Molecular Properties of Pyruvate Formate-Lyase Activating Enzyme†

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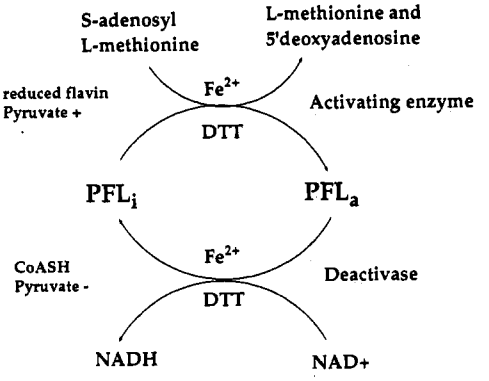
ABSTRACT: Pyruvate formate-lyase is a radical-containing enzyme that catalyzes the nonoxidative cleavage of pyruvate via a postulated homolytic mechanism. The formation of this enzymic radical in vitro requires an activating system composed of PFL-activating enzyme, *S*-adenosylmethionine, ferrous ion, a reduced flavin, DTT, and pyruvate as an allosteric effector. The need for large quantities of PFL-activating enzyme for biochemical and biophysical studies on the mechanism of protein radical formation has prompted us to clone the *act* gene and overexpress the gene product in *Escherichia coli*. Using PCR technology, the *act* gene was isolated and subcloned into various expression vectors. The overexpression of the protein was as high as 30–50% of the total cellular protein. However, the majority of the protein resided in the form of insoluble inclusion bodies. A procedure was developed to denature and isolate the inclusion bodies followed by refolding under anaerobic conditions. This purification method affords 5 mg of purified protein from 1 g of cells. Biochemical characterization demonstrated that the enzyme can bind one Fe(II) per protein monomer, and the protein did not exhibit any visible chromophore as previously observed. Co(II) and Cu(II) can be reconstituted into the protein with similar stoichiometries. Kinetic studies showed that the rate of radical formation was independent of ionic strength and the K_m's for SAM and inactive PFL were determined to be 2.8 and 1.2 μM, respectively. Fluorescent binding data revealed that the K_d for SAM binding to the activating enzyme alone was comparable to the K_m for SAM in the PFL activation indicating that the binding site for SAM resides on AE. Protein radical formation requires Fe(II). Several other divalent metal ions were found to be inhibitory to protein radical formation with Cu(II), Zn(II), and Cd(II) exhibiting the most potent inhibition. The putative Fe(II) binding domain shares homologies with two other proteins found in the protein databases.

Pyruvate formate-lyase (PFL,[1] EC 2.3.1.54) is a key enzyme of anaerobic glycolysis in facultative bacteria, such as *Escherichia coli*, and in some lower eucaryotes (Kalnitsky & Werkman, 1943; Utter & Werkman, 1944; Knappe & Sawer, 1990). It catalyzes the CoA-dependent, nonoxidative cleavage of pyruvate to acetyl-CoA and formate. The enzyme possesses an organic free radical at the active site (Knappe et al., 1984), and the mechanism of action has been proposed to be a homolytic process with the participation of the organic free radical (Brush et al., 1988; Unkrig et al., 1989). Recent findings suggest that the enzyme-stabilized radical is located on the α-carbon of glycine 734 in the protein backbone (Wagner et al., 1992), thus identifying PFL as the first enzyme of this type.

PFL is synthesized in the inactive, nonradical form under conditions of both aerobic and anaerobic growth. Under anaerobic conditions, however, transcription of the PFL gene is greatly increased and PFL is posttranslationally activated by an activating enzyme (AE) (Sawer & Bock, 1988, 1989). The activation reaction is SAM-dependent and also requires ferrous iron, DTT, and a reduced flavin under in vitro conditions (Scheme I). Pyruvate is a positive allosteric effector of the activation but can be replaced by oxamate (Conradt et al., 1984). The catalytic generation of the enzymic radical at the active site of PFL is the end-product of the activation process (Unkrig et al., 1989). Recently, a deactivating system has been discovered which reversibly inactivates PFL, presumably by quenching the radical. This reaction requires the presence of Fe(II), NAD, CoA, and pyruvate which serves as a negative effector. The deactivase activity is a response to

Scheme I: Regulation of PFL Activity by Activating Enzyme and Deactivase[a]



[a] PFL_i (nonradical form) is converted to PFL_a (radical form) by the action of activating enzyme with concomitant cleavage of SAM to L-methionine and 5′-deoxyadenosine. PFL_a is converted to PFL_i by the action of deactivase which converts NAD to NADH. Both reactions require Fe(II) and anaerobic conditions.

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[1] Abbreviations: PFL, pyruvate formate-lyase; AE, pyruvate formate-lyase activating enzyme; SAM, *S*-adenosyl-L-methionine; DTT, dithiothreitol; CoA, coenzyme A; EDTA ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; PMSF, phenylmethanesufonylfluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography; MOPS, 3-(*N*-morpholino)propanesulfonic acid; BSA, bovine serum albumin; LB, Luria-Bertani medium; TBE, Tris-borate EDTA buffer; TE, Tris-HCl and EDTA buffer; NAD, nicotinamide adenine dinucleotide, oxidized; EPR, electron paramagnetic resonance.

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the depletion of glucose during anaerobic growth (Kessler et al., 1991). The reversible formation of the active site radical is tightly regulated by activating enzyme and deactivase which in turn are sensitive to the metabolic status of the microorganism.

The activation of PFL is an intriguing reaction that represents a novel enzymatic system for the net synthesis of a catalytically important protein-based radical. Aside from the observation that one molecule of SAM is stoichiometrically reduced to methionine and 5′-deoxyadenosine with each formation of active dimeric PFL (Knappe & Schmitt, 1976), little is known about the mechanism of radical formation. Evidence for the incorporation of one radical per protein dimer has been further supported by EPR analysis and quantitation (Unkrig et al., 1989).

The physical and kinetic properties of AE have not been extensively characterized due to the low abundance of the protein present in *E. coli* (0.05% of the total soluble protein in *E. coli*). In this report, we describe the cloning of *act* using PCR methods, the overexpression of AE in *E. coli* utilizing various expression systems, and the subsequent purification and refolding of AE from inclusion bodies into active enzyme. We also report the characterization of the kinetic properties of recombinant AE and its binding of metal ions and of SAM.

