Studies of the Redox Properties of CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1) and CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase (E_3): Two Important Enzymes Involved in the Biosynthesis of Ascarylose†

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ABSTRACT: Studies of the biosynthesis of ascarylose, a 3,6-dideoxyhexose found in the lipopolysaccharide of *Yersinia pseudotuberculosis* V, have shown that the C-3 deoxygenation is a process consisting of two enzymatic steps. The first enzyme involved in this transformation is CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1), which is a pyridoxamine 5′-phosphate dependent iron−sulfur protein. The second catalyst, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase, formally called CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3), is an NADH dependent plant type [2Fe-2S] containing flavoenzyme. To better understand the electron transfer carried out by these two enzymes, the potentials of the E_1 and E_3 redox cofactors were determined spectroelectrochemically. At pH 7.5, the midpoint potential of the E_3 FAD was found to be −212 mV, with the FAD_{ox}/FAD_{sq} couple ($E_1^{\circ\prime}$) and the FAD_{sq}/FAD_{hq} couple ($E_2^{\circ\prime}$) calculated to be −231 and −192 mV, respectively. However, the $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ of the FAD in E_3(apoFeS) at pH 7.5 were estimated to be −215 and −240 mV, respectively, which are quite different from those of the holo-E_3, suggesting a significant effect of the iron−sulfur center on the redox properties of the flavin coenzyme. Our data also showed that the midpoint potential of the E_3 iron−sulfur is −257 mV and that of the E_1 [2Fe-2S] center is −209 mV. These values indicated a thermodynamic barrier to the proposed electron transfer of NADH → FAD → E_3[2Fe-2S] → E_1[2Fe-2S] at pH 7.5. Regulation of electron transfer by several mechanisms is possible and experiments were performed to examine ways of overcoming the unfavorable electron transfer energetics in the E_1/E_3 system. It was found that both binding of E_3 with NAD^+ and complex formation between E_3 and E_1 showed no effect on the midpoint potentials of the E_3 FAD and iron−sulfur center. Interestingly, the midpoint potential of the E_3 FAD shifts dramatically to −273 mV ($E_1^{\circ\prime} \approx -345$ mV and $E_2^{\circ\prime} \approx -200$ mV) at pH 8.4, with very little semiquinone stabilization (<5%). The potential of the E_3 [2Fe-2S] center at pH 8.4 was also found to undergo a negative shift to −279 mV, and that of the E_1 iron sulfur center remained essentially the same at −206 mV. These data indicated that the redox properties of this system may be regulated by pH and the electron transfer between the E_3 redox centers may be prototropically controlled. These results also demonstrated that E_3 is unique among this class of enzymes.

The 3,6-dideoxyhexoses are found in the lipopolysaccharide of a number of Gram-negative bacteria where they have been shown to be the dominant antigenic determinants (Lindberg, 1990; Bishop, 1982; Williams, 1980; Hanessian, 1966; Lüderitz, 1966). Studies of the biosynthesis of ascarylose (**1**, in Scheme 1), the 3,6-dideoxy-L-*arabino*-hexopyranose derived from CDP-4-keto-6-deoxy-D-glucose (**2**),[1] revealed that the hydroxyl group at C-3 of **2** is replaced by a hydrogen atom in a novel deoxygenation process consisting of two enzymatic steps (Gonzalez-Porqué & Strominger, 1972; Gonzalez-Porqué, 1986; Liu & Thorson, 1994). The first step is catalyzed by CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E_1), a pyridoxamine 5′-phosphate (PMP) dependent enzyme which also contains a catalytically important [2Fe-2S] cluster (Thorson & Liu, 1993a; Rubenstein & Strominger, 1974a). The second step is carried out by an NADH-dependent reductase, CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase, formerly known as CDP-6-deoxy-$\Delta^{3,4}$-glucoseen reductase (E_3), which contains both an FAD and a plant-type ferredoxin [2Fe-2S] center (Lo et al., 1994; Miller et al., 1993).

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[1] Abbreviations: DP, cytidine 5′-diphosphate; 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_3(apoFeS), iron−sulfur deleted E_3; $E_1^{\circ\prime}$, formal potential of first electron transfer; $E_2^{\circ\prime}$, formal potential of second electron transfer; E_m, midpoint potential; EPR, electron spin resonance spectroscopy; FAD, flavin adenine dinucleotide; FAD_{ox}, oxidized flavin adenine dinucleotide; FAD_{sq}, semiquinone flavin adenine dinucleotide; FAD_{hq}, hydroquinone flavin adenine dinucleotide; FMN, flavin mononucleotide; FNR, ferredoxin $NADP^+$ reductase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MMO, methane monooxygenase; NAD^+ (NADH), β-nicotinamide adenine dinucleotide (reduced form); $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; PDR, phthalate dioxygenase reductase; PDR(-FeS), iron−sulfur domain deleted phthalate dioxygenase reductase; PMP, pyridoxamine 5′-phosphate; TMD, trimethylamine dehydrogenase; UV, ultraviolet; XO, xanthine oxidase.

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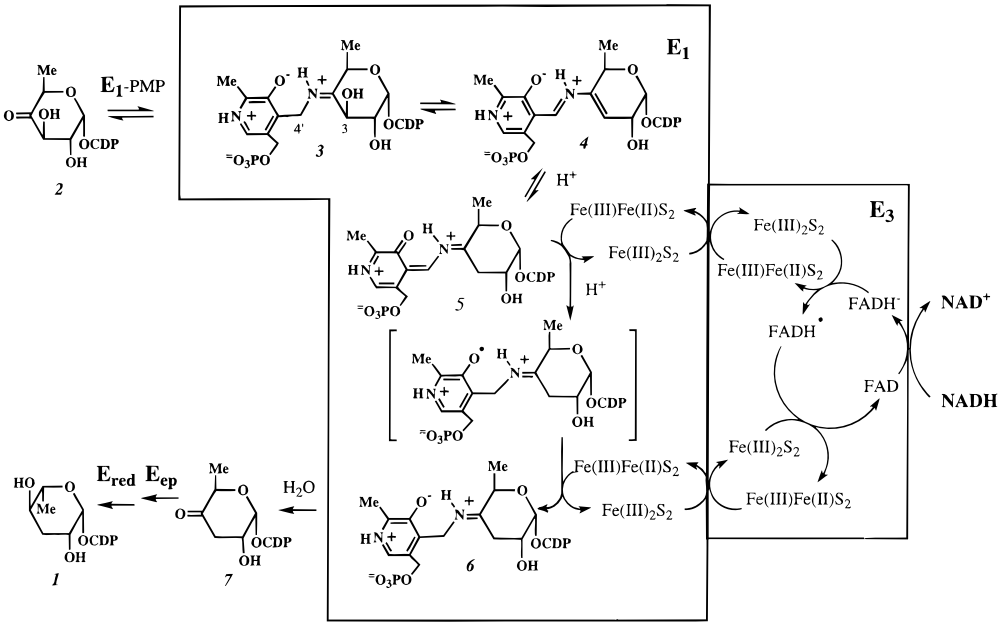
Scheme 1



The proposed mechanism of this reaction is depicted in Scheme 1. The PMP coenzyme of E_1 is responsible for the dehydration in which the *pro*-S proton is abstracted from the C-4′ position of **3** triggering the expulsion of the C-3 hydroxyl group (Rubenstein & Strominger, 1974a; Weigel et al., 1992a,b). Reduction of the nascent intermediate **4** initiated by E_3 gives **6** (Rubenstein & Strominger, 1974b; Pieper et al., 1995), which after hydrolysis yields the deoxygenation product, CDP-3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**7**). Early studies have established that the reductive half-reaction is initiated with hydride reduction of the E_3 FAD by NADH, followed by electron transfer from the reduced flavin to E_3's [2Fe-2S] cluster which then relays the reducing equivalents via the [2Fe-2S] center in E_1 to **4** (Miller et al., 1993). Since these [2Fe-2S] centers are well known one-electron carriers and are essential for both E_1 and E_3 activities, the reduction of the PMP-glucoseen intermediate **4** must proceed via a radical mechanism (Thorson & Liu, 1993b).

