Photolysis of Adenosylcobalamin and Radical Pair Recombination in Ethanolamine Ammonia-Lyase Probed on the Micro- to Millisecond Time Scale by Using Time-Resolved Optical Absorption Spectroscopy[†]

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ABSTRACT: The quantum yield and kinetics of decay of cob(II)alamin formed by pulsed-laser photolysis of adenosylcobalamin (AdoCbl; coenzyme B_{12}) in AdoCbl-dependent ethanolamine ammonia-lyase (EAL) from *Salmonella typhimurium* have been studied on the $10^{-7}-10^{-1}$ s time scale at 295 K by using transient ultraviolet−visible absorption spectroscopy. The aim is to probe the mechanism of formation and stabilization of the cob(II)alamin−5′-deoxyadenosyl radical pair, which is a key intermediate in EAL catalysis, and the influence of substrate binding on this process. Substrate binding is required for cobalt−carbon bond cleavage in the native system. Photolysis of AdoCbl in EAL leads to a quantum yield at 10^{-7} s for cob(II)alamin of 0.08 ± 0.01, which is 3-fold smaller than for AdoCbl in aqueous solution (0.23 ± 0.01). The protein binding site therefore suppresses photoproduct radical pair formation. Three photoproduct states, P_f, P_s, and P_c, are identified in holo-EAL by the different cob(II)alamin decay kinetics (subscripts denote fast, slow, and constant, respectively). These states have the following first-order decay rate constants and quantum yields: 2.2×10^3 s^{-1} and 0.02 for P_f, 4.2×10^2 s^{-1} and 0.01 for P_s, and constant amplitude, with no recombination, and 0.05 for P_c, respectively. Binding of the substrate analogue (*S*)-1-amino-2-propanol to EAL eliminates the P_f state and lowers the quantum yield of P_c (0.03) relative to that of P_s (0.01) but does not significantly change the quantum yield or decay rate constant of P_s, relative to those of holo-EAL. The substrate analogue thus influences the quantum yield at 10^{-7} s by changing the cage escape rate from the geminate cob(II)alamin−5′-deoxyadenosyl radical pair state. However, the predicted substrate analogue binding-induced increase in the quantum yield is not observed. It is proposed that the substrate analogue does not induce the radical pair stabilizing changes in the protein that are characteristic of true substrates.

Coenzyme B_{12}-dependent enzymes catalyze radical-mediated rearrangement reactions in both bacteria and mammals (*1*−*3*). The first step in the catalytic cycle is the homolytic cleavage of the cobalt−carbon (Co−C) bond in coenzyme B_{12} [adenosylcobalamin (AdoCbl)[1] (Figure 1)], which results in the formation of the cob(II)alamin−5′-deoxyadenosyl radical pair. The C5′ radical center of the 5′-deoxyadenosyl moiety then migrates over 5−6 Å (*4, 5*) to abstract a hydrogen atom from the substrate, which activates the substrate for rearrangement. Cleavage of the Co−C bond is accelerated by $>10^{11}$-fold in the enzymes, relative to the cleavage in solution (*6*−*8*). A long-standing issue in AdoCbl-dependent enzyme catalysis is the molecular mechanism of rate acceleration (*9, 10*), and how substrate binding, which is required for cleavage, is coupled to the reaction (*1*−*3*). To address the mechanism of formation and stabilization of the cob(II)alamin−5′-deoxyadenosyl radical pair in the protein, we use pulsed-laser photolysis of the Co−C bond to prepare the radical pair population, followed by a UV−visible absorption probe of its time evolution. This method overcomes the kinetic complexity and asynchrony of steady-state kinetic studies, and the evolution of the radical pair can be observed on time scales that are several orders of magnitude shorter than those in previous stopped-flow studies of cob(II)alamin formation in the AdoCbl-dependent enzymes, methylmalonyl-CoA mutase (*11*), glutamate mutase (*12*), ribonucleotide triphosphate reductase (*13*), and ethanolamine ammonia-lyase (*14*). The photolysis measurements are performed by using ethanolamine ammonia-lyase (EAL) [EC 4.3.1.7; cobalamin (vitamin B_{12})-dependent enzyme superfamily (*15, 16*)] from *Salmonella typhimurium* (*1, 17*). EAL catalyzes the conversion of aminoethanol and 2-aminopropanol to the corresponding aldehydes and ammonia in bacteria (*18*).

Figure 2 shows a simplified kinetic scheme of the canonical states and steps involved in the photolysis experiment. Following photoexcitation, a fraction of the photoproducts relaxes to the ground state in $<10^{-9}$ s (*19, 20*), forming the geminate cob(II)alamin−5′-deoxyadenosyl radical pair. The excited-state formation and sequence of early

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[1] Abbreviations: AdoCbl, adenosylcobalamin; EAL, ethanolamine ammonia-lyase; EPR, electron paramagnetic resonance; GluM, glutamate mutase.

