Fumarase A from *Escherichia coli*: Purification and Characterization as an Iron–Sulfur Cluster Containing Enzyme†

Dennis H. Flint,*,‡ Mark H. Emptage,‡ and John R. Guest§

E. I. du Pont de Nemours and Company, Central Research and Development, Experimental Station, Wilmington, Delaware 19880-0328, and Department of Microbiology, University of Sheffield, Sheffield S10 2TN, U.K.

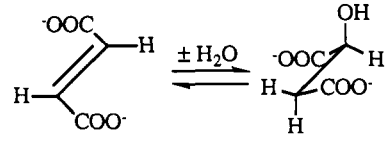
Received April 15, 1992; Revised Manuscript Received July 20, 1992

ABSTRACT: It has been shown previously that *Escherichia coli* contains three fumarase genes designated *fumA*, *fumB*, and *fumC*. The gene products fumarases A, B, and C have been divided into two classes. Class I contains fumarases A and B, which have amino acid sequences that are 90% identical to each other, but have almost no similarity to the sequence of porcine fumarase. Class II contains fumarase C and porcine fumarase, which have amino acid sequences 60% identical to each other [Woods, S.A., Schwartzbach, S.D., & Guest, J. R. (1988) *Biochim. Biophys. Acta 954*, 14–26]. In this work it is shown that purified fumarase A contains a [4Fe-4S] cluster. This conclusion is based on the following observations. Fumarase A contains 4 Fe and 4 S^{2-} per mole of protein monomer. (The mobility of fumarase A in native polyacrylamide gel electrophoresis and the elution volume on a gel permeation column indicate that it is a homodimer.) Its visible and circular dichroism spectra are characteristic of proteins containing an Fe–S cluster. Fumarase A can be reduced to an EPR active-state exhibiting a spectrum consisting of a rhombic spectrum at high fields (g-values = 2.03, 1.94, and 1.88) and a broad peak at $g = 5.4$. Upon addition of substrate, the high field signal shifts upfield (g-values = 2.035, 1.92, and 1.815) and increases in total spins by 8-fold, while the $g = 5.4$ signal disappears. This is consistent with the $[4Fe\text{-}4S]^+$ cluster existing in a mixture of $S = 1/2$ and $S = 3/2$ ground states, while the cluster–substrate complex has only $S = 1/2$ ground state. The redox potential of the Fe–S cluster in the presence of substrate is –480 mV. The activity of enzyme is dramatically effected (10–50-fold decrease) by photoreduction, which lowers the V_{max} and increases the K_m for malate. Fumarase A can also be oxidized with O_2 and $K_3Fe(CN)_6$ to an inactive form which has an axial EPR signal at $g = 2.02$, consistent with the presence of a $[3Fe\text{-}4S]^+$ cluster. The facile oxidation of the cluster by O_2 is the basis of the instability of the activity of fumarase A in air. The activity of partially inactivated fumarase A can be restored by incubation with iron and reductant. These results provide strong evidence that, like aconitase, fumarase A contains a catalytically active [4Fe-4S] cluster that can be readily oxidized to an inactive [3Fe-4S] cluster.

Fumarase (EC 4.2.1.2) catalyzes the reversible addition of water to fumarate to form *S*-malate as shown in Scheme I. The fumarase reaction in the direction from left to right is a component of the citric acid cycle. The fumarase reaction also plays an additional role under anaerobic conditions in *E. coli* where it functions in the opposite direction to which it occurs in the citric acid cycle, i.e., from right to left in Scheme I. In the latter case, *S*-malate is dehydrated to fumarate which then acts as an electron sink and is reduced to succinate.

Because of the central role that the citric acid cycle plays in aerobic metabolism, fumarase is widely distributed in a variety of organisms including animals, plants, invertebrates, molds, yeast, and bacteria (Hill & Teipel, 1971). Porcine fumarase was crystallized in 1951 (Massey, 1952) and has been extensively studied. It is a tetramer of identical 48 500-Da subunits and displays no requirements for metals or cofactors. Porcine fumarase shows structure similarity and extensive sequence homology (Sacchettini et al., 1988) to the fumarases found in humans (Kinsella & Doonan, 1986), rat (Suzuki et al., 1989), yeast (Wu & Tzagoloff, 1987), and the bacterium *Bacillus subtilis* (Miles & Guest, 1985). The two

Scheme I



fumarate *S*-malate

isozymes of fumarases found in yeast and mammals (mitochondrial and cytosolic) are highly homologous and apparently originate from a single gene (Wu & Tzagoloff, 1987; Suzuki et al., 1989).

In an effort to clone the fumarase gene(s) of *E. coli*, Guest's laboratory (Guest & Roberts, 1983; Guest et al., 1985) discovered three separate genes (named *fumA*, *fumB*, and *fumC*) which code for proteins with fumarase activity. The conditions for the expression of each of the three fumarases are different. Fumarase A is expressed under aerobic conditions; fumarase B is expressed under anaerobic conditions and is controlled by the anaerobic transcriptional activator FNR (Woods & Guest, 1987); and fumarase C is not influenced by different growth conditions and appears to be constitutive. While the deduced amino acid sequences of fumarase A and fumarase B are 90% identical to each other, they have little similarity with other known fumarases (Miles & Guest, 1984; Bell et al., 1989). In contrast, the deduced amino acid sequence of fumarase C (Woods et al., 1986) is highly homologous (60% identical) to the mammalian fumarases (Sacchettini et al., 1988).

† This is Contribution 6212 from the Central Research and Development Department, E. I. du Pont de Nemours and Co., Wilmington, DE 19898. A preliminary account of this work was presented previously (Flint et al., 1989).

* Author to whom correspondence should be addressed.

‡ E. I. du Pont de Nemours and Company.

§ University of Sheffield.

0006-2960/92/0431-10331$03.00/0

Because of the existence of the sequence Cys-Pro (which is often found in sequences of Fe–S proteins) in the primary structure of fumarase A, it was suggested that it may belong to the Fe-dependent class of hydrolyases (Woods et al., 1988). Aconitase is the best-characterized enzyme in this class. It contains a [4Fe-4S] cluster which participates in substrate binding and in catalysis by removing and adding a hydroxyl group to the substrate (Emptage, 1988). Subsequently, it was reported that the predominant fumarase in aerobically grown *E. coli* (presumably fumarase A) required activation by incubation with Fe and a thiol, suggesting that it contained an Fe–S cluster (Yumoto & Tokushige, 1988). In a preliminary communication (Flint et al., 1989) we have reported that purified fumarase A contains a [4Fe-4S] cluster in its active form and a [3Fe-4S] cluster in its inactive form. Recently, additional data have also been presented consistent with fumarase A containing a [4Fe-4S] cluster in its active form (Ueda et al., 1991). Here we present details of the purification and physical properties of *E. coli* fumarase A.