MATERIALS AND METHODS

Restriction enzymes and T4 ligase were purchased from New England Biolabs or Promega. Malate dehydrogenase, citrate synthase, SAM, malate, NAD, $Fe(NH_4)_2(SO_4)_2$, pyruvate, and DTT were obtained from Sigma. Primers were synthesized at the Protein/DNA Facility of the University of Maryland. Plasmid pKK223-3 and *E. coli* JM105 were purchased from Pharmacia. Plasmid pMG27NS and *E. coli* N4830 were the gifts of Professor John Gerlt (University of Maryland), and plasmid pBAce and *E. coli* cell line Sø606 were graciously provided by Dr. C. C. Wang (UCSF). Guanidine hydrochloride (ultrapure) was purchased from Research Organics. 5-Deazariboflavin was the generous gift of D. T. Ashton, MSD Research Laboratories, Rahway, NJ. All other reagents were of the highest purity commercially available. Analysis of protein composition and N-terminal sequencing were performed at the Protein Sequencing Laboratory of the Medical Biotechnology Center, University of Maryland School of Medicine.

Molecular Biology. *E. coli* genomic DNA was isolated by a modification of earlier published procedures (Marmur, 1961; Hall et al., 1981). The genomic DNA was digested with *Hin*dIII and used for subsequent PCR reactions. The following primers corresponding to the 5′ and 3′ ends of *act* were synthesized on the basis of the known DNA sequence (Rodel et al., 1988). The 5′ primer had the following sequence: GGCCATATGTCAGTTATTGGTCGCAT which incorporated a *Nde*1 site at the start codon. The 3′ primer had the following sequence: TAAGCGATTATTTAAAAAAT. The PCR reaction consisted of 100 mM Tris-HCl, pH 9.0, 500 mM KCl, 1% Triton X-100, 20 mM $MnCl_2$, 2.5 mM dNTP solution, 1 mM each of the 5′ primer and 3′ primer, 1 μl of template (nanogram amounts of *Hin*dIII digested genomic DNA), and 2 units of *Taq* DNA polymerase (Promega) in a final volume of 100 μL. Amplification was performed in an Eppendorf Microcycler using 30 cycles of 5 min at 74 °C, 1 min at 94 °C, and 2 min at 37 °C. Hard copies of *act* from PCR amplification were made by direct ligation of the unpurified PCR product into the vector pCR-1000 (Invitrogen) and transformed into *E. coli* strain INFα according to the manufacturer's protocol. The resultant plasmid was designated pCRA1-2.

The placement of *act* under the control of the *tac* promoter was accomplished by digesting the plasmid, pCRA1-2, with *Nde*1 and subsequently filling in with Klenow. This was followed by digestion with *Hin*dIII. The 1 kb fragment containing the *act* gene was isolated and purified by low-melting agarose (IBI) gel electrophoresis and Gene-Clean (BIO 101), respectively. The expression plasmid pKK223-3 was digested with *Sma*1 and *Hin*dIII, isolated, and purified in the same manner. The two fragments were ligated unidirectionally using T4 DNA ligase overnight at 15 °C, and the reaction was transformed into JM105 cells using the $CaCl_2$ method (Maniatis et al., 1982). Positive ligation product was determined by restriction analysis using *Eco*R1 and *Hin*dIII and double-stranded DNA sequencing. The resultant plasmid was designated pKKA1-2. The placement of *act* under the control of the λ promoter was accomplished by digesting the plasmid pCRA1-2 with *Nde*1 and *Hin*dIII, and the 1 kb fragment was ligated into the *Nde*1 and *Hin*dIII sites of pMG27NS and transformed into N4830 cells using methods described above. The resultant plasmid was designated pMGA1-2. The placement of *act* behind a slow inducing promoter *pho*A was accomplished by digestion of pCRA1-2 with *Nde*1 and *Dra*1. The 1 kb fragment was ligated into the *Nde*1 and *Eco*RV sites of pBAce (Graig et al., 1991) and transformed into Sø606 cells as described above. The resulting plasmid was designated pBA1-2. Double-stranded DNA sequencing was performed according to the protocol from United States Biochemical using [α-^{35}S] dATP and Sequenase. DNA bands were resolved on either denaturing 6% bisacrylamide (using 1× TBE buffer) or 5% hydrolink (using 0.6× TBE buffer) using the Sequi-Gen Nucleic Acid Sequencing System (Bio-Rad) and exposed on X-ray film (Fuji).

Protein Induction Experiments. Cells harboring the plasmids, pMGA1-2 and pKKA1-2 were grown at 30 and 37 °C, respectively, overnight in 4 mL of LB containing ampicillin (50 μg/mL) and reinoculated in the same media the next day. The cells were grown at the designated temperature until late log phase (2–3 h). Induction was accomplished with either the addition of IPTG to a final concentration of 1 mM for pKKA1-2 plasmid or increasing the temperature to 42 °C for pMGA1-2 plasmid for 2 h. The cells were collected by centrifugation at 16000*g*, resuspended in extraction buffer (50 mM MOPS, pH 6.5, 2 mM EDTA, 5 mM DTT, and 1 mM PMSF), and sonicated using a Brandson Sonifier 250 equipped with a microtip set at 20% duty cycle. The cell extract was then centrifuged at 16000*g* for 20 min. Both the pellet and supernatant were analyzed by SDS–PAGE. Protein induction of the cells harboring *act* ligated into the pBAce vector has been described previously (Graig et al., 1991), and protein fractions were analyzed by SDS–PAGE.

Purification of Inclusion Bodies. N4830 cells harboring the pMGA1-2 plasmid were grown overnight in 4 mL of LB and ampicillin (50 μg/mL) media and inoculated into 200 mL of the same media the next day. The cells were allowed to grow until late log phase (3–4 h) and then induced at 42 °C for 2 h in a water shaker bath and subsequently collected by centrifugation. The pellet (0.8 g) was resuspended in 2 mL of extraction buffer and sonicated using a Brandson Sonifier 250 equipped with a microtip at 20% duty cycle setting. The inclusion bodies were collected along with other cell debris by centrifugation at 16000*g* for 20 min and washed with 50 mM Tris-HCl, pH 7.2. The suspension was collected again

by centrifugation and dissolved in 1.5 mL of denaturing buffer (50 mM Tris-HCl, 6 M guanidine-HCl, and 100 mM DTT, pH 7.2). The solution was maintained at ambient temperature for 1 h. Insoluble material was pelleted by centrifugation and the clear supernatant loaded unto a Superose-12 gel filtration column (HR16/50, Pharmacia) that had previously been equilibrated with 50 mM Tris-HCl, 6 M guanidine-HCl, and 1 mM DTT, pH 7.2. Protein fractions (1.5 mL) were eluted at 0.3 mL/min with the same buffer using the FPLC system (Pharmacia). Fractions corresponding to purified denatured AE, as judged by SDS–PAGE, were pooled and used for subsequent refolding experiments. The typical yield of purified, denatured inclusion bodies from 0.8 g of cells was approximately 10 mg.