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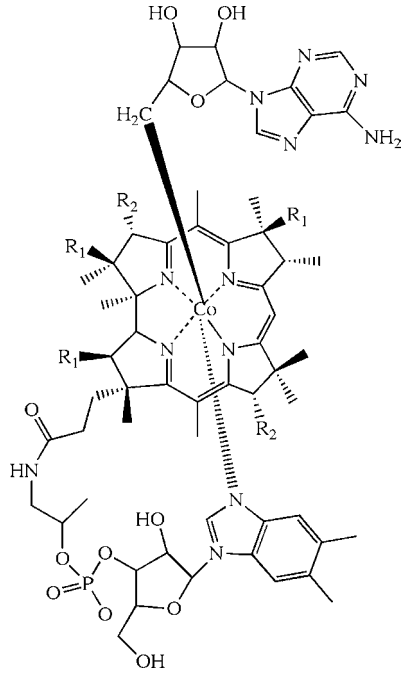

FIGURE 1: Depiction of the structure of AdoCbl. The β-axial ligand is 5′-deoxyadenosyl. R_1 and R_2 represent acetamide and propionamide side chains, respectively. The dimethylbenzimidazole α-axial ligand of the coenzyme remains coordinated when the coenzyme is bound to EAL (*53*, *54*).

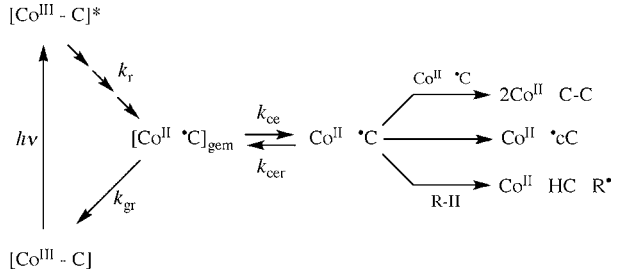

FIGURE 2: Simplified schematic diagram of the states and pathways of formation following photolysis of AdoCbl in solution. The cobalamin and 5′-deoxyadenosyl moiety are represented by cobalt (Co) and C5′-methylene center (C), as follows: [Co^{III}−C], intact coenzyme; [Co^{III}−C]*, excited singlet state; [Co^{II} •C]$_{gem}$, geminate radical pair; Co^{II} •C, cage escape radical pair. The cage escape radical pair can recombine to re-form the geminate radical pair or can undergo radical−radical annihilation (top), undergo internal rearrangement to form cycloadenosyl or other species (middle), or react with solvent (R-H) by hydrogen atom abstraction (bottom), which leads to irreversible cob(II)alamin formation (*19*, *50*). Intermediate excited and relaxed states (*20*, *26*), which are not shown, are represented by the sequence of arrows leading from [Co^{III}−C]*. Rate constants are defined as follows: k_r, excited to ground-state relaxation; k_{gr}, geminate recombination; k_{ce}, cage escape; k_{cer}, re-formation of the geminate radical pair from the cage escape radical pair.

photoproduct intermediates for alkylcobalamins in solution, which are not shown explicitly in Figure 2, have been described by Sension and co-workers (*20*, *21*). The geminate radical pair exists within a "cage" of surrounding solvent molecules and can recombine promptly (geminate recombination) or diffuse apart (cage escape) and recombine on a slower time scale (*22*). The radical pair formation and decay are detected optically with relatively high sensitivity by monitoring the UV−visible absorption changes associated with the interconversion of the cobalamin between the Co^{III} state (visible wavelength maximum, $\lambda_{max} = 525$ nm in water) and Co^{II} state ($\lambda_{max} = 470$ nm in water). In studies of AdoCbl, MeCbl, and other alkylCbl in solution, time scales from electronic excited-state formation and decay (femtoseconds) to solvent separation of the cob(II)alamin radical pairs (milliseconds) have been monitored (*19*, *20*, *23*−*33*). Following AdoCbl photolysis in water, the geminate cob(II)alamin−5′-deoxyadenosyl radical pair recombination and cage escape occur with rate constants of 1.4×10^9 and 0.6×10^9 s^{-1}, respectively, leading to a fraction (0.29) of radical pairs that escape the solvent cage (*20*, *31*).

Quantitative time-resolved measurements of AdoCbl photolysis and recombination in proteins have thus far been performed only for AdoCbl-dependent glutamate mutase (GluM) on the ultrafast time scale (<9 ns) (*34*, *35*). The results showed that binding of AdoCbl to GluM led to a reduction in the quantum yield of cob(II)alamin (at 9 ns) from 0.23 in solution to 0.05 in the protein. This was caused primarily by a decrease in the cage escape rate constant to $5-6 \times 10^7$ s^{-1} (*34*, *35*), relative to the value of 5.7×10^8 s^{-1} reported for pure water. The protein reduced the geminate recombination rate by only 30% (*23*). These results show that AdoCbl can be photolyzed in situ and that the protein influences (reduces) the quantum yield. However, these studies were restricted to ultrafast time scales that do not address recombination of the cage escape radical pair. If the cage escape 5′-deoxyadenosyl radical diffuses along the native radical pair separation coordinate (*36*−*38*) in the active site, then the time scale of recombination may be governed by the activation free energy barriers that control the native radical pair separation process on the micro- to millisecond time scales. The measurements in GluM were also carried out with holoenzyme in the absence of substrate. Therefore, the influence of substrate binding on the photoproduct yields and decay reactions was not addressed.

