High-energy radical species are widespread in biology as transient reaction intermediates and free radicals. The control of these extremely reactive molecules is crucial to limit oxidative stress and promote healthy physiological function.[1] In some cases this reactivity can be harnessed by a system to do useful work.[2] Over two decades ago Rétey discussed how energy can be "borrowed" from an enzyme to effect an unlikely chemical change.[3] In the case of coenzyme B_{12}-dependent enzymes,[4] this energy is "lent" in the form of radical intermediates, which are formed upon substrate binding by Co–C bond homolysis in the cofactor. The energy is then returned by radical pair recombination after turnover (Scheme 1).

The key is the way in which the protein generates the radicals and then prevents these high-energy intermediates from stabilising (i.e., dropping to a lower energy) through side reactions. Thus, the borrowed energy is channelled to the desired chemical change. Despite ample data demonstrating the role of radicals in coenzyme B_{12}-dependent enzymes, there is less direct evidence for how the protein controls them.[5] Herein, we present data that suggest that a single point mutation can have a significant impact on Co–C bond homolysis and the control of the radical trajectory and reactivity in coenzyme B_{12}-dependent ethanolamine ammonia lyase (EAL). The data show that the size and negative charge of an active-site glutamate (E287, Figure 1) contribute variously to 1) apparent substrate affinity, 2) promotion of rapid Co–C bond homolysis, 3) control of the reactivity of both the 5'-deoxyadenosyl and cob(II)alamin radicals, 4) the kinetics of the rate-limiting steps. The data also indicate that local flexibility in EAL might facilitate the approach of the charged E287 towards the polar ribose of the 5'-deoxyadenosyl, potentially offering a unified dynamic/electrostatic model of radical generation and control.
After substrate binding to EAL, and Co–C bond homolysis, the 5′-deoxyadenosyl radical abstracts hydrogen from the α carbon of 2-aminoethanol to give the substrate radical. This undergoes intramolecular rearrangement—that is, a 1,2-shift of the amine—to the product-like radical, which abstracts hydrogen back from the 5′-deoxyadenosine and then dissociates into products (see Figure S1 in the Supporting Information). Kinetic isotope effect (KIE) and magnetic field effect experiments showed that Co–C bond homolysis and initial H-abstraction are kinetically coupled, and went some way to explaining the fleeting existence of the 5′-deoxyadenosyl radical. Until crystal structures of EAL were published recently, however, we had only limited insight into how the protein might both create and control the radical intermediates. These structures identified a mobile, active-site glutamate residue (E287) that only becomes localised upon substrate binding (Figure 1). We then proposed a dynamic “substrate trigger” mechanism for coupled Co–C bond homolysis and H abstraction, whereby substrate binding draws the negatively charged E287 past the polar ribose of the 5′-deoxyadenosyl, initiating homolysis and guiding the radical towards the substrate.

To investigate the “substrate trigger” hypothesis set out above for Co–C bond homolysis in EAL, we made three separate single-point mutations (see the Experimental Section and Figure S2). The electrostatic contribution was assessed with E287Q (i.e., glutamine), which maintains the approximate volume and H-bonding of E287, whilst removing the formal negative charge. The contribution of residue size and proximity (H-bonding distance, van der Waals contact) were investigated by using the E287D variant, which incorporates the smaller, negatively charged aspartate. E287A was also investigated because it removes most of these interactions. The peak of the ω absorption band for free coenzyme B12 in aqueous buffer is at 525 nm and that for methylcobalamin is at 521 nm. These peaks were found to blue-shift by varying the hydrophobic environment for the B12 chromophore when bound to WT, E287D and E287Q. Such a shift suggests a more hydrophobicity of residue 287 (Asp < Glu < Gln < Ala). The ± 2 nm error was based on the breadth of the peaks. See text for discussion.