Refolding of Denatured AE. The purified, denatured AE (10 mg in 10 mL) from the Superose-12 gel filtration step was allowed to refold by a 100-fold dilution into 50 mM Tris-HCl, pH 7.2, and 0.5 mM DTT that had been purged with argon for 2 h. Fe(II) at concentrations of 0.02–0.1 mM can be added to give iron-reconstituted protein. Refolding was performed at 4 °C under anaerobic conditions for approximately 14 h. The refolded protein solution was clarified by centrifugation, concentrated to 100 mL using an Amicon ultrafiltration apparatus equipped with a PM-10 membrane, and dialyzed against 50 mM K^+MOPS, pH 7.2, and 0.1 mM EDTA. After dialysis the solution was further concentrated to approximately 10 mL. The recovery was approximately 3–5 mg from 10 mg of denatured AE corresponding to a 30–50% yield of solubilized AE.

Chromatofocusing. Chromatofocusing of AE was performed using a Mono P (HR 5/10) chromatofocusing column equilibrated with 20 mM imidazole-HCl, pH 7.2, and developed by a pH gradient using polybuffer 74 (1:10 dilution), pH 4.0, with a flow rate of 0.5 mL/min. Fractions of 1 mL were collected, and the pH was measured using an Orion 811 pH meter. The pI of AE was determined from a plot of pH versus fraction number.

Protein Gel Electrophoresis. SDS–PAGE was performed on a discontinuous 10% acrylamide gel and ran at a constant 25 mA using the Mighty Small II gel apparatus (Hoefer Scientific). Protein was stained with Coomassie brilliant blue and calibrated using low molecular weight markers (Bio-Rad). Protein concentrations were determined by the method of Bradford (1976) provided in a kit from Bio-Rad using BSA as a standard.

Assay of AE Activity. The activity assay for AE was a modification of published methods that was based upon the appearance of PFL activity (Brush et al., 1988; Conradt et al., 1984). The assay mixture contained 100 mM Tris-HCl, pH 7.6, 100 mM KCl, 0.2 mM SAM, 15 μM 5-deazariboflavin, 5 mM DTT, 10 mM oxamate, 40 μg of inactive PFL, and 30 μL of AE sample in a final volume of 450 μL in a 1.5-mL septum sealed vial (Pierce). The mixture was degassed and purged with oxygen-free argon, and 5 μL of Fe(II) was added to a final concentration of 0.2 mM via a gas tight syringe. The sample was then degassed and purged with oxygen-free argon again and incubated at 30 °C in a water bath until anaerobic as indicated by a clear or greenish solution color. The reaction was initiated by photoreduction of the 5-deazariboflavin using a 300-W halogen bulb situated 5 cm away from the sample. After 20 min, an aliquot was removed via a gas tight syringe and assayed for PFL activity using the coupled enzyme system consisting of malate dehydrogenase and citrate synthase as described previously (Brush et al., 1988; Conradt et al., 1984). One unit of AE activity will catalyze the formation of 1 pmol of active PFL per min at 30 °C using 0.47 μM inactive PFL (Conradt et al., 1984). One picomole of PFL equals 50 units, and one unit of PFL will convert 1 μmol of pyruvate per min at 25 °C and pH 8.1 (Knappe et al., 1974; Ulissi-DeMario, 1991). The specific activity of AE is expressed as pmol/min per mg of AE. The initial rate data were fitted to the Michaelis–Menten equation using the Hypero Fortran program of Cleland (1979).

Absorption Spectroscopy. UV–vis absorption spectroscopy was performed on a Hewlett Packard 8450A diode-array spectrometer. Iron, copper, and cobalt reconstituted AE were concentrated to 0.5 mg/mL, and their respective metal content was quantitated by flame atomic absorption at 248.3 nm for Fe, 324.8 nm for Cu, and 240.7 nm for Co, using a Perkin-Elmer 2380 spectrometer equipped with an air/acetylene flame. Calibration curves were determined from known concentrations of aqueous $Fe(NH_4)_2(SO_4)_2$, $CuSO_4$, and $CoSO_4$ standards that were prepared fresh. Protein concentration was determined by the Bio-Rad protein assay. Proteins of known iron content (i.e., myoglobin and BSA) were also analyzed in the same manner and served as controls.

Fluorescent Binding Studies. Experiments were performed on a Fluorolog SPEX 1681 fluorimeter (Spex Industries, Inc.) connected to a water bath set at 25 °C in a 3-mL quartz cuvette. Typical experiments involved the addition of SAM from 0 to 120 μM to a solution of AE (3.5 μM) in 50 mM Tris-HCl, pH 7.2. Fluorescence measurements at an excitation wavelength of 295 nm were used to selectively excite tryptophan and were monitored with 20-s integrations at 350 nm. *S*-Adenosylmethionine does not absorb light at 295 or 350 nm thus eliminating the complication of an inner filter effect. The excitation maximum wavelength of the AE was 280 nm with the corresponding emission maximum wavelength at 350 nm. PFL has an emission maximum at 340 nm. The fluorescence values upon addition of SAM were normalized for dilution effects. The amount of fluorescence quenching should be proportional to the percentage of binding sites occupied by the ligand, SAM. For each concentration of SAM, the extent of quenching at 350 nm was recorded. The binding constant for SAM was then calculated using eq 1, where ΔF

$$\Delta F = \Delta F_{max} - K_s(\Delta F/[\text{SAM}]) \quad (1)$$

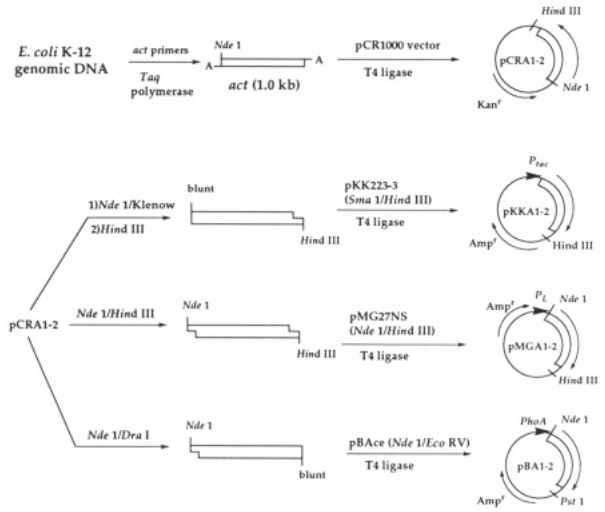
is the change in fluorescence at a specific concentration of SAM, ΔF_{max} is the maximum change in fluorescence when all binding sites are occupied, and K_s is the binding constant for the ligand, SAM.

