons E_1{\cdot}nE_3$), and if one assumes that there are *n* mol of E_3 bound to E_1 at equilibrium, the apparent dissociation constant (K_d) of this complex should be $K_d = ([E_1] - c)([E_3] - nc)/c$, where $[E_1]$ = the initial E_1 concentration in the reaction mixture, $[E_3]$ = the initial E_3 concentration, n = the ratio between E_3 and E_1 in the complex, and c = the concentration of the postulated $E_1{\cdot}nE_3$ complex at equilibrium. Since the overall reaction rate v is proportional to the concentration of the E_1·E_3 complex, the data collected in this experiment could be analyzed by fitting the plot of v versus $[E_1]$ to eq 1 (in

$$v = \frac{k\{(n[E_1] + [E_3] + K_d) - [(n[E_1] + [E_3] + K_d)^2 - 4n[E_1][E_3]]^{1/2}\}}{2n} \quad (1)$$

which v = the observed reaction rate in μM min^{-1}, k = the first-order rate constant in min^{-1}, K_d = the apparent dissociation constant of the E_1·E_3 complex in nM, and the remaining symbols have the same definition as indicated above) to estimate the stoichiometry and the affinity of the E_1·E_3 complex.

Detection of in Vivo E_1·E_3 Complex Formation by the Two-Hybrid System. The two vectors used in the two-hybrid system assay were pBTM116, which contains the DNA-binding domain of bacterial LexA protein, and pVP16, which harbors the nuclear localized VP16 acidic activation domain of herpes virus protein. The selectable marker of pBTM116 is the yeast *TRP*1 gene, and that of pVP16 is the *LEU*2 gene. Both genes are impaired in the host strain *S. cerevisiae* L40. The complementarity achieved after transformation with these plasmids allows the yeast host to grow on selective media lacking tryptophan, leucine, and uracil. The reporter genes carried by the yeast host strain are the *lacZ* and *HIS*3 genes. Both contain an upstream binding site for the LexA protein.

(1) Plasmid Constructs. The genes of E_1 (*asc*C) and E_3 (*asc*D) were amplified by PCR using the respective plasmids pJT18 (Thorson et al., 1994a) and pOP1 (Ploux et al., 1995) as the templates. Two sets of oligonucleotides, 5′-GCGC-GAATTCAGTCAAGAAGAATTA-3′/5′-CGACGGATC-CCTTAAAAATTCAAAC-3′ and 5′-CGGTGGATCCCCT-CATTAAATGTTAAG-3′/5′-GCGGGAATTCTTAG-CTATTTTGATGGCA-3′, were used as the forward/halt primers for the *asc*C and *asc*D genes, respectively. In each case, the start primer contains a restriction site for *Eco*RI, and the halt primer includes a *Bam*HI site immediately downstream from the target gene. In addition, the start codon ATG of each desired gene was deleted to facilitate the subsequent in-frame cloning. The resulting PCR-amplified *asc*C gene was cloned into pBTM116 to give the recombinant plasmid pBTM116-E_1. Similarly, the gene of E_3 (*asc*D) was introduced into vector pVP16 to give plasmid pVP16-E_3. These recombinant plasmids were separately transformed and maintained in *E. coli* strain HB101.

(2) Yeast Transformation. The yeast strains used in this work were grown and manipulated according to standard procedures (Ausubel et al., 1989). Plasmid DNAs were

purified from *E. coli* HB101 using standard miniprep protocols, and the transformations were carried out by the high-efficiency method of Schiestl and Gietz (1989) using Li^+ to prepare the competent yeast cells. Two hybrid plasmids, pBTM116-E_1 and pVP16-E_3, were introduced simultaneously into the host cells at equal quantity. Synthetic media with 2% glucose as the carbon source but lacking tryptophan, leucine, and uracil were used in selection of yeast cells cotransformed with both hybrid plasmids. Control strains were also constructed by transforming various plasmids (pLexA-Ras, pVP-CYR, pBTM116, pVP16, pBTM116-E_1, and pVP16-E_3) in combinations into L40 cells.

(3) β-Galactosidase Assay. Plates prepared with synthetic media (Trp^-, Leu^-, and Ura^-) containing 2% sucrose as the carbon source and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal, 40 μg/mL) as the substrate for β-galactosidase were used to detect the transformed yeast cells that expressed β-galactosidase activity. Transformants were first allowed to grow in synthetic selective media in the presence of 2% glucose at 30 °C for 2 days and then replica-plated to the above X-gal plates for further incubation at 30 °C. The cells that expressed β-galactosidase activity produced blue colonies. A filter assay (Breedan & Nasmyth, 1985) was also used to confirm the results.