It is worth noting that E_3 is closely related to a number of flavoprotein reductases found in multicomponent hydroxylating systems (Mason & Cammack, 1992), such as methane monooxygenase (MMO) reductase (Lipscomb, 1994, Stainthorpe et al., 1990; Prince & Patel, 1986; Lund et al., 1985), phthalate dioxygenase reductase (PDR) (Gassner et al., 1994, 1995; Batie et al., 1987, 1991), and xylene monooxygenase reductase (Shaw & Harayama, 1992; Suzuki et al., 1991). In fact, E_3 can be included in a distinct family of flavoprotein reductases represented by ferredoxin $NADP^+$ reductase (FNR) (Correll et al., 1992; Karplus et al., 1991; Ploux et al., 1995). All members of this class contain six highly conserved peptide fragments involved in flavin and pyridine nucleotide recognition (Karplus et al., 1991). While E_3 clearly shares features with a group of reductases, E_1 can be considered singular in its own class. Although coenzyme B_6 phosphate-dependent enzymes are known to mediate a diverse repertoire of reactions primarily involved in the metabolism of amino acids and amines (Evangelopoulos, 1984; Dolphin et al., 1986; Christen & Metzler, 1985), there is no precedence for the role that the PMP cofactor plays in E_1 catalysis. The discovery of a catalytically active [2Fe-2S] center in E_1 further distinguishes this enzyme as a unique catalyst (Thorson & Liu, 1993a).

It has been speculated that reduction of **4** could occur by a number of different pathways. However, preliminary EPR data are most compatible with a mechanism initiated by a tautomerization step to generate a PMP-quinonoid intermediate **5** (Thorson & Liu, 1993b). This pseudo-*ortho*-quinonoid species is expected to have the desirable redox properties to serve as the ultimate electron acceptor. Clearly, the electron relay through the flavin and iron−sulfur centers in E_3 and E_1 to reduce the putative quinonoid intermediate **5** is the culminating step in C-3 deoxygenation. In an effort to better understand this process, the midpoint potentials of the redox coenzymes in E_3 and E_1 were determined. Experiments were also conducted to elucidate whether binding with E_1 or NAD^+ would affect the midpoint potentials of the redox centers in E_3. In addition, the effects of pH on the potentials of redox centers in E_1 and E_3 and the possible consequences for enzyme regulation were also examined. The results of these studies and a comparison with kinetic data as well as the redox properties of related flavoprotein reductases are presented here.

EXPERIMENTAL PROCEDURES

Enzyme Preparation. The E_3 was purified from an *Escherichia coli* pOPI/JM105 overproducing strain according to the method of Ploux et al. (1995). Its activity was determined as previously described (Lo et al., 1994). Purification of E_1 followed the method of Lei et al. (1995) using the recombinant *E. coli* pJT18/JM105 overproducing strain (Thorson et al., 1994). The E_1 activity was determined by an E_1/E_3 coupled assay described by Lei et al. (1995). Protein concentrations were estimated by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. The Bradford reagent was obtained from Bio-Rad (Richmond, CA). The iron content of the purified enzymes was quantitated by the method of Fish (1988).

Preparation of E_3(apoFeS). The E_3(apoFeS) was prepared by a previously reported procedure (Miller et al., 1993) with

some modification. A 0.5 mL aliquot of 149 mM sodium mersalyl acid solution in 50 mM potassium phosphate buffer, pH 7.5 (80 equiv) was mixed with purified E_3 (0.94 μmol, in 5 mL of the same buffer). This solution was incubated for 30 min at room temperature, concentrated, and loaded onto a Sephadex G-25 column (1.5 × 10 cm) pre-equilibrated with 50 mM potassium phosphate buffer, pH 7.5. Excess mersalyl acid was removed by eluting the column with the same buffer. The protein-containing fractions were pooled, concentrated, and incubated with 2-mercaptoethanol (15 μL) for 20 min at 4 °C. The solution was again placed on a pre-equilibrated Sephadex G-25 column (1.5 × 10 cm) and eluted with 50 mM potassium phosphate buffer, pH 7.5. The fractions containing E_3(apoFeS) were collected and concentrated by Centricon 10 (Amicon, Beverly, MA). The extent of iron depletion was determined by measuring the iron content of samples before and after treatment. To determine the amount of FAD bound to E_3(apoFeS), a sample of the enzyme was first boiled at 100 °C to liberate the FAD. The denatured protein was removed by centrifugation and the supernatant which contained free FAD was analyzed with a Perkin-Elmer LS 50B spectrofluorimeter (λ_{ex} = 450 nm, λ_{em} = 529 nm). The concentration of FAD extracted from the apoenzyme was deduced by correlating the reading of the sample solution against a standard curve (0−5 μM of FAD in 50 mM potassium phosphate buffer, pH 7.5).

Determination of Molar Absorptivities of Oxidized and Reduced Coenzymes in E_3. The molar absorptivity of the oxidized flavin in E_3 was determined by the method of Williamson and Engel (1984) using a double sector cuvette. In one side of the cuvette was placed E_3(apoFeS) in 50 mM potassium phosphate buffer (pH 7.5), and in the other side was a solution of 10 M guanidinium HCl in the same buffer. These two solutions were then mixed to denature the protein and liberate FAD. The absorption was taken prior to and after mixing, and the concentration of the released FAD was calculated based on the known extinction coefficient of free FAD in 10 M guanidinium HCl ($\epsilon_{450} = 1.18 \times 10^4$ M^{-1} cm^{-1}) (Thorpe et al., 1979). Based on a 1:1 ratio between FAD and E_3 (Miller et al., 1993), the FAD concentration, which equals the concentration of E_3(apoFeS), was used directly to calculate the molar absorptivity of enzyme-bound FAD_{ox}. Reduction of E_3(apoFeS) with dithionite allowed the molar absorptivities of the reduced flavin hydroquinone (FAD_{hq}) and blue neutral semiquinone (FAD_{sq}) to be determined. The molar absorptivity of the oxidized iron−sulfur cluster ($[2Fe\text{-}2S]_{ox}$) in E_3 was deduced from the difference spectrum obtained by subtracting the absorption of the oxidized E_3(apoFeS) from that of the oxidized holo-E_3. Analogously, subtracting the absorption of the reduced E_3(apoFeS) from that of the reduced holo-E_3 led to a difference spectrum from which the molar absorptivity of reduced E_3 iron−sulfur center ($[2Fe\text{-}2S]_{red}$) was calculated. For each coenzyme at different oxidation states, the molar extinction coefficients at four different wavelengths (395, 428, 455, and 474 nm) were calculated.

Reductive Titration of E_3 and E_1. Both coulometric and dithionite titrations were carried out to determine the number of reducing equivalents required to reduce E_3 and E_1. All experiments were performed at 4 °C and followed previously described procedures (Stankovich, 1980; Stankovich & Fox, 1983; Einarsdottir et al., 1988). In the coulometric titrations, the sample solution containing E_3 (8 μM) and methyl viologen (100 μM) in 3 mL of potassium phosphate buffer (pH 7.5) was reduced using a three-electrode system. In dithionite titrations, E_3 (10 μM) or E_1 (55 μM) was reduced with sodium dithionite (3−6 mM in 50 mM potassium phosphate buffer, pH 7.5) which was standardized with lumiflavin-3-acetate (a generous gift from Dr. Sandro Ghisla, University of Konstanz, Germany). All solutions were made anaerobic by 8−10 cycles of argon and vacuum carried out on a Schlenk line over a period of at least 1 h. Spectrophotometric measurements were taken using a Perkin-Elmer Lambda 2S or 12S UV-visible spectrophotometer, interfaced with an IBM-compatible computer.

