Redox Properties of the PutA Protein from *Escherichia coli* and the Influence of the Flavin Redox State on PutA−DNA Interactions†

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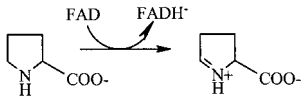
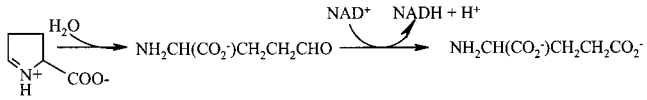
ABSTRACT: The PutA flavoprotein from *Escherichia coli* is both a transcriptional repressor and a membrane-associated proline dehydrogenase. PutA represses transcription of the *putA* and *putP* genes by binding to the control region DNA of the *put* regulon (*put* intergenic DNA). Previous work has shown that FAD has a role in regulating the transcriptional repressor and membrane binding functions of the PutA protein. To test the influence of the FAD redox state on PutA−DNA interactions, we characterized the redox properties of the PutA flavoprotein from *E. coli*. At pH 7.5, an E_m(E−FAD/E−FADH$_2$) of −0.076 V for the two-electron reduction of PutA-bound FAD was determined by potentiometric titrations. Stabilization of semiquinone species was not observed during potentiometric measurements. Dithionite reduction of PutA, however, caused formation of red anionic semiquinone. The E_m value for the proline/Δ^1-pyrroline-5-carboxylate couple was determined to be −0.123 V, demonstrating the reduction of PutA by proline is favored by a potential difference ($\Delta E^{\circ\prime}$) of more than 0.045 V. Characterization of the PutA redox properties in the presence of *put* intergenic DNA revealed an E_m(E_{DNA}−FAD/E_{DNA}−FADH$_2$) of −0.086 V. The 10 mV negative shift in E_m corresponds to just a 2.3-fold increase in the dissociation constant of PutA with the DNA upon reduction of FAD. Thus, it appears the FAD redox state has little influence on the overall PutA−DNA interactions.

Enteric bacteria can use proline as the sole source of carbon, nitrogen, and electrons. To use proline, expression of the *putP* and *putA* genes of the proline utilization (*put*) regulon is required. The *putP* gene product is necessary for L-proline transport, and the *putA* gene encodes the multifunctional flavoprotein, PutA (*1*, *2*). PutA has been most extensively characterized from *Escherichia coli* and *Salmonella typhimurium* and is a peripherally membrane-bound dehydrogenase that catalyzes the two step oxidation of L-proline to glutamate (*2*−*7*). In the first step, flavin adenine dinucleotide (FAD)[1] is required for the dehydrogenation of L-proline to Δ^1-pyrroline-5-carboxylate (P5C) in which two electrons are transferred from proline to the FAD cofactor presumably as a hydride (*4*, *5*).



Electrons from reduced FAD are then transferred to an acceptor in the electron transport chain. In the second step, P5C is first hydrolyzed to give a glutamic semialdehyde which is subsequently oxidized to glutamate by coupling with nicotinamide adenine dinucleotide (NAD^+) reduction (*2*, *3*):



In addition to catalysis, PutA is an autogenous transcriptional regulator of the *put* regulon (*1*, *8*, *9*). Transcription of the *putP* and *putA* genes occurs in opposite directions from the central *put* intergenic control region. In both *E. coli* and *S. typhimurium*, PutA represses the divergent transcription of genes *putP* and *putA* by binding to specific promoter sequences in the *put* intergenic region (*7*, *10*). It was demonstrated that PutA has multiple binding sites in the *put* intergenic region and that upon binding PutA bends the DNA, conceivably forming a complex PutA−DNA structure (*10*). More recently, PutA has also been shown to be an autorepressor of *putA* gene transcription in *Pseudomonas putida* and *Sinorhizobium meliloti* (*11*, *12*). In these soil bacteria, PutA appears to have a critical role in root colonization since proline is an important energy source during root nodule formation (*11*, *13*). PutA is one of only two known flavoproteins that is also a transcriptional regulator. The nitrogen fixation regulatory protein L (NifL) from *Azotobacter vinelandii* is the other flavoprotein that acts as a transcriptional regulator (*14*, *15*). NifL represses transcription of the nitrogen fixation (*nif*) genes in *A. vinelandii* by regulating the activity of the transcriptional

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[1] Abbreviations: FAD, flavin adenine dinucleotide; *put*, proline utilization; NAD^+, nicotinamide adenine dinucleotide; P5C, Δ^1-pyrroline-5-carboxylate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactoside; DCPIP, dichlorophenolindophenol; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; E_m, reduction potential; $\Delta E^{\circ\prime}$, potential difference.

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activator nitrogen fixation regulatory protein A (NifA) through protein−protein interactions in response to alterations in the flavin redox state (*14*, *15*). Unlike PutA, however, NifL does not interact with regulatory DNA sequences.

PutA from *E. coli*, the subject of this study, is a single polypeptide of 1320 amino acids and purifies as a homodimer with an apparent molecular mass of 293 kDa determined from sedimentation and diffusion coefficients and contains 2 mol (per 293 kDa unit) of noncovalently bound FAD (*7*, *16*). Because there is no PutA crystal structure and no significant homology with a protein whose crystal structure is known, domain structure analysis of PutA has been based on sequence comparisons. Sequence analysis has identified residues 340−590 and 650−1130 as the proline and P5C dehydrogenase domains, respectively (*16*). Residues 315−357 are thought to comprise the FAD-binding motif since they are similar to the consensus FAD binding region of the succinate dehydrogenase family (*16*). Sequence analysis has not revealed putative DNA and membrane binding domains.