Effect of Ionic Strength on the Catalytic Activity of the E_1·E_3 Complex. The effect of buffer ionic strength on E_1·E_3 complex formation was deduced from the determination of its effect on the activity of the resulting complex using the E_1-E_3 coupled assay. The assay mixture consisted of 100 μM substrate (**2**), 25 μM PMP, 200 μM NADH, 2.0 μM E_1, and 0.98 μM E_3 in 800 μL of 50 mM potassium phosphate buffer (pH 7.5). The concentration of KCl in the assay solutions was varied from 0 to 1 M. The corresponding ionic strengths were calculated to be 0.02−1.02 M on the basis of $I = (1/2)(\sum z_i^2 m_i)$, where z_i is the charge of ion i and m_i is the molarity of ion i (Noggle, 1985). The catalytic activity of the enzyme complex was monitored by the rate of NADH consumption as described above. The effect of ionic strength on the NADH oxidase activity of E_3 (Han et al., 1990) alone was also tested.

Circular Dichroism Spectra of the E_1·E_3 Complex at Different Ionic Strengths. The conformational change of E_1 upon interaction with E_3 and the effect of buffer ionic strength on the conformational integrity of the E_1·E_3 complex were assessed by circular dichroism spectroscopy. Samples of E_1 (3.15 μM) or E_3 (6.30 μM) were made in 50 mM potassium phosphate buffer (pH 7.5). Mixtures of E_1 (3.15 μM) and E_3 (6.30 μM) in the same buffer, with and without 1 M potassium chloride, were also prepared. The circular dichroism spectra of these samples were recorded by scanning each solution in a 0.1 mm cell over the far-UV range (195−250 nm) using a Jasco J-710 spectropolarimeter (Jasco Inc., Easton, MD).

Cross-Linking Experiments. The cross-linking mixture contained E_1 (98.7 μg, 1 nmol), E_3 (36 μg, 1 nmol), and 50 mM EDC [1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide] in 50 μL of 50 mM potassium phosphate buffer, pH 7.5 (Fox et al., 1991). The reaction was incubated at room temperature for 10 min and quenched by 20 μL of electrophoresis tracking buffer (100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol in 50 mM Tris−HCl, pH 6.8). This mixture was analyzed by gel electrophoresis as described earlier. Other conditions attempted included (1) changing the E_1:E_3 ratio from 1:1 to 1:2 to 1:3, (2) varying the incubation time from 10 to 60 min, (3) altering the temperature from room temperature to 37 °C, (4) changing the buffer pH to 8.0 and 8.5, (5) using buffer of different ionic strengths (10 and 50 mM potassium phosphate buffer, pH 7.5), and (6) including E_1 substrate (**2**) and/or NADH in the reaction mixture. Experiments were also performed with dimethyl suberimidate (DMS) as the cross-linking reagent.

Dansylation of E_3. A batch of E_3 (3.42 mg in 3 mL) was dialyzed for 2 h against 100 mL of 10 mM sodium bicarbonate buffer (pH 10) at 4 °C with two changes. To 1 mL of this protein solution was added 15 μL of 6 mM dansyl chloride (3 molar equiv) in acetone with slow agitation under N_2 (Kincaid et al., 1982). After incubation for 2 h at 30 °C, the mixture was dialyzed against 500 mL of 50 mM potassium phosphate buffer (pH 7.5) at 4 °C for 3 h. The labeled protein was slightly yellow with an absorption maximum around 320 nm. The labeling reaction was also carried out in 50 mM potassium phosphate buffer at pH 7.5. Nearly identical degrees of protein dansylation were obtained at both pHs. A control was run without the labeling reagent. The activity of the dansylated E_3 (∼7 units/mg) was significantly lower than the native E_3 (47.5 units/mg) and the control sample (14.4 units/mg). Fluorescence measurements were made at room temperature in 50 mM potassium phosphate buffer, pH 7.5, using a Perkin-Elmer Model LS50B spectrofluorometer (F_{ex} = 315 nm, F_{em} = 490 nm). The excitation and emission slits were 6 and 16 nm, respectively.

