Published in final edited form as:

Biochemistry. 2007 November 13; 46(45): 12889–12895. doi:10.1021/bi701745h.

Binding energy in the one-electron reductive cleavage of *S*-adenosylmethionine in lysine 2,3-aminomutase, a radical SAM enzyme[†]

Susan C. Wang and Perry A. Frey

Department of Biochemistry, University of Wisconsin-Madison, 1710 University Avenue, Madison, WI 53726

Abstract

The common step in the actions of members of the radical SAM superfamily of enzymes is the one-electron reductive cleavage of *S*-adenosyl-L-methionine (SAM) into methionine and the 5'-deoxyadenosyl radical. The source of the electron is the [4Fe-4S]¹⁺ cluster characterizing the radical SAM superfamily, to which SAM is directly ligated through its methionyl carboxylate and amino groups. The energetics of the reductive cleavage of SAM is an outstanding question in the actions of radical SAM enzymes. The energetics is here reported for the action of lysine 2,3-aminomutase (LAM), which catalyzes the interconversion of L-lysine and L-β-lysine. From earlier work, the reduction potential of the [4Fe-4S]^{2+/1+} cluster in LAM is −0.43 V with SAM bound to the cluster (Hinckley, G.T. and Frey, P.A. (2006) *Biochemistry* 45, 3219–3225), 1.4 V higher than the reported value for trialkylsulfonium ions in solution. The mid-point reduction potential upon binding L-lysine has been estimated to be −0.6 V from the values of mid-point potentials measured with SAM bound to the cluster and L-alanine in place of L-lysine, with *S*-adenosyl-L-homocysteine (SAH) bound to the cluster in the presence of L-lysine, and with SAH bound to the cluster in the presence of L-alanine or of L-alanine and ethylamine in place of L-lysine. The reduction potential for SAM has been estimated to be −0.99 V from the measured value for *S*-3',4'-anhydroadenosyl-L-methionine. The reduction potential for the [4Fe-4S] cluster is lowered 0.17 V by the binding of lysine to LAM, and the binding of SAM to the [4Fe-4S] cluster in LAM elevates its reduction potential by 0.81 V. Thus, the binding of L-lysine to LAM contributes 4 kcal mol^{−1}, and the binding of SAM to the [4Fe-4S] cluster in LAM contributes 19 kcal mol^{−1} toward lowering the barrier for reductive cleavage of SAM from 32 kcal mol^{−1} in solution to 9 kcal mol^{−1} at the active site of LAM.

Members of the radical SAM¹ superfamily catalyze a diverse array of biochemical processes through mechanisms with free radical intermediates (1–10). Radical SAM enzymes participate in amino acid metabolism; biosynthesis of coenzymes such as biotin, lipoic acid, thiamine pyrophosphate, molybdopterin, heme in anaerobic bacteria, and pyrroloquinoline quinone among others; DNA biosynthesis and repair; and the biosynthesis of a variety of antibiotics. The radical SAM enzymes incorporate specialized iron-sulfur clusters nucleated by the cysteine motif CxxxCxxC, and the three cysteine-residues within this motif account for three

[†]This research was supported by NIH grant DK28607 from the National Institute of Diabetes and Digestive and Kidney Diseases

*To whom correspondence should be addressed. Department of Biochemistry, University of Wisconsin-Madison, 1710 University Ave., Madison, WI 53726, USA; Tel: (608) 262-0055. Fax: (608) 265-2904. E-mail: frey@biochem.wisc.edu.

¹Abbreviations. Ado •, the 5'-deoxyadenosyl radical; anAdo •, the 3',4'-anhydro-5'-deoxyadenosyl radical; anATP, 3', 4'-anhydroadenosine triphosphate; anSAM, *S*-3',4'-anhydroadenosyl-L-methionine; EDTA, ethylenediaminetetraacetic acid; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; G, Gauss; EPR, electron paramagnetic resonance; HPLC, high performance liquid chromatography; LAM, lysine 2,3-aminomutase; Met, methionine; PLP, pyridoxal-5'-phosphate; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; SeSAM, Se-adenosyl-L-selenomethionine.

ligands to iron in the [4Fe–4S] centers. The fourth ligand is SAM itself, with the methionyl amino and carboxylate groups serving as a bidentate ligand to the non-cysteine-ligated iron of the cluster (11–13).

Very few of the reaction mechanisms in the radical SAM superfamily are known in detail. Available information indicates that radical SAM enzymes share one process in common, the one-electron reductive cleavage of SAM to the 5'-deoxyadenosyl radical and methionine (1–10). The electron for reductive cleavage arises from the [4Fe–4S]¹⁺ cluster, according to eq. 1,

where Ado • represents the 5'-deoxyadenosyl radical and Met is methionine. Ado • initiates catalysis by abstracting a hydrogen atom from a substrate to generate 5'-deoxyadenosine and a substrate-related radical as initial intermediates. Subsequent steps in the mechanisms of the overall reactions are as diverse as the reactions themselves. The most thoroughly documented radical mechanism among members of this family is that of lysine 2,3-aminomutase (LAM) shown in Fig. 1 (3,5). Three of the four radical intermediates have been identified and characterized spectroscopically and kinetically.