Pivotal results on how the PutA protein switches between its two mutually exclusive functions as a membrane-bound proline dehydrogenase and a transcriptional regulator have been gained by assessing the effects of proline and the FAD redox state on PutA associations with the membrane and the *put* intergenic DNA. It has been demonstrated that in the presence of proline, PutA associates with the membrane (*6*, *17*). Surber and Maloy (*17*) reported that PutA association with inverted membrane vesicles is increased by 40% in the presence of proline (*17*). Since proline reduces the FAD, the FAD redox state is implicated as a regulatory signal for the association of PutA with the membrane. Other observations that support a FAD redox-dependent mechanism are that the midpoint for the association of PutA with the membrane and the reduction of the FAD occur at approximately the same concentration of proline, 0.1 mM (*18*). Furthermore, the addition of an electron acceptor (2,3-dimethoxy-5-methyl-1,4-benzoquinone) to proline-reduced PutA disrupts PutA−membrane associations, presumably through reoxidation of the FAD (*17*). Evidence that PutA−DNA interactions are also redox dependent is more ambiguous. Maloy et al. (1997) showed that the addition of proline causes PutA to release the *put* intergenic DNA, but only in the presence of membrane vesicles (*19*). Just a small decrease (2-fold) in the binding constant of PutA to the *put* intergenic DNA was detected in the presence of proline (*20*). Yet in gel-shift assays employing sodium dithionite as a reducing agent, the *put* intergenic DNA was released from PutA, suggesting a much larger change in the PutA−DNA binding constant upon reduction of the FAD (*21*).

To determine whether PutA−DNA binding is redox-dependent, we have characterized the redox properties of PutA in the absence and presence of the *put* intergenic DNA. We report that only a slight change in the overall affinity of PutA for the *put* intergenic DNA occurs upon reduction of the FAD. Our results support the proposal that redox-dependent changes in PutA affinity for the membrane regulate PutA location and function (*17*, *18*).

MATERIALS AND METHODS

Enzymes and Chemicals. T4 DNA ligase, calf intestinal alkaline phosphatase, T4 DNA kinase, and Taq DNA polymerase were purchased from Promega and were used according to the recommendations of the manufacturer. Restriction endonucleases were purchased from Fermentas. β-Agarase was purchased from New England Biolabs. DNA sequences were determined using the Sequenase Version 2.0 DNA sequencing kit obtained from U.S. Biochemical Corp. [γ-^{32}P]dATP and [α-^{32}P]dATP were purchased from Amersham Corp. Sequence specific synthetic oligonucleotides were purchased from Oligos Etc. Inc. (Bethel, ME). DL-P5C was synthesized as previously described and quantitated using *o*-aminobenzaldehyde as described by Strecker (*22*−*24*). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from NovachemBiochem Corp. All other chemicals and buffers were obtained from either Fisher or Sigma Chemical Co.

Bacterial Strains and Plasmids. The *E. coli* strains JM109 and Stratagene XL-blue were used for the subcloning of the *put* intergenic region and the *putA* gene. *E. coli* strain BL21 DE3 pLysS (Novagen) was used as the host for the expression of PutA. *E. coli* strain JT31 and the pML9 construct were generous gifts from J. Wood (University of Guelph, Guelph, ON). The pGEM-T and pET-3a vectors were obtained from Promega and Novagen, respectively.

Put Intergenic DNA Preparation. Oligonucleotides 5′-GGTGGATCCCATGCCATTACTCCT-3′ and 5′-GTGC-GAATTCCCATCTAAAGTCTCCAA-3′ served as primers in a polymerase chain reaction (PCR) using genomic DNA from *E. coli* strain JT31 as the template. The genomic DNA was purified using the Qiagen Genomic DNA purification kit. The PCR was performed using a Perkin-Elmer thermal cycler and Taq DNA polymerase. The conditions for the PCR were as follows: denaturation at 94 °C, annealing at 50 °C, and elongation at 72 °C. The PCR product was cloned into a pUC18 vector using *Bam*HI and *Eco*RI. The DNA sequence of the PCR product was determined using two universal primers (M13 reverse and −40) and was shown to match the sequence of the *put* intergenic region. The new construct was designated pUTC01.

E. coli strain XL blue containing pUTC01 was grown in Terrific broth (100 μg of ampicillin/mL) at 37 °C until the OD reached ∼2.0−3.0. pUTC01 was isolated and purified using the Wizard Plus Megaprep DNA Purification System from Promega. Typical yields were 2−4 μg of plasmid DNA/mL of culture. Purified pUTC01 was cut with *Bam*HI and *Eco*RI, and the *put* intergenic DNA was separated by electrophoresis in a 0.8% low-melting agarose gel. A 432 bp fragment containing the *put* intergenic region (419 bp) was then excised from the gel and isolated by digestion with β-agarase at 37 °C according to the manufacturer's (New England Biolabs) recommendations. The 432 bp fragment was resuspended in water and lyophilized. The lyophilized product was then resuspended in 70 mM Tris buffer (pH 7.5) and 10% glycerol. The DNA was quantitated by UV−visible absorption at 260 and 280 nm ($A_{260}/A_{280} = 1.8-1.9$) and verified by agarose gel electrophoresis.

PutA Preparation. Ling and Wood created the pML9 construct by inserting a *Hin*dIII−*Sma*I 5.7 kb fragment, which contains the *putA* gene, into pT7-6 (*16*). We modified pML9 by positioning an *Nde*I site at the translation start codon and a *Bam*HI site immediately after the translation stop codon. The *Nde*I and *Bam*HI sites were introduced using synthetic oligonucleotides and the QuikChange (Stratagene)

site-directed mutagenesis kit. The *putA* gene (3.963 kb) was then subcloned into a pET-3a vector using *Nde*I and *Bam*HI for expression in *E. coli* strain BL21(DE3) pLysS. PutA is expressed from the ∅*10*T7 promoter in the pET-3a vector by inducing with isopropyl β-D-thiogalactoside (IPTG) when the culture reaches an OD between 0.6 and 1.0. Purified PutA originated from cultures grown in Terrific broth and induced with IPTG for 3 h at 37 °C.