MATERIALS AND METHODS

Deferoxamine mesylate (pharmaceutical grade) was obtained from Ciba-Geigy. DEAE-Sepharose, phenyl-Sepharose, and Superdex column packings were purchased from Pharmacia. Matrix Gel Red A was from Amicon. Deazaflavin sulfonate was the kind gift of Dr. H. Beinert, Medical College of Wisconsin, Milwaukee, WI. Triquat[1] and 2-hydroxy-3-nitropropionate were prepared according to literature procedures (Salmon & Hawkridge, 1980; Shechter & Conrad, 1953). [^{17}O]Carboxyl-labeled malate was prepared using 50% enriched $H_2{}^{17}O$ from MSD Isotopes as described previously, and complete isotopic exchange between the water and the carboxyl groups was assumed (Emptage et al., 1983a). The reagents for the Bradford protein assay was purchased from Bio-Rad. The other chemicals used were obtained from Sigma.

Assays. The enzyme activity was measured in 100 mM phosphate buffer, pH 7.5. On most occasions the activity was measured by following the decrease in absorbance at 300 nm (ϵ = 0.033 mM^{-1}) that occurs as fumarate is converted to malate. On some occasions the enzyme activity was measured by following the increase in absorbance at 240 nm (ϵ = 2.4 mM^{-1}) that occurs as *S*-malate is converted to fumarate. A unit of activity was defined as the amount of enzyme that converts 1 μmol/min fumarate to malate at 25 °C.

Iron (Kennedy et al., 1984), labile sulfur (Beinert, 1983), protein (Bradford, 1976), and protein dry weight (Nozaki, 1986) analyses were performed as has been described.

Enzyme Purification. The strain used for purifying fumarase A was *E. coli* strain JRG1905. This strain is a transformant of *E. coli* JH400, a *fumAC fumB* deletion strain, containing the *fumA* plasmid pGS57 (Woods et al., 1988). The specific activity of fumarase A in this strain is approximately 30-fold above wild type.

E. coli JRG1905 cells grown aerobically on Luria broth were harvested, resuspended in 3 vol of 50 mM Tris-HCl and 10 mM $MgCl_2$, pH 8.0 (hereafter referred to as Tris–Mg^{2+} buffer), and sonicated. The fumarase activity in the supernatant was stable at 4 °C for a few hours. Sufficient protamine sulfate (10 mg/mL, adjusted to pH 8) was added to the crude supernatant to reach a final protamine sulfate concentration of 1.4 mg/mL, and the precipitate was quickly removed by centrifugation. Immediately after centrifugation, oxygen was removed from the protamine sulfate supernatant by placing it in a semiclosed container and then sweeping the headspace with argon for 30 min while the solution was being stirred.

Because the activity of fumarase A is unstable in the presence of oxygen and this instability increases with increasing purification, all subsequent operations were performed either in an anaerobic glovebox (Coy Laboratories) or on a Pharmacia FPLC protein chromatography system which has been modified to allow anaerobic purification of proteins. The two main modifications are the replacement of all the plastic tubing with stainless steel to prevent oxygen diffusion into the system and continuous sparging of the input buffers with purified argon (Gas Technics). Fractions were collected in serum stoppered bottles filled with argon.

The deoxygenated protamine sulfate supernatant was loaded onto the bottom of a DEAE-Sepharose column equilibrated with Tris–Mg^{2+} buffer. Fumarase A was eluted upward with an increasing KCl gradient. The dark brown band of fumarase A could readily be seen as it moved through this column and the columns that follow. The brown-colored fumarase A containing fractions were pooled, and $(NH_4)_2SO_4$ was added to a final concentration of 1.5 M. This material was loaded on the top of a phenyl-Sepharose column equilibrated with Tris–Mg^{2+} buffer plus 1.5 M $(NH_4)_2SO_4$. Fumarase A was eluted with a decreasing $(NH_4)_2SO_4$ gradient, and fractions that contained fumarase activity were pooled and concentrated to about 20 mg of protein/mL. The dark brown sample was loaded onto a Superdex 35/600 column and eluted with Tris–Mg^{2+} buffer containing 500 mM KCl. The fumarase fractions were pooled and concentrated to about 50 mg of protein/mL and then frozen as beads in liquid nitrogen.

Partially inactivated fumarase A could be reactivated by anaerobic incubation with 10 mM dithiothreitol and 0.5 mM ferrous ammonium sulfate. Maximum activation required 1–2-h incubation at room temperature.

Redox Chemistry. Photoreductions for activity studies were carried out as described previously (Flint & Emptage, 1988). *S*-Malate rather than fumarate was used as the substrate.

Redox titrations were carried out entirely within an anaerobic glovebox (Coy Laboratories). In a small stirred vessel was placed 1.5 mL of 90 mM Tris–Mg^{2+}, pH 8, 10 mM malate, 0.1 mM methyl viologen (E_m = –440 mV), 0.1 mM Triquat (E_m = –540 mV), and 6 mg/mL fumarase A. The potential of the solution was measured using a platinum foil electrode and a Ag/AgCl reference electrode connected to a CV-27 voltammograph (Bioanalytical Systems). The system redox potential was lower by adding small aliquots of 20 mM sodium dithionite. After the potential had stabilized, a 0.2-mL aliquot was removed, transferred to a quartz EPR tube, and frozen immediately by emersion in liquid nitrogen. The extent of reduction was quantitated by electron paramagnetic resonance (EPR) spectroscopy and compared to a control aliquot with excess dithionite added.

The $Fe(CN)_6{}^{3+}$ titrations were done on a sample of 20 μM fumarase A in Tris–Mg^{2+} buffer. $Fe(CN)_6{}^{3+}$ additions were made sequentially, and after each addition a 200 μL sample was withdrawn and frozen in an EPR tube.