Homology Search. The protein sequence of AE was used to search the protein databases using the Geneman program (DNASTAR, Ltd.). The threshold, gap penalty, and ktuple were set at 10%, 4, and 2, respectively. Multiple sequence alignments were carried out using the Megalign program (DNASTAR, Ltd.) employing the Clustal algorithm. Hydrophobicity analysis was performed using the Protean program (DNASTAR, Ltd).

RESULTS

Cloning and Overexpression of act. The *act* gene coding for AE was cloned from *E. coli* genomic DNA using PCR. Primers were synthesized on the basis of the published DNA sequence for *act* (Rodel et al., 1988). To facilitate subcloning, a *Nde*1 site was engineered near the ATG site of the gene. The *act* gene amplified by *Taq* polymerase containing 3′-dA overhangs was ligated directly into pCR1000 vector which contained the complementary 5′-T overhangs (Scheme II).

Scheme II: Cloning Strategies for *act*[a]



[a] The *act* gene was first isolated from genomic DNA by PCR amplification and ligated directly into pCR1000 plasmid. Subcloning of *act* into pKK223-3, pMG27NS, and pBAce expression vectors was performed as described under Materials and Methods.

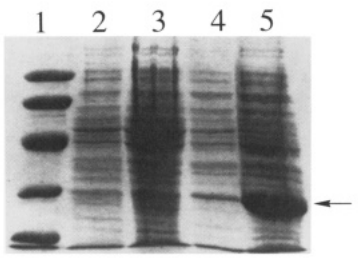
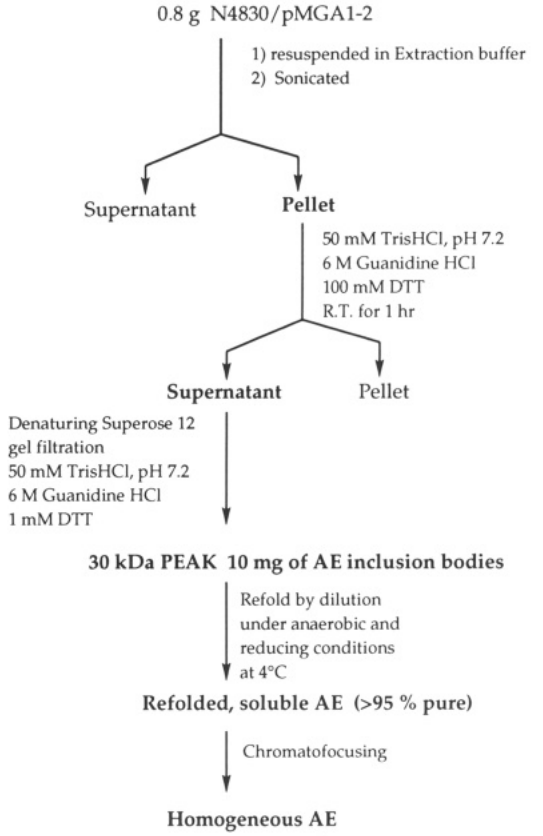


FIGURE 1: Overexpression of AE analyzed by 10% SDS–PAGE. Lane 1 is molecular weight markers, 97.4 kDa (phosphorylase b), 66.2 kDa (bovine serum albumin), 45 kDa (ovalbumin), 31 kDa (carbonic anhydrase), and 21.5 kDa (soybean trypsin inhibitor). Lanes 2 and 3 are soluble and insoluble cell extracts, respectively, from JM105/pKKA1-2 cells induced with 1 mM IPTG for 2 h. Lanes 4 and 5 are soluble and insoluble cell extracts, respectively, from N4830/pMGA1-2 cells induced for 2 h at 42 °C. The arrow indicates the position of migration for AE.

The resultant plasmid, designated pCRA1-2, had no *Taq*-induced mutations in the *act* coding region and was used subsequently for subcloning into expression vectors. *Act* was initially subcloned into the *Sma*1/*Hin*dIII sites of pKK223-3, but induction experiments showed that expression was low (Figure 1, lane 2). Subcloning *act* to the *Nde*1/*Hin*dIII sites of pMG27NS, a heat-inducible expression vector (Scheme II; Gross et al., 1985), gave substantially higher levels of expression, approximately 30–50% of the total cellular protein. However, greater than 90% of the recombinant protein resided in the insoluble portion of the cell extract, presumably in the form of inclusion bodies (Figure 1, lane 5). A variety of methods to solubilize the inclusion bodies were tested without satisfactory result. Among them were the placement of *act* behind the slow promoter of pBAce (Graig et al., 1991; Chen et al., 1991), the use of brief intervals of induction, and the inclusion of detergents (Frankel et al., 1991).

Isolation, Purification, and Refolding of AE from Insoluble Inclusion Bodies. The method of purification of the AE inclusion bodies under denaturing conditions followed by in vitro refolding of the protein yielded active enzyme (Martson & Hartley, 1990; Kohno et al., 1990). Scheme III outlines the procedure for solubilization of the inclusion bodies, purification, and in vitro refolding of AE. One advantage offered by the formation of inclusion bodies is that the relative

Scheme III: Denaturation, Purification, and Refolding of AE Inclusion Bodies[a]



[a] The AE inclusion bodies were isolated and dissolved in denaturing buffer after sonication. Denatured AE was purified via a denaturing gel filtration. Refolding by dilution under anaerobic and reducing conditions afforded soluble AE which was greater than 95% pure. A subsequent chromatofocusing step afforded homogeneous AE.