RESULTS

Physical Evidence of E_1·E_3 Complex Formation. Probing the possible complex formation between E_1 and E_3 was first attempted by gel filtration. The retention times of E_1 and E_3 obtained by eluting each protein from a FPLC Superdex 200 column were determined to be 28.0 and 31.1 min, respectively (Figure 1). Since E_3 has a calculated molecular mass of 36 150 Da based on its translated nucleotide sequence (Miller et al., 1993; Thorson et al., 1994a), the deduced molecular mass of 37.9 kDa from its retention time calibrated against the standard curve was clearly in close agreement. An excellent correspondence was also found between the calculated molecular mass of E_1 (98 650 Da) (Thorson & Liu, 1993a; Thorson et al., 1994a) and that from the calibration curve (97.7 kDa). When a mixture containing equal moles of E_1 and E_3 was incubated with E_1 substrate **2** and analyzed under identical conditions, a clearly discernible new species in addition to free E_1 and E_3 was observed with a retention time of 26.4 min, although it was not well resolved from E_1 (Figure 1). The apparent molecular mass of this new species was estimated to be 151.9 kDa from the same calibration curve. This number is greater than that expected for a 1:1 complex (135.6 kDa), albeit smaller than the calculated molecular mass for a 1:2 E_1·E_3 complex (173.5 kDa). It is conceivable that this fraction was a mixture of both the 1:1 and 1:2 protein complexes that were not resolved by the FPLC column. A similar situation was also observed under other incubation conditions, such as in the presence of both substrate **2** and PMP, in the absence of substrate, with altered E_1:E_3 ratios, or with varied lengths of incubation time. The coexistence of free E_1 dimer, E_3, and the E_1·E_3

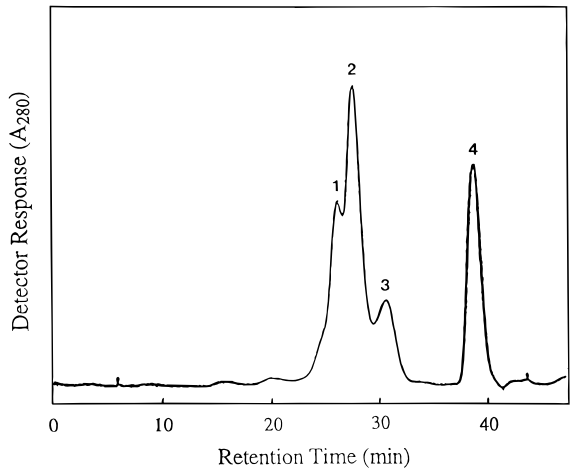


FIGURE 1: Size-exclusion chromatography of E_1 complexed with E_3 on a FPLC Superdex 200 HR 10/30 column. Native E_1 and E_3 (20 μM each in 50 μL of 50 mM potassium phosphate buffer, pH 7.5) were mixed with E_1 substrate, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**2**, 100 μM), and incubated at room temperature for 1 h prior to being loaded on the column preequilibrated with 50 mM potassium phosphate buffer (pH 7.5). Elution was carried out with the same buffer at a flow rate of 0.5 mL/min, and the detector was set at 280 nm. The peaks identified are the (1) E_1·E_3 complex, (2) E_1, (3) E_3, and (4) the E_1 substrate **2**. The same result was also obtained in the absence of substrate **2** (not shown). Details are presented under Materials and Methods.

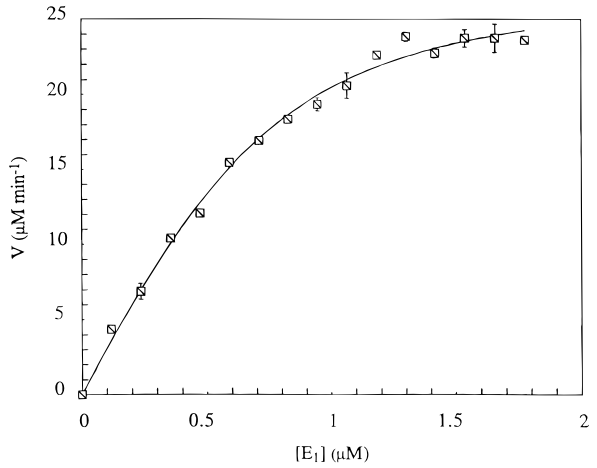


FIGURE 2: Rate of NADH oxidation catalyzed by the E_1·E_3 complex as a function of E_1 concentration. A fixed amount of E_3 (1.18 μM) was mixed with E_1 in various concentrations (from 0.12 to 1.78 μM) in 50 mM potassium phosphate buffer, pH 7.5. Reaction velocity is given in micromolar NADH consumption per minute determined as described under Materials and Methods.

complexes in the chromatographic mixture revealed moderate binding between E_1 and E_3.