Here, we report transient optical absorption measurements of the quantum yield and radical pair recombination kinetics following phototolysis of AdoCbl on time scales from 10^{-7} to 10^{-1} s in solution and in EAL. The influence of substrate on the quantum yield and radical pair recombination kinetics is assessed by using (*S*)-1-amino-2-propanol, an inactive substrate analogue, which binds to the substrate binding site in EAL but does not form the cob(II)alamin−substrate radical pair state. This analogue has a methyl group at the pro-*S* position of stereospecific hydrogen atom abstraction from the carbinol carbon of the native substrate (*39*, *40*), which blocks the hydrogen atom transfer reaction. A branched kinetic mechanism is proposed to account for the results, in which the observed transients represent metastable cob(II)alamin−5′-deoxyadenosyl radical pair states. We conclude that the substrate analogue partially fulfills the native function by suppressing nonproductive reactions of the radical pair but is not capable of inducing the full "substrate trigger" of Co−C bond cleavage that is characteristic of the native substrates.

EXPERIMENTAL PROCEDURES

Enzyme Preparation. EAL was purified from the *Escherichia coli* overexpression strain incorporating the cloned *S. typhimurium* EAL coding sequence (*41*) essentially as described previously (*42*), the exception being that the enzyme was dialyzed against buffer containing 100 mM

HEPES (pH 7.5), 10 mM potassium chloride, 5 mM dithiothreitol, 10 mM urea, and 10% glycerol (*43*). Enzyme activity was determined as described previously (*44*) by using the coupled assay with alcohol dehydrogenase/NADH. The specific activity of the purified enzyme with aminoethanol as the substrate was 35−45 μmol min^{-1} mg^{-1}.

Sample Preparation. Adenosylcobalamin and (*S*)-1-amino-2-propanol were purchased from Sigma-Aldrich Chemical Co. Samples of enzyme (20−100 μM active sites) with cofactor (10−50 μM) bound were prepared in 10 mM potassium phosphate (pH 7.5) and sonicated at 277 K to reduce light scattering. Samples with substrate analogue bound were prepared as described above with 10 mM substrate analogue (*S*)-1-amino-2-propanol (dissociation constant for interaction with holo-EAL, 39 μM) present. Anaerobic solutions of AdoCbl were prepared by placing small volumes of the cofactor stock solution under vacuum, followed by backfilling with argon before addition to anaerobic buffer. Argon bubbling for 1 h was utilized to degas the buffer solution. Components were injected into a 3 mL quartz anaerobic cuvette with a sealed septum that had been flushed with argon for at least 5 min prior to sample mixing.

Static Absorption Spectra. Static absorption spectra from 300 to 650 nm were collected by using a Shimadzu UV-1601 absorption spectrometer with a wavelength accuracy of 0.5 nm. Spectra were collected before and after transient absorption measurements and again after laser saturation of the sample. A scattering baseline of each enzyme sample was taken before the addition of cofactor. All measurements were performed at 295 ± 1 K.

Transient Absorption Spectroscopy. Transient absorption of the cob(II)alamin state was monitored at 470 nm (probe) following laser pulse photolysis at 532 nm. Measurements were taken with dwell times of 0.1−10 μs and corresponding time constants of 0.01−1 μs. All measurements were performed at 295 ± 1 K.

Transient Absorption Spectrophotometer. Transient absorption measurements were performed by using a transient spectrophotometer designed and constructed in house. The transient absorption spectrometer has a sensitivity to change in absorbance (A) of 2×10^{-3} over 300−650 nm, and a dead time of ≤20 ns. The second harmonic output (532 nm) of a Nd:YAG laser (SpectraPhysics GCR-10; 10 ns pulse width), with the pulse energy adjusted by a glan prism polarizer/half-wave plate, is used as the actinic source. The pump pulse is reduced and aligned along the same axis through the sample as the probe beam. A computer-controlled shutter controls admittance of the pump pulses into the spectrometer. After passage through the sample, the pump pulse is collected in a beam dump. The probe beam of the spectrometer is provided by a low-noise 300 W xenon arc lamp (Hamamatsu, L2480). The broadband output of the lamp is reduced before entering a monochromator (Applied Photophysics, F-34) to create a probe beam with a bandwidth of approximately 25 nm. The probe beam is directed through a computer-controlled shutter before entering the spectrometer housing. The probe beam shutter minimizes the time that the probe beam interacts with the sample. The probe beam is focused through a brass sample holder and into a small adjustable aperture before being passed through a second monochromator (Jobin Yvon, H-20). The second monochromator provides a resolution of 1 nm for the detection of the probe beam and contributes to the elimination of any stray photons from the intense laser pulse. The probe beam is directed through two laser line reject filters (Omega Optical, 532 nm) and onto the active area of the photomultiplier tube (Hamamatsu model R928; range, 300−650 nm). The photomultiplier tube output is digitized and temporarily stored in a digital sampling oscilloscope (Tektronix, TDS 520B). The spectrometer sample holder is associated with a liquid flow system for temperature control, a computer-controlled magnetic stirrer, and sample and sample space gas flow lines. The spectrometer's pump and probe pulse timing sequences and data collection are controlled by a Macintosh computer with LabVIEW (National Instruments) via a GPIB/IEEE-488.2 interface. Data fitting and analysis were performed by using Matlab (Natick, MA) routines that were run on personal computers.