EPR-Spectroelectrochemical Experiments on E_3. Collecting redox potential data for E_3 was initially attempted by an EPR-spectroelectrochemical method described earlier by Paulsen et al. (1992, 1993). All experiments were performed at 4 °C in 20 mM HEPES buffer at pH 7.5. A set of indicator dyes were employed to cover a broad potential range. These included anthraquinone-1,5-disulfonic acid (ICN Pharmaceuticals, Costa Mesa, CA), −174 mV; riboflavin (Sigma, St. Louis, MO), −198 mV; phenosafranin (Sigma), −244 mV; safranine O (Fisher, Pittsburgh, PA), −289 mV; and benzyl viologen (Sigma), −352 mV. The sample solution contained 120 μM E_3 and 36 μM indicator dyes in 1 mL of buffer. The cell was made anaerobic as described above for the spectroelectrochemical experiments. Potentials were measured with an Orion Research Model 601A digital ionalyzer and reported versus the standard hydrogen electrode. A Varian E-109 EPR spectrometer equipped with an Oxford Instruments ESR-910 liquid helium cryostat (Fox et al., 1989) in the laboratory of Dr. John Lipscomb at the University of Minnesota were used for X-band EPR measurements.

Potentiometric Titrations of E_3 and E_1. The redox potentials for the FAD of E_3(apoFeS), the FAD of E_3, and the [2Fe-2S] centers of E_3 and E_1 were determined at two different pHs (7.5 and 8.4) and 4 °C by potentiometric titrations (Stankovich, 1980; Stankovich & Fox, 1983; Einarsdottir et al., 1988). A typical incubation mixture contained 8 μM E_3 or 33 μM E_1 in the presence of 2−5 mM indicator dyes. The indicator dyes used were riboflavin and lumiflavin-3-acetate which have midpoint potentials of −198 and −234 mV, respectively, at pH 7.5. These midpoint potentials shift to −230 and −260 mV, respectively, at pH 8.4. The titration involved successive addition of sodium dithionite (3−6 mM) and measurement of the potential after each addition with an Orion Research Model 601A digital ionalyzer. The system was considered to be at equilibrium when the change in the measured potential at each point in the titration was less than 1 mV per 10 min. The equilibration typically took about 1 h. Spectrophotometric measurements were taken using a Perkin-Elmer 2S or 12S UV-visible spectrophotometer and the pH measurements, were made with an Accumet extra-long calomel micro probe combination electrode.

For E_3 titrations, the concentrations of the five redox species which coexisted at each point (FAD_{ox}, FAD_{sq}, FAD_{hq}, $[2Fe\text{-}2S]_{ox}$, and $[2Fe\text{-}2S]_{red}$) were deduced by simultaneously solving five equations using the computer program Mathmatica version 1.0 (Wolfran Research). The first equation is a mass balance equation. The remaining four equations utilize Beer's Law, relating the absorbance to the concentrations of those species at four different wavelengths. Since

the molar absorptivity for each species at these wavelengths could be deduced from the above experiments, these five equations are solvable. Once the concentration of each species was determined, the Nernst equation (eq 1) was applied to calculate the midpoint potential (E_m) of FAD in E_3 from the plot of E versus log([ox]/[red]). In this equation, E is the measured equilibrium potential at each point in the

$$E = E_m + (0.054/n) \log([ox]/[red]) \quad (1)$$

titration, [ox] and [red] represent the concentrations of FAD_{ox} and FAD_{hq} at each point, and $n = 2$ is the number of electrons required to interconvert FAD between these two redox states. Based on the midpoint potential of the FAD (E_m) determined above and the amount of stabilized semiquinone formed thermodynamically (M), the values of $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ can be calculated using the following equations (Clark, 1960):

$$E_1^{\circ\prime} + E_2^{\circ\prime} = 2E_m \quad (2)$$

$$E_1^{\circ\prime} - E_2^{\circ\prime} = 0.12 \log[2M/(1 - M)] \quad (3)$$

The formal potential values ($E^{\circ\prime}$) of the [2Fe-2S] clusters in E_3 and E_1 were calculated similarly ($n = 1$). Nernstian behavior is observed if the experimental n value is close to the theoretical value.

Effect of E_1-E_3 Interaction on E_3's [2Fe-2S] Potential. The potential of the E_3 iron−sulfur center determined spectroelectrochemically was verified by an EPR-spectroelectrochemical experiment (Paulsen et al., 1992, 1993). In this experiment, the E_3 (120 μM) was incubated with lumiflavin-3-acetate (400 μM) in 50 mM potassium phosphate buffer, pH 7.5, at 4 °C. Three samples were prepared in which the incubation mixture was reduced with sodium dithionite (3−6 mM) to a measured potential of −230, −239, and −257 mV, respectively. The iron−sulfur center in E_3 should be 24, 32, and 50% reduced at these potentials, as predicted by the formal potential determined spectroelectrochemically. A fully reduced sample was also taken to be used as a reference. After the equilibrium was reached, a 250 μL aliquot of each experimental solution was anaerobically transferred to an EPR tube and frozen in liquid nitrogen. A second set of samples were also collected in a separate experiment in which the E_3 concentration was 187 μM. Although the reduced E_3 [2Fe-2S] signal is overlapped with the flavin semiquinone signal in the EPR spectrum, the first peak (g_z component) of the rhombic [2Fe-2S] signal is well resolved from the rest of spectrum (see Figure 4). Thus, the intensity of this peak, which is indicative of the extent of metal center being reduced, was determined by measuring the integration of this signal. The percent reduction of the E_3 [2Fe-2S] center was calculated using the integration of the fully reduced sample as the reference.

The same experiment was repeated in which the incubation was conducted in the presence of lumiflavin-3-acetate (1.6 mM) and E_1. Again, two sets of samples from the incubation of 490 μM E_1/120 μM E_3 and 560 μM E_1/187 μM E_3 were prepared and measured. These experiments were undertaken to test whether the interaction between E_1 and E_3 would affect the potential of the E_3 [2Fe-2S] center. The extent of reduction of the E_3 [2Fe-2S] center in the absence and presence of E_1 was compared as judged by the intensity of the first EPR peak of E_3's rhombic metal signal which is well resolved from those of the flavin semiquinone and the iron−sulfur center of E_1. A change in the integration of this peak would indicate a change in the E_3 [2Fe-2S] potential.

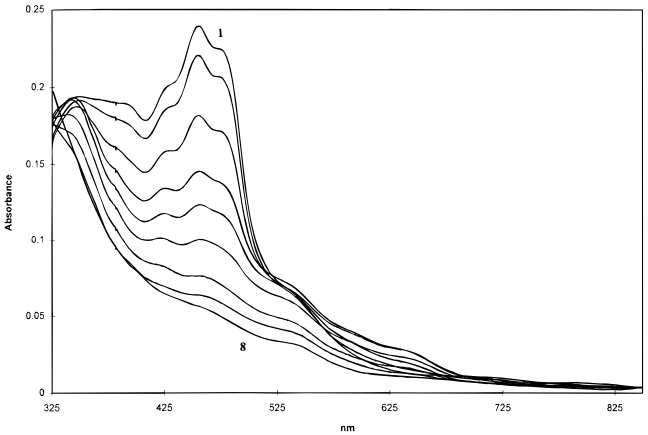


FIGURE 1: Dithionite reduction of E_3 (10.5 μM) in 50 mM potassium phosphate buffer, pH 7.5, at 4 °C. Curves 1−8: spectrum 1 is fully oxidized and spectrum 8 is fully reduced.

Effect of NAD^+ Binding on E_3. The effect of NAD^+ binding on the potential of the E_3 FAD was assessed spectroelectrochemically. The reaction mixture contained E_3 (8 μM), lumiflavin-3-acetate (3 μM), methyl viologen (100 μM), and 50 mM potassium phosphate buffer, pH 7.5. Under anaerobic conditions, the solution was reduced coulometrically at 4 °C, and the potential was poised at −206 mV. At this potential, only FAD was reduced, while the iron−sulfur center remained unchanged. A spectrum was recorded when the potential had reached equilibrium. A degassed solution of NAD^+ was then added to the cell via a gas tight syringe to a final concentration of 560 μM, and the potential was again poised at −206 mV. Once the potential reached equilibrium, another spectrum was recorded. The absorbances at 454 nm of each spectrum were compared. A change in absorbance would indicate a shift in the potential of the FAD in E_3.