Purification of PutA was similar to that reported previously except for a few modifications and the exclusion of the hydroxyapatite column (*7*, *18*). Pelleted *E. coli* cells were resuspended in 70 mM Tris (pH 8.1) buffer containing 10% glycerol, 1 mM FAD, and protease inhibitors phenylmethanesulfonyl flouride (0.5 mM), L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-HCl (0.13 mM), L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone (0.08 mM), ϵ-amino caproic acid (5 mM), and leupeptin (1 μM). The resuspended cells were disrupted by sonication at 4 °C using a pulse sequence of 15 s on, 45 s off (8 min total pulse time). The broken cells were spun at 30 000 rpm for 30 min. The resulting supernatant was then applied to a high-performance Q-Sepharose column equilibrated with 70 mM Tris (pH 8.1) and 10% glycerol. A 1 L gradient from 70 to 500 mM KCl in 70 mM Tris (pH 8.1) and 10% glycerol eluted PutA. Fractions containing proline dehydrogenase activity were then pooled and precipitated with 50% ammonium sulfate. The resulting 50% ammonium sulfate pellet was resuspended in 70 mM Tris buffer (pH 7.5) with 10% glycerol and 5 mM EDTA containing 1.5 mM CHAPS and applied to a Superose-6 gel filtration column equilibrated with 70 mM Tris (pH 7.5), 10% glycerol, and 5 mM EDTA. Eluted PutA was then pooled and concentrated using a 50 mL Amicon ultrafiltration cell with a 30 kDa molecular mass cutoff. Purified PutA was stored at −70 °C. Typical yields of PutA were ∼30 mg/L of culture.

Characterization of PutA. Activity assays for PutA were performed as described previously (*4*, *7*, *18*). Proline dehydrogenase activity was determined using dichlorophenolindophenol (DCPIP) as the terminal electron acceptor. One unit of proline dehydrogenase activity is the quantity of enzyme that transfers electrons from 1 μmol of proline to dichlorophenol indolphenol (DCPIP)/min at 25 °C. P5C dehydrogenase activity was determined by monitoring the reduction of NAD^+ at 340 nm. One unit of P5C dehydrogenase activity is the quantity of enzyme that reduces 1 μmol of NAD^+/min at 25 °C. The concentrations of oxidized PutA were determined spectrophotometrically using an extinction coefficient of 12 700 M^{-1} cm^{-1} at 452 nm and by the Bradford method using the Bio-Rad reagent with bovine serum albumin as the standard (*18*, *25*). To estimate the molar ratio of FAD to polypeptide, PutA was denatured in 6 M guanidinium chloride and the spectrum was recorded from 600 to 250 nm. The total amount of polypeptide was determined using the predicted molar extinction coefficient for denatured PutA of 122 562 M^{-1} cm^{-1} at 278 nm in 6 M guanidinium chloride and the molar extinction coefficient for uncomplexed FAD in guanidinium chloride of 11 800 M^{-1} cm^{-1} at 450 nm (*26*). The N-terminal amino acid sequence of purified PutA was determined at the Protein Core Facility at Washington University (St. Louis, MO).

Gel-Shift Assays. The interactions of PutA with *put* intergenic DNA were characterized using nondenaturing gel electrophoresis as described previously (*7*). The *put* intergenic DNA was labeled at the 5′ end with [γ-^{32}P]ATP using a labeling protocol from Promega. PutA (0−200 nM) was incubated with ^{32}P-labeled *put* intergenic DNA in 70 mM Tris buffer (pH 7.5) containing 10% glycerol and 5 mM EDTA at 20 °C for 15 min prior to electrophoresis. Calf thymus competitor DNA (100 μg/mL) was added to the PutA−DNA binding mixture to prevent nonspecific PutA−DNA interactions. The PutA−DNA binding mixture was then electrophoresed for 3 h at 4 °C in a 3.5% polyacrylamide gel in Tris-borate EDTA buffer (89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA) at a constant voltage (8 V/cm). Addition of CHAPS, methyl viologen, indigo disulfonate, or pyocyanine to binding assays did not disrupt the formation of the PutA−DNA complex.

After electrophoresis, the gels were analyzed by a Storm 860 Phosphoimager (Molecular Dynamics). The positions of the uncomplexed and PutA-complexed DNA in the gels were confirmed in a separate experiment by using a 50 bp ladder (Promega) and by staining PutA with Coomassie Blue G-250, respectively. In addition to the uncomplexed and PutA-complexed DNA bands, the ^{32}P label was also found in the wells which was not unexpected since it was observed in previously reported gel-shift experiments with PutA (*10*, *21*). The variation between the lanes in the amount of ^{32}P label that did not enter the gel was typically <10% as estimated by phosphorimaging analysis. Despite the loss of the ^{32}P label in the wells, the uncomplexed and PutA-complexed DNA bands were clearly visible in the gel-shift assays.

The overall dissociation constant of oxidized PutA with the *put* intergenic DNA was determined by nonlinear regression methods assuming one independent PutA binding site per *put* intergenic DNA using eq 1

$$\text{fraction of DNA bound} = n[\text{L}]/(K_{\text{dox}} + [\text{L}]) \quad (1)$$

where n is the number of PutA binding sites and [L] is the total concentration of PutA. The disappearance of the uncomplexed DNA band was followed during titrations with PutA to calculate the fraction of DNA bound at each PutA concentration. The radioactivity of the uncomplexed DNA band was quantitated using a Storm 860 Phosphoimager (Molecular Dynamics). The errors in the estimation of K_{dox} and n were determined by the method of least squares in which the total squared deviation is the sum of the squared deviations of the experimental data to the best fit of the data. Although PutA has multiple binding sites in the *put* intergenic DNA, the data fit a simple model of one PutA binding site per *put* intergenic DNA and showed no complications from multiple binding sites or cooperativity (*10*). Constraining the number of PutA binding sites to higher values such as 2 did not generate a reasonable fit to the experimental data and significantly increased the error (>30%) in the estimation of K_{dox}. Inspection of the data by Scatchard or Hill plot analysis showed no evidence of site heterogeneity or cooperativity, respectively.

UV−Visible Spectroscopy and Spectroelectrochemistry. Potentiometric and coulometric measurements of PutA were performed using a spectroelectrochemical cell as previously described under an argon atmosphere (*27*). Experiments with DNA-complexed PutA utilized a spectroelectrochemical cell

in which the solution volume was reduced from 3.5 to 0.7 mL. Experimental conditions were 20 °C and pH 7.5−7.55 in 70 mM Tris buffer containing 10% glycerol, 5 mM EDTA, and 1.5 mM CHAPS. Sodium dithionite solutions were made fresh before each experiment in 0.1 M sodium pyrophosphate buffer (pH 9.0) and standardized by titrating FAD. Sodium sulfite solutions were made in 70 mM Tris buffer (pH 7.5). All potential values are reported versus the standard hydrogen electrode. Methyl viologen (0.1 mM) was used as the mediator dye, while pyocyanine (5 μM) and indigo disulfonate (2−3 μM) were used as indicator dyes. The redox potential values(E_m) for pyocyanine and indigo disulfonate were determined to be −40 and −109 mV, respectively, under the same experimental conditions used in the potentiometric titrations of PutA (70 mM Tris, pH 7.5, and 20 °C). PutA−FAD concentrations in the spectroelectrochemistry experiments ranged from 10 to 20 μM. The *put* intergenic DNA concentrations were in 2-fold excess over that of the PutA dimer in the DNA-complexed PutA experiments. Using the macroscopic dissociation constant from this work for *put* intergenic DNA with PutA (K_{dox} = 45 nM), the amounts of PutA dimer bound to DNA during the experiment were calculated at lower limits of 95%. To test nonspecific PutA−DNA interactions, potentiometric titrations of PutA were performed in the presence of calf thymus DNA (1.5 mg/mL).