Quantum Yield Calculation. Quantum yield values were obtained by using laser pulse energies of 1.0, 1.5, and 2.0 mJ. Quantum yield measurements as a function of pulse energies up to 10 mJ were performed to determine the pulse energy threshold for multiple-photon absorption. The 470 nm probe beam was used to detect the formation of cob(II)alamin. Quantum yield measurements were made with a dwell time of 0.2 μs and a time constant of 0.02 μs. The quantum yield (ϕ) is defined as the concentration of cob(II)alamin photoproduct formed by the laser pulse, divided by the absorbed photon ($h\upsilon$) concentration, $[h\upsilon]_{abs}$. The concentration of photoproduct is found by using the previously measured difference extinction coefficients for adenosylcob(III)alamin to cob(II)alamin conversion, $\Delta\varepsilon$ (M^{-1} cm^{-1}), for AdoCbl (*45*). The concentration of photoproduct is calculated by using the following expression

$$[\text{photoproduct}] = \log\left(\frac{\upsilon_{\text{initial}}}{\upsilon_{\text{final}}}\right) \times \Delta\varepsilon^{-1} \quad (1)$$

where $\upsilon_{initial}$ and υ_{final} are the initial (prelaser pulse) and final (postphotolysis) detector voltages, respectively, and the sample path length is 1 cm. The ratio $\upsilon_{initial}/\upsilon_{final}$ is directly proportional to the ratio of the initial and final transmittance (T) values. The concentration of incident photons, $[h\upsilon]_{incident}$, is obtained from the following expression

$$[h\upsilon]_{\text{incident}} = \frac{E_{\text{pulse}}}{E_{\text{photon}} V_{\text{beam}} N_{\text{A}}} \quad (2)$$

where E_{photon} is the photon energy at 532 nm, V_{beam} is the pump excitation volume (1.3×10^{-4} L), N_A is Avogadro's number, and E_{pulse} is the laser pulse energy. The value of $[h\upsilon]_{abs}$ is obtained from $[h\upsilon]_{incident}$ after scaling by the probability of photon absorption at 532 nm, which is given by $1 - T$.

Temperature Dependence of the First-Order Rate Constant. The temperature dependence of the first-order rate constant, k, is given by the Arrhenius expression (*46*):

$$k(T) = A\mathrm{e}^{-E_a/RT} \quad (3)$$

where E_a is the activation energy, R is the gas constant, and A is a prefactor that represents the value of k as $E_a \rightarrow 0$. The value of A is typically approximated as k_BT/h, where k_B is Boltzmann's constant and h is Planck's constant.

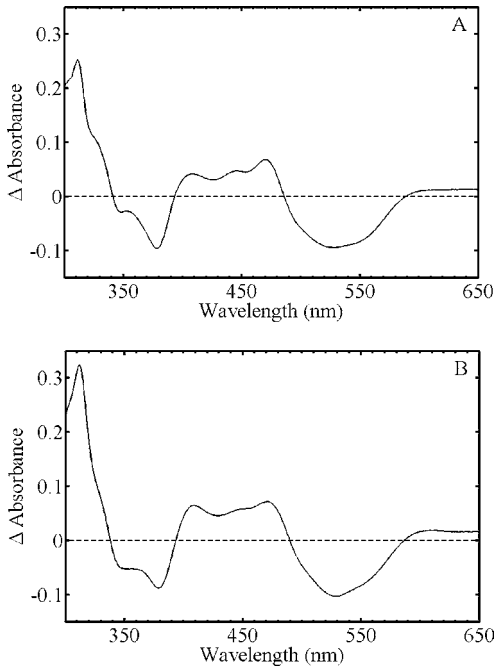

FIGURE 3: Absorption difference spectra of photolyzed minus prephotolysis AdoCbl. (A) AdoCbl in EAL in an aerobic solution. (B) AdoCbl in an anaerobic solution.

RESULTS

Pre- and Postphotolysis Absorption of AdoCbl in Solution and in EAL. The photoproduct difference spectra (postphotolysis minus prephotolysis) for AdoCbl in solution and in EAL are presented in Figure 3. The difference spectra in panels A and B of Figure 3 are characterized by the loss of the AdoCbl $\alpha\beta$-band maximum around 525 nm (negative feature) and the rise of the long-wavelength maximum of cob(II)alamin around 470 nm (positive feature). The comparable spectral features indicate that the difference spectrum for AdoCbl photolysis in EAL in an aerobic solution in Figure 3A represents AdoCbl minus cob(II)alamin, because it is the same as the reference [AdoCbl minus cob(II)alamin] difference spectrum obtained in an anaerobic solution (Figure 3B). Small differences in the line shape and peak positions are caused by the different cofactor environments in aqueous solution and in EAL, as found for other AdoCbl-dependent enzymes (*9, 10, 47*).