Catalytic Competence of the E_1·E_3 Complex. The catalytic competence of the E_1·E_3 complex was assessed by the E_1−E_3 coupled assay that monitors the consumption of NADH. As illustrated in Figure 2, with the E_3 concentration kept constant, the rate of the enzymatic reaction exhibits pseudosaturation kinetics with the increase of E_1 concentration in the assay mixture. This result indicates that the capability of this system to reduce intermediate **3** is limited by the amount of one enzyme (e.g., E_3) which becomes catalytically competent only after forming a complex with the second enzyme (e.g., E_1). As expected, the addition of excess E_1 showed no effect on catalysis when all of the available E_3 had already associated with E_1 (Figure 2).

Table 1: Transcriptional Activation by Hybrid Proteins Assayed by Synthetic Medium Containing X-gal

plasmid	transcriptional activation
1. pLexA-RAS, pVP-CYR[a]	+ (control)
2. pBTM116, pVP16	−
3. pBTM116-E_1, pVP16-E_3	+
4. pBTM116-E_1, pVP16	−
5. pBTM116, pVP16-E_3	−
6. pBTM116-E_1, pVP-CYR	−
7. pLexA-RAS, pVP16-E_3	−
8. pVP16-E_3[b]	−
9. pVP-16	−
10. pLexA-VP[c]	+ (control)
11. pLex A-lamin	− (control)
12. pBTM116-E_1	−
13. pBTM116	−

[a] For entries 1−7, medium lacks L-tryptophan, L-leucine and uracil. [b] For entries 8 and 9, medium lacks L-leucine. [c] For entries 10−13, medium lacks L-tryptophan.

Estimation of the Dissociation Constant and Stoichiometry of the E_1·E_3 Complex. Studies of the electron transfer of the reductive half-reaction by stopped-flow spectrophotometry had revealed that the association and dissociation of E_1 and E_3 are rapid compared to the chemical steps of the reduction of intermediate **3** and the release of final product **4** (Gassner et al., 1996; Johnson et al., 1996). The gel filtration experiment described earlier also pointed out that the E_1·E_3 complex is in equilibrium with both free E_1 and E_3 molecules. Hence, the E_1·E_3 complex formation is expected to follow the classical equilibrium for a two-component system (eq 1). When a nonlinear least-squares procedure was used to fit the curve of Figure 2 to eq 1, it was found that the curve was best fitted with an apparent dissociation constant of 288 ± 22 nM, indicating a modest affinity between these two proteins. An apparent stoichiometry of 1.7 between E_3 and E_1 was also deduced from this analysis, which again indicates the coexistence of a mixture of E_3·E_1·E_3 and E_1·E_3 complexes.

Analysis of in Vivo Protein Interaction by the Two-Hybrid System. The yeast two-hybrid system, originally developed by Fields and Song (1989) and later modified by Vojtek et al. (1993), was used to examine the interaction between E_1 and E_3 *in vivo*. In these experiments, the pBTM116-E_1 plasmid expressed the E_1 gene as a fusion protein to the LexA protein, and the pVP16-E_3 plasmid gave a product with E_3 fused to the VP16 transcriptional activating domain. Individually, these hybrids are unable to activate transcription. However, if E_1 and E_3 have a strong interaction when coexpressed in the *S. cerevisiae* L40 strain, they could reconstitute the VP16 activating domain with the LexA DNA-binding domain and allow transactivation of the reporter *lac*Z gene to synthesize β-galactosidase. Several control experiments were also performed to distinguish false positives due to nonspecific protein interactions. For example, pLexA-RAS and pVP16-CYR, which contain genes encoding the wild-type H-Ras protein and the interacting leucine-rich repeat region of yeast adenylyl cyclase, respectively, were used as a positive control (Vojtek et al., 1993). The pLexA-lamin is a negative control fusion, and pLexA-VP, which has both LexA and VP16 domains fused into one open reading frame, is another positive control (Vojtek et al., 1993). Our results are summarized in Table 1.

As expected, the transcription of the *lac*Z gene was not activated by the negative control (entry 11), by the yeast

