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Photolysis and Recombination of Adenosylcobalamin Bound to Glutamate Mutase

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Adenosylcobalamin-dependent (coenzyme B_{12}, AdoCbl) enzymes catalyze a variety of chemically difficult reactions that proceed by mechanisms involving organic radicals.[1−3] Radicals are initially generated by fission of the coenzyme cobalt−carbon bond to generate adenosyl radical and cob(II)alamin. In free solution at room temperature, the bond dissociation energy of the Co−C bond is 32 kcal/mol and the half-time for homolysis is estimated to be about 6 months.[4] In contrast, when bound to an enzyme, homolysis occurs on the millisecond time scale in response to substrate binding, and the internal equilibrium constant for homolysis is close to one.[5−8] The unusual nature of the homolysis reaction has prompted numerous investigations on model compounds, coenzyme analogues, and the enzymes themselves, aimed at understanding how the enzymes achieve the $\sim 10^{12}$-fold acceleration in the rate of AdoCbl homolysis.[7,9−19] However, this aspect of catalysis remains poorly understood.

The relatively weak AdoCbl cobalt−carbon bond readily undergoes photolysis when exposed to light to generate Cbl(II) and adenosyl radical.[20−27] These are the same products that are formed in enzyme-catalyzed homolysis. We have recently measured the kinetics of AdoCbl photolysis and recombination in free solution using ultrafast spectroscopic techniques.[27] Although photolysis is not the physiological mechanism by which radicals are generated at the enzyme active site, it provides a sensitive probe with which to investigate how the protein environment may influence the reactivity of the cobalt−carbon bond.

Here we describe ultrafast spectroscopic experiments to measure the kinetics of homolysis and recombination for AdoCbl bound in the active site of glutamate mutase,[28,29] the first such measurement on an AdoCbl-dependent enzyme. We were able to detect a very short-lived intermediate formed prior to Cbl(II) that was not observed upon photolysis of AdoCbl in free solution. Overall, though, the rate constants for photolysis and recombination are not greatly different from those measured in free solution, suggesting the protein does not greatly perturb the stability of the cobalt−carbon bond upon binding the coenzyme.

Transient absorption difference spectra and single wavelength transient absorption kinetics of AdoCbl bound to glutamate mutase were obtained using femtosecond Ti:Sapphire laser systems.[27,30] A summary of the spectral evolution from excitation to 1.5 ns is plotted in Figure 1. The data show an initial bleaching of the ground-state absorption spectrum of AdoCbl, a slower growth of the bleaching signal on a time scale of 125 ps, and a subsequent decay of the absorption on a 900 ps time scale.

The transient absorption data between 5 ps and 2 ns were analyzed using a singular value decomposition algorithm.[31−34] This analysis yielded two dominant spectral components. This decomposition is summarized in Figure 2. At early times the difference spectrum contains contributions from production of an early cob-

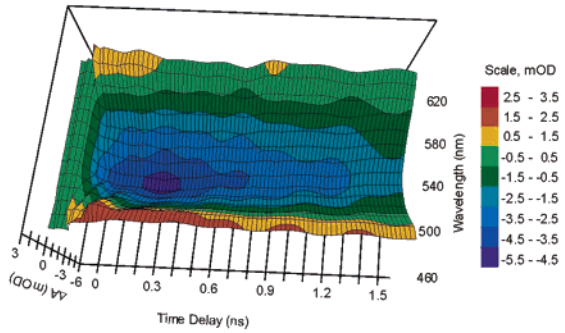

Figure 1. Evolution of the difference spectrum obtained following the photolysis of adenosylcobalamin bound to glutamate mutase at 400 nm.

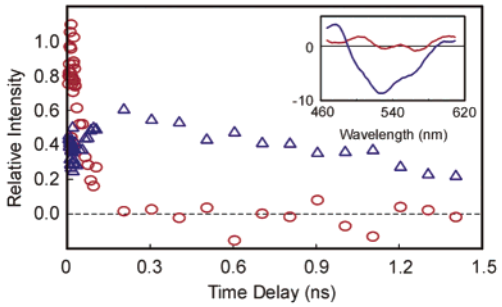

Figure 2. Singular value decomposition of the transient absorption spectra obtained between 5 ps and 2 ns. The spectra are well represented as linear combinations of two component spectra. The temporal evolution of these two components is plotted in the figure. The spectra are plotted in the inset.

(III)alamin-like intermediate and from production of the cob(II)alamin product. At later times the only contribution is from the cob(II)alamin homolysis product.