A general mechanistic problem in the radical SAM superfamily is the one-electron reductive cleavage of SAM. The reduction potentials (2+/1+) of the [4Fe–4S] clusters in the family are far less negative than the reported values for the irreversible one-electron reduction of trialkyl sulfonium ions such as SAM, which are approximately –1.8 V (14–16). In the case of LAM, the (2+/1+)-reduction potential of the [4Fe–4S] cluster with SAM ligated to the cluster is –430 mV (17), so that the barrier to the one-electron reductive cleavage of SAM should be about –1.4 V, corresponding to 32 kcal mol^{–1}. The barrier must be much lower in the active site of LAM; otherwise the rate constant for the slowest step in the mechanism would have to be about 4×10²² s^{–1}, many orders of magnitude larger than typical bond vibrational frequencies.² We report the effect of binding lysine to the active site on the reduction potential (2+/1+) of the [4Fe–4S] cluster, we measure the equilibrium constant for reductive cleavage of *S*-3',4'-anhydroadenosyl-L-methionine (*anSAM*) at the active site in the presence of lysine, we calculate the corresponding equilibrium constant for SAM, and we estimate the energetic contributions of binding lysine and SAM to decreasing the energy barrier to reductive cleavage of SAM.

EXPERIMENTAL PROCEDURES

Materials

4,4'-Dimethyl-1,1'-trimethylene-2, 2'-dipyridyl bromide and 1, 1'-trimethylene-2,2'-dipyridyl bromide were synthesized as described (18). *anATP* was synthesized previously (19) and stored at –70° C until use. *anSAM* was used either as a stock solution frozen at –70° C or synthesized and frozen at –70° C without further purification. Other reagents were purchased from Thermo Fisher Scientific (Waltham, MA), MP Biomedicals (Irvine, CA), or Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. SAM synthetase was purified as described (20,21). 4,4'-Dimethyl-1,1'-tetramethylene-2,2'-dipyridyl bromide was synthesized according

²If the one-electron reductive cleavage of SAM at the active site is fast and at equilibrium during catalysis, the estimated ΔE° of –1.4 V in solution means that the equilibrium constant for the formation of Ado • from SAM at the active site should be 10^{–23} if the reduction potential of SAM at the active site is the same as in solution. The turnover number for LAM is about 40 s^{–1} under initial rate conditions, that is, in the absence of the product. Then at saturating lysine, 1 μM LAM will generate β-lysine at the rate of 40 μM s^{–1}, and this rate must be attained with the Ado •-containing intermediate at a concentration of about 10^{–23} μM, given the postulated equilibrium constant for its formation. If this were the case, the rate constant for the reaction of Ado • in the irreversible formation of the product would be 40 μM s^{–1}/10^{–23} μM, or 4×10²² s^{–1}, an unreasonable value. Thus, the equilibrium constant for the formation of Ado • cannot be 10^{–23} and the value of ΔE° at the active site cannot be the solution value of –1.4 V.

to previously reported methods (18,22) with the following modifications: Stoichiometric amounts of the starting materials, 4,4'-dimethyl-2,2'-dipyridyl (0.5 g) and 1,4-dibromopropane (300 μ L) were mixed and heated in anisole (28 mL) at reflux for ~24 h under Ar in the dark. The resulting solid was filtered and washed with acetone. The dried product was dissolved in methanol (0.5 mL) and recrystallized using acetone (~10 mL).

Enzyme production, purification, reconstitution, and characterization

Recombinant LAM was purified and reconstituted as described (17,23,24). Reconstitution of iron-sulfur clusters was performed for 12–15 h at room temperature in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI). Amicon Ultra-15 Centrifugal Filter Units (30,000 MWCO) from Millipore (Billerica, MA) placed in sealing 250 mL centrifuge bottles were used to concentrate the protein. Iron and sulfide analysis were performed as described (25,26). Protein concentrations were determined by comparing the absorbance at 280 nm with the extinction coefficient for reconstituted LAM as determined by amino acid analysis ($\epsilon = 7.63 \times 10^4$ subunit $\text{M}^{-1} \text{cm}^{-1}$; (27). LAM was assayed for activity as described (28) with minor modifications.

Anhydroadenosyl radical formation

Due to the instability of *anSAM*, the cofactor analogue was prepared *in vitro* and stored at -70°C without further purification. The reaction was performed in the anaerobic chamber and monitored by HPLC. Each reaction contained 50 mM EPPS (pH 8.0); 20 mM MgSO_4 ; 3.25 mM *anATP*; 3.52 mM methionine; 40 μ g inorganic phosphatase (Sigma-Aldrich, St. Louis, MO); and sufficient SAM synthetase to ensure that the reaction had gone to completion after 30 min. The reaction was fast-frozen in liquid nitrogen prior to storage.