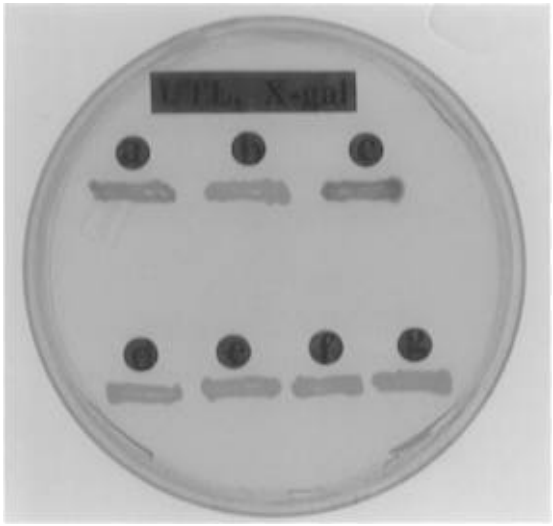


FIGURE 3: Interaction of LexA-E_1 fusion protein with VP16-E_3 fusion protein detected by the two-hybrid system: assay for β-galactosidase on X-gal plates. The *S. cerevisiae* reporter strain L40 was transformed with plasmid DNAs: (a) pLexA-Ras and pVP16-CYR; (b) pBTM116 and pVP16; (c) pBTM116-E_1 and pVP16-E_3; (d) pBTM116-E_1 and pVP16; (e) pBTM116 and pVP16-E_3; (f) pBTM116-E_1 and pVP16-CYR; (g) pLexA-Ras and pVP16-E_3. The strain cotransformed with pLexA-Ras and pVP16-CYR was used as the positive control, while cells containing vector plasmids pBTM116 and pVP16 were used as the negative control. Cotransformants d, e, f, and g were used to test the possible existence of nonspecific interaction between the E_1 or E_3 fusion proteins with the LexA DNA-binding domain or VP16 acidic activation domain. To assay the β-galactosidase activity, individual transformants were grown on synthetic medium plates lacking tryptophan and leucine at 30 °C for 2 days and then replica-plated to X-gal plates for further incubation at 30 °C. Strains a and c turned blue within 3 days. Details are presented under Materials and Methods.

strains transformed with either vector alone (entries 9 and 13), or by the single recombinant plasmids (entries 8 and 12). Those cotransformed with both vectors (entry 2) or with pBTM116-E_1 and pVP16 or pBTM116-E_1 and pVP-CYR (entries 4 and 6) also failed to activate the transcription. Similar outcomes were again noted for the cases of pVP16-E_3 with pBTM116 or pLexA-RAS (entries 5 and 7). These results clearly indicated that no interaction exists between the LexA and VP16 protein fragments, either singly or in their fusion forms. However, when the adenylyl cyclase and H-Ras hybrid proteins were coexpressed in the reporter strain, transactivation of the *Lac*Z gene occurred (entry 1). A more distinct color change was observed with the pLexA-VP positive control (entry 10). Most importantly, yeast cells cotransformed with pBTM116-E_1 and pVP16-E_3 were able to grow in the absence of histidine (data not shown) and were blue when tested for β-galactosidase activity on a X-gal plate (entry 3). This finding provided convincing evidence indicating that the elements for transcription initiation encoded by the plasmid vectors were brought together only by the specific E_1-E_3 interaction. Figure 3 presents the results of the plating assay, showing the color changes of a few representative transformants described above. It is worth mentioning that no exogenous CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**2**) was included in the media for the above experiments. This again demonstrated that these two enzymes could form a complex even in the absence of E_1 substrate. Thus, the interacting domains of both proteins are not expected to undergo significant conformational change upon substrate binding.

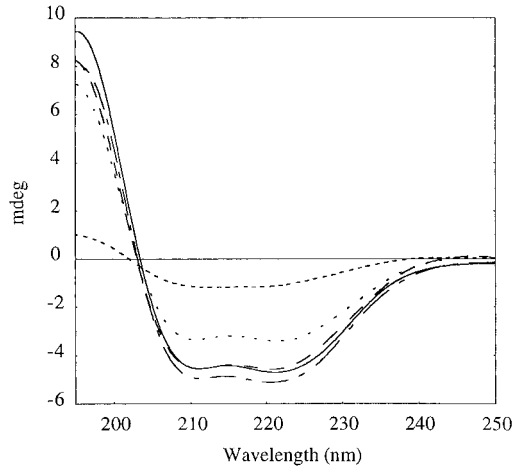


FIGURE 4: Circular dichroism spectra of E_3 (6.30 μM, - - -), E_1 (3.15 μM, ···), mixture of E_1 (3.15 μM) and E_3 (6.30 μM) in 50 mM potassium phosphate buffer, pH 7.5 (—), and mixture of E_1 (3.15 μM) and E_3 (6.30 μM) in the same buffer containing 1 M potassium chloride, pH 7.5 (— - —). The simple sum of E_1 and E_3 is also shown for comparison (— —).

