Tritium Isotope Effects in Adenosylcobalamin-Dependent Glutamate Mutase: Implications for the Mechanism†

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ABSTRACT: The transfer of tritium between adenosylcobalamin and substrate in the reaction catalyzed by glutamate mutase was examined to investigate the possibility of a protein-based radical intermediate. There was no evidence that tritium was transferred to the protein during the reaction, as tritium neither became stably bound to the protein nor exchanged with water. The kinetics of tritium transfer from adenosylcobalamin to 3-methylaspartate was investigated. Both the transfer of tritium to product and the exchange of enzyme-bound and free coenzyme contribute to the kinetics of tritium loss from adenosylcobalamin. By varying the experimental conditions, the rates of both coenzyme exchange and tritium transfer could be measured. Exchange of adenosylcobalamin with enzyme is very slow, k_{off} = 0.01 s^{-1}, which may reflect a conformational change in the coenzyme and/or protein involved in forming active holo enzyme. The rate constants for the loss of tritium from adenosylcobalamin and the appearance of tritium in 3-methylaspartate are much faster and very similar, $k = 0.67 \pm 0.05$ s^{-1} and $k = 0.50 \pm 0.05$ s^{-1}, respectively, consistent with the transfer of tritium occurring directly between coenzyme and substrate. The isotope effect, calculated from the rate constants for tritium transfer, and k_{cat}, determined for the overall reaction under the same conditions, are between 13.5 and 18. These values are typical of primary isotope effects seen for enzymes in which hydrogen transfer is substantially rate limiting. A protein radical, therefore, appears unlikely to feature in the mechanism of this enzyme.

Organic-based free radicals are increasingly seen to play an important part in the catalysis of a variety of enzymic reactions (Stubbe, 1989; Marsh, 1995). Well-studied examples include the ribonucleotide reductases (Reichard, 1993; Stubbe, 1990), pyruvate formate-lyase (Knappe et al., 1993), lysine 2,3-aminomutase (Frey, 1993), and the adenosylcobalamin (AdoCbl)[1] -dependent isomerases (Ochiai, 1994). The focus of this study is glutamate mutase, one of the group of AdoCbl-dependent isomerases that catalyze unusual skeletal rearrangements: in this case, the interconversion of L-glutamate and L-*threo*-3-methylaspartate (Barker et al., 1964).

Glutamate mutase comprises two readily separable subunits or components designated MutE and MutS, both of which have recently been cloned, sequenced, and overexpressed (Marsh & Holloway, 1992; Holloway & Marsh, 1993, 1994). MutE is a dimeric protein of subunit M_r 53708, whereas MutS is a monomer of M_r 14748, which shows sequence similarity to other cobalamin-dependent enzymes (Marsh & Holloway, 1992; Beatrix et al., 1994). Association of the two subunits has been shown to be necessary before the enzyme can bind AdoCbl (Holloway & Marsh 1994).

In this and other AdoCbl-dependent reactions, AdoCbl acts as a source of 5′-deoxyadenosyl radical that is unmasked by homolysis of the labile cobalt−carbon bond of the coenzyme (Halpern, 1985). The first step in the rearrangement involves abstraction of the migrating hydrogen from the substrate to give a substrate radical that subsequently undergoes rearrangement (Figure 1). In the simplest mechanism, the migrating hydrogen is abstracted directly by the 5′-deoxyadenosyl radical; this is consistent with experiments on many enzymes that demonstrate exchange of tritium between substrate and the 5′-position of AdoCbl (Babior & Krower, 1979).

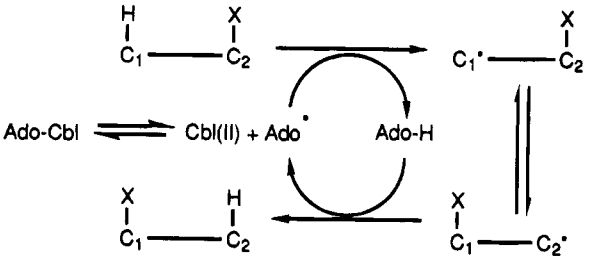

FIGURE 1: Minimal mechanistic scheme for the rearrangements catalyzed by AdoCbl-dependent isomerases. X is an electron-withdrawing group which may be OH, NH_2, or a carbon-containing fragment, as is the case in the isomerization catalyzed by glutamate mutase.