amounts of other contaminating proteins are few in the insoluble fraction. AE constituted at least 50% of the total protein present in the insoluble cell extract when overexpressed from pMGA1-2 (Figure 1, lane 5) and pBA1-2 plasmids (data not shown). For convenience, the heat-inducible expression plasmid pMGA1-2 was used as the source of AE inclusion bodies. Guanidine-HCl was used as the denaturant. The protein was successfully purified by a denaturing gel filtration column affording AE that was nearly homogeneous (>90%). The denatured protein was refolded under anaerobic conditions by a 100-fold dilution in the presence of 0.5 mM DTT. Fe(II) is not required for refolding but can be included at concentration of 0.02–0.1 mM to obtain Fe(II)-reconstituted enzyme. We have taken advantage of this step for the reconstitution of various divalent metals [i.e., Co(II) and Cu(II)] into AE. The final yield of the refolded protein was approximately 3–5 mg from 10 mg of denatured protein The specific activity was approximately 1300 units/mg, and the protein was greater than 95% pure as judged by SDS–PAGE. A chromatofocusing step can be used to obtain homogeneous AE (Figure 2, lane 1), but substantial loss of protein was observed due to precipitation.

Characterization of Recombinant AE. The recombinant AE isolated from this procedure migrated as a 30-kDa species under native and denaturing conditions and exhibited a pI of 5.7 consistent with that of the wild-type enzyme (Conradt et al., 1984). The amino acid composition of the recombinant AE agreed well with the predicted values determined from the DNA sequence, except for cysteine which was experimentally low (data not shown). N-terminal sequencing of

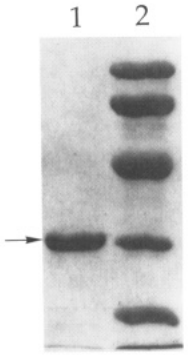


FIGURE 2: Homogeneous AE after Mono P chromatofocusing chromatography analyzed by 10% SDS–PAGE. Lane 1 is purified AE. Lane 2 is molecular weight markers.

Table 1: Atomic Absorption Analysis of Metal Reconstituted AE

sample	amt of protein[a] (nmol)	amt of M(II) (nmol)	stoichiometry (nmol of metal/ nmol of protein)	relative activity in the presence of 0.2 mM Fe(II) (%)
Fe-AE	17.0	12.4	0.7	100
Co-AE	17.0	12.4	0.7	113[b]
Cu-AE	17.0	10.2	0.6	72[b]
myoglobin	15.3	9.2	0.6[d]	
BSA	15.1	0[c]	0[d]	

[a] Protein concentrations were determined by the Bio-Rad protein assay and calculated based on the molecular mass of 28 000 Da. [b] Co-AE and Cu-AE were not catalytically active in the absence of added Fe(II).[c] Below the detectable limit of 0.02 nmol. [d] Determined values match the values determined independently by Sigma.

the recombinant AE yielded the sequence Ser-Val-Ile-Gly-Arg and matched the sequence predicted from the DNA sequence, indicating that the first methionine residue is removed after translation.

The activation of PFL catalyzed by AE is an Fe(II)-dependent reaction and AE has been described to be an Fe(II)-containing protein (Knappe & Sawer, 1990). There is, however, no direct evidence that AE actually binds Fe(II). With the recombinant enzyme reconstituted with Fe(II) in hand, atomic absorption was performed and was consistent with an average of ~1 iron per protein monomer (Table I). Furthermore, spectroscopically active metals such as Co(II) and Cu(II) can be reconstituted into AE with a stoichiometry similar to that for Fe(II)-reconstituted AE. The amounts of Fe(II) in the Co(II)- and Cu(II)-reconstituted AE's were negligible. Both the Co(II)- and Cu(II)-reconstituted AE's were unable to activate PFL in the absence of Fe(II), but when Fe(II) was added, the activities measured were similar to that of the Fe(II)-AE (Table I).

Previous studies with purified enzyme suggested the presence of a covalent chromophore having a peak absorbance at 395 nm and associated with the protein both under native and denaturing conditions (Conradt et al., 1984). In our hands, recombinant AE does not contain a visible chromophore at 395 nm under either native or denaturing conditions. Although refolding in the presence of Fe(II) afforded protein with an UV–vis absorbance shoulder at 300–340 nm (Figure 3A), this absorbance was removed by chromatofocusing with no loss of activity (Figure 3B).

Kinetic Studies. Kinetic studies on the activation of PFL have been difficult due to the low amounts of activating enzyme and the requirement for strict anaerobic conditions. As a result an accurate K_m for SAM has not been reported; moreover, the K_m for inactive PFL is not known. Earlier results using semipurified AE indicated that half-maximal

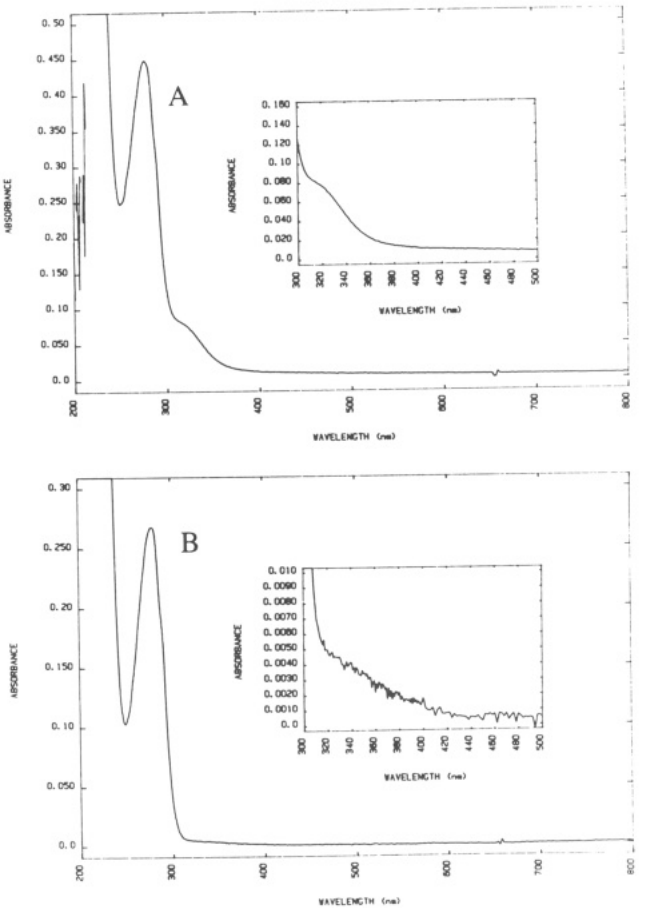


FIGURE 3: UV–vis absorption spectra of AE under aerobic conditions. (A) Fe(II)-reconstituted AE. (B) Apo-AE after chromatofocusing. The inserts are an expanded region of the absorption spectrum between 300 and 500 nm.

activity of activation occurred within 30–80 μM range of SAM concentrations (Chase & Rabinowitz, 1968; Knappe et al., 1969). Using recombinant AE, we have determined the K_m for SAM to be 2.8 ± 0.3 μM (Figure 4A). In addition, we have determined the K_m for inactive PFL to be 1.2 ± 0.4 μM (Figure 4B). The V_{max} for the activation was 1268 ± 277 pmol/(min·mg) at 0.2 mM SAM. The enzyme elutes as a single peak on a chromatofocusing column and the value of 1300 pmole/min mg was invariant from preparation to preparation.