Instrumentation. Optical and EPR spectra were obtained as described previously (Flint & Emptage, 1988). EPR spins were quantified under nonsaturating conditions by double integration of the EPR signal relative to that of a Cu(II)–EDTA standard. Circular dichroism (CD) spectra were recorded on a Jobin Yvon Dichrograph V spectropolarimeter in 1-cm cyclindrical cells. Because of the air sensitivity of

[1] Abbreviations: CD, circular dichroism; EPR, electron paramagnetic resonance; ENDOR, electron-nuclear double resonance; ES, enzyme–substrate; kDa, kilodaltons; SDS, sodium dodecyl sulfate; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Triquat, 1,1′-trimethylene-2,2′-bipyridinium dibromide; Tris, tris(hydroxymethyl)aminomethane.

Table I: Purification of *E. coli* Fumarase A

fraction	vol, mL	protein, g	act. kilounits	sp act., units/mg of protein	yield, %	purification factor
crude extract	600	14	1200	85	100	1
protamine sulfate supernatant	670	12	1400	120	116	1.4
DEAE-Sepharose	300	0.9	1300	1400	108	16
phenyl-Sepharose	110	0.41	850	2100	71	25
Superdex 35/600	26	0.28	720	2600	60	31
Matrix Gel Red A	15	0.24	660	2800	55	32

fumarase A, the cells were filled in an anaerobic glove and fitted with a rubber septum. Aliquots of anaerobic stock solutions of substrate or inhibitors were then added via a gas-tight syringe.

RESULTS

Purification of Fumarase A. Table I shows the course of a typical purification of fumarase A. Since the JRG1905 strain overproduces fumarase A, the purification factor required to reach homogeneity is not large. Nevertheless, the purification of this enzyme remains a challenge because of its instability. Fumarase A purified through the procedure outlined in Table I was judged to be over 90% pure based on SDS–polyacrylamide gel electrophoresis.

Physical Properties. A monomer molecular mass of 60 163 Da for the fumarase A polypeptide is predicted from its gene sequence (Miles & Guest, 1985). The purified enzyme migrates with a molecular mass of 60 kDa on SDS gels and predominantly 126 kDa on native gels. When purified fumarase A is run on native gels, under aerobic conditions, a minor amount of fumarase A protein runs at 250 and 375 kDa, but this seems to be an oxidation artifact since it is almost completely eliminated by running the gels in the presence of 2-mercaptoethanol. On a calibrated Superdex 35/600 column, fumarase A elutes at a volume characteristic of globular proteins with a molecular mass of 100 kDa. These results reveal that in its native state fumarase A is a dimer of identical subunits.

A dry weight protein analysis was conducted on a fumarase A sample and compared to a Bradford protein analysis. Using BSA as a protein standard, the analysis by the Bradford method gave a protein amount that was 1.07 times that determined by dry weight analysis. Thus, the amount of fumarase A in a purified sample was determined by measuring the protein with the Bradford method using a BSA standard and dividing the result by 1.07.

The Fe content of fumarase A was determined in two samples of enzyme and found to be 4.2 and 4.0 Fe/mol of monomer. The labile sulfide content of these two samples was found to be 3.7 and 3.5 S^{2-}/mol of monomer. These results provide strong evidence that fumarase A contains a [4Fe-4S] cluster that is stable to the conditions used in our purification procedure. The Fe/S^{2-} ratio from these samples is 1.14, which is higher than the expected value of 1. The Fe/S^{2-} could be high due to either the presence of adventitious iron or the loss of volatile sulfide due to cluster destruction during purification or storage.

The midpoint potential for the reduction of fumarase A was determined to be –480 mV. This is similar to the values obtained for other Fe–S-containing hydrolyases; approximately –500 mV estimated for aconitase (Emptage et al., 1983b) and –470 mV determined for dihydroxy-acid dehydratase (Flint & Emptage, 1988).

We also examined the effect of reduction on the kinetics of the malate dehydration reaction. This was accomplished by photoreduction in an anaerobic cuvette as outlined previously (Flint & Emptage, 1988). The fumarase A activity

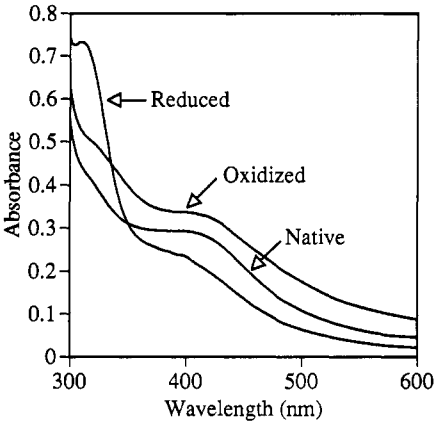


FIGURE 1: UV–visible absorbance spectrum of native, reduced, and oxidized fumarase A. 15 μM native fumarase A is as isolated in Tris–Mg^{2+} buffer, 15 μM reduced fumarase A containing 5 equiv of sodium dithionite per equiv of enzyme, and 15 μM oxidized fumarase A containing 4 equiv of $K_3Fe(CN)_6$ per equiv of enzyme.

is less stable to photoreduction and air oxidation than either beef heart aconitase (Emptage et al., 1983) or dihydroxy-acid dehydratase (Flint & Emptage, 1988). In several experiments at different enzyme and malate concentrations and different irradiation times, the enzyme activity was reduced to 1–4% of the initial activity upon photoreduction, and 50–70% of the initial activity was recovered upon air oxidation. Our data indicate that upon reduction of the Fe–S cluster, the k_{cat} and K_m for malate dehydration which is respectively 3100 and 0.7 mM in the native enzyme (Flint, unpublished data) changes to approximately 300 for the k_{cat} and approximately 3 mM for the K_m in the reduced enzyme.

Spectral Properties. The UV–visible spectra of fumarase A in its native form, after reduction with sodium dithionite, and after oxidation with potassium ferricyanide are shown in Figure 1. The ratio of absorbance values at 400 and 280 nm for the native protein is 0.145. These spectra are typical of proteins with [4Fe-4S] clusters (Palmer, 1973) and support the idea that fumarase A contains a $[4Fe\text{-}4S]^{2+}$ cluster in its native state.