RESULTS

Spectral Properties of E_3. E_3 is a monomeric protein containing one FAD and one plant-type ferridoxin [2Fe-2S] center. The highly purified E_3 used in these studies had 1.9 mol of Fe per mol of E_3 and a specific activity of 70 μmol mg^{-1} min^{-1}. Figure 1 shows the UV−visible spectrum of oxidized E_3. Reduction of these chromophores could be accomplished by successive addition of sodium dithionite or by coulometric titration. Both methods gave the same spectral changes depicted in Figure 1. The EPR spectrum of reduced E_3 had been reported in which the flavin semiquinone and reduced [2Fe-2S] signals overlapped (Miller et al., 1993). Further analysis revealed that the flavin signal was saturated at all conditions within the limits of the EPR instrument, with a lowest power of 4 μW and highest temperature at 100 K. This prevented us from using EPR to quantitate the flavin semiquinone and thus to determine the potentials of the redox centers of E_3. However, it was noted that the flavin semiquinone signal appeared first followed by the iron−sulfur signal during the reduction of E_3 by dithionite (Miller et al., 1993); hence the iron−sulfur center must have a more negative potential than the flavin.

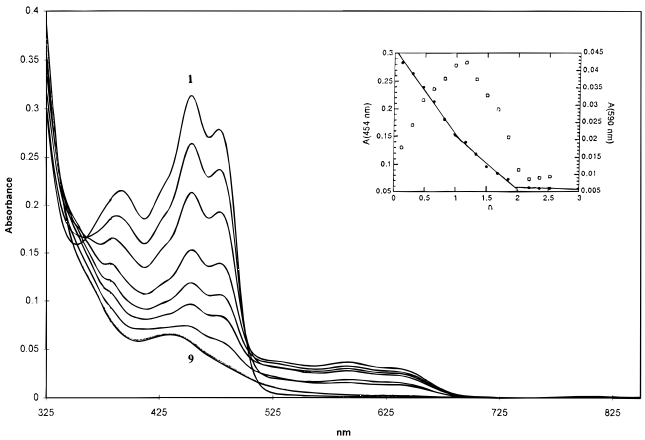


FIGURE 2: Dithionite reduction of E_3(apoFeS) (26.8 μM) in 50 mM potassium phosphate buffer, pH 7.5, at 4 °C. Inset is a plot of *A*454 or *A*590 versus the number of reducing equivalents added. Curves 1−9: spectrum 1 is fully oxidized and spectrum 9 is fully reduced.

E_3(apoFeS). As shown in Figure 1, the electronic absorptions of the flavin and iron−sulfur center overlap. In order to study the oxidation−reduction properties of these redox coenzymes by the spectroelectrochemical method, the spectrum of E_3 needs to be deconvoluted. Thus, the iron−sulfur center was removed by treatment with mersalyl acid to generate E_3(apoFeS). The resulting bright yellow protein contained approximately one FAD per molecule, which was released by denaturing the protein. The concentration of FAD extracted from the apoenzyme was determined fluorimetrically. Assuming a 1:1 stoichiometry between FAD and protein, the fraction of E_3(apoFeS) containing a bound FAD was found to be approximately 85%. Not surprisingly, the absorption features of bound FAD (see Figure 2) in E_3(apoFeS) are quite distinct as compared to the free FAD. In fact, the UV−visible absorption of the oxidized E_3(apoFeS) resembles those of oxidized adrenodoxin reductase (Lambeth & Kamin, 1976) and PDR(-FeS) (Gassner & Ballou, 1995), both of which contain one FAD per molecule. Figure 2 also shows the bleaching of the FAD of E_3(apoFeS) by dithionite reduction. The absorption at 590 nm is characteristic for the blue neutral semiquinone (FAD_{sq}) (Massey & Palmer, 1966). From the plot of absorbance at 454 nm versus the total equivalents of electrons added (Figure 2, inset), it is evident that two electrons are required to fully reduce E_3(apoFeS). This result clearly indicates the absence of redox active cofactor other than FAD in the apoenzyme. The theoretical absorbance of 100% stabilized semiquinone was extrapolated from the absorbance at 590 nm. Based on this absorbance, the amount of blue neutral semiquinone stabilized kinetically was calculated to be approximately 66%.

Molar Absorptivity Determination. According to the method of Williamson and Engel (1984), we determined the molar absorptivity of enzyme bound FAD_{ox} based on the concentration of FAD released from E_3(apoFeS) and the absorption spectra of oxidized E_3(apoFeS) shown in Figure 2. The molar absorptivities of FAD_{hq} at different wavelengths were calculated directly from the corresponding absorbance of the reduced E_3(apoFeS) (Figure 2). Since about 66% of the FAD in E_3(apoFeS) is in the blue neutral semiquinone form whereas the remaining flavin is expected to be equally distributed between FAD_{ox} and FAD_{hq}, the extinction coefficient of FAD_{sq} at a given wavelength can be calculated according to

Table 1: Molar Absorptivity Values (M^{-1} cm^{-1}) of E_3 at Various Wavelengths

	474 nm	455 nm	428 nm	395 nm
FAD_{ox}	10400	11900	8100	7800
FAD_{sq}	4900	5000	3600	3900
FAD_{hq}	1500	1900	2100	2300
$[2Fe\text{-}2S]_{ox}$	10500	10600	10700	9900
$[2Fe\text{-}2S]_{red}$	3300	3500	4000	5800

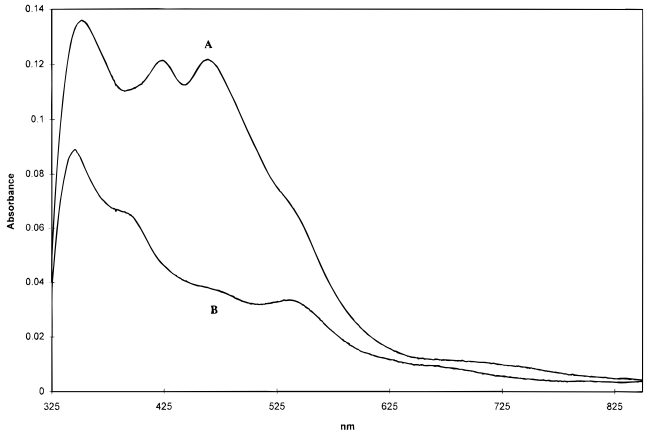


FIGURE 3: Oxidized (A) and reduced (B) spectra of the [2Fe-2S] center in E_3. The spectra were obtained by subtracting the spectra of the oxidized and reduced E_3(apoFeS) from those of the oxidized and reduced holo-E_3.

$$A/[E_3] = 0.17\epsilon_1 + 0.17\epsilon_2 + 0.66\epsilon_3 \qquad (4)$$

where *A* is the absorbance, $[E_3]$ is the E_3(apoFeS) concentration, and ϵ_1, ϵ_2, and ϵ_3 are the molar absorptivity values of FAD_{ox}, FAD_{hq}, and FAD_{sq}. The resulting values are listed in Table 1 and are typical for flavoproteins. The molar absorptivities of the oxidized and reduced [2Fe-2S] center were obtained by subtracting the absorption of oxidized and reduced E_3(apoFeS) from that of the holo E_3 (Figure 3). The calculated values shown in Table 1 are consistent with those found for other plant type iron−sulfur clusters.