Hogenkamp and co-workers have previously shown that aquocob(III)alamin arises from the reaction of cob(II)alamin with dioxygen (O_2) (*48*). The results therefore show that the binding of the native AdoCbl cofactor in EAL leads to a state in which O_2 is prevented from interacting with the photoproduct cob(II)alamin.

Quantum Yield of Cob(II)alamin Formation following Photolysis of AdoCbl in Solution and in EAL. The quantum yield of photolysis of AdoCbl was determined on the 10^{-7} s time scale under different conditions by using low-pulse energy ($\leq$2 mJ) excitation from the 532 nm output of the pulsed Nd:YAG laser, and a continuous-wave probe at 470 nm. The low energies were selected to prevent multiple-photon absorption by AdoCbl, as described in Experimental Procedures. The ratio of the concentration of AdoCbl to EAL active sites was $\leq$0.5, to prevent interference from the free cofactor. A dissociation constant (K_D) for AdoCbl binding to EAL of 0.5 μM is estimated from the Michaelis constant

Table 1: Quantum Yield of Cob(II)alamin at 10^{-7} s following Photolysis of AdoCbl in an Anaerobic Solution and in Aerobic Solutions of EAL in the Absence and Presence of Substrate Analogue

cobalamin	substrate analogue	environment	quantum yield[a]
adenosylcobalamin	−	solution	0.23 ± 0.01
	−	EAL	0.08 ± 0.01
	(*S*)-1-Am-2-PrOH	EAL	0.04 ± 0.01

[a] Average of at least three determinations ± the standard deviation.

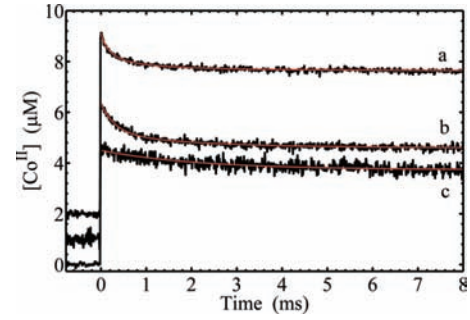

FIGURE 4: Time dependence of cob(II)alamin concentration following pulsed-laser photolysis of AdoCbl, and overlaid best-fit functions (red). The cob(II)alamin concentration was obtained from the absorbance at 470 nm. (a) Anaerobic solution; second-order plus constant fit function. (b) In EAL in an aerobic solution; biexponential plus constant fit function. (c) In EAL in an aerobic solution, with (*S*)-1-amino-2-propanol bound; monoexponential plus constant fit function. The decay has been multiplied by a factor of 2.0 to account for the 2-fold higher concentration AdoCbl in trace b relative to trace c. The fitting parameters are listed in Table 2.

(*42*), which indicates that, under the conditions of the experiments, the free cofactor represents 4% of the total cofactor in the sample. The quantum yield values are presented in Table 1. The measured quantum yield at 10^{-7} s for AdoCbl photolysis in 10 mM potassium phosphate buffer at pH 7.5 of 0.23 ± 0.01 is comparable to previously reported aqueous solution, room-temperature values of 0.23 ± 0.04 (excitation wavelength, λ_{ex} = 532 and 355 nm) (*31*) and 0.24 ± 0.04 (λ_{ex} = 400 and 520 nm) (*20*).

Binding of AdoCbl by EAL leads to a 3-fold reduction in the quantum yield at 10^{-7} s relative to that in solution, to a value of 0.08 ± 0.01. Reduction in the yield of cob(II)alamin at 9 ns has been previously reported for photolysis of AdoCbl bound in glutamate mutase, relative to that in solution (*34*). The yield at 9 ns in glutamate mutase was 0.05 ± 0.03 (*34*). Table 1 shows that, in the presence of bound (*S*)-1-amino-2-propanol, the quantum yield of cob(II)alamin at 10^{-7} s is reduced by 50%, relative to the quantum yield in the holoenzyme.