Prior to use, frozen *anSAM* was thawed in the anaerobic chamber and vacuum purged and flushed with argon (15+ cycles) to remove oxygen. A mix was prepared containing all components except LAM. Aliquots (230 μ L) of the mix were allocated, and addition of the enzyme (20 μ L) initiated the reaction. The resulting mixture was rapidly transferred into an EPR tube and frozen in cold isopentane ($\sim -50^\circ \text{C}$) at time points ranging from 15–75 s. Duplicates were created for each time point, and the average radical concentration is illustrated in Figure 2. Final concentrations of the reaction components were: 34 μ M (subunit) LAM; 200 mM EPPS, pH 8.0; 320 μ M sodium dithionite; 510 μ M *anSAM*; 16 mM lysine (free base). For comparison, samples were set up with no lysine and *anSAM* or with SAM (tosylate salt, 400 μ M) and 4-thia-L-lysine (*S*-2-aminoethyl-L-cysteine hydrochloride, 10 mM). The final concentration of sodium dithionite, which was significantly lower than previously employed (19), was determined by titrating dithionite (100 μ M – 1 mM) against the protein while observing the +1 signal from the iron-sulfur cluster by EPR. It was important to optimize the dithionite concentration because excess dithionite could lead to over-reduction of $[\text{4Fe} - 4\text{S}]^{2+}$ on the right side of eq. 1, with consequent over-production of *anAdo*• from *anSAM*. Controls were performed using SAM generated *in vitro* with SAM synthetase showing that the presence of the additional enzyme and associated components (i.e. Mg^{2+} , methionine, and phosphate) did not interfere with signal intensity.

Spectroelectrochemistry

Spectroelectrochemical titrations were performed as described elsewhere (17) with minor modifications. After reducing the sample for 15 min at a controlled potential, which was monitored for 75 s to track the drift that occurs during sample transfer. The sample was reduced again for 5 min, transferred into the EPR tube, and the transfer time recorded. The corresponding potential from the time course of drift was used for data analysis. A typical mixture contained ~1 mg reconstituted LAM, 150 mM EPPS (pH 8.0), 67 mM K_2SO_4 (as an electrolyte), 100 μ M each of 4,4'-dimethyl-1,1'-trimethylene-2, 2'-dipyridyl bromide and 1,1'-

trimethylene-2,2'-dipyridyl bromide or 4,4'-dimethyl-1,1'-tetramethylene-2, 2'-dipyridyl bromide, and 425 μ M of the appropriate adenosyl cofactor or analogue. Final substrate and substrate analogue concentrations, when present, were as follows: 100 mM alanine, 100 mM ethylamine, 40 mM lysine.

EPR spectroscopy and data analysis

Low-temperature X-band EPR spectra were acquired on a Varian Line spectrometer equipped with a Varian E102 microwave bridge and interfaced with a Linux system. Spectra were obtained and manipulated using the Xemr program (Jussi Eloranta, <http://www.csun.edu/~jeloranta/xemr>). An Oxford Instruments ESR-900 continuous flow helium flow cryostat and Oxford Instruments 3120 temperature controller were used. A Varian gaussmeter and a Hewlett-Packard 5255A frequency converter and 5245L electronic counter were employed to measure the magnetic field strength and the frequency, respectively. A Hewlett-Packard 432A power meter was used to calibrate the microwave power. Spectra were acquired as the average of four 2-min scans. Conditions for iron-sulfur EPR were as follows: 9.252 GHz, 16 G modulation amplitude, 3400 G center field, 1000 G sweep width, 1–5 mW power, 1600–8000 gain, 0.3 s time constant, 10 K. Conditions for radical EPR were 9.252 GHz, 4 G modulation amplitude, 3300 G center field, 400 G sweep width, 15 μ W power, 2000 gain, 0.3 s time constant, 10 K. Spin concentrations were estimated by double integration of spectra using a copper standard (1 mM CuSO₄, 10 mM EDTA) at 15 μ W and 10 K as a reference. Data analysis was performed as described elsewhere (17) using the program PSI-Plot (Poly Software International, Pearl River, NY).

RESULTS

Midpoint reduction potential of [4Fe–4S] bound to LAM in the Michaelis complex

The one-electron mid-point reduction potential of the [4Fe–4S]^{2+/1+} center with SAM bound to the active site as a ligand to the cluster is –430 mV (17). This value ensures that the iron-sulfur cluster can be reduced to the (1+) state *in vivo* by biological systems (29). All reduction potentials and equilibrium constants in this paper refer to pH 8.0; reduction potentials for sulfonium ions are pH-independent (15).

The potential of –430 mV is not necessarily relevant to the reductive cleavage of SAM in the active site because of the absence of the substrate lysine under the conditions of measurement. The most relevant reduction potential for the iron-sulfur cluster would be that of the Michaelis complex, which contains lysine and SAM and is poised for reaction. Measurement of that value would be impractical because with lysine present the reaction would proceed.