UV−visible spectra in each experiment were recorded on a Cary 100 spectrophotometer. The absorbancies at 451 and 476 nm (isobestic point for indigo disulfonate) were used to monitor the amount of oxidized and reduced FAD after correcting the spectra for turbidity. A correction for turbidity was applied by subtracting a light scattering spectrum. The amount of turbidity at each point in the titration was determined by measuring the absorbance at 800−850 nm where all observed increases in absorption are due to scattering. The amount of light scattering subtracted from each spectrum was then based on the increased absorbance at 800−850 nm during the potentiometric titration. The potentials reported were determined in the reductive direction. After each potentiometric experiment, PutA was completely reoxidized using oxygen or ferricyanide (0.1 mM) as a mediator. The equilibrium of the system in the UV−visible potentiometric measurements was considered to be obtained when the measured potential drift was less than 1 mV in 5 min; this was typically around 1−2 h. The reduction potentials (E_m) and n values were calculated from the Nernst equation (eq 2)

$$E = E_m + (0.058/n)\log([ox]/[red]) \quad (2)$$

where E is the measured equilibrium potential at each point in the titration and n is the number of electrons transferred. The typical error in the reported reduction potentials values was ±2−3 mV. All reduction potentials exhibited Nernstian behavior as indicated by their n values.

PutA was titrated with proline at 20 °C in 70 mM Tris buffer (pH 7.5) and 10% glycerol) under anaerobic conditions by degassing both the PutA and proline solutions with argon. At each concentration of proline, PutA was allowed to equilibrate for 5 min before recording the spectrum. The data were analyzed as previously described assuming the formation of a reduced PutA−P5C complex (eq 3) (*18*).

$$PutA_{ox} + \text{proline} \leftrightarrow PutA_{red}{-}P5C \quad (3)$$

Determination of the midpoint potential of the proline/P5C couple in 70 mM Tris (pH 7.5), 10% glycerol, and 5 mM EDTA at 20 °C was performed according to a previously described method (*28*). The total proline/P5C concentration (600 μM) was kept constant, while the ratio of proline/P5C was varied in each experiment. Methyl viologen (0.1 mM) was used as a mediator dye, while indigo disulfonate (10 μM), pyocyanine (12 μM), and anthraquinone-2,6-disulfonate (10 μM) were used as indicator dyes. After the entire contents of the cell had been degassed, the system was electrochemically reduced to a point within ±20 mV of the anticipated equilibrium position. Electrochemical reduction reduced the amount of substrate/product turnover required to reach equilibrium, which could eventually lead to a significant change in the ratio of proline to P5C. After electrochemical reduction, PutA (70 μL of a 50 μM stock) was added from the sidearm of the cell to the solution to yield a final PutA concentration of 1 μM. When the measured potential drift was less than 1 mV in 15 min, the potential was recorded. Due to the instability of P5C at neutral pH, P5C was neutralized with 1 M KOH just prior to the proline/P5C potentiometric experiments.

RESULTS

PutA Properties. Purification of PutA from *E. coli* strain BL21(DE3) pLysS followed a similar protocol to that reported previously (*7*, *18*). SDS−PAGE analysis demonstrated PutA was purified to >90% homogeneity. The nine-amino acid N-terminal sequence of purified PutA (MGTTT-MGVK) matches the predicted sequence from the *putA* gene and the PutA N-terminal sequence published previously except for the first methionine (*7*, *16*). The retention of the first methionine is not surprising since post-translational modifications often do not keep pace with highly efficient protein expression systems. The UV−visible spectrum of PutA is similar to that reported earlier with absorption maxima at 278, 381, and 451 nm but with slightly higher 278/451 and lower 451/381 ratios of 15.0 and 1.05, respectively (*18*). On the basis of the denatured protein absorbance at 278 nm and the amount of released FAD, PutA contains 0.83 mol of FAD per mole of polypeptide. Proline and P5C dehydrogenase specific activities of purified PutA were typically 2−2.5 and 0.2−0.3 units/mg, which is similar to that reported previously with a ratio of proline to P5C dehydrogenase activity of 8−10 (*7*).

Titration of PutA with sodium dithionite (pH 7.5) generated a noticeably visible stabilization of red anionic semiquinone with isobestic points observed at 502 and 406 nm during the transfer of the first electron, consistent with the presence of two species, oxidized FAD and anionic semiquinone (Figure 1). The greatest amount of semiquinone was observed at about 1 equiv of electrons (n = 1) with maximum absorbance occurring at 371 nm. In a plot of the absorbance at 371 and 451 nm versus the number of equivalents of electrons (n) (Figure 1, inset), the intersection of the lines at n = 1 yields the theoretical absorbance values at which 100% of the anionic semiquinone is formed. Using this plot, molar absorptivities of 15 450 and 3540 M^{-1} cm^{-1} were estimated for the red anionic semiquinone at 371 and 451 nm,

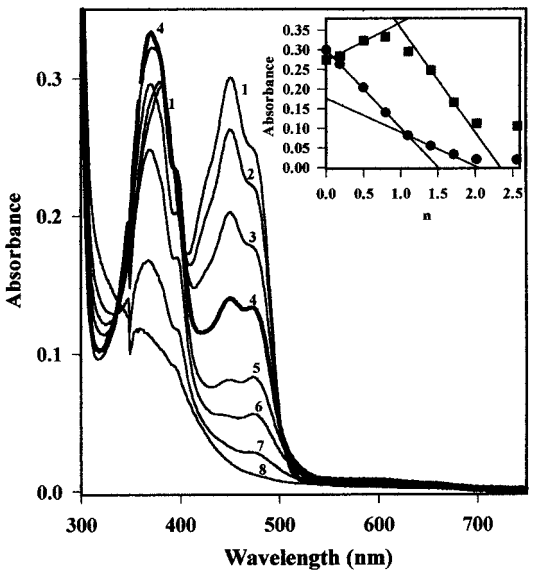

FIGURE 1: Dithionite reduction of uncomplexed PutA (23.7 μM) at 20 °C (pH 7.5) (for curves 1−8, $n = 0$, 0.18, 0.5, 0.79, 1.1, 1.4, 1.7, and fully reduced, respectively). After each addition of sodium dithionite, PutA was allowed to equilibrate for 5 min prior to recording the spectrum. (Inset) Plot of absorbance at 371 (■) and 451 nm (●) vs the number of reducing equivalents.