Time Dependence of Photoproduct Cob(II)alamin following Photolysis of AdoCbl in Solution and in EAL. Figure 4 shows the transient kinetics of cob(II)alamin on the millisecond time scale, following photolysis of AdoCbl in the buffered aqueous solution and in EAL in the absence and presence of bound (*S*)-1-amino-2-propanol. For these experiments, a relatively high laser pulse energy of 10 mJ/pulse was used to enhance the population of cob(II)alamin, by photolyzing AdoCbl that had undergone geminate recombination on the time scale of the 10 ns laser pulse width. Measurements of the decay on shorter time scales of at least 0.1 μs do not show additional kinetic transients. The kinetics in Figure 4 therefore represent the events that occur on the time scale of $\geq$0.1 μs for each condition. This is consistent

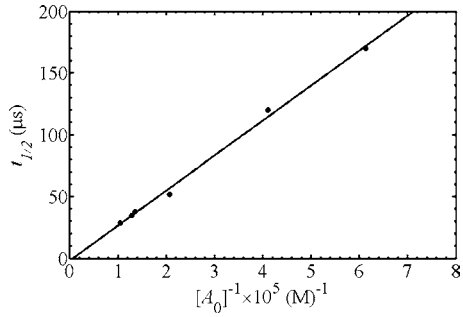

FIGURE 5: Half-time of cob(II)alamin decay as a function of inverse concentration of cob(II)alamin photoproduct following AdoCbl photolysis in an anaerobic solution. The best-fit line corresponds to a second-order rate constant of 3.53×10^9 M^{-1} s^{-1}. Fitting parameters: slope $= 2.83 \times 10^{-10}$, ordinate intercept $= -1.29 \times 10^{-6}$, $R^2 = 0.9963$.

Table 2: Relative Amplitudes and Observed Recombination Rate Constants for Cage Escape Populations in Holo-EAL and in Holo-EAL with Bound (*S*)-1-Amino-2-propanol

condition	population	relative amplitude	k_{decay} (s^{-1})
EAL·AdoCbl	P_f	0.19 ± 0.05	$(2.2 \pm 0.4) \times 10^3$
	P_s	0.13 ± 0.05	$(4.2 \pm 1.5) \times 10^2$
	P_c	0.68 ± 0.08	−
EAL·AdoCbl·substrate analogue	P_f	0	−
	P_s	0.27 ± 0.11	$(4.5 \pm 1.3) \times 10^2$
	P_c	0.73 ± 0.11	−

with previous studies of AdoCbl photolysis in solution (*19*, *45*). The time dependence of the cob(II)alamin photoproduct concentration in solution was fit by a second-order decay plus a constant function. The second-order character of the transient decay was confirmed by the linear dependence of the decay half-time on AdoCbl concentration (*46*), as shown in Figure 5. The linear fit in Figure 5 leads to a second-order rate constant of 3.5×10^9 M^{-1} s^{-1}, which is consistent with the reported diffusion-limited rate of cob(II)alamin recombination with the 5′-deoxyadenosyl radical (*19*). The constant component of the fit of the solution decay in Figure 4 represents cob(II)alamin "stranded" by the rapid radical−radical recombination reaction of 5′-deoxyadenosyl radicals. The radical−radical annihilation reaction proceeds with second-order rate constants of approximately 3×10^9 M^{-1} s^{-1} (*19*) and, therefore, competes favorably with recombination of cob(II)alamin with 5′-deoxyadenosyl radicals. The second-order and constant components of the time-dependent cob(II)alamin signal in solution confirm that the observed cob(II)alamin is the cage escape photoproduct.

Figure 4B shows that the decay of the long-lived cob(II)alamin photoproduct following photolysis of AdoCbl bound to EAL, which also displays transient and constant components. The transient decay was well fit by using a biexponential plus constant function. The single-exponential plus constant function did not provide a satisfactory fit to the transient decay (Figures S1 and S2 of the Supporting Information). Nineteen percent of the long-lived cob(II)alamin decays with a lifetime of 0.46 ms, and a second component (13%) decays with a lifetime of 2.4 ms. The remaining 68% of the cob(II)alamin does not recombine on the longest time scales observed by transient absorption or by subsequent recording of the static spectra of the photolyzed samples (≤300 s). The kinetics and amplitudes of the decay are not influenced by changing the concentrations of

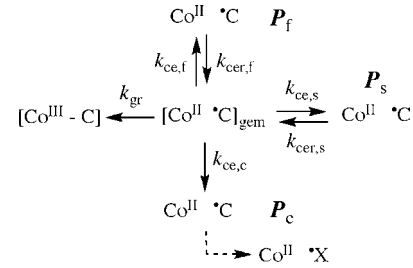

FIGURE 6: Proposed kinetic scheme for reactions of the cob(II)alamin−5′-deoxyadenosyl radical pair states following formation by photolysis in EAL.

AdoCbl or EAL. The presence of the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) in the solution with holo-EAL does not influence the decay kinetics, and no spin-trapped, paramagnetic DMPO adduct species were detected in the solution around the protein following photolysis, by using electron paramagnetic resonance (EPR) spectroscopy. These results indicate that the observed cob(II)alamin recombines with the 5′-deoxyadenosyl radical within the protein interior. Therefore, the transients correspond to first-order kinetic processes.

Figure 4C shows the time dependence of the long-lived cob(II)alamin photoproduct state in EAL in the presence of bound (*S*)-1-amino-2-propanol. The transient decay can be fit by using a single-exponential function plus a constant. A biexponential function did not lead to a significantly better fit (Figure S3 of the Supporting Information). The fit shows that the cob(II)alamin signal decays with a lifetime of 2.2 ms, which accounts for 27% of the long-lived cob(II)alamin. A proportion of 73% of the cob(II)alamin did not recombine at ≤300 s. The results show that (*S*)-1-amino-2-propanol selectively influences the cob(II)alamin photoproduct species, by eliminating the fast decay process, and by reducing the amplitude of the constant phase relative to the slow phase.