The CD spectra of fumarase A are shown in Figure 2. Because CD spectra among Fe–S proteins with [4Fe-4S] clusters are quite different, CD spectroscopy is not useful as a tool to identify the cluster type (Stevens et al., 1978). However, CD spectra are sensitive to environment and ligand changes at the cluster. A dramatic change in the CD spectrum of fumarase A is evident upon the addition of either substrate. A similar change, but larger in magnitude, is observed upon addition of 3-hydroxy-2-nitropropionate. This latter compound is a competitive inhibitor (Flint, unpublished data), which is thought to form a tight-binding reaction intermediate analog when fully deprotonated (Porter & Bright, 1980). The spectral changes upon addition of substrate were used to monitor the enzyme-substrate (ES) complex and thus determine the apparent K_d for the equilibrium mixture of the substrates (*S*-malate and fumarate) as is shown in Figure 3. The data was fitted with a value of 0.7 mM for the K_d. This

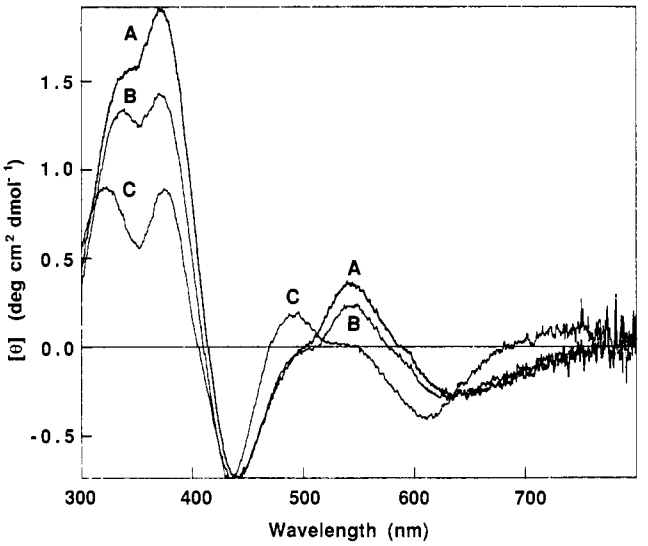


FIGURE 2: Circular dichroism spectra of fumarase A. Native fumarase A (1.5 mg/mL) in Tris–Mg^{2+} buffer. (A) With the addition of sodium 2-hydroxy-3-nitropropionate to 5 mM. (B) With the addition of sodium malate to 13 mM. (C) With no additions. The samples were all anaerobic as described in the Materials and Methods section.

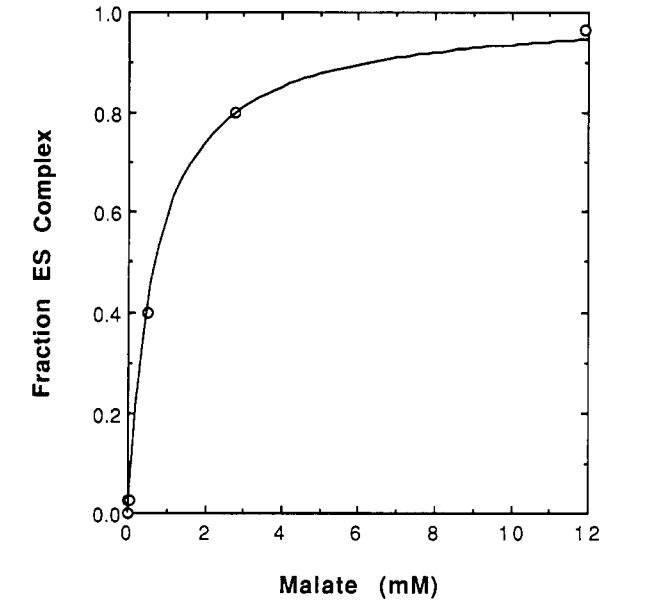


FIGURE 3: Titration of fumarase A with malate as followed by CD spectroscopy. The starting solution contained 1.5 mg/mL fumarase A in 0.1M Tris-HCl, pH 8.0. The change in intensity of the band at 372 nm (see Figure 2) was used to determine the fraction of substrate-bound enzyme. The theoretical curve is that determined for the equation K_d = [S][E]/[ES], where [S] represents free substrate, [E] represents free fumarase A, and [ES] represents substrate-bound fumarase A. The best fit to the data was obtained with K_d = 0.7 mM. Because of the inactivation of fumarase A by air the titration was performed anaerobically described in the Materials and Methods section.

is close to the K_ms for malate and fumarate [0.7 and 0.6 mM, respectively (Flint, unpublished data)].

Fumarase A as isolated produces an axial EPR signal with g-values at 2.02 and 2.00. The intensity of this signal varies from batch to batch between 0.03 and 0.25 spins/mol. The intensity of this signal can be enhanced by the addition of $K_3Fe(CN)_6$ as shown in Figure 4. This EPR signal disappears above 30K due to rapid relaxation effects. These spectral characteristics suggest the presence of variable amounts of a $[3Fe\text{-}4S]^+$ cluster in these preparations (Beinert & Thomson, 1983). Upon reduction of native fumarase A with 10-fold excess sodium dithionite, the g = 2.02 signal disappears and

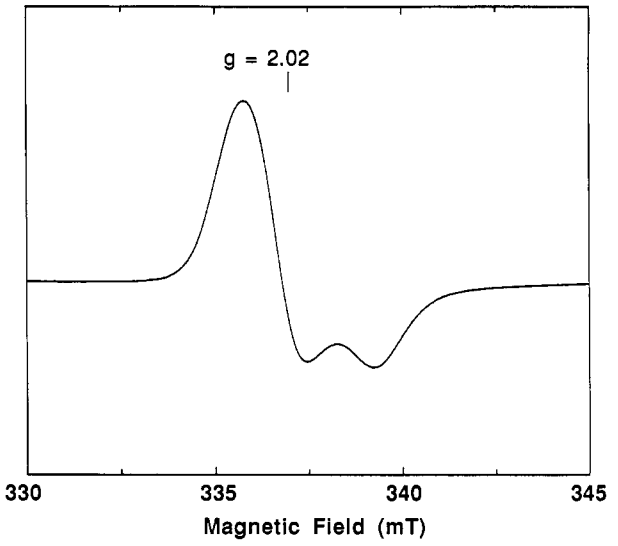


FIGURE 4: EPR spectrum of oxidized inactive fumarase A. Fumarase A (0.8 mg/mL) in 0.1 M Tris-HCl, pH 8.0, plus 50 μM potassium ferricyanide. Experimental conditions for obtaining EPR spectra were 10K, 1 mW microwave power, 0.8 mT modulation amplitude, and 9.526 GHz microwave frequency.