An estimate of the midpoint reduction potential for the iron-sulfur cluster in the Michaelis complex, which may be represented as E–[4Fe–4S]¹⁺–SAM.PLP=Lys, may be obtained from the midpoint potentials for complexes that are not reactive but are structurally similar to the Michaelis complex. Such complexes have SAH in place of SAM, or alanine in place of lysine, or alanine and ethylamine in place of lysine. Alanine is practically unreactive with LAM but is rescued as a substrate by the presence of ethylamine.³ Values of midpoint potentials for such complexes are presented in Table 1. The results show that the binding of lysine or alanine significantly lowers the midpoint potentials. The binding of alanine to LAM with SAM lowers the potential by about 150 mV, and with SAH in place of SAM alanine lowers the potential by about 106 mV. Alanine should not be as effective as lysine, and this expectation is supported by the fact that lysine lowers the midpoint potential of the complex with SAH by 17 mV more

³F.J. Ruzicka and P.A. Frey, manuscript in preparation.

than alanine. Furthermore, the addition of both alanine and ethylamine to the complex with SAH lowers the potential by 28 mV more than the addition of alanine alone.

It appears that the lysyl side chain induces a 17 to 28 mV lowering of the midpoint potential. When employed to adjust the midpoint potential of -582 mV measured for the complex with SAM and alanine, we can expect the value for SAM and lysine to lie between -599 and -610 mV. For the present purpose, shall adopt the value -600 mV as a conservative estimate of the midpoint potential for $[4\text{Fe}-4\text{S}]$ in the Michaelis complex.

The midpoint potential for reductive cleavage of SAM in the active site

We wish to estimate the midpoint reduction potential for one-electron reductive cleavage of SAM at the active site of LAM (E°_2) according to eq. 3. A value can be calculated if we have ΔE° for the overall reaction of eq. 4, because we know E°_1 for the half-reaction in eq. 2 from the foregoing section. A value for the equilibrium constant in the reaction of eq. 4 is needed to calculate $\Delta G^\circ_{\text{pH}8}$ from the relation $\Delta G^\circ_{\text{pH}8} = -RT \ln K_{\text{eq}}$, and $-E^\circ$ can then be calculated from $\Delta G^\circ_{\text{pH}8} = -nF\Delta E^\circ$,

where $n = 1$ electron and F = Faraday constant ($23 \text{ kcal mol}^{-1} \text{ V}^{-1}$). The equilibrium constant for reaction 4 cannot be measured directly because the product reacts forward too rapidly, and in any case the value is too small to be measured by available analytical methods. The value can be estimated from the equilibrium constant for the related reaction 5 and the relative enthalpies of the 5'-deoxyadenosyl radical ($\text{Ado} \bullet$) and the 3',4'-anhydro-5'-deoxyadenosyl radical ($\text{anAdo} \bullet$).

The radical $\text{anAdo} \bullet$ is the EPR spectroscopically observed analog of $\text{Ado} \bullet$ that appears when LAM is activated by anSAM , the corresponding analog of SAM (19,30). The results in Fig. 2 allow the value of K_{eq} for eq. 5 in the reaction of anSAM to be calculated as 6.2×10^{-2} , which corresponds to $\Delta G^\circ_{\text{pH}8} = 1.6 \text{ kcal mol}^{-1}$ for reaction 5. The calculation of 0.062 for the equilibrium constant is based on the subunit concentration of LAM and the assumption that the subunits function independently. LAM is a dimer of intercalated dimeric units, and if it should happen that only one subunit of the intimate dimer is functional, that is if the enzyme functions in a half-of-the-sites mode, then the equilibrium constant would be 0.12. This difference or uncertainty of a factor of two in the equilibrium constant would have a minor affect on the overall energetics in the reductive cleavage of SAM ($\sim 2 \text{ kcal mol}^{-1}$).

We have confidence that the plateau in Fig. 2 signals equilibrium in eq. 5, the formation of $\text{anAdo} \bullet$, because of the available information about this radical (19). anSAM functions normally as a coenzyme in place of SAM, albeit at a much slower rate. The slow rate is due to the stability of $\text{anAdo} \bullet$, which is an allylic radical and much less reactive than $\text{Ado} \bullet$ in abstracting the hydrogen atom from the 3-pro-*R* position of the lysyl side chain. The value of the deuterium kinetic isotope effect in the reaction of deuterio-lysine shows that hydrogen abstraction by $\text{anAdo} \bullet$ limits the rate. The slow overall rate, the large deuterium kinetic isotope effect, and the observation of $\text{anAdo} \bullet$ as the sole free radical in the reaction all indicate equilibrium at the first hydrogen transfer step, the process in eq. 5.

A value of $\Delta G^{\circ}_{\text{pH}8}$ for eq. 4 can be calculated from $\Delta G^{\circ}_{\text{pH}8}$ for eq. 5 and the relative energies of Ado • and anAdo •. From the Co—C5' bond dissociation energy of 31 kcal mol⁻¹ for adenosylcobalamin (31) and the bond dissociation energy of 24 kcal mol⁻¹ for 3',4'-anhydroadenosylcobalamin (32) the enthalpy difference between Ado • and anAdo • is 7 kcal mol⁻¹. If the entropy changes in the reactions of eq. 4 and eq. 5 are the same or very similar, the enthalpy difference will be very near the free energy difference for these radicals at the active site of LAM. Inasmuch as both reactions occur within the confines of the active site, which contains no water or other molecules that might be dissociable (33), the assumption of similar or identical entropy changes in the two reactions seems reasonable. On that basis, a value of $\Delta G^{\circ}_{\text{pH}8}$ for reaction 4 can be estimated by correcting the standard free energy of reaction 5 for the energy difference between the two radicals. An estimate of $\Delta G^{\circ}_{\text{pH}8}$ for reaction 4 is then 8.6 kcal mol⁻¹, and the value of ΔE° is -390 mV. From the values $\Delta E^{\circ} = -390$ mV for eq. 4 and $E^{\circ}_1 = +600$ mV for eq. 2, the midpoint potential for reductive cleavage of SAM in eq. 3 can be calculated as $E^{\circ}_2 = -990$ mV.

