Structural studies of the interaction of *S*-adenosylmethionine with the [4Fe-4S] clusters in biotin synthase and pyruvate formate-lyase activating enzyme

MICHELE M. COSPER,[1] NATHANIEL J. COSPER,[1] WEI HONG,[2] JACOB E. SHOKES,[1] WILLIAM E. BRODERICK,[2] JOAN B. BRODERICK,[2] MICHAEL K. JOHNSON,[1] AND ROBERT A. SCOTT[1]

[1]Department of Chemistry, University of Georgia, Athens, Georgia 30602-2556, USA
[2]Department of Chemistry, Michigan State University, East Lansing, Michigan 48824, USA

Abstract

The diverse reactions catalyzed by the radical-SAM superfamily of enzymes are thought to proceed via a set of common mechanistic steps, key among which is the reductive cleavage of S-adenosyl-L-methionine (SAM) by a reduced [4Fe-4S] cluster to generate an intermediate deoxyadenosyl radical. A number of spectroscopic studies have provided evidence that SAM interacts directly with the [4Fe-4S] clusters in several of the radical-SAM enzymes; however, the molecular mechanism for the reductive cleavage has yet to be elucidated. Selenium X-ray absorption spectroscopy (Se-XAS) was used previously to provide evidence for a close interaction between the Se atom of selenomethionine (a cleavage product of Se-SAM) and an Fe atom of the [4Fe-4S] cluster of lysine-2,3-aminomutase (KAM). Here, we utilize the same approach to investigate the possibility of a similar interaction in pyruvate formate-lyase activating enzyme (PFL-AE) and biotin synthase (BioB), two additional members of the radical-SAM superfamily. The results show that the latter two enzymes do not exhibit the same Fe-Se interaction as was observed in KAM, indicating that the methionine product of reductive cleavage of SAM does not occupy a well-defined site close to the cluster in PFL-AE and BioB. These results are interpreted in terms of the differences among these enzymes in their use of SAM as either a cofactor or a substrate.

Keywords: Biotin synthase (BioB); iron-sulfur cluster; pyruvate formate-lyase activating enzyme (PFL-AE); radical SAM superfamily; X-ray absorption fine structure; X-ray absorption spectroscopy

Supplemental material: See www.proteinscience.org.

Iron-sulfur (Fe-S) clusters constitute one of the most structurally and functionally diverse classes of biological prosthetic groups. The primary involvement of Fe-S clusters in biological systems is the mediation of electron transport. However, alternative functions have emerged in recent years, such as regulation of genes and structural roles (Beinert et al. 1997; Beinert 2000). Recently, an additional new role has been revealed involving the initiation of radical catalysis via the reductive cleavage of S-adenosylmethionine (SAM) by an Fe-S cluster (Cheek and Broderick 2001;

Fontecave et al. 2001; Frey 2001). This class of enzymes, characterized by a C-X$_3$-C-X$_2$-C conserved binding motif and dependence on SAM, has been identified as the radical-SAM superfamily (Sofia et al. 2001). The diverse range of functions in this superfamily is remarkable, as represented by the most well-characterized enzymes, the activatases for pyruvate formate-lyase (PFL; Broderick et. al. 1997; Külzer et al. 1998) and anaerobic ribonucleotide reductase (ANAR; Ollagnier et al. 1997), which are responsible for creation of stable protein-based radicals; KAM, (Lieder et al. 1998), which catalyzes the interconversion of α-L-lysine and β-L-lysine; and BioB (Sanyal et al. 1994; Duin et al. 1997; Guianvarc'h et al. 1997) and lipoate synthase (LipA; Busby et. al. 1999; Ollagnier-de Choudens and Fontecave 1999), which are involved in the terminal step of the biosyntheis of the vitamins, biotin and lipoic acid, respectively.

PFL-AE generates a stable glycyl radical on PFL, which is responsible for the first step in bacterial anaerobic glucose metabolism, the conversion of pyruvate and coenzyme-A to formate and acetyl-CoA (Knappe et al. 1993; Wong and Kozarich 1994; Broderick et. al. 1997). Anaerobically isolated PFL-AE contains mostly cuboidal [3Fe-4S]$^+$ clusters and minor quantities of [4Fe-4S]$^{2+}$, [2Fe-2S]$^{2+}$, and linear [3Fe-4S]$^+$ clusters (Broderick et al. 2000; Krebs et. al. 2000); however, a homogeneous [4Fe-4S]$^{2+,+}$ form is prepared under reducing conditions. The reduced [4Fe-4S]$^+$ cluster in PFL-AE has been demonstrated to be catalytically essential for cleaving SAM and generating the protein-based radical on PFL (Henshaw et. al. 2000).