Circular Dichroism Spectra of E_1, E_3, and the E_1·E_3 Complex. The CD spectra of these samples are presented in Figure 4. They all have two extinction minima in the 210−225 nm region and a maximum at ca. 195 nm, with the overall ellipticity of E_1 much greater than that of E_3. The spectrum for the E_1·E_3 complex is distinct from that of E_1 or E_3 alone. It is also different from the sum of the E_1 and E_3 spectra. These CD results indicate that there are interactions between E_1 and E_3, and the E_1 conformation is altered upon binding with E_3. Whether E_3 also undergoes conformational change is difficult to detect due to the limited contribution from E_3 to the overall spectrum of the complex. Although CD analysis cannot give absolute structural information of a protein, it is very sensitive to the conformational change of a protein under different conditions (Woody, 1995). As shown in Figure 4, the CD spectra of E_1·E_3 samples prepared in buffers of different ionic strengths are not superimposable. These observations clearly illustrate that the conformation of the E_1·E_3 complex is affected by the ionic strength of the buffer.

Effect of Ionic Strength on the Catalytic Activity of the E_1·E_3 Complex. Figure 5 shows the effects of KCl on the E_1-E_3 coupled reaction. The activity of the E_1·E_3 complex decreased as the ionic strength of the buffer was elevated. At 1 M salt concentration, the rate of the reaction was approximately only 50% of that measured under normal assay conditions. Interestingly, the amount of E_1·E_3 complex also diminished significantly when size-exclusion chromatography was carried out in 50 mM potassium phosphate buffer (pH 7.5) containing 1 M KCl (data not shown). Due to the poor resolution of the separation in which the E_1·E_3 complex signal was merely a small shoulder of the E_1 peak, it was difficult to quantitatively correlate the change of E_1·E_3 complexation at low and high salt concentrations. Nevertheless, these data appear to suggest that weakening of the interaction between these two enzymes may have been the cause for the observed reduction of the overall catalytic activity. The variations in the CD spectra of the E_1·E_3 complex at different ionic strengths have provided physical evidence for the alteration of the protein complex conformation. However, it was also found that the activity of E_3 alone, as a NADH oxidase, was reduced by 55% as the ionic

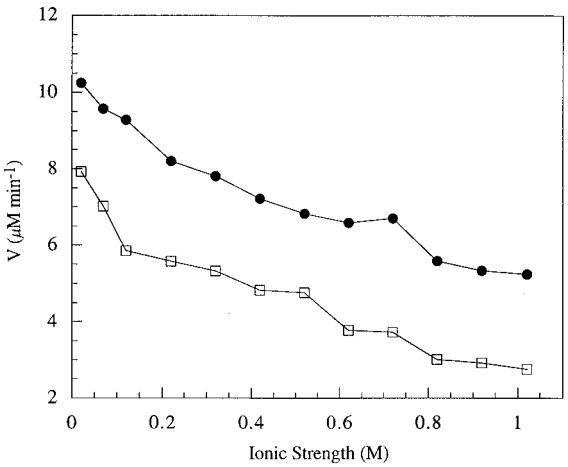


FIGURE 5: Effect of ionic strength on the catalytic activity of E_3 and the E_1·E_3 complex. Activity of E_3 (□) was determined by the NADH oxidase assay, and that of the E_1·E_3 complex (●) was measured by the coupled assay. The data shown are the average of two repeats. Details are described under Materials and Methods.

strength of the buffer increased from 0.02 to 1.02 M (Figure 5). Thus, while causes for the decrease of the E_1·E_3 activity at different ionic strengths may be manyfold, the retardation of the electron transfer process is the most likely contributor.

Other Attempts To Study Complex Formation between E_1 and E_3. Chemical cross-linking of two interactive proteins is a common approach to trap a protein complex. The cross-linking reagents EDC with a "zero length" linkage and DMS with a much longer hinge were used. Unfortunately, despite extensive efforts, no distinct cross-linked protein bands could be detected by denaturing gel electrophoresis. Attempts to determine the affinity between E_1 and E_3 by following the changes of fluorescence upon complex formation also failed. Although the N-terminus of E_3 was successfully labeled with a dansyl group, the dark brown color of the native E_3 disappeared after dansylation, indicating the loss of its [2Fe-2S] center. This dansyl group gave intense fluorescence at 490 nm when excited at 412 nm; however, neither the intensity nor the wavelength of this emission changed upon the addition of E_1.

DISCUSSION

The C-3 deoxygenation catalyzed by E_1 and E_3 is mechanistically the most intriguing step in the biosynthesis of ascarylose. Early experiments had shown that the E_1 iron−sulfur center could be directly reduced by NADH only in the presence of E_3 and thus suggested a relay mechanism of electron transfer from E_3 to E_1 (Miller et al., 1993). The detection of a new species other than E_1 and E_3 when a mixture of these two proteins was analyzed by gel filtration chromatography provided the first physical evidence supporting the expected complex formation. The deviation of the CD spectrum of an E_1−E_3 mixture from the simple sum of the spectra of its components also indicated an E_1·E_3 complex whose formation resulted in the conformational changes of these proteins. It had been well documented that adrenodoxin and adrenodoxin reductase form a complex with a stoichiometric molar ratio of 1:1. The dissociation constant of this complex was approximately 10^{-9} M in a low ionic strength medium (Chu & Kimura, 1973). Due to such a tight binding, a single symmetrical peak was obtained when an equal molar mixture of the reductase and adrenodoxin was passed through a size-exclusion column. No peak corresponding to either the reductase or adrenodoxin was discernible. Hence, the coexistence of the protein complex with each of the individual components at equilibrium in the E_1−E_3 case suggests that the affinity between E_1 and E_3 is not as tight as that between adrenodoxin and adrenodoxin reductase.