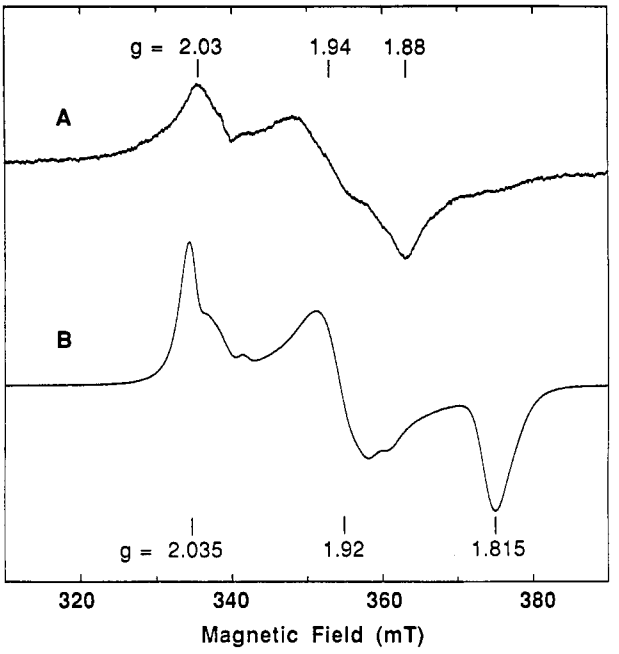


FIGURE 5: EPR spectra of sodium dithionite reduced fumarase A, 310–390 mT. (A) Fumarase A (10 mg/mL) in 0.1 M Taps, pH 8.2, plus 10-fold excess sodium dithionite. (B) Same as (A) except after the addition of 5 mM sodium fumarate added anaerobically to the thawed sample. The gain of spectrum B was decreased by a factor of 0.12. Experimental conditions for obtaining EPR spectra were the same as those listed in the legend to Figure 4.

a rhombic signal appears with g-values at 2.03, 1.94, and 1.88 as shown in Figure 5. This signal integrates to only about 0.05 spins/mol. Furthermore, there is a broad signal at g = 5.4 which disappears upon addition of 5 mM fumarate as is shown in Figure 6. The signal at g = 5.4 is indicative of a $[4Fe\text{-}4S]^+$ cluster with an S = 3/2 ground state (Lindahl et al., 1987; George et al., 1989; Zambrano et al., 1989; Conover et al., 1990). As the g = 5.4 signal disappears in the presence of fumarate, a new rhombic signal appears with g-values at 2.035, 1.92, and 1.815 which is shown in Figure 5. This latter signal is typical of proteins containing a $[4Fe\text{-}4S]^+$ cluster with an S = 1/2 ground state and integrates to 0.4 spins/mol. Features of a second minor signal can be seen at g = 2.02 and 1.90. This latter signal may arise from different ligation at the cluster or a nonspecific effect. The addition of the inhibitor

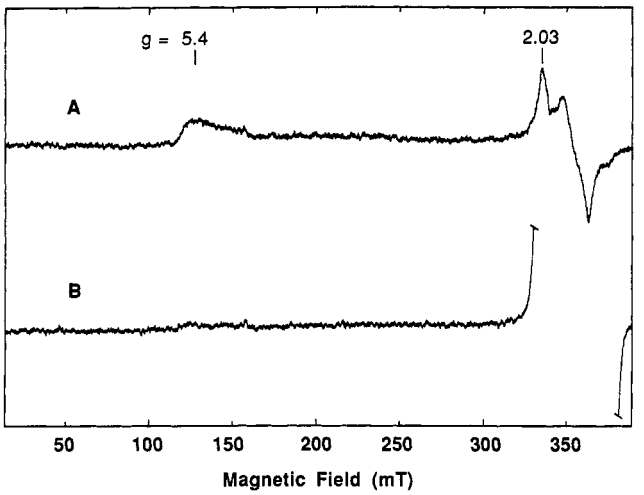


FIGURE 6: EPR spectra of sodium dithionite reduced fumarase A, 0–400 mT. (A) Fumarase A (10 mg/mL) in 0.1 M Taps, pH 8.2, plus 10-fold excess sodium dithionite. (B) Same as A except after the addition of 5 mM sodium fumarate added anaerobically to the thaw sample. Experimental conditions for obtaining EPR spectra were the same as those listed in the legend to Figure 5.

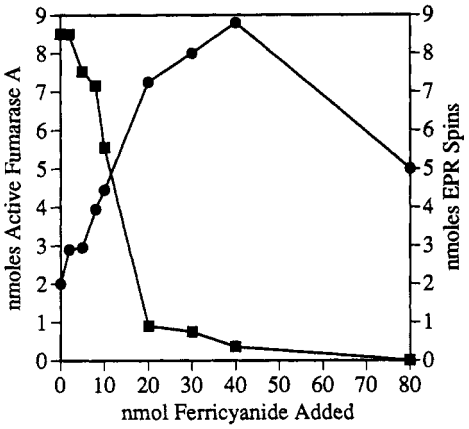


FIGURE 7: Effect of $K_3Fe(CN)_6$ on activity of fumarase A and number of spins. Aliquots of fumarase A were treated with various amounts of $K_3Fe(CN)_6$ as described in the Materials and Methods section. The effect of this oxidant on the activity of the enzyme (■) and the number of spins (●) detected by an EPR spectrometer was measured.

2-hydroxy-3-nitropropionate to the reduced enzyme has a very similar effect on the EPR spectrum as does the addition of fumarate. In the presence of the inhibitor a new $S = 1/2$ species appears with g-values at 2.05, 1.94, and 1.82.

We have also looked for evidence of substrate binding to the reduced cluster by observation of line broadening of the EPR signal in the presence of [^{17}O]carboxyl-labeled malate. While we observe a 2-G broadening of the high field peak at $g = 1.815$, this small effect is at the limits of reliability. However, the sample with [^{17}O]carboxyl-labeled malate has an ^{17}O ENDOR signal similar to that found for reduced aconitase with bound [^{17}O]carboxyl-labeled citrate (Kennedy et al., 1987) providing direct evidence for substrate coordination to the Fe–S cluster of fumarase A (A. L. P. Houseman, B. M. Hoffman, M. H. Emptage, and D. H. Flint, unpublished data).

Upon oxidation of native fumarase A with increasing amounts of potassium ferricyanide, the [3Fe-4S]$^+$ signal (shown in Figure 4) initially increases while the activity of fumarase A decreases. This signal eventually reaches a maximum of 1 spin/mol and then begins to decrease as shown in Figure 7. The increase in the EPR signal concomitant with loss of enzyme activity from fumarase A is consistent with the active site of the native enzyme containing an EPR-silent [4Fe-4S]$^{2+}$ cluster which loses an Fe upon oxidation leading to formation of an EPR-active [3Fe-4S]$^+$ cluster and loss of enzyme activity. The decrease in EPR signal that follows addition of larger amounts of ferricyanide as shown in Figure 7 results from cluster destruction as indicated by a decrease in labile sulfide in the enzyme samples (data not shown). These results are quite similar to those previously reported for aconitase (Kennedy et al., 1983).