The final step of the biotin biosynthetic pathway is catalyzed by BioB and involves the insertion of S into dethiobiotin (DTB) at unactivated C-H bonds. Biotin synthases from *Escherichia coli* (Sanyal et al. 1994), *Bacillus sphaericus* (Méjean et al. 1995), and *Arabidopsis thaliana* (Baldet et al. 1997) have been purified to homogeneity from recombinant strains of *E. coli* or *B. sphaericus*. The *E. coli* enzyme is a 78-kD homodimer and, as isolated, contains one [2Fe-2S]$^{2+}$ cluster per monomer (Sanyal et al. 1994; Ollagnier-De Choudens et al. 2000). The presence of [2Fe-2S]$^{2+}$ clusters was also reported for the *B. sphaericus* (Méjean et al. 1995) and *A. thaliana* (Baldet et al. 1997) enzymes. Spectroscopic studies have demonstrated that anaerobic reconstitution of apo BioB with Fe and S results in the formation of one [4Fe-4S]$^{2+}$ cluster per monomer (Ollagnier-de Choudens et al. 2000). Recently, Fontecave and colleagues (Ollagnier-de Choudens et al. 2002) provided direct evidence that the [4Fe-4S]$^+$ cluster is responsible for reductive cleavage of SAM and is ligated by the cysteines in the C-X$_3$-C-X$_2$-C motif.

Mechanistic details of Fe-S cluster-mediated SAM cleavage are beginning to emerge through spectroscopic studies aimed at examining the nature of the interaction of SAM with the [4Fe-4S]$^{2+,+}$ cluster (Cosper et al. 2000; Krebs et al. 2002; Walsby et al. 2002a,b). Despite the diversity in

functions of the enzymes in the radical-SAM superfamily, cleavage of SAM by a reduced [4Fe-4S] cluster has been postulated to occur by a similar mechanism. Recent experiments have suggested that there is a close interaction of SAM with the [4Fe-4S]$^{2+,+}$ clusters of KAM, PFL-AE, and BioB. A non-cysteinyl-ligated unique Fe site in the [4Fe-4S]$^{2+}$ cluster of PFL-AE has been identified by Mössbauer spectroscopy (Krebs et al. 2002). Moreover, the Mössbauer parameters of this unique Fe can be dramatically affected by the addition of SAM to PFL-AE, suggesting that SAM must be interacting with the unique Fe (Krebs et al. 2002). Furthermore, electron-nuclear double-resonance (ENDOR) spectroscopy has shown that SAM coordinates the unique Fe in the [4Fe-4S]$^{2+,+}$ cluster (Walsby et al. 2002a,b). These studies led to a model in which the amino acid moiety of SAM coordinates the unique Fe, whereas the sulfonium ion interacts with one of the μ_3-bridging sulfides of the [4Fe-4S] cluster (Fig. 1). Similarly, a combination of EPR, resonance Raman, and Mössbauer spectroscopies has provided evidence for the interaction of SAM at a unique Fe site of the [4Fe-4S]$^{2+}$ cluster in BioB (Cosper et. al. 2002).

In contrast, Se XAS studies on KAM; however, have led to the suggestion that interaction between a cluster Fe and the sulfonium ion plays an important role in the reductive cleavage of SAM. Specifically, a 2.7 Å interaction between an Fe atom of the [4Fe-4S]$^{2+}$ cluster and the Se in selenomethionine (SeMet), a cleavage product of Se-adenosyl-L-selenomethionine (Se-SAM), was observed when [4Fe-4S]$^{2+}$ KAM was incubated with Se-SAM, dithionite, and the substrate analog, *trans*-3,4-dehydrolysine. The same interaction was observed upon incubation of KAM with SeMet, deoxyadenosine, and *trans*-3,4-dehydrolysine (Cosper et al. 2000). These observations led to the proposal of a mechanism by which an Fe atom of the [4Fe-4S] cluster interacts directly with the sulfonium ion of SAM, allowing for electron transfer to SAM and subsequent adenosyl radical formation (Cosper et al. 2000). In light of the different inter-
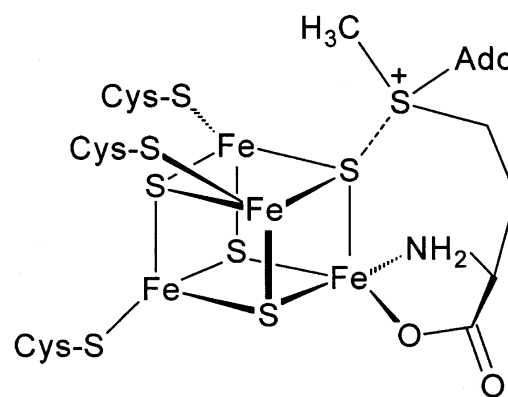
Figure 1. Proposed mode of interaction of SAM with the [4Fe-4S] cluster of PFL-AE. The model is based on electron-nuclear double resonance and Mössbauer studies (Krebs et al. 2002; Walsby et al. 2002a,b).