The catalytic role of Fe(II) in the activation is unclear. Previous studies with semipurified enzyme indicated that Co(II) does not activate PFL but inhibits this process (Knappe et al., 1969). To address the metal requirement in the activation of PFL more thoroughly, a series of metals were tested for their abilities to substitute for Fe(II) in the activation of PFL. As shown in Table II none of the divalent metal ions tested were capable of supporting PFL activation, indicating that the activation of PFL has an absolute requirement for Fe(II); however, several metal ions were found to be inhibitors. Cu(II), Zn(II), and Cd(II) exhibited the best inhibition, 96%, 76%, and 62%, respectively, at 0.2 mM. Co(II) and Hg(II) were modest inhibitors while Ni(II), Mn(II), and Ca(II) had no effect on the rate of activation. Control experiments showed that the metal ions tested did not inhibit the coupling enzymes used to measure PFL activity. Chloride and sulfate salts of the metal ions gave essentially identical results. Inhibition studies with semipurified wild-type enzyme gave similar results (data not shown).

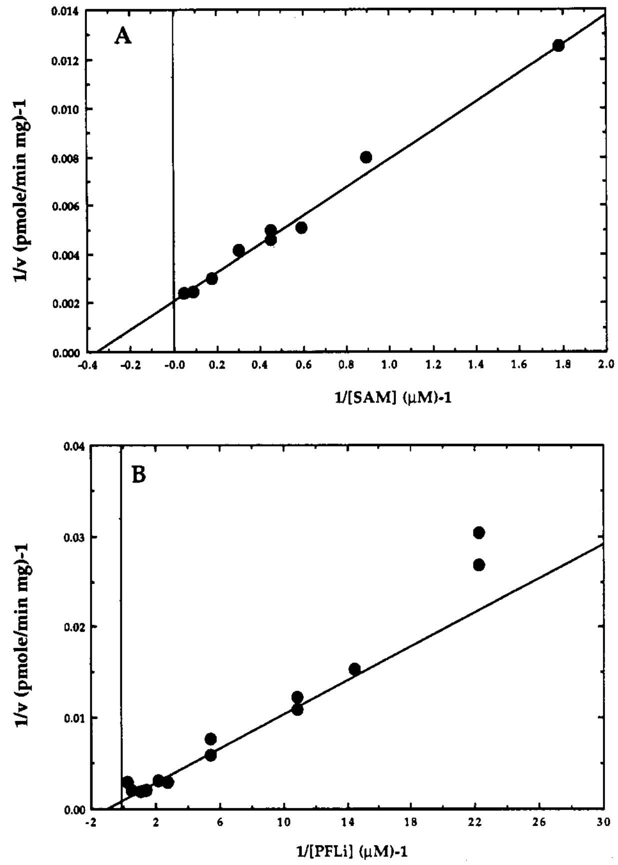


FIGURE 4: Kinetic parameters of SAM and PFL_i. Lineweaver–Burk plots with varying concentration of SAM (A) and PFL_i (B). Experimental conditions are described and Materials and Methods. The experimental values were fitted to the nonlinear regression program of Cleland (Hypero).

Table 2: Metal Inhibition on the Rate of PFL Activation Catalyzed by AE

metal	percentage of inhibition[a]	percentage of PFL activation[b]
Fe(II)		100
Cu(II)	96	0
Zn(II)	76	0
Cd(II)	62	0
Hg(II)	46	0
Co(II)	30	0
Ni(II)	0	0
Mn(II)	0	0
Ca(II)	0	0

[a] Fe(II) concentration is 0.1 mM, and metal inhibitor concentrations are 0.2 mM. Initial rates of PFLa formation were measured using the coupled enzyme assay (see Materials and Methods). Percentages of inhibition were calculated relative to the rate when no inhibitor was added. [b] Alternate divalent metal ions at 0.2 and 2.0 mM concentrations were substituted for Fe(II) in the activation reaction, and the activity of PFL was monitored as a function of time using the coupled enzyme assay (see Materials and Methods). Values obtained are relative to the value with 0.2 mM Fe(II).

Protein–protein interactions are clearly important during the activation reaction since AE must recognize and bind to inactive PFL. The observed insolubility of the recombinant AE suggested that hydrophobic interactions may play a role in such recognition. To test whether electrostatic interactions play a role in protein–protein recognition, the initial rate of PFL activation was measured as a function of ionic strength (0.1–1.6 M KCl). The results showed that the rate of radical formation was independent of ionic strength for both the purified recombinant enzyme and the semipurified wild-type