Early studies also showed that the additions of adrenodoxin in small increments to a constant amount of the oxidized adrenodoxin reductase led to an increase in absorbance at the region of FAD absorption. The increase in absorbance continued until the molar ratio of the adrenodoxin to adrenodoxin reductase reached 1. The sharp inflection point of the titration curve was consistent with the tight binding (10^{-9} M) found for these two protein components (Chu & Kimura, 1973). A similar phenomenon was also observed for the interaction between methane monooxygenase (MMO) hydroxylase and its component B whose dissociation constant had been determined to be 10 nM (Fox et al., 1991). When the change of the integration of the EPR signal at g 1.62 was monitored during the titration of the mixed valent hydroxylase with component B, a sharp break at approximately 2 equiv of component B per hydroxylase was noted. Thus, the nearly linear, albeit biphasic, titration curve with a sharp break appears to be characteristic for high affinity between the protein components. The fact that the titration curve of Figure 2 shows no sharp inflection point again indicates that the interaction between E_1 and E_3 is relatively weak. Indeed, the estimated affinity of 288 ± 22 nM for the E_1·E_3 couple is weaker than those of adrenodoxin reductase−adrenodoxin and MMO hydroxylase−component B, and the estimated stoichiometry of 1.7 (E_3:E_1) suggests a mixture of two types of complexes for this system at equilibrium: E_3·E_1·E_3 and E_1·E_3. The complex formation between E_1 and E_3 was further substantiated by the outcomes of the two-hybrid system, in which the interaction between E_1 and E_3 brings together the LexA and VP16 domains, allowing the reconstitution of a functional transcription complex that activates the expression of the *lac*Z reporter gene. This result provided the most compelling evidence for the formation of the E_1·E_3 complex *in vivo*.

It is worth mentioning that a high affinity is advantageous for a system of soluble components that must function as a complex at a reasonable rate. However, not all multicomponent protein complexes, especially those coupled with electron transfer proteins, have dissociation constants in the lower nanomolar range. For example, the affinity of components in the cytochrome P-450 camphor monooxygenase system is 1−2 orders of magnitude weaker than that of the MMO hydroxylase−component B complex (Sligar et al., 1974; Sligar & Gunsalus, 1974). It was suggested that the relatively facile dissociation resulting from this low affinity could allow putidaredoxin to disengage from P-450 and thus facilitate the transfer of the required two electrons for hydroxylation by the strictly one-electron reduced putidaredoxin, although not necessarily by the same molecule (Tyson et al., 1972). This scenario is particularly attractive for the E_1·E_3 case, since recent studies of the kinetic properties of E_3 showed that the intramolecular electron transfer between coenzymes in E_3 is several orders of magnitude faster than the intermolecular electron transfer from E_3 to E_1 (Gassner et al., 1996; Johnson et al., 1996). It was unfortunate that the complex formation between E_1 and

E_3 showed little effect on the fluorescence of each protein component, even on that of the N-terminal dansylated E_3. The lack of a convenient probe sensitive to the physical changes associated with the complex formation has hampered additional quantitative analysis of the E_1-E_3 interaction.