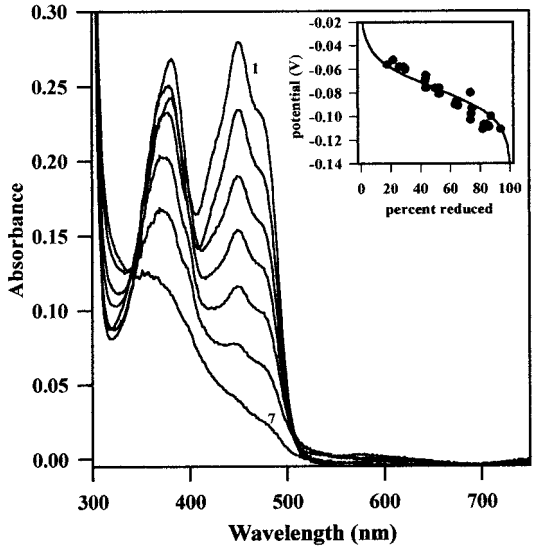

FIGURE 2: Coulometric reduction of uncomplexed PutA (22 μM) with 100 μM methyl viologen at 20 °C (pH 7.5) (for curves 1−7, $n = 0$, 0.33, 0.72, 1.04, 1.37, 1.71, and 2.04, respectively). The inset shows a fit of the reduction potential data for uncomplexed PutA (pH 7.5) to a theoretical curve generated from the Nernst equation for one redox center with a reduction potential of −0.076 V ($n = 2$).

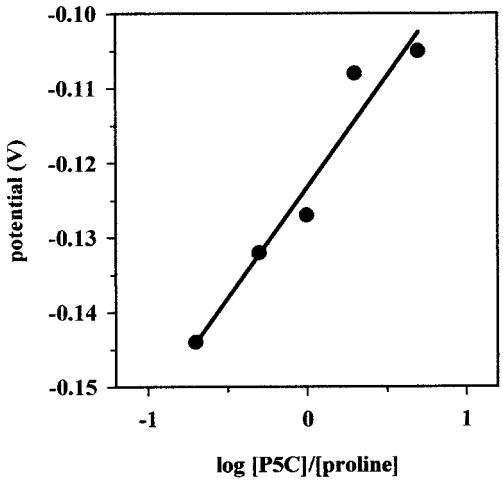

FIGURE 3: Nernst plot of the potentiometric data for the proline/P5C couple in 70 mM Tris buffer (pH 7.5) with 1 μM PutA at 20 °C. Proline to P5C ratios of 5:1, 2:1, 1:1, 1:2, and 1:5 were used, yielding a total proline/P5C concentration of 600 μM in each experiment.

respectively. The molar absorptivities for the fully reduced enzyme at 371 and 451 nm were 4730 and 870 M^{-1} cm^{-1}, respectively. From the molar absorptivities for oxidized FAD, anionic semiquinone, and reduced FAD, the maximum amount of red anionic semiquinone stabilized during the titration with sodium dithionite was calculated to be 65−70% by solving three equations with three unknowns. The anionic semiquinone was observed to decay at ∼0.3%/min by a disproportionation reaction resulting in the formation of oxidized and reduced PutA.

A coulometric titration of PutA showed less than 20% stabilization of the red anionic semiquinone during electrochemical reduction (Figure 2). In both the coulometric and dithionite titrations of PutA, two electron equivalents was required to fully reduce PutA, confirming FAD is the only redox active center. PutA reduced electrochemically or with dithionite was fully reoxidized upon addition of an equal volume of air-saturated buffer with a $t_{1/2}$ of ∼5 min. A reduction potential value of −0.076 V was determined for PutA from potentiometric measurements with no significant stabilization of semiquinone species during the titration (Figure 2, inset). A Nernst plot gave a 34 mV slope for the potentiometric measurement, which is near the theoretical value of 29 mV for a two-electron transfer. Removal of glycerol or CHAPS from the buffer had no effect on the reduction potential of PutA.

A titration of PutA with proline under anaerobic conditions showed that complete reduction of PutA is achieved and that no significant amount of semiquinone species is generated by reduction with proline. The equilibrium constant for the anaerobic reduction of PutA by proline is 36 mM^{-1}, which is 4-fold higher than the previously determined equilibrium constant under aerobic conditions (*18*). PutA reduced with proline was fully reoxidized in 4 h upon the addition of an equal volume of air-saturated buffer with a $t_{1/2}$ of ∼125 min. To assess the potential difference ($\Delta E^{\circ\prime}$) between the proline/P5C couple and the FAD in PutA, the reduction potential for the proline/P5C couple was determined. Potentiometric measurements were taken for various proline/P5C ratios. From a Nernst plot of the measured potentials, a reduction potential of −0.123 V was determined for the proline/P5C couple with a slope of 30 mV, consistent with a two-electron transfer (Figure 3). Thus, the reduction of PutA by the proline/P5C couple is thermodynamically driven by a $\Delta E^{\circ\prime}$ of ∼45−50 mV.