pretations of the SAM-cluster interactions for PFL-AE and BioB relative to KAM, it was of interest to determine whether the phenomenon of the sulfonium-Fe interaction is unique to KAM or is a general feature of the radical-SAM family. We have therefore used Se XAS to probe the interaction between Se-SAM and the [4Fe-4S] clusters in BioB and PFL-AE.

Results and Discussion

Previous Se XAS results for KAM identified a ~2.7 Å FT peak, which was interpreted as an interaction between a Fe atom from the [4Fe-4S] cluster and the Se atom of SeMet (Cosper et al. 2000). This interaction was observed only in the presence of both the cleavage products of Se-SAM and the substrate analog *trans*-4,5-dehydrolysine. Such samples could be prepared either by incubating [4Fe-4S]$^{2+}$-KAM with Se-SAM, reductant and the substrate analog, the combination of which promoted reductive cleavage of Se-SAM to 5′-deoxyadenosine and Se-Met, or by incubating the enzyme with the products of Se-SAM cleavage (SeMet and 5′-deoxyadenosine) and substrate or substrate analog. Of these two methods, the latter provides the simplest assessment of the Se-Fe interaction, because it can be done in the presence of the native substrate rather than the substrate analog. Although there was no evidence for interaction of the Se of Se-SAM with a cluster Fe, the discovery that the Se of SeMet lies close to an Fe site of the cluster (Se-Fe distance of ~2.7 Å), led to the hypothesis that reductive cleavage of SAM involves interaction between a cluster Fe and the sulfonium ion of SAM.

Se XAS data were collected for BioB and PFL-AE using both the turnover technique (in which Se-SAM and substrate are added to the active enzyme) and the addition of products technique. Incubation of BioB with SeMet, 5′-deoxyadenosine and biotin or of PFL-AE with SeMet, 5′-deoxyadenosine and PFL, results in XAS and FT spectra that show no evidence for a close Se-Fe interaction (Fig. 2). The Se EXAFS spectra for both BioB with SeMet, 5′-deoxyadenosine, and biotin and PFL-AE with SeMet, 5′-deoxyadenosine, and PFL can be best fit assuming a Se-C$_2$ coordination environment with a Se-C distance of 1.93–1.96 Å and with reasonable Debye-Waller factor values (Fits 2, 6; Table 1). These fits are similar to those obtained for the model compound, SeMet (Cosper et al. 2000). Attempts to include a 2.7 Å Se-Fe interaction provided unsatisfactory fits. Additionally, no Se-Fe interaction was observed by use of the turnover technique, in which samples of BioB and PFL-AE were incubated with Se-SAM and substrate or when PFL-AE was incubated with Se-SAM alone (see supplemental material).

The differences in the Se-XAS results of KAM (Cosper et al. 2000) compared with those reported herein for PFL-AE and BioB may reflect differences in the involvement of
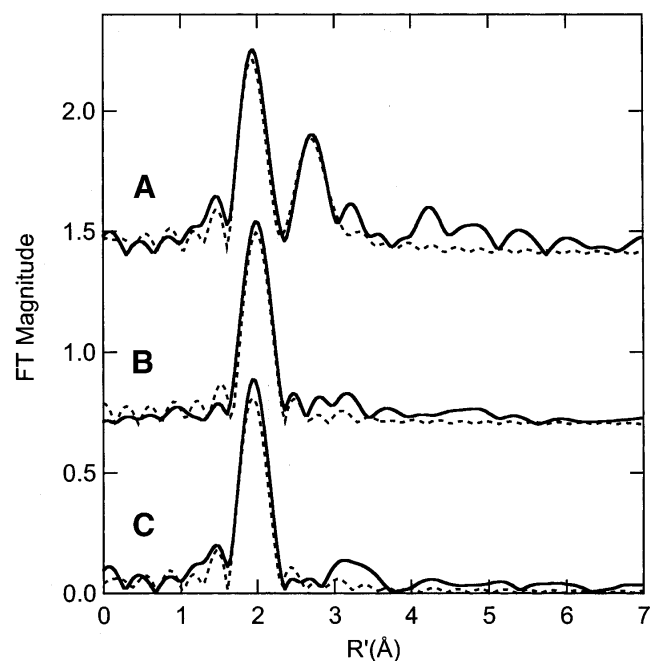
Figure 2. Fourier transforms (over $k = 2$–12.5 Å^{-1}) for (*A*) lysine 2,3-aminomutase incubated with SeMet, 5′deoxyadenosine, and didehydrolysine (solid line) and the calculated spectra for Se-C,Fe (Cosper et al. 2000); (*B*) PFL-AE [4Fe-4S]$^{2+}$ incubated with SeMet and PFL (solid line) and the calculated spectra for Se-C$_2$ (broken line; Fit 2, Table 1); and (*C*) BioB incubated with SeMet, d-biotin, and 5′deoxyadenosine (solid line) and the calculated spectra for Se-C$_2$ (broken line; Fit 6, Table 1).