DISCUSSION

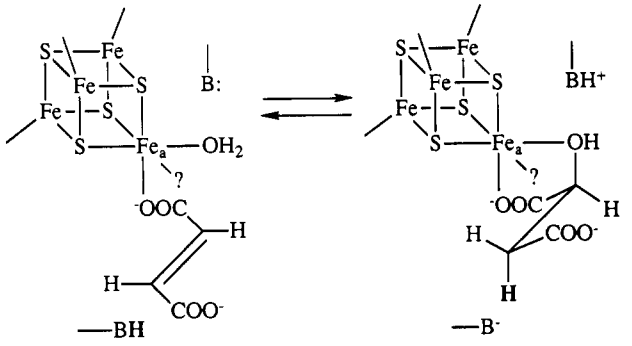
We have presented data demonstrating that *E. coli* fumarase A contains a catalytically active [4Fe-4S] cluster. This places fumarase A in the expanding group of Fe–S-containing hydrolyases of which aconitase is the best characterized (Emptage, 1988). Other Fe–S enzymes in this category are dihydroxy-acid dehydratase (Flint & Emptage, 1988), isopropylmalate isomerase (Emptage, 1990), phosphogluconate dehydratase (Scopes & Griffiths-Smith, 1984), tartrate dehydratase (Kelly & Scopes, 1986), maleate hydratase (Dreyer, 1985), lactyl-CoA dehydratase (Kuchta et al., 1986), 2-hydroxyglutaryl-CoA dehydratase (Schweiger et al., 1987), and serine dehydratase from *Peptostreptococcus asaccharolyticus* (Grabowski & Buckel, 1991).

The data provided in this paper support the proposed division of the fumarases into two classes (Woods et al., 1988). We have found that fumarase A contains a catalytically active [4Fe-4S] cluster, is a homodimer of 120 kDa, and is unstable in the presence of O_2, thus distinguishing it from porcine fumarase. The class of fumarases to which *E. coli* fumarase A belongs was referred to as class I and may also include the following fumarases: *E. coli* fumarase B, *Euglena gracilis* fumarase (Shibata et al., 1985), and *Rhodobacter capsulatus* (Luque-Romera & Castillo, 1991) fumarase. We have obtained preliminary evidence that *E. coli* fumarase B also has a [4Fe-4S] cluster that is unstable in the presence of O_2 (D. H. Flint and M. H. Emptage, unpublished data). This may be a characteristic of all members of this class. In contrast, all class II fumarases that have been studied require no metals or cofactors, are homotetramers of about 200 kDa, and are stable in air. *E. coli* fumarase C and the fumarases from *Bacillus subtilis*, yeast, and mammals appear to belong to this latter class.

Since aconitase is the best characterized of all the Fe–S cluster containing hydrolyases, it instructive to compare the properties of aconitase and fumarase A. The primary sequences of the fumarase A (Miles & Guest, 1984) and aconitase (Zheng et al., 1990) show very little homology. The cluster binding cysteines (C358, C421, and C424) of aconitase are in the middle of the sequence, while two of the likely cluster ligands for fumarase A, C542 and C545, are at the carboxyl terminus of its sequence. Like aconitase, fumarase A is inactivated by oxidation to its [3Fe-4S] form. Aconitase can be titrated stoichiometrically and cleanly to the [3Fe-4S]$^{1+}$ form with 2 oxidizing equiv of $K_3Fe(CN)_6$ which follows the equation: [4Fe-4S]$^{2+} - 2e^- =$ [3Fe-4S]$^+ +$ Fe^{3+} (Kennedy et al., 1983). While a similar oxidation occurs on the addition of $K_3Fe(CN)_6$ to fumarase A, the [3Fe-4S]$^{+1}$ cluster of fumarase A appears to be more susceptible to further oxidation than aconitase, leading to higher stoichiometries and cluster destruction. In the reverse direction, the activation with iron and reductant is much slower, taking more than 1 h to reach maximum activity at room temperature. This contrasts aconitase reactivation which takes place in less than 5 min under similar conditions (Kennedy et al., 1983).

One major difference between fumarase A and aconitase is the relative "stickiness" of the substrates as demonstrated by substrate titration as followed by CD. The K_m/K_d ratio

Scheme II



for fumarase is close to 1 while that for aconitase is greater than 100 (Emptage et al., 1983b; Schloss et al., 1984). This unusually large ratio for aconitase may stem from the need to keep the intermediate *cis*-aconitate in the active site during the conversion of citrate to isocitrate.

The reduced [4Fe-4S] cluster of fumarase A in the frozen state exists as a mixture of $S = 3/2$ and $S = 1/2$ ground states. High-spin ground states have been observed recently in a limited number of proteins containing [4Fe-4S] clusters, namely, the Fe protein of nitrogenase (Lindahl et al., 1987), glutamine phosphoribosylpyrophosphate amidotransferase from *B. subtilis* (Oñate et al., 1989), hydrogenase I of *Clostridium pasteurianum* (Zambrano et al., 1989), *Desulfovibrio africanus* ferredoxin III (George et al., 1989), and the ferredoxin from the extreme thermophile *Pyrococcus furiosus* (Conover et al., 1990). Mixtures of high- and low-spin states have also been observed in model compounds of [4Fe-4S] clusters (Carney et al., 1986). From the study of the these model clusters and from recent developments in the theory of exchange coupling and resonance delocalization in $[4Fe\text{-}4S]^+$ clusters (Noodleman, 1991), it has been shown that the relative energies of the $S = 3/2$ and $S = 1/2$ ground states are quite sensitive to environmental changes. With fumarase A, the binding of substrate eliminates the $S = 3/2$ species and increases the spin quantitation in the $S = 1/2$ species about 8-fold. This change in the spin quantitation suggests that the $S = 3/2$ species may represent about 85% of the total spins in the substrate-free form of the enzyme. Other than the Fe protein of nitrogenase (Lindahl et al., 1987) and fumarase A, we are not aware of additional Fe–S-containing enzymes whose spin state is modulated by substrate binding.