PutA−DNA Interactions. The entire *put* intergenic region was titrated with oxidized PutA to determine the overall dissociation constant of the PutA−DNA complex (K_{dox}). During the titration, a low-mobility PutA−DNA complex formed similar to that observed previously by Brown and Wood (*7*) (Figure 4). A dissociation constant of ∼45 ± 4 nM (Figure 4) was determined by monitoring the disappearance of the uncomplexed DNA at each point in the titration

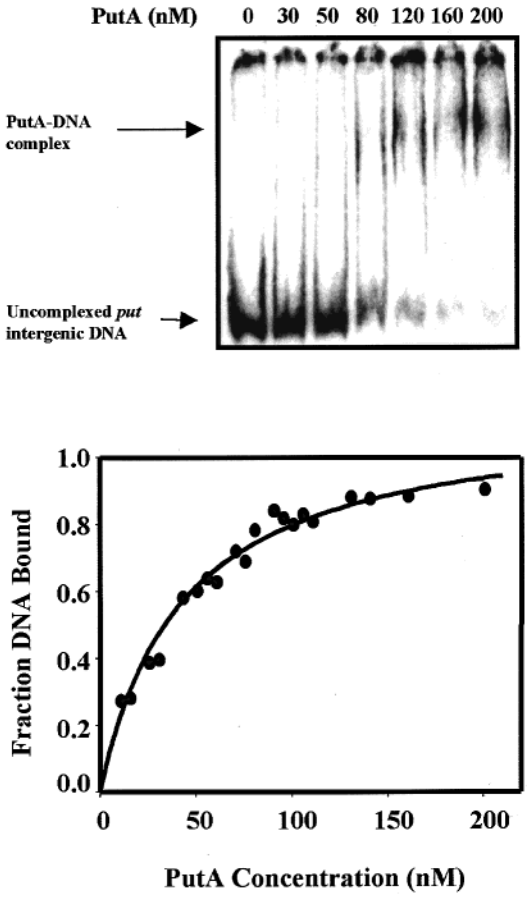

FIGURE 4: Gel mobility shift analysis of PutA and the *put* intergenic DNA. (Top) Representative gel-shift assay in which increasing concentrations of PutA (0−200 nM dimer) were added to binding mixtures containing *put* intergenic DNA (1 nM) and 100 μg/mL nonspecific calf thymus DNA at 20 °C. The complexes were separated using a native polyacrylamide gel (3.5%), and the uncomplexed DNA was quantitated. (Bottom) The plot of the fraction of bound DNA vs PutA dimer concentration from the gel-shift assays. The data were fit to eq 1 (n = 1.1) to yield a dissociation constant of 45 ± 4 nM.

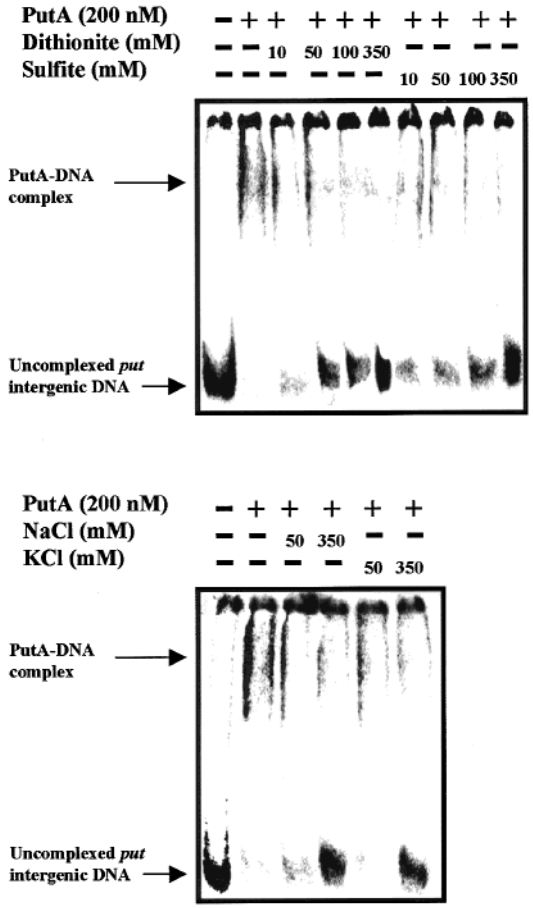

FIGURE 5: Gel mobility shift analysis of the effects of sodium dithionite and changes in ionic strength on PutA−DNA associations. In both gel-shift assays, the binding mixtures contained PutA (200 nM dimer), calf thymus DNA (100 μg/mL), and *put* intergenic DNA (1 nM). (Top) PutA−DNA binding mixtures were titrated with sodium dithionite and sodium sulfite (0−350 mM) at 20 °C. The complexes were separated using a native polyacrylamide gel (3.5%), and the uncomplexed DNA was quantitated. Fifty percent of the *put* intergenic DNA was released at ∼100 mM sodium dithionite and sodium sulfite. (Bottom) PutA−DNA binding mixtures were treated with sodium chloride and potassium chloride (50 and 350 mM) at 20 °C. The uncomplexed and PutA-complexed DNA were separated using a native polyacrylamide gel (3.5%).

and fitting the data to eq 1. The binding data were fit best with one independent PutA binding site (n = 1.1) per *put* intergenic DNA. Since the uncomplexed DNA band was used to determine the amount of free and bound DNA, the dissociation constant we determined is for the first binding event, presumably the event with the highest affinity.

The influence of the FAD redox state on PutA−DNA interactions was first tested by reducing PutA with sodium dithionite and monitoring the disruption of the PutA−DNA complex by gel-shift assays. Previously, it was shown that in the presence of sodium dithionite (350 mM), PutA releases the *put* intergenic DNA, implicating the FAD redox state as a regulator of PutA−DNA binding (*21*). We performed the dithionite reduction of PutA by incubating PutA−DNA complexes with different concentrations of sodium dithionite and quantitating the amount of uncomplexed *put* intergenic DNA (Figure 5, upper gel). As a control, we also titrated the PutA−DNA complex with sodium sulfite since oxidation of dithionite generates bisulfite (*29*). Figure 5 (upper gel) shows that both sodium dithionite and sodium sulfite cause the release of the *put* intergenic DNA. To determine whether sulfite reacted with the FAD in PutA (e.g., addition of sulfite to the flavin N-5 position), we titrated PutA with sulfite up to a concentration of 50 mM (*30*). As expected, we observed no reactivity of FAD with sulfite (e.g., bleaching of the flavin absorbance at 450 nm) because dithionite reduction of PutA is fully reversible (*31*). We then tested the effects of changing ionic strength on PutA−DNA interactions by adding sodium chloride and potassium chloride (0−350 mM) to the PutA−DNA binding mixtures (Figure 5, lower gel). Increasing the ionic strength also disrupted the PutA−DNA complex, demonstrating by gel shift experiments that it is not clear whether dithionite reduction of the FAD influences PutA−DNA interactions.