SAM in the mechanisms of these enzymes. One major difference between KAM and the other well-characterized radical-SAM enzymes is that KAM uses SAM catalytically. In KAM, SAM is cleaved reversibly by the [4Fe-4S]$^{+}$ cluster to generate a 5′-deoxyadenosyl radical intermediate and methionine, and the SAM is regenerated after each interconversion of α-lysine to β-lysine; in other words, one SAM is involved in catalyzing numerous lysine rearrangements (Moss and Frey 1987). In the other well-characterized members of the radical-SAM family, such as PFL-AE and BioB, SAM is a reagent, being converted to methionine and 5′-deoxyadenosine with each substrate turnover (Knappe et. al. 1984; Guianvarc'h et al. 1997).

These differing roles of SAM in KAM compared with BioB and PFL-AE—as a catalytic cofactor in the former and as a substrate in the latter—may be reflected in differences in the mode of SAM binding as suggested previously (Walsby et al. 2002a) and/or differences in the binding affinity of the methionine product. For KAM, the products of SAM cleavage must remain in close proximity in the active site, as completion of catalysis is accompanied by reformation of SAM from methionine and the 5′-deoxyadenosyl radical. Hence, it is possible that interaction between an Fe atom of the [4Fe-4S] cluster in KAM and the methionine thioether serves to increase the methionine-binding affinity,

Table 1. *Curve fitting results for Se EXAFS of PFL-AE and BioB incubated with SeMet*[a]

Sample filename (k range) $\Delta k^3 \chi$	Fit	Shell	R_{as} (Å)	σ_{as}^2 (Å^2)	ΔE_0 (eV)	f'[b]
PFL-AE + SeMet, PFL, 5'deoxyAdo	1	Se-C	1.96	−0.0016	2.07	0.144
EPRPA (2–12.5 Å^{-1})	2	Se-C$_2$	1.96	0.0016	1.05	0.119
$\Delta k^3 \chi = 5.70$	3	Se-C$_3$	1.95	0.0039	−1.26	0.121
	4	Se-C$_4$	1.94	0.0060	−4.41	0.136
BioB + SeMet, d-biotin, 5'deoxyAdo	5	Se-C	1.94	−0.0018	−1.97	0.130
EBBDA (2–12.5 Å^{-1})	6	Se-C$_2$	1.93	0.0012	−4.75	0.106
$\Delta k^3 \chi = 5.34$	7	Se-C$_3$	1.93	0.0035	−6.95	0.111
	8	Se-C$_4$	1.92	0.0054	−8.64	0.129

[a] Shell is the chemical unit defined for the multiple scattering calculation. R_{as} is the metal-scatterer distance. σ_{as}^2 is a mean square deviation in R_{as}. ΔE_0 is the shift in E_0 for the theoretical scattering functions.
[b] f' is a normalized error (χ^2):

$$f' = \frac{\left\{ \sum_i [k^3(\chi_i^{obs} - \chi_i^{calc})]^2 / N \right\}^{1/2}}{[(k^3 \chi^{obs})_{max} - (k^3 \chi^{obs})_{min}]}$$

thereby holding the methionine in place to rejoin the 5'-deoxyadenosyl radical. In contrast, the products of SAM cleavage must leave the active site of PFL-AE and BioB after each turnover, being replaced by a new SAM to be used in the next catalytic cycle. Because no interaction between an Fe atom from the [4Fe-4S] cluster and the Se atom of SeMet was observed for either PFL-AE or BioB, the SeMet is either absent from the active site, bound in a similar configuration, but with the Se atom more distant from a cluster Fe atom, or occupying several different orientations near the [4Fe-4S]$^{2+}$ cluster. In the absence of information on methionine-binding affinity for KAM, PFL-AE, and BioB, it is not possible to discriminate between these possibilities or address the possibility of differences in interaction of SAM with the [4Fe-4S] cluster among members of the radical-SAM superfamily. However, what is clear from the results presented herein, is that a well-defined interaction between the product methionine and an Fe of the [4Fe-4S] cluster as seen in KAM (Cosper et al. 2000) is not a general property of radical-SAM family of Fe-S enzymes. Rather, it may be confined to members of this class, which use SAM in a catalytic role rather than as a substrate.