The spectroscopic data discussed above is quite similar to data previously published on aconitase [for review, see Emptage (1988)]. The binding of substrate and inhibitors to the Fe–S cluster of aconitase has been clearly established by both EPR (Emptage, 1983a) and ENDOR spectroscopic data (Telser et al., 1986; Kennedy et al., 1987; Werst et al, 1990) and the recently reported crystal structure of the isocitrate and nitroisocitrate (1-hydroxy-2-nitro-1,3-propanedicarboxylate) complexes of aconitase (Lauble et al., 1992). While the data presented here for fumarase A is not as complete nor as convincing as that for aconitase, we do have ENDOR experiments in progress which provide additional support for substrate binding to the Fe–S cluster of fumarase A (A. L. P. Houseman, B. M. Hoffman, M. H. Emptage, and D. H. Flint, unpublished data). With this in mind, a mechanism for the role of the Fe–S cluster of fumarase A which (1) parallels that proposed for aconitase (Emptage, 1988), (2) follows the known stereochemistry of the fumarase A reaction (Flint, unpublished), (3) borrows from the crystal structure of the isocitrate–aconitase complex (Lauble et al., 1992) is shown in Scheme II.

Here one carboxylate of fumarate binds to Fe_a of the Fe–S cluster converting that Fe site from what is most likely a 4-coordinate Fe with a water ligand to a 5- or 6-coordinate Fe site. The binding of the carboxylate group to the positively changed Fe–S cluster activates the adjacent olefinic carbon to nucleophilic attack by hydroxide bound to Fe_a. It seems likely that a base (-B:) would be necessary to catalyze the formation of a hydroxyl group by removing a proton from a H_2O bound to the cluster. An additional base (-BH) is necessary to protonate the C-3 carbon. In the reverse direction the Fe–S cluster will act as a Lewis acid to facilitate the dehydration reaction. While we have no evidence for a sixth ligand to Fe_a in Scheme II and show a question mark in its place, the crystal structure of isocitrate bound to aconitase shows a water molecule bound as a sixth ligand to Fe_a (Lauble et al., 1992).

It was pointed out in the introduction that there are three proteins in *E. coli* that catalyze the fumarase reaction, fumarases A, B, and C. Because of the considerable sequence homology of fumarase C to porcine fumarase and the stability of fumarase C to oxygen, in all probability it does not contain an Fe–S cluster. If this is the case, then fumarases A and C are mechanistically distinct since an Fe–S cluster is involved in the mechanism of fumarase A but not C. Although there are known examples of groups of enzymes catalyzing analogous transformations using metals as part of the mechanism in some instances and not using metals in others [e.g., class I and class II aldolases (Kadonaga & Knowles, 1983), serine, sulfhydryl, metallo, and acid proteases (Walsh, 1977)], these transformations are either on the same substrate in different cells or on different substrates in the same cell. The fumarase reaction in *E. coli* is the only case we are aware of where the same reaction is the same cell is catalyzed by enzymes with significant mechanistic differences.

Since *E. coli* produces both types of fumarases and varies the amounts of each depending upon the metabolic circumstances (Woods & Guest, 1987), it would seem that each of these enzymes must have some uniquely useful properties. Just what these are is not yet clear. It should be remembered that the direction of the fumarase reaction is toward malate during aerobic growth and toward fumarate during anaerobic growth. The existence of both fumarase A and fumarase B may be related to this situation, which is analogous to the existence of succinate dehydrogenase and fumarate reductase. However, this does not explain the existence of fumarase C. In contrast to the situation with fumarases, all the aconitases examined to date, including representatives from bacteria (Kingman & Sonenshein, 1987), yeast (Suzuki et al., 1973), higher plants (Brouquisse, et al., 1986), and mammals (Kennedy et al., 1972) are Fe–S proteins. An interesting possible property in relation to the presence of an Fe–S cluster in fumarase A (and B) is they may function in a regulatory role in addition to their catalytic role as appears to be the case with cytoplasmic aconitase which functions in mammals as an iron-responsive element binding protein as well as a hydrolyase catalyst (Kaptain et al., 1991).

It has been shown that antibodies to Euglena fumarase cross react with fumarase A (Shibata et al., 1985; Woods, et al., 1988). This, along with the instability of the activity of *Euglena* fumarase and its similarity in molecular weight to fumarase A, suggests that it may be closely related to fumarase A and may contain an Fe–S cluster. Since *Euglena* is a photosynthetic organism, this brings up the possibility that higher plants may also contain a class I fumarase with an Fe–S cluster. The little information that is in the literature regarding higher plant fumarase shows it to be a very unstable

enzyme (Shih & Barnett, 1967), which would be consistent with it containing an Fe–S cluster.

ACKNOWLEDGMENT

We express appreciation to Edith DuPlessis, Joseph Tuminello, Caroline Braun, and William Barney for excellent technical assistance.