The substrate-related radical in reaction of 4-thia-L-lysine

LAM activated by SAM catalyzes the transformation of 4-thia-L-lysine into ethylamine and formylacetate (malonate semialdehyde). The cleavage products arise from the chemical decomposition of the enzymatic product, 4-thia-β-lysine, generated by the action of LAM on 4-thia-L-lysine as a substrate (28,34). In the steady state of the reaction of 4-thia-L-lysine, the only observable free radical is the 4-thialysyl-3-yl species, the 4-thia-analog of the substrate-related radical **1** (Fig. 1), with the unpaired electron formally on C3 of the lysyl side chain. LAM activated by anSAM also accepts 4-thia-L-lysine as a substrate, and in the steady state a mixture of the substrate-related radical and anAdo • are observed by EPR spectroscopy, indicating that the two radicals are of comparable stability (19). The measured concentration of the 4-thia-analog of radical **1** in Fig. 1 in the reaction of 34 μM LAM activated by SAM is 2.9 μM, which corresponds to $K_{\text{eq}} = 0.087$ in the formation of the 4-thialysine-3-yl radical. Comparison of this value with 0.062 for the generation of anAdo • confirms the relative stabilities of the 4-thialysyl-3-yl and anAdo • radicals at the active site of LAM.

DISCUSSION

The one-electron reductive cleavage of trialkyl sulfonium ions leads to alkyl radical products. Both electrolytic reduction at electrodes and chemical reduction by low potential iron sulfur clusters of the type [4Fe-4S(SR)₄] in acetonitrile effect electron transfer-induced cleavages to radicals (14,16,35). The chemical cleavages by [4Fe-4S(SR)₄] in acetonitrile appear similar to the processes in radical SAM enzymes, but they are mechanistically unlike the enzymatic reactions. The chemical reactions of [4Fe-4S(SR)₄] involve very low potential clusters ($E^{\circ} = -1.2$ V in acetonitrile) and highly reactive sulfonium ions ($E^{\circ} = -0.9$ V). Reductive cleavages can take place through energetically downhill electron transfer in the absence of a kinetic barrier (35). The enzymatic reactions occur with [4Fe-4S]^{2+/1+} transitions displaying $E^{\circ} = -0.4$ to -0.5 V (17,36,37); they involve SAM as the trialkylsulfonium ion, with $E^{\circ} \sim -1.8$ V as discussed in a following section; and SAM is ligated to the cluster, allowing inner sphere electron transfer (11-13,32,37-41).

The present results allow us to describe how binding energy is employed to decrease the energy barrier in the one-electron reductive cleavage of SAM at the active site of LAM. The barrier arises because of the difference between the one-electron reduction potential of the [4Fe-4S]^{2+/1+} cluster with SAM ligated to the unique iron (-430 mV) and the one-electron irreversible reduction potentials for trialkylsulfonium ions in solution, which can be estimated to be -1800 mV. The potential -1800 mV may be accepted as reasonable based on the reported value of -1850 mV for trimethylsulfonium and values of -1600 to -1650 mV for methyl alkyl

aryl sulfonium ions (14–16). Aryl substituents elevate the potentials due to inductive electronic effects, and higher alkyl groups lead to less unstable radicals than methyl groups. The sulfonium group in SAM does not include an aryl substituent but does include two higher alkyl substituents, so the reduction potential will be slightly less negative than that of trimethylsulfonium ion (–1850 mV) but lower than if there were an aryl, vinyl, or allyl substituent. The value may lie between –1750 and –1800 mV, and we adopt –1800 as a rounded value in two significant figures.

The foregoing considerations lead to the free energy levels illustrated in Fig. 3. The reduction potential of the iron-sulfur cluster in the resting enzyme, with cysteine as the ligand to the unique iron in the cluster is –484 mV (17). When SAM binds to the cluster, the potential is elevated to –430 mV (Table 1), making reduction of the cluster to the 1+ state readily accessible to biological reducing systems (29). Upon binding lysine, the potential is lowered to –600 mV, still 1.2 V more positive than that of a free trialkylsulfonium ion. The reduction potential of free SAM may be estimated as that for one-electron irreversible reduction of a trialkylsulfonium ion, –1800 mV. However, when ligated to the [4Fe–4S] cluster in the active site of LAM, the calculated value is –990 mV, 810 mV higher. The difference between midpoint potentials for SAM and the [4Fe–4S] cluster in the active site of LAM is 390 mV, 1400 mV less than it would be in solution. While the values of E° and $\Delta G^\circ_{\text{pH}8}$ in this paper refer to pH 8.0, they are pH-independent and should be the same at pH 7.0.