### Table 1. Steady-state turnover kinetic parameters for the EAL wild-type and E287 variants with 2-aminoethanol at 298 K under aerobic and anaerobic conditions.

|            | $k_{cat}$ [s$^{-1}$] | $K_m$ [mM] | $k_{cat}/K_m$ [M$^{-1}$ s$^{-1}$] |
|------------|----------------------|------------|----------------------------------|
| WT$^{[a]}$ | 33.5 ± 0.62          | (1.97 ± 0.12) x 10$^{-3}$ | (1.71 ± 0.07) x 10$^2$ |
| + O$_2$    | D 0.34 ± 0.01        | 2.02 ± 0.18 | 170 ± 11                        |
| Q          | 0.041 ± 0.007        | 1.47 ± 0.19 | 28.7 ± 3.3                     |
| A          | D 0.34 ± 0.002       | 2.01 ± 0.11 | 171 ± 8                         |
|            | Q 0.059 ± 0.004      | 1.42 ± 0.09 | 41.8 ± 2.3                     |
| −O$_2$     | A − − −              | − − −      | − − −                            |

[a] The WT EAL steady-state parameters are invariant in the presence and absence of oxygen.

Steady-state kinetic parameters (Table 1) were calculated for WT EAL and each E287 variant with 2-aminoethanol (see the Experimental Section and Figure S4). EAL protects against the efficient reaction between the cob(II)alamin radical and O$_2$ both after Co–C bond homolysis through substrate binding and Co–C bond photolysis. Initially, therefore, these data were acquired under aerobic conditions. There is a substantial decrease in the apparent $k_{cat}$ in both E287D (−100-fold) and E287Q (−820-fold) compared to WT. This can be explained by Co–C bond homolysis and/or H-transfer from the substrate becoming at least partially rate limiting during turnover in E297D and E297Q. Alternatively, E287 could play an important role in radical rearrangement and/or the second H transfer, which are thought to be rate limiting in the WT (Figure S1).

No turnover was observed for E287A. Similar data from coenzyme B12-dependent ornithine 4,5-aminomutase (OAM) and methylmalonyl-CoA mutase (MCM) show a more modest decrease in $k_{cat}$ with equivalent variants, and retention of activity after the alanine mutation. Moreover, the glutamine variant has a higher turnover number than the aspartate for both OAM and MCM, whereas the opposite is true for EAL (see later discussion).
The apparent substrate affinity is significantly lower in E287D and E287Q (K_m is approximately three orders of magnitude higher). This is perhaps unsurprising considering that the crystal structure of EAL from E. coli shows two H-bonds between E287 and the substrate.\(^{(1)}\) The K_m values are very similar between the two active variants of EAL, despite the significantly lower k_cat for E297Q. Electrostatics, therefore, has more of an influence on turnover than apparent affinity for substrate. The catalytic efficiency, k_cat/K_m, is reduced by approximately five orders of magnitude in the active EAL variants; this is substantial for a single point mutation.

When the steady-state data were acquired under anaerobic conditions, the kinetic parameters for E287D remained the same within error (Table 1). However, k_cat for E287Q increased by ~45% with K_m remaining the same within error. Oxygen, therefore, appears only to influence enzyme turnover. We also investigated the effect of oxygen on the pre-steady-state data by stopped-flow (see the Experimental Section). Figure 3A shows the change in absorbance at 525 nm after rapidly mixing E287D with 2-aminoethanol under anaerobic (main panel) and aerobic (inset) conditions. The absorbance decrease at 525 nm in both cases is consistent with the conversion of 5’-deoxyadenosylcob(III)alamin to the cob(II)alamin radical. Both data sets also follow the same single-phase kinetics: \(k_{\text{obs}} = (7.29 \pm 0.18) \text{s}^{-1}\) (anaerobic) and \((7.24 \pm 0.31) \text{s}^{-1}\) (aerobic). Figure 3B shows the equivalent data for E287Q. Under anaerobic conditions, the accumulation of cob(II)alamin is again observed (main panel, \(k_{\text{obs}} = (3.13 \pm 0.83) \text{s}^{-1}\)). However, there is an increase in absorbance under aerobic conditions (inset), which is consistent with a reaction between cob(II)alamin and O_2 that generates cob(III)alamin species with higher extinction coefficients at 525 nm than co-enzyme B_12.\(^{(1)}\) The more hydrophobic environment produced by the glutamine in E287Q compared to either glutamate (WT) or aspartate (E287D) appears to allow oxygen to access the active site, thus quenching the cob(III)alamin radical.