Electrostatic interactions have been suggested to play a major role in the formation of many protein complexes. For example, cytochrome P-450 and P-450 reductase are held together by the interaction of complementary charged residues (Bernhardt et al., 1988; Nadler & Strobel, 1988; Shimizu et al., 1991), and complexes of P-450 and putidaredoxin or adrenodoxin are also formed by attraction of oppositely charged amino acid residues (Stayton et al., 1989; Stayton & Sligar, 1990; Tsubaki et al., 1989; Tuls et al., 1989). Since electrostatic interactions are sensitive to the shielding of charges by adding ions (Moulis et al., 1995), the progressive increase in the salt concentration gradually neutralizes these interactions by lowering the activity coefficients of the ions (Voznesensky & Schenkman, 1994). If the protein complex is held mainly by electrostatic attraction, a decrease in the activity coefficients will weaken the protein−protein interactions and render the catalyst less effective. Indeed, early studies had shown that spinach ferredoxin−$NADP^+$ reductase and ferredoxin changed from a tightly bound state ($K_d < 10^{-8}$ M) to a loosely bound state ($K_d \sim 2\ \mu$M) when the ionic strength of the buffer increased (Nakamura & Kimura, 1971). A similar pattern was also found for the adrenodoxin reductase−adrenodoxin complex, which has a much greater dissociation constant (10^{-6} M), in a high ionic strength medium (Chu & Kimura, 1973). Since the E_1-E_3 interaction could not be directly probed as mentioned in the preceding paragraphs, a study of its nature was attempted by examining the effect of salt concentration on the overall catalytic activity. While the results shown in Figures 4 and 5 suggested a possible charge pairing association between E_1 and E_3, the fact that the NADH oxidase activity of E_3 alone greatly decreased at high salt concentration strongly argues that the slowing down of the electron relay mediated by E_3, instead of the weakening of the interactions between these two enzymes, is likely the major cause for lowering the E_1·E_3 catalytic activity at high ionic strength. Further definition of the amino acid residues involved and the molecular nature of the interaction must await a full crystallographic characterization of these two enzymes.

All of the above data support the notion that E_3 indeed forms a complex with E_1 during catalysis. However, a study of the inactivation of E_3 by DTNB showed that E_1 cannot protect E_3 from inactivation (Ploux et al., 1995). Since the extent of the E_3 thiol modification by DTNB was identical in either the presence or absence of E_1, these results implied that the conformation of E_3 experiences little change, at least around the DTNB-sensitive cysteine residues, upon coupling with E_1. Furthermore, the midpoint potentials of the redox centers in E_1 and E_3 at pH 7.5 were recently determined to be −212 mV for the E_3 FAD, −257 mV for the E_3 iron−sulfur center, and −209 mV for the E_1 [2Fe-2S] center (Burns et al., 1996). Notably, these potentiometric data at pH 7.5 do not support the thermodynamic favorability of the proposed electron flow, NADH → FAD → E_3 [2Fe-2S] → E_1 [2Fe-2S] (Miller et al., 1993; Gassner et al., 1996). A similar unfavorable electron flow was found in the adrenodoxin reductase−adrenodoxin system. Through binding with adrenodoxin reductase, the formal potential of the [2Fe-2S] center of adrenodoxin shifts negatively by 30−40 mV (Lambeth & Kamin, 1976). On the contrary, no potential change of the E_3 iron−sulfur center was observed in an EPR spectroelectrochemical experiment, in which an excess of E_1 was incubated with E_3 (Burns et al., 1996). Apparently, the interaction between E_1 and E_3 imposes no significant effect on the E_3 [2Fe-2S] potential and thus plays little role in the regulation of the redox properties of this system. Although the electrochemical properties of E_3 appear to be insensitive to complex formation, the specificity of the protein−protein recognition between these two enzymes is well demonstrated by the *in vivo* two-hybrid system and the general impotence of other biological electron donors in this catalysis (Weigel et al., 1992b). The inability of other electron transfer proteins to replace E_3 in this electron transport relay clearly indicates that they lack the specificity inherent to E_3 which makes it the preferred reductase for E_1 in the biosynthesis of ascarylose.

In summary, E_1, together with E_3, catalyzes a novel deoxygenation reaction essential for the biosynthesis of 3,6-dideoxyhexoses. There are a number of enzymes like E_3 that are involved in electron transfer from reduced nicotinamide coenzyme (NAD[P]H) via flavin and [2Fe-2S] redox centers to a terminal acceptor (Mason & Cammack, 1992). Interestingly, all of them are members of multicomponent oxygenase systems in which the terminal acceptor is typically an iron-containing oxygenase. While E_1 shows little resemblance to the oxygenase components of these enzymes, the action mode of the E_1·E_3 couple is closely related to these multicomponent oxygenase systems (Miller et al., 1993; Gassner et al., 1996). The present results, in conjunction with the insights gained from early mechanistic studies (Liu & Thorson, 1994) and a recent kinetic characterization of the organic radical intermediate found in E_1·E_3-catalyzed reaction (Johnson et al., 1996), lend further credence to the significance of the E_1·E_3 complex formation for catalysis and provide compelling evidence substantiating the mechanism proposed for the overall deoxygenation process. Thus, the E_1·E_3 system represents an intriguing example of an iron−sulfur flavoprotein (E_3) that forms an electron transport conduit with a protein other than a metallooxygenase and delivers electrons from NADH to an organic structure as complex as the PMP-glucoseen intermediate **3**.

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