DISCUSSION

Model for the Formation and Reaction of Cage Escape Co^{II} Radical Pair Populations following AdoCbl Photolysis in EAL. Three cage escape photoproduct states are identified from the different components of the decay of cob(II)alamin that are observed in the transient absorption measurements (Figure 4 and Table 2), as follows: (a) fast transient (corresponding to photoproduct population, P_f), (b) slow transient (photoproduct population, P_s), and (c) constant amplitude (photoproduct population, P_c). Figure 6 shows the kinetic model that is proposed to account for the formation and time dependence of the cob(II)alamin photoproduct states in holo-EAL, in which three pathways from the geminate radical pair state lead to formation of cage escape photoproducts, which remain within the protein interior. We favor this branched reaction mechanism of separate paths from the geminate radical pair to the P_f, P_s, and P_c states because it is the most simple mechanism that is consistent with the data. A mechanism in which cage escape leads to sequential formation of the P_f state and then the P_s state is not favored, because the decay rate constant assigned to P_s in holo-EAL is maintained in the absence of P_f in (*S*)-1-amino-2-propanol-bound holo-EAL. This is not expected for a sequential mechanism, $A_1 \leftrightarrows A_2 \leftrightarrows A_3$, where A_1 denotes the geminate radical pair and A_2 and A_3 are sequential cage escape states,

Table 3: Absolute Quantum Yield Values at 10^{-7} s and Estimated Rate Constants and Activation Energies for the Cage Escape Population for Different Escape Populations in Holo-EAL and in Holo-EAL with Bound (*S*)-1-Amino-2-propanol

condition	population	ϕ_i	$k_{ce,i}$ (s^{-1})[a]	$E_{a,ce,i}$ (kcal/mol)[a]
EAL·AdoCbl	P_f	0.015 ± 0.004	2×10^7	8
	P_s	0.010 ± 0.004	2×10^7	8
	P_c	0.054 ± 0.009	6×10^7	7
EAL·AdoCbl·substrate analogue	P_f	0	−	
	P_s	0.011 ± 0.005	1×10^7	8
	P_c	0.029 ± 0.009	3×10^7	7

[a] k_{ce} calculated by assuming $k_{gr} = 1 \times 10^{-9}$ s^{-1}.

because the two relaxation rate parameters, λ_1 and λ_2, for the linear two-step mechanism are functions of all four rate constants [$\lambda = \lambda(k_{ij},k_{ji})$, where $i = 1$, 2, or 3, $j = 1$, 2, or 3, $i \neq j$, and k_{ij} and k_{ji} are the forward and backward rate constants for each step, respectively] (*46*). In the general case, this mechanism leads to the prediction that the single relaxation rate constant, which is measured upon elimination of the terminal state, is different from λ_1 and λ_2.

Substrate Analogue Binding Influences the Cage Escape Process. Table 3 shows the quantum yields for states P_f, P_s, and P_c in holo-EAL. These values were obtained by scaling the relative amplitudes in Table 2 by using the quantum yield value of 0.08 ± 0.01 (Table 1). Table 3 also shows the quantum yields in holo-EAL in the presence of bound (*S*)-1-amino-2-propanol. The absence of the P_f state and the decrease in the amplitude of the P_c state relative to P_s (see Table 2) in the presence of (*S*)-1-amino-2-propanol account for the substrate analogue-induced decrease in the quantum yield of the cage escape population. Therefore, the decrease in quantum yield of the total cage escape population is caused by an effect of (*S*)-1-amino-2-propanol on the cage escape rate constant, rather than by an effect on the subnanosecond quantum yield of the geminate radical pair state, or by a change in the rate constant for geminate recombination. The latter two effects would cause a uniform decrease in the amplitudes of all three P states, with preservation of the relative amplitudes.

We speculate that P_f and P_c may represent states on nonproductive pathways of the cob(II)alamin−5′-deoxyadenosyl radical pair reaction. If so, this would indicate that one role of protein changes induced by substrate binding is to suppress nonproductive pathways of radical pair reaction (*49*). The P_c state may represent an intermediate in which the 5′-deoxyadenosyl radical has undergone internal radical rearrangement or has reacted with the protein by hydrogen atom abstraction or radical substitution. These are reactions with chemical precedent in solution homolyses (*50*). P_s, whose yield is not suppressed by substrate analogue binding, may thus resemble a productive radical pair state.