Photolysis of coenzyme B_12 also generates the cob(II)alamin/5’-deoxyadenosyl radical pair. Under aerobic conditions, O_2 is thought to react with both radicals.\(^{(1)}\) The inset of Figure 3B provides evidence for a similar oxidative reaction with cob(II)-alamin following the substrate-driven, thermal Co–C bond homolysis by E287Q. Quenching of the cob(II)alamin radical in this way will prevent it from recombining with the 5’-deoxyadenosyl radical. If radical pair recombination is required after each turnover, one might expect either deactivation after a single turnover or a substantial reduction in turnover of E287Q under aerobic conditions. Instead, we only see a slight reduction in k_cat upon the introduction of oxygen to the reaction sample, this suggests a significant proportion of radical pairs remain dissociated until the substrate is exhausted. Furthermore, it is unlikely that the reaction between O_2 and 5’-deoxyadenosyl radical is a dominant aerobic pathway in this EAL variant. If it were, one would expect E287Q to deactivate immediately following homolysis under aerobic conditions because the organic radical is required for H abstraction from the substrate. Therefore, the size and H-bonding of the glutamine residue in E287Q provides at least partial control over the path of the 5’-deoxyadenosyl radical towards the substrate.

The k_obs values for the anaerobic data from E287D ((7.29 ± 0.18) s\(^{-1}\)) and E287Q ((3.13 ± 0.83) s\(^{-1}\)) with 2-aminoethanol are around two orders of magnitude slower than k_obs estimated for the WT at 298 K.\(^{(1)}\) Moreover, both k_cat and the amplitude of the absorbance change at 525 nm for E287D are ~2.5 times those of E287Q. This suggests that the equilibrium for Co–C bond homolysis is further towards the intact cofactor in E287Q. Unlike the WT data (at 278 K), which exhibit multiphasic kinetics,\(^{(1)}\) both anaerobic data sets in Figure 3 fit well to a single exponential function. Although the substantial rise in K_m indicates that the apparent substrate affinity might be significantly compromised, this is unlikely to limit the pre-steady-state kinetics because the experiments were conducted under saturating substrate concentrations (Figure S5). The single-phase behaviour therefore suggests that one of the chemical steps that contribute to this signal (i.e., homolysis or H-transfer) has become sufficiently slow compared to the other to dominate the pre-steady-state kinetics.

The k_obs values for E287D and E287Q are lower than k_cat for the WT enzyme, but are ~20 and ~50 times higher, respectively, than their own k_cat values. The homolysis/H-transfer steps that contribute to the pre-steady-state signal are therefore unlikely to be rate limiting for steady-state turnover in these var-
iants. This indicates a substantial effect on subsequent steps in the catalytic cycle (Figure S1), which is supported by the fact that $k_{\text{obs}}$ for E287D and E287Q are closer in value than are their respective $k_{\text{cat}}$ values. EPR data indicate the substrate-like radical accumulates during turnover; this suggests that radical rearrangement could be rate limiting. However, a deuteron KIE of −7 has been reported on turnover of 2-aminoethanol by EAL, thus indicating that H transfer from the coenzyme to the product-like radical must also contribute, at least partially, to the overall rate. The effect on these steps warrants further investigation.

E287A does undergo substrate-induced Co–C bond homolysis, albeit very slowly, despite being ostensibly inactive (Figure 4). These data suggest that E287 is not an absolute requirement for Co–C bond homolysis in EAL, but that it is required for homolysis to happen with any efficiency. It also appears crucial for activity. Although E287A can trigger Co–C bond homolysis upon substrate binding, we did not observe turnover for this variant. The absence of charge and the small size of the alanine side chain appear to result in a loss of control over the high-energy 5′-deoxyadenosyl radical. The UV–visible spectra in Figure 4 show the accumulation of cob(II)alamin despite the fact that the data were acquired under aerobic conditions. The spectra are similar if acquired in the absence of oxygen (not shown). This is perhaps surprising considering the result in Figure 3B, which shows a very efficient side reaction between the cob(II)alamin radical and $O_2$ immediately following Co–C bond homolysis in E287Q. This side reaction is sufficiently rapid that it seems highly unlikely that product release is conditional. This apparent disparity may be explained by the fact that E287Q fills a similar space to E287 in the WT, whereas E287A leaves a void that can be occupied by neighbouring polar/charged side chains or water molecules. The result would be the retention of the hydrophilic environment around cob(II)alamin that limits the approach of $O_2$.