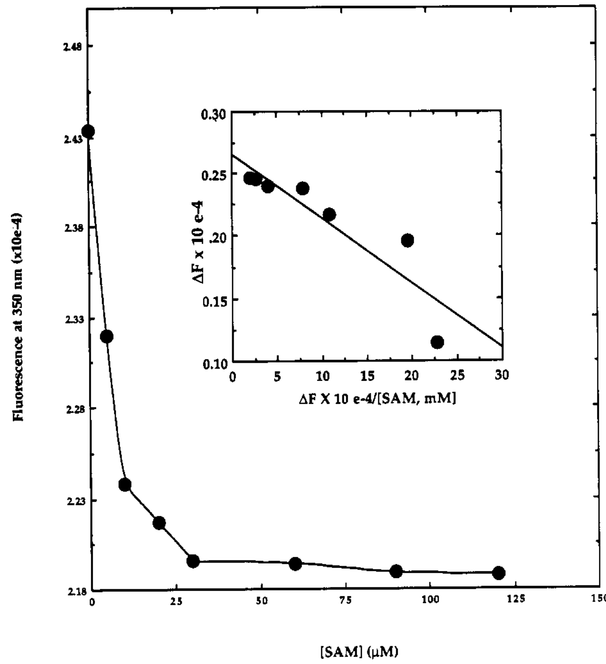


FIGURE 5: Fluorescence titration of AE with SAM. The addition of SAM to AE causes the quenching of the tryptophan fluorescence of the protein that is saturable (see Material and Methods). The plot of ΔF versus ΔF/[SAM] yielded a line where the binding constant K_s was derived from the slope of the line (inset).

```
         6                                  42
AE       IHSFESCGTVDGPGIRFITFFQGCLMRCLYCHNRDTW
         5                                  41
55.9     YDRIYPCDFVNGPGCRVVLFVTGCLHKCEGCYNRSTW
         776                               992
DNAPolα  VSAEVPPESLICLSCRAAGLPSPCLHKCILPKVIRGL
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FIGURE 6: Sequence homologies at the putative metal binding domain of AE. Gene 55.9 from T4 bacteriophage and DNA polymerase α from *T. crusei* shared significant homologies at the cysteine cluster (in bold) to AE. Identical residues are boxed.

AE (data not shown). Although the data do not directly address the role of hydrophobic contributions to the protein–protein interaction, it does suggest that electrostatic interactions play a relatively minor role in the activation.

Fluorescent Binding Studies. While the direct binding of SAM to AE has been inferred in the literature, it is not known if SAM binds exclusively to AE or if both AE and PFL are required. To address this question, we investigated the fluorescent quenching of the enzyme upon binding of SAM in the absence of inactive PFL. The SAM-dependent quenching of Fe(II)-AE fluorescence was saturable (Figure 5) and was consistent with a single SAM binding site per protein monomer. The data yielded a K_s of 5.2 ± 1.1 μM for SAM, in close agreement with the steady-state K_m value of 2.8 ± 0.3 μM.

Homology. The N-terminal domain of AE containing a cluster of cysteine residues postulated to be the metal binding region shared significant homology with two other proteins found in the Swiss protein database. The first protein is an open reading frame of gene 55.9 in bacteriophage T4. The cysteine cluster in gene 55.9 is also in the N-terminal. This gene is situated downstream from the Sun Y gene, which shares extensive homology to the *E. coli* anaerobic ribonucleotide reductase. The cysteines in this domain are highly conserved (Figure 6). The second protein is DNA polymerase α from *Trypanosoma crucei.* The homologous region is found in the middle of the protein and shared a lesser degree of homology to AE and gene 55.9, but two out of the three cysteines appeared to be conserved.

DISCUSSION

The AE-dependent formation of the active site radical in PFL requires the reductive cleavage of SAM. The site for this radical has recently been localized on glycine 734 (Wagner et al., 1992). This unusual reaction has only one other postulated example, the *E. coli* anaerobic ribonucleotide reductase. This reductase is an iron–sulfur enzyme that requires activation by SAM, although the reductive cleavage has not been demonstrated (Mulliez et al., 1993). The activation results in the formation of an organic radical that appears to be spectroscopically equivalent to the radical in active PFL. A related radical formation involving reductive cleavage of SAM in anaerobic bacteria has also been established for lysine 2,3-aminomutase from *Clostridium* SB_4. In this case, a postulated adenosyl radical intermediate is believed to function catalytically as a B_{12} surrogate (Baraniak et al., 1989; Moss & Frey, 1987). In addition, transient radical intermediates generated subsequent to the cleavage of SAM have been observed by EPR (Ballinger et al., 1992). These radical intermediates have been demonstrated to reside on the backbone of lysine during amino group migration. The detailed mechanism of radical incorporation into PFL by AE is unknown but has been postulated to occur via a similar adenosyl radical intermediate, although in this case cleavage of SAM is directly linked to PFL activation and only indirectly to pyruvate turnover (Knappe & Schmitt, 1976). Thus, the cleavage of SAM is catalytic with respect to pyruvate turnover. Our recent findings have revealed that the abstraction of an α-hydrogen from glycine 734 to generate active PFL is stereospecific (pro-*S*) and is rate-limiting (Murray et al., 1993).

The elucidation of the mechanism of this intriguing reaction has been hampered by the low abundance of AE in *E. coli* which is synthesized constitutively under both aerobic and anaerobic conditions. Although the purification of AE has been reported (Conradt et al., 1984), our attempts to purify AE to homogeneity following that procedure were not successful. As a first step toward this goal, the *act* gene encoding for AE was cloned from genomic *E. coli* DNA using PCR methods. Subcloning of *act* into various expression vectors afforded high levels of protein overexpression. However, the majority of the recombinant protein resided in the form of insoluble aggregates with only a small amount in the soluble fraction which was not amenable to purification due to precipitation during chromatographic steps. This problem was resolved by purification of the inclusion bodies under denaturing conditions followed by in vitro refolding (Gross et al., 1985; Marton & Hartley, 1990). The conditions for high recovery of AE required the presence of DTT and the absence of oxygen during the refolding step. Using this method, 3–5 mg of AE can be obtained from 1 g of cells requiring only 3 days. A previous purification protocol for the wild-type AE reported a yield of only 4 mg of protein from approximately 500 g of *E. coli* K-12 cells (Conradt et al., 1984) requiring a minimum of 8 days.

The insolubility properties observed when proteins are heavily expressed are not surprising and are usually due to an insufficient time for correct folding into the native state (Williams et al., 1982; Holladay et al., 1973). However, our extensive attempts to modulate transcription using in vivo methods to allow time for proper folding were unsuccessful and suggest that the observed insolubility may be predominately an intrinsic property of AE instead of a folding problem. Once purified using the method described, the recombinant AE was soluble at a concentration of 0.5 mg/mL; however, at concentrations greater than 0.5 mg/mL, precipitation was observed over time indicating that the protein may indeed be intrinsically hydrophobic. Analysis of the secondary structure of the protein revealed hydrophobic domains at the amino terminus, between residues 1 and 40, that may explain the observed insolubility of AE. Furthermore, the hydrophobic domains may play a role in the function of AE, since AE must interact with PFL during protein radical formation. It is conceivable, then, that hydrophobic recognition with PFL is important for activation. Along these lines, electrostatic protein–protein interactions appear not to be important in the activation of PFL since the rate of activation remains constant over a broad range of ionic strengths (i.e., 0.1–1.6 M KCl). At high concentrations of AE in the absence of PFL, hydrophobic interactions between molecules of AE may explain the aggregation properties of AE. Although the data cannot rule out the contribution of hydrogen bonding, it is certainly suggestive that hydrophobicity does plays a role in the activation reaction. This is in contrast to the cytochrome P_{450} reductase system where electrostatic interactions are the predominant force between the reductase and P_{450} proteins and this interaction is sensitive to ionic strength (Strobel & Coon, 1971; Bosterling & Trudell, 1982; Strobel et al., 1989).