Fig. 3 shows that the binding of both lysine and SAM to LAM contribute to decreasing the barrier to the reductive cleavage of SAM. Lysine binding contributes 170 mV or 4 kcal mol^{–1}, and the binding of SAM to the [4Fe–4S]¹⁺ cluster contributes 810 mV or 19 kcal mol^{–1} to decreasing the barrier from 32 to 9 kcal mol^{–1}.

The elevated reduction potential of SAM in the active site of LAM can be attributed largely to its ligation to the [4Fe–4S] cluster. Ligation to Fe^{2.5+} can by itself be expected to elevate the potential to some degree, even though the locus of ligation through the methionyl amino and carboxyl groups appears chemically remote from the sulfonium center. A further consideration in this phenomenon is the mechanism of electron transfer, shown in Scheme 1 (11,33,38). In the mechanism, electron transfer is accompanied by the transformation of pentacoordinate iron in [4Fe–4S]¹⁺ into hexacoordinate iron in [4Fe–4S]²⁺, a more favored coordination state for iron. The coordination transition can be expected to contribute to easing electron transfer to the sulfonium group. Not least important is the fact that the mechanism facilitates inner-sphere electron transfer. The structure shows Se in SeSAM within van der Waals distance of Fe in the cluster (33) so that the electron is transferred in the process of bond formation between Fe and Se, or Fe and S in the case of SAM, and does not have to traverse a significant distance through a medium.

The present work also measures the effect of the binding of lysine to lowering the reduction potential of the [4Fe–4S] center without explaining the molecular basis. The structure shows lysine bound as the external aldimine to PLP in very close proximity to the complex of SAM and the [4Fe–4S]. The presence of lysine in the site can be expected to limit exposure of the iron-sulfur cluster to the solvent. The question of whether this brings about a lowering of the reduction potential remains for future research.

ACKNOWLEDGMENTS

We thank Glen T. Hinckley for insightful discussions and synthesizing 4,4'-dimethyl-1,1'-trimethylene-2,2'-dipyridyl bromide and 1,1'-trimethylene-2,2'-dipyridyl bromide; Frank Ruzicka for supplying LAM and for experimental assistance; Olafur Magnusson for a sample of *an*SAM and for supplying *an*ATP; Dawei Chen for supplying SAM synthetase; Russell Poyner and George Reed for assistance with the operation of the EPR spectrometer.

REFERENCES

1. Sofia HJ, Chen G, Hetzler BG, Reyes-Spindola JF, Miller NE. Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res* 2001;29:1097–1106. [PubMed: 11222759]
2. Cheek J, Broderick JB. Adenosylmethionine-dependent iron-sulfur enzymes: versatile clusters in a radical new role. *J. Biol. Inorg. Chem* 2001;6:209–226. [PubMed: 11315557]
3. Frey PA, Booker SJ. Radical mechanisms of *S*-adenosylmethionine-dependent enzymes. *Adv Protein Chem* 2001;58:1–45. [PubMed: 11665486]
4. Fontecave M, Mulliez E, Ollagnier-de-Choudens S. Adenosylmethionine as a source of 5'-deoxyadenosyl radicals. *Curr. Opin. Chem. Biol* 2001;5:506–511. [PubMed: 11578923]
5. Frey PA, Magnusson OTh. *S*-Adenosylmethionine: A wolf in sheep's clothing or a rich man's adenosylcobalamin? *Chem. Rev* 2003;103:2129–2148. [PubMed: 12797826]
6. Marsh EN, Patwardhan A, Huhta MS. *S*-adenosylmethionine radical enzymes. *Bioorg Chem* 2004;32:326–440. [PubMed: 15381399]
7. Jarrett JT. The generation of 5'-deoxyadenosyl radicals by adenosylmethionine-dependent radical enzymes. *Curr. Opin. Chem. Biol* 2003;7:174–182. [PubMed: 12714049]
8. Jarrett JT. The novel structure and chemistry of iron-sulfur clusters in the adenosylmethionine-dependent radical enzyme biotin synthase. *Arch. Biochem. Biophys* 2005;433:312–321. [PubMed: 15581586]
9. Layer G, Kervio E, Morlock G, Heinz DW, Jahn D, Rétey J, Schubert WD. Structural and functional comparison of HemN to other radical SAM enzymes. *Biol. Chem* 2005;386:971–980. [PubMed: 16218869]
10. Wang S, Frey PA. *S*-Adenosylmethionine as an oxidant: The radical SAM superfamily. *Trends Biochem. Sci* 2007;32:101–110. [PubMed: 17291766]
11. Chen D, Walsby C, Hoffman BM, Frey PA. Coordination and mechanism of reversible cleavage of *S*-adenosylmethionine by the [4Fe-4S] center in lysine 2,3-aminomutase. *J. Am. Chem. Soc* 2003;125:11788–11789. [PubMed: 14505379]
12. Walsby CJ, Hong W, Broderick WE, Cheek J, Ortillo D, Broderick JB, Hoffman BM. Electron-nuclear double resonance spectroscopic evidence that *S*-adenosylmethionine binds in contact with the catalytically active [4Fe-4S]⁺ cluster of pyruvate formate-lyase activating enzyme. *J. Am. Chem. Soc* 2002;124:3143–3151. [PubMed: 11902903]
13. Walsby CJ, Ortillo D, Broderick WE, Broderick JB, Hoffman BM. An anchoring role for FeS clusters: Chelation of the amino acid moiety of *S*-adenosylmethionine to the unique iron site of the [4Fe-4S] cluster of pyruvate formate-lyase activating enzyme. *J. Am. Chem. Soc* 2002;124:11270–11271. [PubMed: 12236732]
14. Colichman EL, Love DL. Polarography of sulfonium salts. *J Org. Chem* 1953;18:40–46.
15. Grimshaw, J. Electrochemistry of the sulphonium group. Chapter 7. In: Stirling, CJM.; Patai, S., editors. *The Chemistry of the Sulphonium Group*. Wiley & Sons Ltd.; 1981. p. 141-155.
16. Saeva FD, Morgan BP. Mechanism of one-electron electrochemical reductive cleavage reactions of sulfonium salts. *J. Am. Chem. Soc* 1984;106:4121–4125.
17. Hinckley GT, Frey PA. Cofactor-dependence in reduction potentials for [4Fe-4S]^{2+/1+} in lysine 2,3-aminomutase. *Biochemistry* 2006;45:3219–3225. [PubMed: 16519516]
18. Salmon RT, Hawkrigde FM. The electrochemical properties of three dipyridinium salts as mediators. *J. Electroanal. Chem* 1980;112:253–364.
19. Magnusson, OTh; Reed, GH.; Frey, PA. Characterization of an allylic analogue of the 5'-deoxyadenosyl radical: An intermediate in the reaction of lysine 2,3-aminomutase. *Biochemistry* 2001;40:7773–7782. [PubMed: 11425303]
20. Markham GD, Hafner EW, Tabor CW, Tabor H. *S*-Adenosylmethionine synthetase from *Escherichia coli*. *J. Biol. Chem* 1980;255:9082–9092. [PubMed: 6251075]
21. Lees NS, Chen D, Walsby CJ, Behshad E, Frey PA, Hoffman BM. How an enzyme tames reactive intermediates: positioning of the active-site components of lysine 2,3-aminomutase during enzymatic