Redox Potentials of E_3. From the spectroelectrochemical potentiometric titrations of holo-E_3 and analysis of the data using the molar absorptivities given in Table 1, the midpoint potential for the two-electron reduction of FAD was determined to be −212 mV at pH 7.5 and 4 °C with a slope of 30 mV, which compares quite well with the theoretical slope of 27 mV for a two-electron transfer. The formal potentials of the FAD_{ox}/FAD_{sq} couple ($E_1^{\circ\prime}$) and that of the FAD_{sq}/FAD_{hq} couple ($E_2^{\circ\prime}$) were then calculated to be −231 and −192 mV, respectively. Likewise, the potential of the [2Fe-2S] center in E_3 was determined to be −257 mV with a slope of 47 mV, which is very close to the theoretical value of 54 mV for a one-electron transfer. These data are summarized in Table 2. It is worth mentioning that the maximal amount of blue neutral semiquinone thermodynamically stabilized was less than 20%, whereas a maximum of about 37% semiquinone was kinetically stabilized in dithionite titrations. Interestingly, potentiometric titrations of E_3(apoFeS) in the presence of two indicator dyes, riboflavin and lumiflavin-3-acetate, led to an $E_1^{\circ\prime}$ value of approximately −215 mV and an $E_2^{\circ\prime}$ of −240 mV at pH 7.5 and 4 °C. Approximately 55% blue neutral semiquinone was thermodynamically stabilized during this potentiometric titration.

EPR of E_3 Reduced at Controlled Potentials. As the midpoint potentials of the E_3 redox centers are now available,

Table 2: Comparison of the Redox Potentials of E_3 with Potentials of Other Closely Related [2Fe-2S]-Containing Flavoproteins

	flavin			[2Fe-2S]
	$E_1°'$ (mV)	$E_2°'$ (mV)	E_m (mV)	$E°'$ (mV)
E_3 pH 7.5	−231	−192	−212	−257
E_3 pH 8.4	∼−345	∼−200	−273	−279
E_3 (apoFeS)	−215	−240	−227	
PDR[a]	−174	−287	−230	−174
MMO *Methylococcus capsulatus*[b]	−150	−260	−205	−220
MMO *Methanobacterium* CRL26[c]	−195	−250	−222	−247
xylene monooxygenase[d]	−244	−297	−270	−171

[a] Gassner et al. (1995). [b] Lund and Dalton (1985). [c] Prince and Patel (1986). [d] Shaw and Harayama (1992).

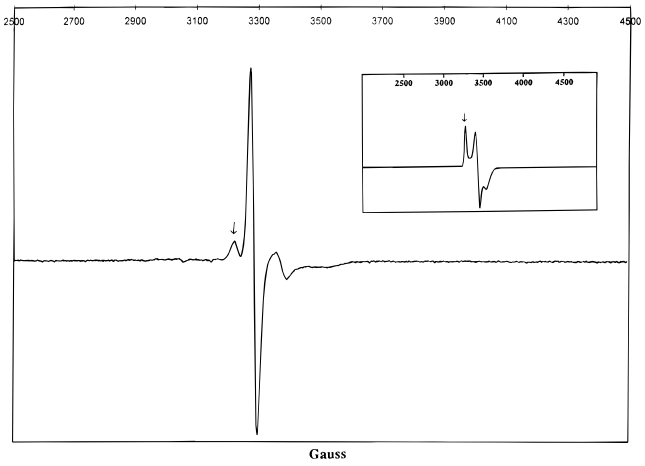


FIGURE 4: X-band EPR spectra recorded during an EPR-spectroelectrochemical experiment under nonsaturated conditions for the [2Fe-2S] center in E_3. Spectrum of E_3 (120 μM) in 50 mM potassium phosphate buffer, pH 7.5, which was poised at −230 mV at 4 °C (9.214 GHz, 15 mW, 4×10^3 gain, 10 K, modulation amplitude 10 G). The first peak, indicated with an arrow, is the signal arising from the g_z component in the rhombic EPR iron signal of E_3, which was used to monitor the extent of E_3 [2Fe-2S] center reduction. Inset: spectrum of E_3 (120 μM) in 50 mM potassium phosphate buffer, pH 7.5, which was fully reduced at 4 °C.

one can control the extent of reduction of these coenzymes by poising the potential of E_3. A good correlation between the concentration of reduced coenzymes and the potential at which E_3 was in equilibrium would further confirm the midpoint potentials of these redox centers obtained from the spectroelectrochemical studies. In these experiments, the system was reduced with potentials poised at −230, 239, and 257 mV, where the E_3 [2Fe-2S] center should be approximately 24%, 32%, and 50% reduced, respectively. The peak integration of the g_z component of the EPR iron−sulfur signal was measured, and the spectrum of a fully reduced sample was also recorded for comparison. The spectra are shown in Figure 4. As depicted in Figure 5, the resulting metal cluster was indeed 25% (−230 mV), 31% (−239 mV), and 50% (−257 mV) reduced as compared to the fully reduced reference spectrum. These results provide strong evidence corroborating the midpoint potential (−257 mV) determined spectroelectrochemically for the E_3 [2Fe-2S] center.

Spectral and Redox Properties of E_1. E_1 is a [2Fe-2S] containing enzyme which is also PMP dependent (Miller et al., 1993; Lei et al., 1995). The E_1 used was determined to contain 0.84 mol of Fe/mol of E_1 monomer with a specific activity of 119 nmol mg^{-1} min^{-1}. However, as in the case of E_3(apoFeS), only protein with bound cofactors absorbs in the UV−visible region of interest. Hence, the presence of not fully constituted apoprotein imposed no complication to our spectroelectrochemical experiments. The oxidized E_1 has a maximum absorbance at 450 nm with an estimated molar absorptivity of 5,450 M^{-1} cm^{-1}. This was calculated from the absorption spectrum shown in Figure 6. Figure 6 also shows the spectral changes associated with successive titration with dithionite at pH 7.5 and 4 °C. The oxidized and reduced spectra are characteristic for a vertebrate-type [2Fe-2S] center. One electron is required to reduce the E_1 [2Fe-2S] center as seen in the inset of Figure 6. The formal potential of E_1 was determined to be −209 mV with a slope of 52 mV at pH 7.5 and 4 °C, which is very close to the theoretical value of 54 mV for a one-electron transfer at this temperature.

Effect of E_1−E_3 Interaction on E_3's [2Fe-2S] Potential. Since both E_1 and E_3 participate in electron transfer to reduce the dehydration product **4**/**5** (Scheme 1) (Miller et al., 1993),

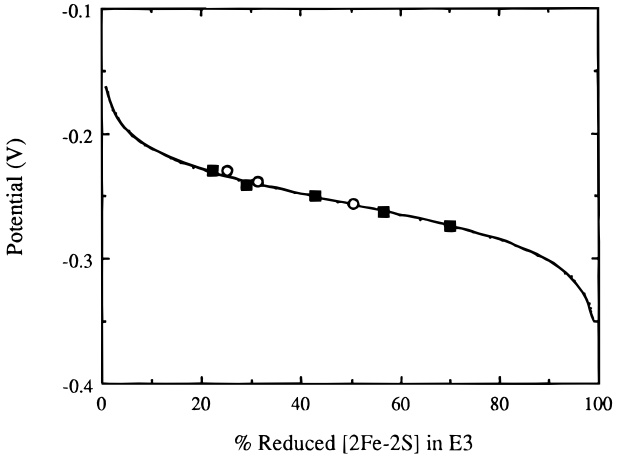


FIGURE 5: Plot of the potential versus the percent reduced [2Fe-2S] in E_3. The points were collected during EPR-spectroelectrochemical titrations in 50 mM potassium phosphate buffer, pH 7.5, at 4 °C. The theoretical curve deduced from the spectrochemically determined potential of the [2Fe-2S] in E_3 is plotted as the solid line. The percent of reduced [2Fe-2S] in E_3 determined by EPR is represented by the open circles (○). Those determined spectroelectrochemically are represented by the solid boxes of (■).