The formal negative charges of the glutamate in WT EAL and aspartate in the E287D variant appear to play a more prominent role in EAL than in the mutants studied in ref. [12]. This is an interesting result because published computational data suggest that the electrostatic contribution to Co–C bond homolysis is dominant in coenzyme $B_{12}$-dependent enzymes. The authors of ref. [12] argue that, assuming Co–C bond homolysis is rate limiting during turnover, the low $k_{\text{cat}}$ for the aspartate variant of both OAM and MCM might be explained by a decreased charge–dipole interaction between the aspartate and polar 5′-deoxyadenosyl. The magnitude of this interaction follows an inverse $r^2$ relationship, in which $r$ = distance between the residues and 5′-deoxyadenosyl. $k_{\text{cat}}$ and $K_m$ are both affected significantly by E287 mutations. However, there are a similar order between E287D and E287Q, thus suggesting a substantial impact of electrostatics on the chemistry later in the catalytic cycle, as demonstrated by the difference in $k_{\text{cat}}$.

It is also possible that the apparent flexibility of EAL ensures that protection against the side reaction between cob(II)alamin and $O_2$ is maintained in E287D. The aspartate is brought adequately close to the cofactor to keep the environment of the central Co hydrophilic. The fact that aerobic addition of substrate to E287A results in the accumulation of cob(II)alamin, with no evidence of reaction with $O_2$, is again consistent with an active site that is flexible enough to compress and fill the void left by the glutamate-to-alanine substitution. These neighbouring residues might also facilitate substrate-triggered Co–C bond homolysis in E287A. A nearby arginine, R160 (Figure 1), has previously been shown as playing a critical role in EAL catalysis. Again, the formal (positive) charge of R160 is significant, with R160K retaining activity with a modest drop in $k_{\text{cat}}/K_m$ (~180-fold) and R160A resulting in the accumulation of cob(II)alamin but not facilitating turnover. There was also evidence for the involvement of more than one residue from our stopped-flow FTIR data that estimated the movement of approximately seven peptide bonds alongside the pre-steady-state chemistry. Such a movement might not directly involve seven side chains, but does suggest a flexible, dynamic active site in EAL. This picture supports the model for Co–C bond homolysis in EAL proposed by Robertson et al.: coordinated protein motions that guide the cleavage of the Co–C bond along the reaction coordinate.

Structural and computational studies indicate that large-scale motions help prime $B_{12}$-dependent enzymes, such as OAM, lysine 5,6-aminomutase, diol dehydratase, and MCM, for radical-mediated catalysis. There is an increasing body of evidence that suggests that localised motions are
then intimately involved in controlling the trajectory and reactivity of the activated radical intermediates (e.g., ref. [5b], [21]). However, EAL shows no evidence of large-scale motions upon substrate binding.\textsuperscript{[7]} It therefore achieves the substrate trigger of Co–C bond homolysis and subsequent radical control by using active-site interactions and dynamics alone. This has been borne out here, with E287 apparently providing both electrostatic and dynamical contributions to Co–C bond homolysis and subsequent radical control in EAL.

Experimental Section

Refer to the Supporting Information for Experimental details.

Acknowledgements

We thank the University of Manchester, UK Biotechnology and Biological Sciences Research Council (BBSRC), Natural Science Foundation of China (No. 31071495) and Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions for funding. S.H. is a BBSRC David Phillips Fellow. N.S.S. holds a Royal Society Wolfson Merit Award and an Engineering and Physical Sciences Research Council (EPSRC) Established Career Fellowship.

Keywords: coenzyme B\textsubscript{12} \cdot electrostatics \cdot enzymes \cdot protein dynamics \cdot radicals

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Received: June 27, 2013
Published online on August 19, 2013

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