- turnover as determined by ENDOR spectroscopy. *J. Am. Chem. Soc* 2006;128:10145–10154. [PubMed: 16881644]
22. Spotswood TM, Tanzer CI. N.M.R. Spectra and Stereochemistry of the Bipyridyls. *Aust. J. Chem* 1967;20:1213–1225.
23. Ruzicka FJ, Lieder KW, Frey PA. Lysine 2,3-aminomutase from *Clostridium subterminale* SB4: Mass spectral characterization of cyanogen bromide-treated peptides and cloning. *J. Bacteriol* 2000;182:469–476. [PubMed: 10629195]
24. Petrovich RM, Ruzicka FJ, Reed GH, Frey PA. Metal cofactors of lysine 2,3-aminomutase. *J. Biol. Chem* 1991;266:7656–7660. [PubMed: 1850415]
25. Beinert H. Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins. *Anal. Biochem* 1983;131:373–378. [PubMed: 6614472]
26. Kennedy MC, Kent TA, Emptage M, Merkle H, Beinert H, Munck E. Evidence for the formation of a linear [3Fe-4S] cluster in partially unfolded aconitase. *J. Biol. Chem* 1984;259:14463–14471. [PubMed: 6094558]
27. Chen D, Frey PA, Lepore BW, Ringe D, Ruzicka FJ. Identification of structural and catalytic classes of highly conserved amino acid residues in lysine 2,3-aminomutase. *Biochemistry* 2006;45:12647–12653. [PubMed: 17042481]
28. Miller J, Bandarian V, Reed GH, Frey PA. Inhibition of lysine 2,3-aminomutase by the alternative substrate 4-thialysine and Characterization of the 4-thialysyl radical intermediate. *Arch. Biochem. Biophys* 2001;387:281–288. [PubMed: 11370852]
29. Brazeau BJ, Gort SJ, Jessen HJ, Andrew AJ, Liao HH. Enzymatic activation of lysine 2,3-aminomutase from *Porphyromonas gingivalis*. *Appl Environ Microbiol* 2006;72:6402–6404. [PubMed: 16957271]
30. Magnusson, OTh; Reed, GH.; Frey, PA. Spectroscopic evidence for the participation of an allylic analogue of the 5'-deoxyadenosyl radical in the reaction of lysine 2,3-aminomutase. *J. Am. Chem. Soc* 1999;121:9764–9765.
31. Finke RG, Hay BP. Thermolysis of adenosylcobalamin: a product, kinetic, and cobalt-carbon (C5') bond dissociation energy study. *Inorg. Chem* 1984;23:3041–3043.
32. Magnusson, OTh; Frey, PA. Synthesis and characterization of 3',4'-anhydroadenosylcobalamin: A coenzyme B₁₂ analogue with unusual properties. *J. Am. Chem. Soc* 2000;122:8807–8813.
33. Lepore BW, Ruzicka FJ, Frey PA, Ringe D. The x-ray crystal structure of lysine-2,3-aminomutase from *Clostridium subterminale*. *Proc. Natl. Acad. Sci. U.S.A* 2005;102:13819–13824. [PubMed: 16166264]
34. Wu W, Lieder KW, Reed GH, Frey PA. Observation of a second substrate radical intermediate in the reaction of lysine 2,3-aminomutase: A radical centered on the β-carbon of the alternative substrate, 4-thia-l-lysine. *Biochemistry* 1995;34:10532–10537. [PubMed: 7654708]
35. Dayley CJA, Holm RH. Reactivity of [Fe₄S₄(SR)₄]^{2-/3-} clusters with sulfonium cations: Analogue reaction systems for the initial step in biotin synthase catalysis. *Inorg. Chem* 2001;40:2785–2793. [PubMed: 11375696]
36. Ugulava NB, Gibney BR, Jarrett JT. Biotin synthase contains two distinct iron sulfur binding sites: chemical and spectroelectrochemical analysis of iron-sulfur interconversions. *Biochemistry* 2001;40:843–851.
37. Pierrel F, Hernandez HL, Johnson MK, Fontecave M, Atta M. MiaB protein from *Thermotoga maritima*. Characterization of an extremely thermophilic tRNA-methylthiotransferase. *J. Biol Chem* 2003;278:29515–29524. [PubMed: 12766153]
38. Cospier NJ, Booker SJ, Ruzicka F, Frey PA, Scott RA. Direct FeS cluster involvement in generation of a radical in lysine 2,3-aminomutase. *Biochemistry* 2000;39:15668–15673. [PubMed: 11123891]
39. Layer G, Moser J, Heinz DW, Jahn D, Schubert WD. Crystal structure of coproporphyrinogen III oxidase reveals cofactor geometry of Radical SAM enzymes. *EMBO J* 2003;22:6214–6224. [PubMed: 14633981]
40. Berkovitch F, Nicolet Y, Wan JT, Jarrett JT, Drennan CL. Crystal structure of biotin synthase, an S-adenosylmethionine-dependent radical enzyme. *Science* 2004;303:767–769.