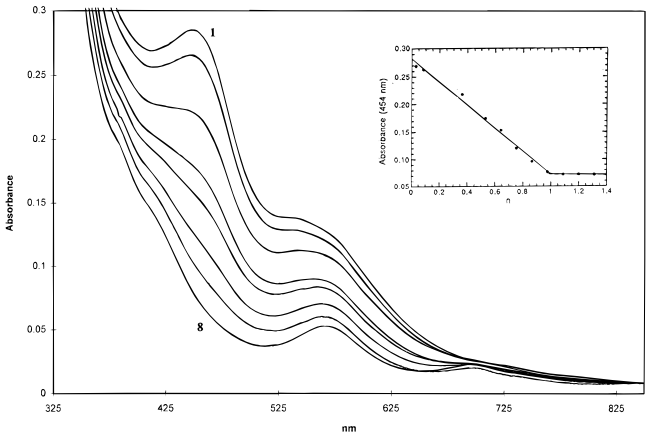


FIGURE 6: Dithionite reduction of E_1 (51.9 μM) in 50 mM potassium phosphate buffer, pH 7.5 at 4 °C. Curves 1−8: spectrum 1 is fully oxidized and spectrum 8 is fully reduced. Inset: plot of A_{450} versus the number of reducing equivalents added.

it is imperative to examine whether binding with E_1 would alter the redox potential of the [2Fe-2S] center in E_3. Since the first peak (g_z component) in the rhombic EPR iron signal of E_3 does not overlap with those arising from the reduced E_1 metal center, the EPR-spectroelectrochemical method described above was also used to probe the effect of E_1-E_3 interaction on the potential of the E_3 [2Fe-2S] center. The incubation conditions were the same as in the aforementioned EPR study to confirm the potential of E_3's iron−sulfur center, except for the addition of 3 molar equivalents of E_1 and an increased concentration of indicator in this experiment. Again, the potential was poised at −230, −239, and −257 mV with EPR samples taken at each potential. The integration of the first peak in the EPR signals of E_1 and E_3 was measured and compared to the data obtained from the E_3 alone experiment (see Figure 4). Since the results of these two set of experiments were nearly identical, it was concluded that there was no shift in the E_3 iron−sulfur potential upon complexing with E_1.

Effect of E_3−NAD^+ Interaction on E_3's Flavin Potential. Since E_3 is a NADH dependent reductase, the effect of nicotinamide binding on the potential of its flavin coenzyme was also examined. NAD^+, instead of NADH, was used to prevent E_3 from being reduced by NADH. Poised at −206 mV, the spectra of a sample before and after the addition of NAD^+ were recorded. An increase in the absorbance at 454 nm after the addition of NAD^+ would indicate a negative shift of the FAD potential, whereas a decrease in the absorbance at 454 nm would be indicative of a positive shift. The fact that there was no change in the absorbance at 454 nm (data not shown) clearly demonstrated no change in the concentration of reduced FAD, and thus no shift in the potential.

Potentials of Redox Centers in E_1 and E_3 at pH 8.4. Spectroelectrochemical potentiometric titrations at pH 8.4 were performed to determine the potential of each redox coenzyme in E_1 and E_3. Since the potential of riboflavin was too positive to be an effective indicator for E_3 at this pH, lumiflavin-3-acetate was used instead. It was found that the midpoint potential of FAD shifts negatively to −273 mV in which the $E_1^{\circ\prime}$ and $E_2^{\circ\prime}$ can be calculated to be approximately −345 and −200 mV, respectively (Table 2). The amount of neutral blue semiquinone stabilized was minimal (estimated at <5%). Interestingly, the potential of the [2Fe-2S] center in E_3 also underwent a negative shift to −279 mV. However, the potential of the E_1 [2Fe-2S] determined spectroelectrochemically at pH 8.4 using riboflavin as the indicator was found to be −206 mV, which is essentially the same as that determined at pH 7.5. All the potential data reported here and above are believed to be accurate, because the Nernst plots of all species are close to ideal and this is the most rigorous criterion. However, the errors in potentials determined for the cofactors in E_3 are probably slightly higher than those enzymes with only one chromophoric cofactor, such as E_1, because any inaccuracy in determining the concentration of one species causes deviation in the properties of the other, thus compounding errors.

DISCUSSION

Previously, studies of C-3 deoxygenation catalyzed by E_1 and E_3 have led to an intriguing mechanism involving the transfer of reducing equivalents from NADH via an electron conduit consisting of the flavin and iron−sulfur centers in the active sites of E_1 and E_3 to reduce the E_1-bound dehydration product **4**/**5**. To further our understanding of the mechanism of this unique C−O bond cleavage event, it is essential to characterize this electron transport system in detail. While an EPR-spectroelectrochemical method would allow the formal potential values of each redox center in a multicomponent redox system to be determined directly, the technical complications associated with the inherent properties of the E_3 system prevented us from using this newly developed technique.

In order to use the more conventional spectroelectrochemical method to measure the potentials of these redox centers, we prepared E_3(apoFeS) and determined the molar absorptivities of flavin at different redox states, a prerequisite for analyzing the complex spectra. The data shown in Table 1 agree well with those obtained for other flavoproteins. Interestingly, the spectrum of the apoenzyme is similiar to that of holoenzyme, but much different than the absorption of free FAD, which typically exhibits two absorption maxima around 370 and 450 nm. Not only does the peak at 370 nm in free FAD red shift to 390 nm in the E_3 bound FAD, but the 450 nm maximum becomes well resolved into two distinct peaks at 455 and 474 nm in the E_3(apoFeS) spectrum. In addition, the molar absorptivity of the peak in the near UV region (330−400 nm) decreases significantly in the E_3-bound form. The apparent spectral changes of FAD in the E_3-bound spectrum suggested a hydrophobic environment, as judged from the absorption features in the visible region (450 nm range), with possible hydrogen bonding to the active site residues based on the spectral shift in the near UV region (330−400 nm) (Harbury et al., 1959).

Such hydrophobic interactions and hydrogen bonding between FAD and the active site residues of E_3 may also affect the redox properties of this coenzyme. As shown in Table 2, the midpoint potential of FAD in E_3(apoFeS) was estimated to be −227 mV ($E_1^{\circ\prime}$= −215 mV, $E_2^{\circ\prime}$ = −240 mV), which is indeed different from a value of −216 mV reported for free FAD at pH 7.4 ($E_1^{\circ\prime}$= −241 mV, $E_2^{\circ\prime}$ = −185 mV) (Draper & Ingraham, 1968). Interestingly, the midpoint potential of FAD in holo E_3 (-212 mV) was found to be closer to that of free FAD. The holo-E_3 potential is 15 mV more positive than the E_3(apoFeS), with a notable change in the values of the FAD_{ox}/FAD_{sq} and FAD_{sq}/FAD_{hq} couples ($E_1^{\circ\prime}$ = −231 mV, $E_2^{\circ\prime}$ = −192 mV). Furthermore, a maximum of 37% of the flavin in holo-E_3 was stabilized kinetically in its blue neutral semiquinone form, but less than 20% was thermodynamically stabilized during potentiometric titrations at pH 7.5. In contrast, approximately 66% of the blue neutral semiquinone was stabilized in E_3(apoFeS) under kinetic conditions; while 55% stabilization was observed under thermodynamic conditions. Clearly, deletion of the iron−sulfur center has a significant impact on the redox properties of the flavin coenzyme.

The redox properties of the flavin coenzyme in a closely related enzyme, PDR, were recently studied in which the iron−sulfur binding domain of PDR was removed proteolytically to form a truncated PDR(-FeS) (Gassner & Ballou, 1995). The fully oxidized and fully reduced spectra of E_3(apoFeS) and PDR(-FeS) are similar in appearance, and their flavin redox potentials and semiquinone stabilization properties are also quite comparable. However, in the case

of PDR the formal potential of the FMN_{ox}/FMN_{sq} couple remained constant while the FMN_{sq}/FMN_{hq} couple increased by 52 mV (from −285 to −235 mV) when the holoenzyme was converted to PDR(-FeS) (Gassner & Ballou, 1995). In contrast, the changes seen in E_3 are in the opposite direction with the FAD_{sq}/FAD_{hq} couple being 48 mV more negative in the E_3(apoFeS) (-240 mV) than the holo-E_3 (−192 mV) (Table 2). The difference in the redox potentials between these two related systems are quite dramatic with an almost 100 mV difference between the $E_2^{o\prime}$ of the two holo enzymes. Interestingly, the domain organizations of PDR and E_3 are quite different. The iron−sulfur binding domain of PDR is located at the carboxy terminus of the NAD binding domain (Correll et al., 1992, 1993) whereas the iron−sulfur domain of E_3 is linked to the amino terminus of the flavin binding fold (Ploux et al., 1995). Whether the divergence seen in the flavin potentials between these two holoenzymes is related to the distinction of the domain linkage must await a full characterization of the E_3 structure and further investigation of its structure−redox property relationship.