Estimation of Cage Escape Rate Constants and Activation Free Energy Barriers. The rate constants for cage escape in EAL can be estimated from the measured quantum yield values, if a value for the geminate recombination rate constant (k_{gr}) is assumed. The work of Sension and coworkers has shown that the geminate recombination rate constants for the cob(II)alamin−5′-deoxyadenosyl radical pair are comparable in a variety of solvents, as shown by the following values: 1.43×10^9 s^{-1} for pure water (*23*), 1.34×10^9 s^{-1} for ethylene glycol (*23*), and 1.08×10^9 s^{-1} for the protein in glutamate mutase (*34*). From these measurements, it was concluded that the rate constant for geminate recombination is "at most weakly dependent on solvent" (*28*), even for different alkyl axial ligands (*28*). If it is assumed that the geminate recombination rate constant in EAL is 1×10^9 s^{-1} (Arrhenius activation energy barrier, $E_{a,gr}$, of 5 kcal/mol), then the quantum yield values for P_f, P_s, and P_c (ϕ_f, ϕ_s, and ϕ_c, respectively) can be used in the following system of equations to solve for the corresponding cage escape rate constants ($k_{ce,f}$, $k_{ce,s}$, and $k_{ce,c}$, respectively), as follows:

$$\varphi_i = \frac{k_{ce,i}}{k_{gr} + k_{ce,f} + k_{ce,s} + k_{ce,c}} \quad (4)$$

In eq 4, the index, i, corresponds to f, s, or c. The values of the three $k_{ce,i}$ values and the corresponding Arrhenius activation energies, $E_{a,i}$, are listed in Table 3.

The decays of P_f and P_s through the geminate radical pair each proceed by a two-step mechanism, in which k_{ce} and k_{cer} represent an equilibration step prior to the essentially irreversible geminate recombination (k_{gr}). The estimated k_{ce} values are 16−90-fold slower than k_{gr}, and therefore, $k_{gr} \gg k_{ce}$. Under these conditions, a steady-state approximation is appropriate for the geminate radical pair during the decay, and the observed monoexponential decay rate constant is equal to k_{cer} within approximately 1% (*46*). The k_{cer} values in Table 2 correspond to E_a values of 13−14 kcal/mol. Therefore, if a cob(II)alamin−5′-deoxyadenosyl radical pair successfully escapes from the cage, then it is stabilized, by kinetic and/or thermodynamic factors, against recombination, for times that are commensurate with the lifetimes of hydrogen atom transfer reactions between carbon atoms ($E_a \approx 13-14$ kcal/mol) (*51*). Transfer of a hydrogen atom from the substrate C−H group to the C5′ radical center follows radical pair separation in EAL.

Implications for the Substrate Trigger Mechanism of Cob(II)alamin−5′-Deoxyadenosyl Radical Pair Formation in EAL. The competition between geminate recombination and cage escape controls the microsecond quantum yield. The two reactions occur on a time scale of $10^{-9}-10^{-7}$ s, and on the scale of relatively short cobalt−carbon distances [r(Co−C)]. These distances lie between the equilibrium Co−C bond length of 2.0 Å and approximately 4 Å, which corresponds to the diameter of one methylene carbon atom. Influences of substrate analogue binding on cage escape are therefore characterized by changes in protein−coenzyme interactions that act at relatively short r(Co−C) values. (*S*)-1-Amino-2-propanol binding affects cage escape at short r(Co−C) values, as demonstrated by the elimination of P_f and the decrease in the amount of P_c relative to P_s. However, substrate analogue binding to EAL fails to increase the quantum yield, as predicted, based on the requirement of substrate binding for Co−C bond cleavage and radical pair separation (the "substrate trigger model"). We do not believe that this is because (*S*)-1-amino-2-propanol does not support the hydrogen atom transfer, because the time and r(Co−C) distance scales of the hydrogen atom transfer are $\sim 10^{-3}$ s (*14*, *52*) and ~7 Å (*4*, *5*), respectively. Rather, the substrate analogue may not fully induce the changes in protein−cofactor interactions that alter the cob(II)alamin−5′-deoxyadenosyl radical pair energetics (increased stabilization free energy

or decreased cage escape activation free energy barrier) that are characteristic of the true substrates, because of steric disruption of substrate−protein interactions by the 1-methyl group. According to the mechanism in Figure 6, and the assumed value for k_{gr} of 1×10^9 s^{-1} (*28*), a decrease in the value of $E_{a,s}$ by 3 kcal/mol would increase the quantum yield of P_s to 0.5, from the observed value of 0.01. Thus, it is reasonable that a specific interaction of the protein with the true substrate would be reflected in an enhanced quantum yield. We will test this proposal by performing the photolysis measurements on the EAL·AdoCbl·substrate ternary complex, which is stabilized against turnover, in a recently developed cryosolvent system (*52*).

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SUPPORTING INFORMATION AVAILABLE

Monoexponential plus constant fit to the postphotolysis cob(II)alamin decay for AdoCbl in EAL, residuals for the biexponential and monoexponential fits to the postphotolysis cob(II)alamin decay for AdoCbl in EAL, and residuals for the biexponential and monoexponential fits to the postphotolysis cob(II)alamin decay for AdoCbl in EAL with bound (*S*)-1-amino-2-propanol. This material is available free of charge via the Internet at http://pubs.acs.org.

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