41. Hänzelmann P, Schindelin H. Crystal structure of the *S*-adenosylmethionine dependent enzyme MoaA and its implications for molybdenum cofactor deficiency in humans. *Proc. Natl. Acad. Sci. U.S.A* 2004;101:12870–12875. [PubMed: 15317939]

Fig. 1. The currently accepted mechanism of action of LAM

Kinetic and EPR spectroscopic evidence for the radical complexes **1** and **3** and the radical Ado• as intermediates has been published (3,5,19). The present work relates to the energetics for the reductive cleavage of SAM ligated to the FeS-cluster.

Fig. 2. Equilibrium in the reductive cleavage of *an*SAM at the active site of LAM

*an*SAM in place of SAM at the active site of LAM is reductively cleaved to the radical *an*Ado • upon reaction with lysine (19). The figure shows the increase with time of the EPR signal for *an*Ado • after addition of lysine to 34 μM LAM. The reaction proceeds to equilibrium at 2 μM *an*Ado •. Although lysine is a poor substrate and reacts forward very slowly, the cleavage of *an*SAM to the radical *an*Ado • approaches equilibrium. The detailed composition of the reaction mixture is given in the Experimental section.

Fig. 3. Energetics of one-electron reversible cleavage of SAM at the active site of LAM

The blue scale on the left illustrates the mid-point reduction potentials of the $[4\text{Fe-4S}]^{2+/1+}$ cluster with cysteine as the ligand, with SAM as the ligand, and with lysine bound and SAM as the ligand. The red scale shows the potentials for irreversible one-electron reduction of trialkylsulfonium ions, such as SAM, in solution and for reversible one-electron reductive cleavage of SAM bound to the $[4\text{Fe-4S}]$ cluster in the active site of LAM.

Scheme 1.

Table 1Reduction potentials for $[4\text{Fe-4S}]^{2+/1+}$ in selected complexes of LAM.

Complex	E^* (mV) ^a	Reference
E-[4Fe-4S]-SAM.PLP	-430 ± 2	17
E-[4Fe-4S]-SAH.PLP	-460 ± 3	"
E-[4Fe-4S]- <i>aza</i> SAM.PLP	-497 ± 10	"
E-[4Fe-4S]- <i>an</i> SAM.PLP	-484 ± 5	This work
E-[4Fe-4S]-SAM.PLP=Ala	-582 ± 6	This work
E-[4Fe-4S]-SAH.PLP=Ala	-566 ± 6	"
E-[4Fe-4S]-SAH.PLP=Ala/EtNH ₃ ⁺	-594 ± 17	"
E-[4Fe-4S]-SAH.PLP=Lys	-583 ± 21	"

^a All mid-point potentials are measured at pH 8.0 and are pH-independent.