We have demonstrated that AE can be reconstituted with Fe(II) having a stoichiometry of approximately 1:1. Protein radical formation has an absolute requirement for Fe(II) as no other divalent metal was a viable substitute. Previous studies have shown that Co(II) does not activate PFL but inhibits the activation of PFL (Knappe et al., 1969). Of the eight divalent metals tested, Cu(II), Zn(II), and Cd(II) did exhibit very potent inhibitory effects on the rate of PFL activation. Reconstitution of AE with Cu(II) or Co(II) also yielded a 1:1 stoichiometry suggesting that these alternate metal ions may bind to the same metal binding site, thus inhibiting activation of PFL by competing for the same site. The fact that Co(II) and Cu(II) reconstituted AE's displayed no significant loss of activity in the presence of excess Fe(II) suggests that the bound metal ion can exchange freely with the metal ions in solution under anaerobic conditions. Although there is no direct evidence that Fe(II) undergoes oxidation during protein radical formation, it is conceivable that Fe(II) is oxidized to Fe(III) during protein radical formation. The release of Fe(III) followed by binding of another molecule of Fe(II) would allow the enzyme to continue through another catalytic cycle. Competitive binding studies would be required to prove that these metals actually are binding to the Fe(II) site.

The metal binding site on AE has been postulated to reside on a stretch of polypeptide at the N-terminal portion of the protein (Rodel et al., 1988). As discussed previously, this region has a high predicted hydrophobicity index. Within this domain is a cluster of closely positioned cysteines. Our findings that thiophilic metals such as Cu(II), Zn(II), Hg(II), and Cd(II) are inhibitors of the activation process are consistent with the view that cysteines are the metal ligands. Sequence analysis of this putative metal binding domain reveals two other proteins that share significant sequence homologies with AE within this region. The first protein is an ORF of gene 55.9 in bacteriophage T4. This protein displays marked similarities at the three cysteine residues proposed to be the metal chelation sites. The gene 55.9 is situated in the T4 bacteriophage genome upstream to two other ORF's, gene 55.10 and SunY. SunY has an unknown function but shares extensive homologies with the anaerobic ribonucleotide reductase of *E. coli* (Sun et al., 1993). Recent findings that the

E. coli anaerobic ribonucleotide reductase may contained a glycyl radical at the active site suggest that, on the basis of sequence homology, SunY may also be a glycyl radical-containing anaerobic ribonucleotide reductase of T4 bacteriophage (Sun et al., 1993). Similarly, gene 55.10, whose function is unknown, contains the sequence VIGY at the N-terminal end of the protein and shares homology to the putative glycyl radical sequences TCGY, VCGY, and VSGY for the SunY, anaerobic ribonucleotide reductase, and PFL proteins, respectively. Conceivably, the 55.9 protein may be the corresponding activating enzyme for the SunY and 55.10 gene products. The second sequence with homologies with AE is DNA polymerase α from *T. crucei*. The enzyme contains two clusters of cysteine residues and has be postulated to form zinc fingers (Leegwater et al., 1991). There is no direct evidence that this cysteine-rich region in AE binds to zinc, but it is consistent with our observation that zinc is a potent inhibitor of the activation of PFL.

There has been some controversy as to whether AE contains a covalent organic chromophore (Conradt et al., 1984; Knappe & Sawer, 1990). The recombinant apo and denatured enzyme did not exhibit a UV–vis spectrum consistent with the existence of a covalent chromophore with an peak absorbance at 390 nm (Conradt et al., 1984) and agreed with the later conjecture of Knappe and Sawer (1990) that the enzyme has one tightly bound Fe(II), although no experimental evidence was presented. Our data provide direct evidence that AE can bind to one mole of Fe(II) per mole of protein and verify the assertion of Knappe and Sawer (1990). We have no evidence in support of any organic cofactor associated with AE. Furthermore, AE synthesized via in vitro translation of *act* exhibited PFL activation activity thus excluding the *absolute* requirement for an intrinsic cofactor (data not shown).

Kinetic analysis demonstrated that the activation reaction follows Michaelis–Menten kinetics with respect to the substrates SAM and inactive PFL. The K_m of 1.2 μM for inactive PFL is consistent with the amount of PFL present in the aerobic *E. coli* (Knappe & Sawer, 1990). Thus, AE would be operating under V/K conditions upon switching to anaerobic conditions. Fluorescent binding studies suggest that AE is capable of binding to SAM in the absence of inactive PFL with a dissociation constant that is very close to the steady-state K_m value. Furthermore, anaerobic conditions are not a prerequisite for the binding of SAM. Previous observations with semipurified enzyme indicated that reductive cleavage of SAM occurred, albeit slowly, in the absence of inactive PFL (Knappe & Schmitt, 1976). Our findings along with Knappe and Schmitt's observations suggest that SAM and inactive PFL may bind in an ordered fashion with SAM binding first, followed by inactive PFL. A random ordered binding mechanism is also possible, where both inactive PFL and SAM can bind to free enzyme. However, we have no evidence that AE is capable of binding to inactive PFL in the absence of SAM. Furthermore, the ability to separate the two proteins via aerobic gel filtration suggests that a complex may not form under aerobic conditions (Knappe et al., 1974). The roles of Fe(II) and reduced flavin are still unknown in the formation of this "activation complex", and additional studies are required to determine the kinetic mechanism of complex formation.

In summary, with the availability of recombinant AE, we have demonstrated that AE is exclusively proteinaceous and contains one iron binding site. Protein radical formation is strictly an Fe(II)-dependent process that follows Michaelis–Menten kinetics with respect to SAM and inactive PFL. Finally, SAM can bind to the free form of AE under aerobic conditions suggesting an ordered kinetic mechanism. Studies are underway using EPR spectroscopy to determine the metal chelation environment at the AE active site. Biochemical and biophysical studies into the mechanism of protein radical formation are also in progress.

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