The formal potential of the iron−sulfur center in E_3 was determined to be −257 mV, and that of E_1 was found to be −209 mV. The potential of the E_3 [2Fe-2S] determined spectroelectrochemically was confirmed by correlating the extent of reduction of this metal center to the potential at which this enzyme was poised. As shown in Figure 5, the percent reduction of the E_3 [2Fe-2S] center at given potentials, deduced from the EPR spectra, fits remarkably well with the theoretical reduction curve derived from the formal potential determined spectroelectrochemically for the E_3 iron−sulfur center. It has been suggested that the electron transfer in E_1/E_3 catalyzed reaction follows the sequence: NADH → FAD → E_3 [2Fe-2S] → E_1 [2Fe-2S] (Scheme 1). However, our potential data of these redox centers at pH 7.5 do not support the thermodynamic favorability of the proposed electron flow. As listed in Table 2, the potential of −257 mV of the E_3 [2Fe-2S] center is clearly out of line to serve as a mediator receiving reducing equivalent from FAD (E_m = −212 mV) and then passing on to the E_1 [2Fe-2S] center (−209 mV). The existence of such a barrier to electron transfer between the E_3 FAD and iron−sulfur center is puzzling. Although reduction with the appropriate kinetics may overcome such a relatively small barrier permitting electron transfer between the E_3 FAD and [2Fe-2S] center to occur at a reasonable rate, the unusual redox properties of this system implies that the E_1/E_3 catalysis may be regulated by a specific mechanism. For example, this thermodynamic barrier could serve as a control prohibiting electron transfer unless an excess of NADH is readily accessible. Only under such conditions, the gap between the redox potentials of the FAD and iron−sulfur center could be overcome by the sheer reductive force of the excess reducing equivalents. Since the O-antigen is not essential for cell growth and function, the biosynthesis of its sugar component may thus be controlled by the effectiveness of E_3, which is in turn regulated by the availability of NADH.

A few scenarios are also conceivable in which the midpoint potentials of either the E_3 FAD or iron−sulfur center may be altered, making the electron transfer thermodynamically feasible. For instance, the spectral properties of E_3(apoFeS) closely resembles those of adrenodoxin reductase, which contains one FAD per molecule and has a midpoint potential of −291 mV at pH 7.5. Interestingly, in the presence of $NADP^+$, the midpoint potential of adrenodoxin reductase shifts positively to −198 mV. It was suggested that this large potential shift is important for the reduction of this flavoprotein by NADPH (Lambeth & Kamin, 1976). Since FAD is the immediate acceptor of the hydride from NADH in E_3 catalysis, it is possible that NADH/NAD^+ binding to E_3 may also play a role in a manner similar to the adrenodoxin reductase case by lowering the FAD potential to facilitate this otherwise unfavorable electron transfer process. However, the absorbance at 454 nm was unaffected by the addition of excess NAD^+ when E_3 was partially reduced at −206 mV. Thus, it appears that the midpoint potential of E_3's FAD is not regulated by NAD^+ binding. Similar results were also found for PDR(-FeS) (Gassner & Ballou, 1995) and MMO reductase from strain *Methylococcus capsulatus* (Bath) (Lund & Dalton, 1985), both of which are closely related to E_3.

Since the electron relay proceeds through the iron−sulfur center of E_3 to the iron−sulfur center of E_1, these two centers must have intimate interaction during the electron transfer. Hence, it is also a possibility that the protein−protein interactions between E_3 and E_1 may cause the midpoint potential of the E_3 [2Fe-2S] center to shift to a more positive value allowing a thermodynamically favorable transfer of electrons. In fact, this has precedence in the adrenodoxin reductase:adrenodoxin system in which the formal potential of the [2Fe-2S] center of adrenodoxin is shifted negatively by 30−40 mV upon binding with adrenodoxin reductase (Lambeth et al., 1976). However, no change of potential was noted when an EPR spectroelectrochemical experiment was applied to qualitatively determine if the midpoint potential of the E_3 iron−sulfur center was shifted in the presence of excess E_1. Clearly, the interaction between E_1 and E_3 imposes no effect on the E_3 [2Fe-2S] potential and most likely plays no role in regulation of this system. It should be noted that E_1 and E_3 have been shown to form a complex not only *in vitro* but also *in vivo* (unpublished data).

Another common mode of regulating electron transfer in biological systems is through substrate/product binding. A good example is that the midpoint potential of the FAD in medium-chain acyl-CoA dehydrogenase undergoes a positive shift from −136 to −26 mV when it is bound with substrate (Lenn et al., 1990). Such a large shift brings its potential in line with the substrate/product couple (in a range of −38 to −45 mV) and the electron transfer flavoprotein (−25 mV) which is the proximate electron acceptor. From these data an isopotential delivery of electrons from substrate into the electron-transport chain has been proposed (Lenn et al., 1990). However, the substrate **2** for the E_1/E_3 reaction is bound only in the active site of E_1, and the reducing equivalents are thought to be transferred from E_3 to the substrate through the E_1 [2Fe-2S] center (Scheme 1). Since there is no immediate interaction between E_3 and its sugar substrate/product, their binding to E_1 could only affect the potentials of the E_3 cofactors indirectly, possibly by changing the conformation of E_1 such that its interactions with E_3 may induce a potential shift in a favorable direction. Unfortunately, the complications associated with monitoring the changes of overlapping chromophores from three redox centers in E_1 and E_3 and the lack of a suitable competitive inhibitor that would bind to E_1 during potentiometric titration under equilibrium conditions have both hampered the attempts to test the effect of substrate/product binding on the

redox potentials of this system. Thus, this remains a possible scenario.

There exists yet another mechanism whereby the redox potentials of a biological system can be regulated. Early studies of xanthine oxidase (XO), which contains two [2Fe-2S] clusters, one FMN, and a molybdenum center, showed that when XO is two-electron-reduced at lower pH, its flavin is fully reduced and its iron−sulfur centers remain oxidized. In contrast, the flavin semiquinone and reduced iron−sulfur prevail when XO is reduced at higher pH (Hille, 1994; Rohlfs & Hille, 1991). The same results were also reported for trimethylamine dehydrogenase (TMD) which is a FMN and [4Fe-4S] containing enzyme (Rohlfs & Hille, 1991). In both cases, the redox potential of the flavin semiquinone/hydroquinone couple was found to be higher (more positive) than the iron−sulfur center(s) at lower pH but lower (more negative) than the metal center(s) at higher pH. Interestingly, a similar effect on the midpoint potential of the E_3 FAD was also observed when the pH of the system was increased to 8.4. Since the redox potential of flavin is known to be pH sensitive and becomes more negative at higher pH, the observed change of E_3's midpoint potential from −212 to −273 mV is therefore not surprising. However, in the E_3 case the FAD_{ox}/FAD_{sq} couple shifts most dramatically rather than the semiquinone/hydroquinone couple as was noted above for TMD and XO. The formal potential of the E_3 [2Fe-2S] center was also found to be more negative at higher pH (−257 mV at pH 7.5 versus −279 mV at pH 8.4), whereas the potential of the E_1 iron−sulfur center at pH 8.4 (−206 mV) is essentially the same as that at pH 7.5 (−209 mV). It is thus clear that while there exists a thermodynamic barrier to electron transfer at pH 7.5, this barrier is considerably reduced at elevated pH as the midpoint potential of FAD approaches that of the iron−sulfur center in E_3. Such a pH-induced modulation of the redox properties of the flavin and iron−sulfur centers allows a thermodynamically favorable delivery of electrons through the E_1/E_3 conduit.