Materials and methods

E. coli [4Fe-4S]$^{2+}$ BioB (Ollagnier-de Choudens et al. 2000; Cosper et. al. 2002) and Se-SAM (Park et al. 1996; Walsby et al. 2002a), were prepared as described previously. SeMet, 5'-deoxyadenosine, biotin, and DTB were purchased from Sigma-Aldrich. Four BioB samples were prepared in 50 mM Hepes (pH 7.5) as follows: (1) 1.3 mM [4Fe-4S]$^{2+}$ BioB; (2) 1.3 mM [4Fe-4S]$^{2+}$ BioB, 3.0 mM 5'-deoxyadenosine, and 1.3 mM SeMet; (3) 1.3 mM [4Fe-4S]$^{2+}$ BioB, 3.2 mM 5'-deoxyadenosine, 1.3 mM SeMet, and 2.7 mM biotin; (4) 1.5 mM [4Fe-4S]$^{2+}$ BioB, 1.6 mM Se-SAM, and 3.0 mM DTB. Four additional BioB samples were prepared in 50 mM Tris-HCl (pH 8.5) with 0.2 M NaCl, 1 mM DTT as follows: (1) 1.3 mM [4Fe-4S]$^{2+}$ BioB; (2) 1.3 mM [4Fe-4S]$^{2+}$ BioB

with 1.0 mM Se-SAM; (3) 2.8 mM [2Fe-2S]$^{2+}$/[4Fe-4S]$^{2+}$ BioB; and (4) 2.8 mM [2Fe-2S]$^{2+}$/[4Fe-4S]$^{2+}$ BioB with 2.1 mM Se-SAM. After each sample was prepared and incubated at ambient temperature for 30 min, ethylene glycol was added to a final concentration of 20%. All preparation and manipulation of the eight BioB samples was performed inside a Vacuum Atmosphere glove box with O$_2$ levels <5 ppm.

E. coli PFL-AE was prepared as described previously (Broderick et al. 2000; Walsby et al. 2002a). Five different PFL-AE samples were prepared in 50 mM HEPES (pH 7.5) as follows: (1) 1 mM PFL-AE, 25% (v/v) glycerol, 1 mM sodium dithionite, 200 μM 5-deazariboflavin, and 0.95 mM Se-SAM; (2) 1 mM PFL-AE, 25% (v/v) glycerol, and 0.95 mM Se-SAM; (3) 1 mM PFL-AE, 25% glycerol, 0.95 mM SeMet, and 4 mM 5'-deoxyadenosine; (4) 0.44 mM PFL-AE, 25% glycerol, 0.38 mM SeMet, 4 mM 5'-deoxyadenosine, and 1.1 mM PFL; and (5) 0.4 mM PFL-AE, 25% (v/v) glycerol, 0.8 mM sodium dithionite, 100 μM 5-deazariboflavin, 0.4 mM PFL, and 0.35 mM Se-SAM. Samples 1 and 5 were prepared by combining PFL-AE, glycerol, dithionite, and 5-deazariboflavin and illuminating for 30–60 min to generate the [4Fe-4S]$^+$ state of PFL-AE. Following illumination, Se-SAM (1) or Se-SAM and PFL (5) were added. Sample 5 represents the turnover sample, whereas sample 4 represents the product addition method sample. All samples were prepared in an inert atmosphere box (Mbraun) operating at <3 ppm O$_2$.

XAS data were collected at Stanford Synchrotron Radiation Laboratory (SSRL), beamline 7-3, with the SPEAR storage ring at 3.0 GeV and 60–100 mA. Fluorescence data were collected using a Ge solid-state array detector and a Si(220) double-crystal monochromator that was 50% detuned for harmonic rejection. Calibration was achieved using a powdered elemental Se standard (first inflection, 12658 eV). EXAFS analysis was performed with the EXAFSPAK software (www-ssrl.slac.stanford.edu/exafspak.html), according to standard procedures (Scott 1985). Fourier transform plots were generated with sulfur-based phase correction. Other XAS parameters were as described previously (Cosper et al. 2000, 2001).

Electronic supplemental material

Plots of XAS data for BioB and PFL-AE are available.

Acknowledgments

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