To more carefully evaluate the effect of the FAD redox state on the overall PutA−DNA interactions, we measured the redox properties of PutA in the presence of *put* intergenic DNA. No change in the absorbance spectrum of FAD was detected upon complexation of PutA with the *put* intergenic DNA except for the contribution of DNA to the absorbance at less than 400 nm (Figure 6). Coulometric reduction of PutA in the presence of DNA was similar to the reduction of uncomplexed PutA with no significant stabilization of

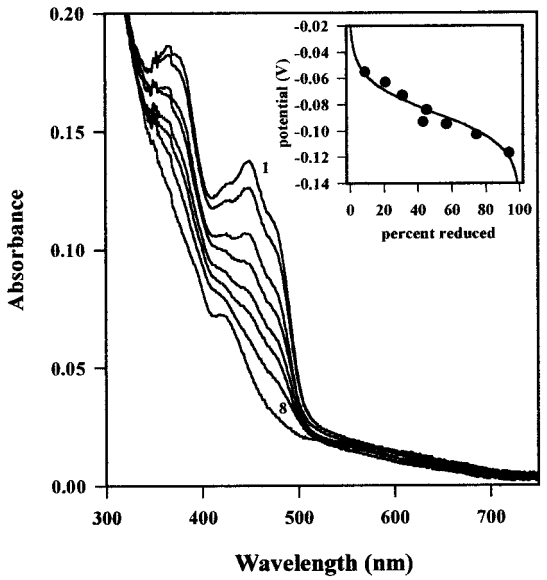

FIGURE 6: Coulometric reduction of PutA (5.4 μM dimer) in the presence of *put* intergenic DNA (7.9 μM or 2.2 mg/mL) with 100 μM methyl viologen at 20 °C (pH 7.5) (for curves 1−8, n = 0, 0.31, 0.66, 1.00, 1.31, 1.46, 1.61, and fully reduced, respectively). The inset shows a fit of the reduction potential data for DNA-complexed PutA (pH 7.5) to a theoretical curve generated from the Nernst equation for one redox center with a reduction potential of −0.086 V (n = 2).

semiquinone species (Figure 6). An E_m value of −0.086 V was determined for PutA in the presence of DNA (Figure 6, inset). A slope of 30 mV was obtained from a Nernst plot indicating a two-electron transfer. The reduction potential is 10 mV more negative than the reduction potential for uncomplexed PutA. To test whether the decrease in the reduction potential was due to the specific interaction of PutA with *put* intergenic DNA, we measured the reduction potential of PutA in the presence of nonspecific DNA. The E_m value for PutA in the presence of nonspecific calf thymus DNA was −0.078 V with a Nernst slope of 35 mV. Thus, the slight negative shift in the E_m value of PutA in the presence of *put* intergenic DNA can be attributed to specific PutA−DNA binding interactions. The 10 mV negative shift in the reduction potential, however, correlates to an only 2.3-fold increase in the overall dissociation constant of PutA with the *put* intergenic DNA upon reduction of FAD (K_{dred} ∼ 100 nM).

DISCUSSION

Important results from the laboratories of Maloy and Wood have established that proline reduction of the FAD regulates the macromolecular associations of PutA (*6*, *17*−*19*, *21*). It is evident that proline enhances PutA binding to the membrane. The understanding of PutA regulation, however, has been hindered by the lack of studies on the electrochemical properties of the bound FAD. Thus, our objective was to characterize the electrochemical properties of PutA to contribute new insights into the regulation of PutA associations with the *put* intergenic DNA and the membrane.

In both potentiometric and coulometric reductions of PutA, the reversibility of PutA reduction was demonstrated by fully reoxidizing bound FAD. Both uncomplexed and DNA-complexed PutA showed no significant stabilization of semiquinone species during electrochemical reduction. Stabilization of the red anionic semiquinone was only achieved when PutA was titrated with sodium dithionite, indicating the second electron transfer is kinetically slow using a negatively charged reductant. The reduction potentials of PutA and the proline/P5C couple demonstrate a substantial thermodynamic driving force for the reduction of PutA by proline. The oxygen reactivity of proline-reduced PutA ($t_{1/2}$ ∼ 125 min) is considerably slower than electrochemically reduced PutA ($t_{1/2}$ ∼ 5 min), suggesting that proline/P5C binding decreases the oxygen reactivity of the FAD. This would ensure the efficient delivery of electrons from FAD to the electron transport chain during catalysis. On the basis of kinetic studies, it has been proposed that P5C remains bound to the enzyme and is channeled to the P5C dehydrogenase active site (*32*).

The binding of PutA to the *put* intergenic DNA (419 bp) has been shown to be complex with significant curvature of the *put* intergenic region, suggesting both protein−protein and protein−DNA interactions occur in the transcriptional regulation of the *put* regulon (*10*). The stoichiometry of PutA binding to the DNA is unknown. At least five putative binding sites with dyad symmetry, however, have been identified in the *put* intergenic region (*10*, *33*). Although PutA−DNA binding appears to be complex, a simple approach of one independent PutA binding site per *put* intergenic DNA best described the PutA−DNA binding data. The dissociation constant we determined is an overall dissociation constant of oxidized PutA with the *put* intergenic DNA, a value which is similar to that reported previously for PutA from *S. typhimurium* (*20*).

The effect of the FAD redox state on PutA−DNA interactions was first evaluated by reducing the PutA−DNA complex with sodium dithionite. In the gel shift experiments, we observed that sodium dithionite causes PutA to release the *put* intergenic DNA as previously reported (*21*). However, because we also observed that increasing the ionic strength interferes with PutA−DNA binding it is evident that the disruption of the PutA−DNA complex by dithionite cannot be attributed to the reduction of the FAD. To test the influence of the FAD redox state on PutA−DNA interactions more explicitly, we determined the reduction potential of PutA in the presence of the *put* intergenic DNA. The reduction potential of PutA was found to become only 10 mV more negative upon binding the *put* intergenic DNA. This negative shift corresponds to just a 2.3-fold increase in the dissociation constant of PutA with the DNA upon reduction of the FAD. Our results demonstrate that the redox state of bound FAD has an almost negligible effect on the apparent affinity of PutA for the *put* intergenic region. Earlier work by Ostrovsky and Maloy (*20*) reported a 2-fold decrease in the PutA−DNA binding affinity in the presence of proline. Thus, it appears proline/P5C binding does not have any additional influence on PutA−DNA binding affinity.