Recently, the kinetics of the reductive half-reaction of E_3 was studied using excess NADH as the reductant (Gassner et al., manuscript submitted). It was found that the extent of the intramolecular electron transfer from FAD_{hq} to the [2Fe-2S] center in E_3 is prototropically controlled by an ionizable group with a pK_a of about 7.3. At pH 7.0, a significant fraction of the enzyme remains stabilized in the oxidized [2Fe-2S] and FAD_{hq} state, but at pH 10 intramolecular electron transfer gives a high yield of the reduced [2Fe-2S] and FAD_{sq}. Based on the pH-dependent distribution of electrons between the iron−sulfur center and FAD of E_3, it was concluded that the FAD_{sq}/FAD_{hq} couple potential ($E_2^{\circ\prime}$) is more positive than that of the iron−sulfur potential at pH 7, and the trend is reversed at pH 10 with the FAD_{sq}/FAD_{hq} couple having a lower potential than the iron−sulfur center (Gassner et al., manuscript submitted). Interestingly, in the potentiometric titration experiments, very little FAD_{sq} was stabilized at pH 8.4. Further analysis revealed that the decrease of the overall midpoint potential the E_3 FAD comes from the extreme shift in the $E_1^{\circ\prime}$ value from −231 mV at pH 7.5 to −345 mV at pH 8.4. The discrepancy between the stopped-flow kinetic measurements and the potentiometric results indicated that the kinetic intermediate states may differ in both their structure and electronic properties from the intermediate states that are stabilized at equilibrium. Nevertheless, the insights gained from both the kinetic and the potentiometric studies provide compelling evidence supporting that the electron transfer in the E_1/E_3 system is regulated by pH.

As mentioned earlier, there are a number of enzymes that, like E_3, also contain flavin and [2Fe-2S] centers and function as electron transport proteins (Mason & Cammack, 1992). The list includes PDR, benzoate-1,2-dioxygenase reductase (Niedle et al., 1991; Yamaguchi & Fujisawa, 1978), 4-chlorophenylacetate-3,4-dioxygenase reductase (Schweizer et al., 1987), 4-sulfobenzoate-3,4-dioxygenase reductase (Locher et al., 1991), toluate-1,2-dioxygenase reductase (Zeyer et al., 1985; Niedle et al., 1991), 4-methoxybenzoate monooxygenase (putidamonooxin) reductase (Twilfer et al., 1981), vanillate demethylase reductase (Brunel & Davison, 1988), MMO reductase, xylene monooxygenase reductase, and phenol hydroxylase reductase (Nordlund et al., 1990). While all of these enzymes are involved in electron transfer from reduced nucleotides (NAD[P]H) via flavin and [2Fe-2S] redox centers to a terminal acceptor, typically a metallooxygenase, only a few have their redox properties characterized in detail. Shown in Table 2 is the comparison of the redox potentials of PDR, xylene monooxygenase reductase and MMO reductase from *Methanobacterium* CRL26 and *M. capsulatus* (Bath). Unlike E_3, whose $E_2^{\circ\prime}$ is more positive than $E_1^{\circ\prime}$ with $E_1^{\circ\prime}$ being greater than the [2Fe-2S] potential, the redox potentials of other enzymes listed in Table 2 are all compatible with a thermodynamically favorable electron transfer since their flavin $E_2^{\circ\prime}$ values are isopotential with or lower than their [2Fe-2S] centers. In addition, in all four examples the $E_1^{\circ\prime}$ values are 50 to over 100 mV higher than their corresponding $E_2^{\circ\prime}$ values, indicating a greater extent of flavin semiquinone stabilization in each case. Indeed, it was found that 80% of the blue neutral semiquinone was stabilized in PDR during NADH reduction (Gassner et al., 1994), while only 37% was stabilized kinetically and less than 20% was stabilized thermodynamically in E_3 during reductive titration. Apparently, despite their structural and mechanistic similarities, the energetics of the electron transfer of E_3 are distinct from these iron−sulfur flavoprotein reductases.

While the E_3 redox behaviors are unique among its immediate peers, similar properties have been reported for the adrenodoxin reductase−adrenodoxin complex (Lambeth & Kamin, 1979). Since this system is a 1:1 complex of a low molecular mass iron−sulfur protein (adrenodoxin) and a high molecular mass flavoprotein (adrenodoxin reductase), it is effectively a structural mimic of E_3. It has been shown that, at lower pH (6.0−7.0), the midpoint potential of flavin in adrenodoxin reductase is higher than the iron−sulfur center in adrenodoxin. However, as the pH is increased, the potential difference between the flavin and iron−sulfur centers narrows and thus allows an isopotential flavin-to-[2Fe-2S] electron transport. Judging from the rate of reduction of NADPH-cytochrome *c* by the adrenodoxin reductase-adrenodoxin complex, a linear correlation was also found between the potential difference of the flavin and iron−sulfur centers and the rate of electron transfer between these redox centers. Such a pH effect on the redox potential as well as the electron transfer rate led to the postulation that both changes are regulated by the protonation state of an amino acid residue with a pK_a of 6.8 in the proximity of the iron−sulfur center (Lambeth & Kamin, 1979). Interestingly, the potentials of the flavin and iron−sulfur centers in

E_3 determined in this work and the rate of equilibrating electrons between these redox centers deduced from a kinetic study of E_3 (Gassner et al., manuscript submitted) were both also found to be pH dependent. A recent study of the prototropic control of intramolecular electron transfer in trimethylamine dehydrogenase revealed that the electron transfer from the two-electron-reduced FMN_{hq} to the iron−sulfur center of this enzyme may be regulated by the protonation state of the N-1 position of its 6-cysteinyl FMN moiety (Rohlfs et al., 1995), which has a pK_a value of approximately 6 (Rohlfs & Hille, 1994). Deprotonation at N-1 of FMN_{hq} decreases the reduction potential for the FMN_{sq}/FMN_{hq} couple ($E_2^{\circ\prime}$) below that for the iron−sulfur center, so that at pH 8 the intramolecular electron distribution favors FMN_{sq} and reduced iron−sulfur center. It is therefore likely that the prototropic control of electron transfer in E_3 by an ionizable group with a pK_a of 7.3 under kinetic conditions may also be ascribed to the ionization at the N-1 position of the FAD in this enzyme (Gassner et al., manuscript submitted). However, the effect found for E_3 under equilibrium conditions is that the potential of the FAD_{ox}/FAD_{sq} couple ($E_1^{\circ\prime}$) becomes lower than that of iron−sulfur center at elevated pH. Considering that the midpoint potential of the E_3 FAD is much more sensitive to the pH variation, the ionizable group responsible for inducing the potential change of E_3 must be on or proximal to the flavin coenzyme. Whether the prototropic regulation of the E_3 potential is still related to the ionization state at N-1 of FAD, or is controlled by a different mechanism, must await further investigation.

In summary, the redox properties of E_3 are clearly distinct from those of the closely related [2Fe-2S] containing flavoprotein reductases listed in Table 2. The midpoint potentials along with the kinetic data support the hypothesis that electron transfer between the E_3 redox centers may be prototropically controlled. Although there is precedence for pH control, such as trimethylamine dehydrogenase and adrenodoxin reductase-adrenodoxin, the details of the mode of such regulation may be different for E_3 than for these systems. The present results in conjunction with the insights gained from the kinetic studies of the reductive half-reaction of E_3 (Gassner et al., manuscript submitted) revealed a sophisticated equilibrium for the distribution of electrons between the E_3 redox centers and thus implied a complicated mechanism for the delivery of reducing equivalents catalyzed by the E_1/E_3 complex.

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