The photolysis dynamics observed for excitation of AdoCbl bound to glutamate mutase differ significantly from those found for free AdoCbl in aqueous solution or in ethylene glycol. Kinetic traces obtained for a probe wavelength of 540 nm are compared in Figure 3. The appearance of the cob(II)alamin photoproduct is delayed for bound AdoCbl. The delayed appearance of the cob(II)alamin species is seen more clearly in the data plotted on a logarithmic time scale in the inset of Figure 3. The kinetic trace can be described by a minimum of five distinct decay components. Global analysis of the data obtained for seven different probe wavelengths yields four exponential decay constants of 9.6 ± 1.0 ps^{-1} (104 fs), 0.18 ± 0.02 ps^{-1} (5.5 ps), 8.0 ± 0.3 ns^{-1} (125 ps), and 1.1 ± 0.1 ns^{-1} (900 ps), as well as a small plateau which does not decay over our 9 ns time window. Analysis of the recombination dynamics for AdoCbl bound to glutamate mutase, as described in ref 27, yields an intrinsic recombination rate of 1.08 ± 0.10 ns^{-1}. This is 16% slower than the recombination rate for free adenos-

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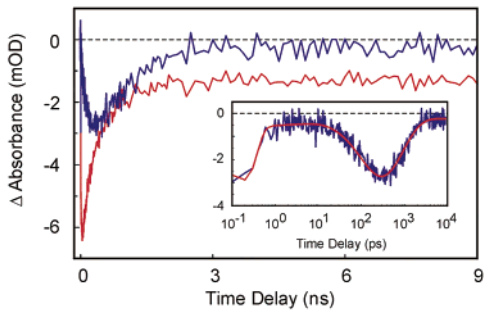

Figure 3. Typical transient absorption traces obtained following the excitation of adenosylcobalamin at 400 nm. These traces were obtained by using a 540 nm probe. The blue trace was obtained for B_{12} bound to glutamate mutase, while the red curve was obtained for B_{12} in aqueous solution. The inset shows the trace obtained for B_{12} bound to glutamate mutase and the fit to this trace. The data in the inset are plotted on a logarithmic scale to emphasize the range of kinetic components.

Table 1. Comparison of Recombination for Bound and Free B_{12}

	ϕ_{9ns}[a]	k_R (ns^{-1})[b]	k_E (ns^{-1})[b]
water[c]	0.28 ± 0.02	1.39 ± 0.06	0.57 ± 0.06
ethylene glycol[c]	0.08 ± 0.02	1.39 ± 0.06	0.11 ± 0.03
bound to protein	0.05 ± 0.03	1.08 ± 0.10	0.06 ± 0.04

[a] Quantum yield of cob(II)alamin remaining at 9 ns. [b] Rate constants for geminate recombination of the radical pair (k_R) or formation of long-lived cob(II)alamin (k_E). [c] Water and ethylene glycol data from ref 27.

ylcobalamin in water or ethylene glycol. The comparison is summarized in Table 1.

Our experiments constitute the first measurement of photolysis and recombination rates for AdoCbl bound to an enzyme, and as such have shed light on some long-standing mechanistic questions. A central problem in understanding catalysis is how the protein activates the Co−C bond toward homolysis. A priori, two mechanisms may be envisaged: one, the mechanochemical hypothesis,[14,35] postulates that an enzyme-induced distortion of the corrin ring weakens the Co−C bond; the other possibility, less extensively discussed in the literature, is that the protein differentially stabilizes the products of homolysis, making recombination less favorable.

Although our experiment does not directly measure the Co−C bond dissociation energy, we would expect photolysis and recombination rates to be sensitive to perturbations in the reactivity of this bond. We find that the rate constants for both homolysis and recombination are very little changed by the protein. This suggests that the protein neither distorts the coenzyme toward homolysis nor stabilizes the products of homolysis against recombination. These results are consistent with previous resonance Raman experiments on glutamate mutase and methylmalonyl-CoA mutase that found little perturbation of the ground-state Co−C stretching frequency upon coenzyme binding to enzyme.[36,37] Taken together, these results mediate against any significant activation of the coenzyme in the absence of substrate.

However, the protein does appear to influence the pathway for photolysis, as evidenced by the observation of a new transient intermediate species with a Cob(III)-like spectrum. It is unclear whether this represents a very short-lived, chemically distinct species or, more likely, an electronically excited state of AdoCbl stabilized by the protein. The significance of this observation for the physiological mechanism of homolysis is unknown.

Finally, it has been known for a long time that enzyme-bound AdoCbl is much less light sensitive than AdoCbl in free solution. For glutamate mutase, our experiments demonstrate that this protective effect arises primarily because the adenosyl radical is "caged" by the protein, resulting in a higher quantum yield for recombination, rather than any intrinsic increase in stability toward photolysis imparted by the protein. Most interesting is the small, but real, fraction of the coenzyme that does not recombine on the 9 ns time scale. We think that this is unlikely to be due to diffusion of adenosyl radical away from the protein, but it may be consistent with a conformational change in the adenosyl moiety that has been implicated in transfer of the radical to the substrate.[38]

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