REFERENCES

Beinert, H. (1983) *Anal. Biochem. 131*, 373–378.
Beinert, H., & Thomson, A. J. (1983) *Arch. Biochem. Biophys. 222*, 333–361.
Bell, P. J., Andrews, S. C., Sivak, M. N., & Guest, J. R. (1989) *J. Bacteriol. 171*, 5928–5932.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Brouquisse, R., Gaillard, J., & Douce, R. (1986) *Plant Physiol. 81*, 247–252.
Carney, M. J., Holm, R. H., Papaefthymiou, G. C., & Frankel, R. B. (1986) *J. Am. Chem. Soc. 108*, 3519–3521.
Conover, R. C., Kowal, A. T., Fu, W., Park, J.-B., Aono, S., Adams, M. W. W., & Johnson, M. K. (1990) *J. Biol. Chem. 265*, 8533–8541.
Dreyer, J.-L. (1985) *Eur. J. Biochem. 150*, 145–154.
Emptage, M. H. (1988) in *Metal Clusters in Proteins* (Que, L., Jr., Ed.) pp 343–371, American Chemical Society, Washington, D.C.
Emptage, M. H. (1990) in *Biosynthesis of Branched Chain Amino Acids* (Barak, Z., Chipman, D. M., & Schloss, J. V., Eds.) pp 315–328, VCH, New York.
Emptage, M. H., Kent, T. A., Kennedy, M. C., Beinert, H., & Münck, E. (1983a) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4674–4678.
Emptage, M. H., Dreyer, J. L., Kennedy, M. C., & Beinert, H. (1983b) *J. Biol. Chem. 258*, 11106–11111.
Flint, D. H., & Emptage, M. H. (1988) *J. Biol. Chem. 263*, 3558–3564.
Flint, D. H., Emptage, M. H., & Guest, J. R. (1989) *J. Inorg. Biochem. 36*, 306.
George, S. J., Armstrong, F. A., Hatchikian, E. C., & Thomson, A. J. (1989) *Biochem. J. 264*, 275–284.
Grabowski, R., & Buckel, W. (1991) *Eur. J. Biochem. 199*, 89–94.
Guest, J. R., & Roberts, R. E. (1983) *J. Bacteriol. 153*, 588–596.
Guest, J. R., Miles, J. S., Roberts, R. E., & Woods, S. A. (1985) *J. Gen. Microbiol. 131*, 2971–2984.
Hill, R. L., & Teipel, J. W. (1971) in *The Enzymes* (Boyer, P. D., Ed.) pp 539–571, Academic Press, New York.
Kadonaga, J. I., & Knowles, J. R. (1983) *Biochemistry 25*, 130–136.
Kaptain, S., Downey, W. E., Tang, C., Philpott, C., Haile, D., Orloff, D. G., Harford, J. B., Rouault, T. A., Klausner, R. D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10109–10113.
Kelly, J. M., & Scopes, R. K. (1986) *FEBS Lett, 202*, 274–276.
Kennedy, C., Rauner, R., & Gawron, O. (1972) *Biochem. Biophys. Res. Commun. 47*, 740–745.
Kennedy, M. C., Emptage, M. H., Dreyer, J. L., & Beinert, H. (1983) *J. Biol. Chem. 258*, 11098–11105.
Kennedy, M. C., Kent, T. A., Emptage, M., Merkle, H., Beinert, H., & Munck, E. (1984) *J. Biol. Chem. 259*, 14463–14471.
Kennedy, M. C., Werst, M., Telser, J., Emptage, M. H., Beinert, H., & Hoffman, B. M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8854–8858.
Kingman, D. W., & Sonenshein, A. L. (1987) *J. Bacteriol. 169*, 3062–3067.
Kinsella, B. T., & Doonan, S. (1986) *Biosci. Rep. 6*, 921–929.
Kuchta, R. D., Hanson, G. R., Holmquist, B., & Abeles, R. H. (1986) *Biochemistry 25*, 7301–7307.
Lindahl, P. A., Gorelick, N. J., Münck, E., & Orme-Johnson, W. H. (1987) *J. Biol. Chem. 262*, 14945–14953.
Luque-Romero, M. M., & Castillo, F. (1991) *Arch. Microbiol. 155*, 149–152.
Massey, V. (1952) *Biochem. J. 51*, 490–494.
Miles, J. S., & Guest, J. R. (1984) *Nucleic Acids Res. 12*, 3631–3642.
Miles, J. S., & Guest, J. R. (1985) *Nucleic Acids Res. 13*, 131–140.
Noodleman, L. (1991) *Inorg. Chem. 30*, 256–264.
Nozaki, Y. (1986) *Arch. Biochem. Biophys. 249*, 437–446.
Oñate, Y. A., Vollmer, S. J., Switzer, R. L., & Johnson, M. K. (1989) *J. Biol. Chem. 264*, 18386–18391.
Palmer, G. (1973) in *Iron–Sulfur Proteins* (Lovenberg, W., Ed.) pp 285–325, Academic Press, New York.
Porter, D. J. T., & Bright, H. J. (1980) *J. Biol. Chem. 255*, 4772–4780.
Robbins, A. H., & Stout, C. D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3639–3643.
Sacchettini, J. C.; Frazier, M. W., Chiara, D. C., Banazak, L. J., & Grant, G. A. (1988) *Biochem. Biophys. Res. Commun. 153*, 435–440.
Salmon, R. T., & Hawkridge, F. M. (1980) *J. Electroanal. Chem. 112*, 253–264.
Schloss, J. V., Emptage, M. H., & Cleland, W. W. (1984) *Biochemistry 23*, 4572–4580.
Schweiger, G., Dutscho, R., & Buckel, W. (1987) *Eur. J. Biochem. 169*, 441–448.
Scopes, R. K., & Griffiths-Smith, K. (1984) *Anal. Biochem. 136*, 530–534.
Shechter, H., & Conrad, F. (1953) *J. Am. Chem. Soc. 75*, 5610–5613.
Shibata, H., Gardiner, W. E., & Schwartzbach, S. D. (1985) *J. Bacteriol. 164*, 762–768.
Shih, D. S., & Barnett, L. B. (1967) *Arch. Biochem. Biophys. 123*, 558–564.
Stephens, P. J., Thomson, A. J., Dunn, J. B. R., Keiderling, T. A., Rawlings, J., Rao, K. K., & Hall, D. O. (1978) *Biochemistry 17*, 4770–4778.
Suzuki, T., Yamazaki, O., Nara, K., Akiyama, S., Nakao, Y., & Fukuda, H. (1973) *Agric. Biol. Chem. 37*, 2211–2212.
Suzuki, T., Sato, M., Yoshida, T., & Tuboi, S. (1989) *J. Biol. Chem. 264*, 2581–2586.
Telser, J., Emptage, M. H., Merkle, H., Kennedy, M. C., Beinert, H., & Hoffman, B. M. (1986) *J. Biol. Chem. 261*, 4840–4846.
Ueda, Y., Yumoto, N., Tokushige, M., Fukui, K., & Ohya-Nishiguchi, H. (1991) *J. Biochem. (Tokyo) 109*, 728–733.
Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, W. H. Freeman and Co., San Francisco.
Werst, M. M., Kennedy, M. C., Beinert, H., & Hoffman, B. M. (1990) *Biochemistry 29*, 10526–10532.
Woods, S. A., & Guest, J. R. (1987) *FEMS Microbiol. Lett. 48*, 219–224.
Woods, S. A., Miles, J. S., Roberts, R. E., & Guest, J. R. (1986) *Biochem. J. 237*, 547–557.
Woods, S. A., Schwartzbach, S. D., & Guest, J. R. (1988) *Biochim. Biophys. Acta 954*, 14–26.
Wu, M., & Tzagoloff, A. (1987) *J. Biol. Chem. 262*, 12275–12282.
Yumoto, N., & Tokushige, M. (1988) *Biochem. Biophys. Res. Commun. 153*, 1236–1243.
Zambrano, I. C., Kowal, A. T., Mortenson, L. E., Adams, M. W. W., & Johnson, M. K. (1989) *J. Biol. Chem. 264*, 20974–20983.
Zheng, L., Andrews, P. C., Hermodson, M. A., Dixon, J. E., & Zalkin, H. (1990) *J. Biol. Chem. 265*, 2814–2821.