The observation that PutA−DNA binding is not redox-dependent suggests transcriptional regulation of the *put* regulon is essentially unresponsive to changes in the redox state of FAD. However, it is still plausible that transcription of the *put* regulon is highly redox sensitive despite small changes in PutA−DNA binding affinity. Our work supports the model in which an increase in the affinity of PutA for the membrane occurs upon reduction of FAD, causing PutA to release the *put* intergenic DNA and alter its intracellular

location from the cytoplasm to a peripheral position on the membrane (*17*, *18*). As a consequence, the *put* regulon is transcriptionally activated in response to the redox state of FAD. Thus, the transcriptional regulation of the *put* regulon is redox-dependent but is primarily mediated by changes in PutA−membrane binding affinity. The mechanism by which the affinity of PutA for the membrane increases upon reduction of the FAD has been proposed to be a conformational change in which the overall hydrophobicity of PutA is increased, leading to an enhanced association with the membrane (*17*, *18*, *21*). This model is analogous to pyruvate oxidase in which reduction of the FAD cofactor by pyruvate exposes a lipid binding domain, causing pyruvate oxidase to peripherally associate with the membrane (*34*−*36*). The association of PutA with the membrane has been shown to be based on general protein−lipid interactions rather than specific PutA membrane binding sites (*17*).

One caveat concerning our study and previous studies, however, is that the entire *put* intergenic region was used; thus, the reported parameters represent global modifications in PutA−DNA binding affinity. It is plausible that the redox dependence of PutA−DNA binding varies among different binding sites. In our potentiometric studies with DNA-complexed PutA, we measured the overall effect of protein−protein and protein−DNA interactions on the reduction potential of bound FAD. Thus, studies that evaluate PutA binding to a single site on the *put* intergenic DNA will be the focus of future work to verify our conclusions using the entire *put* intergenic region.

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REFERENCES

1. Wood, J. M. (1981) *J. Bacteriol. 146*, 895.
2. Menzel, R., and Roth, J. (1981) *J. Biol. Chem. 256*, 9755.
3. Menzel, R., and Roth, J. (1981) *J. Biol. Chem. 256*, 9762.
4. Abrahamson, J. L. A., Baker, L. G., Stephenson, J. T., and Wood, J. M. (1983) *Eur. J. Biochem. 134*, 77.
5. Graham, S., Stephenson, J. T., and Wood, J. M. (1984) *J. Biol. Chem. 259*, 2656.
6. Wood, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 373.
7. Brown, E., and Wood, J. M. (1992) *J. Biol. Chem. 267*, 13086.
8. Menzel, R., and Roth, J. (1981) *J. Mol. Biol. 148*, 21.
9. Maloy, S., and Roth, J. R. (1983) *J. Bacteriol. 154*, 561.
10. Ostrovsky De Spicer, P., O'Brian, K., and Maloy, S. (1991) *J. Bacteriol. 173*, 211.
11. Vílchez, S., Molina, L., Ramos, C., and Ramos, J. L. (2000) *J. Bacteriol. 182*, 91.
12. José Soto, M., Jiménez-Zurdo, J. I., van Dillewijn, P., and Toro, N. (2000) *J. Bacteriol. 182*, 1935.
13. Jiménez-Zurdo, J. I., Garcia-Rodriguez, F. M., and Toro, N. (1997) *Mol. Microbiol. 23*, 85.
14. Hill, S., Austin, S., Eydmann, T., Jones, T., and Dixon, R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2143.
15. Macheroux, P., Hill, S., Austin, S., Eydmann, T., Jones, T., Kim, S.-O., Poole, R., and Dixon, R. (1998) *Biochem. J. 332*, 413.
16. Ling, M., Allen, S. W., and Wood, J. M. (1994) *J. Mol. Biol. 245*, 950.
17. Surber, M. W., and Maloy, S. (1999) *Biochim. Biophys. Acta 1421*, 5.
18. Brown, E. D., and Wood, J. M. (1993) *J. Biol. Chem. 268*, 8972.
19. Muro-Pastor, A. M., Ostrovsky, P., and Maloy, S. (1997) *J. Bacteriol. 179*, 2788.
20. Ostrovsky, P. C., and Maloy, S. (1995) *Genes Dev. 9*, 2034.
21. Ostrovsky De Spicer, P., and Maloy, S. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4295.
22. Mezel, V. A., and Knox, W. E. (1976) *Anal. Biochem. 74*, 430.
23. Mixon, A. J., and Phang, J. M. (1988) *J. Biol. Chem. 263*, 10720.
24. Strecker, H. J. (1957) *J. Biol. Chem. 225*, 825.
25. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
26. Thorpe, C., Matthews, R. G., and Williams, C. H. J. (1979) *Biochemistry 18*, 331.
27. Stankovich, M. T. (1980) *Anal. Biochem. 109*, 295.
28. Stankovich, M. T., and Soltysik, S. (1987) *Biochemistry 26*, 2627.
29. Mayhew, S. G. (1978) *Eur. J. Biochem. 85*, 535.
30. Massey, V., and Hemmerich, P. (1980) *Biochem. Soc. Trans. 8*, 246.
31. Jorns, M. S. (1985) *Biochemistry 24*, 3189.
32. Surber, M. W., and Maloy, S. (1998) *Arch. Biochem. Biophys. 354*, 281.
33. Nakao, T., Yamato, I., and Anraku, Y. (1987) *Mol. Gen. Genet. 210*, 364.
34. Russell, P., Hager, L. P., and Gennis, R. B. (1977) *J. Biol. Chem. 252*, 7877.
35. Schrock, H. L., and Gennis, R. B. (1977) *J. Biol. Chem. 252*, 5990.
36. Schrock, H. L., and Gennis, R. B. (1980) *Biochim. Biophys. Acta 615*, 10.

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