An alternative proposal is that a protein-based radical intermediate may operate in the mechanism of these isomerases (Cleland, 1982). Such an intermediate was originally postulated to explain the extremely large 'isotope effects' for the transfer of tritium between AdoCbl and product, of 160 and 125, respectively, measured for the deaminations and dehydrations catalyzed by ethanolamine ammonia-lyase and diol dehydrase (Weisblat & Babior, 1971; Essenberg et al., 1971). In the case of ethanolamine ammonia-lyase, a protein-based radical is further supported by experiments that demonstrate the release of tritium onto a labile site in the protein (O'Brien et al., 1985) and EPR studies with isotopically-labeled substrates (Tan et al., 1986). One attraction of this proposal is that it would provide a mechanistic link

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[1] Abbreviations: AdoCbl, adenosylcobalamin; TFA, trifluoroacetic acid.

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with AdoCbl-dependent ribonucleotide reductase in which a protein-based intermediate radical is an established feature (Booker et al., 1994).

To determine whether protein-based radicals are a general feature of AdoCbl-mediated isomerizations, I have examined the transfer of tritium between substrate, coenzyme, and product in the glutamate mutase reaction. The results presented here are consistent with hydrogen transfer occurring directly between coenzyme and product and provide no evidence for the formation of a protein radical. An argument based on chemical considerations is presented to explain why protein radicals may operate in some AdoCbl-catalyzed reactions but not in others.

MATERIALS AND METHODS

Materials. The purification of MutE and MutS proteins from recombinant *Escherichia coli* strains has been described previously (Holloway & Marsh, 1994). 3-Methylaspartase was purified from *Clostridium tetanomorphum* as described by Hsiang and Bright (1967). L-[U-^{14}C]Glutamic acid and L-[G-^{3}H]glutamic acid were purchased from Amersham PLC, and AdoCbl was supplied by Fluka Chemical Co. The sources of other materials have been described previously (Holloway & Marsh, 1994) or were purchased from commercial suppliers.

Enzymic Synthesis of 5′-^{3}H-AdoCbl. Uniformly tritiated glutamate was used as a substrate with glutamate mutase to catalyze the exchange of tritium into the 5′-adenosyl carbon of AdoCbl. Reactions were set up at room temperature, under argon, in light-proofed 2.5 mL glass vials fitted with septa. Typically, the following concentrations of enzyme, coenzyme, and substrate were used in a final volume of 500 or 1000 μL of 50 mM potassium phosphate buffer, pH 8.0: MutS protein, 10 μM; MutE protein, 10 μM; AdoCbl, 50 μM; unlabeled sodium glutamate, 250 μM; L-[G-^{3}H]glutamic acid, 50 μCi. The final specific activity of glutamate in the reaction was 80 Ci/mol in the exchangeable position. Tritium was allowed to equilibrate between AdoCbl and glutamate for 30 min, and then AdoCbl was recovered by reverse-phase HPLC. Under the acidic conditions used in chromatography, AdoCbl eluted as the yellow 'base-off' form and was therefore neutralized by the addition of potassium phosphate, pH 7.0, to a final concentration of 0.1 M. The concentration of AdoCbl was determined by measuring absorbance at 522 nm where $\epsilon = 8000\ M^{-1}$ (Barker et al., 1960), and the specific activity was determined by scintillation counting small portions. The specific activity was generally adjusted to give a working stock at 10^5 dpm/nmol, and the tritiated AdoCbl was stored at −70 °C.

Purification of AdoCbl by HPLC. AdoCbl, MutS, MutE, and the degradation product, aquocobalamin, were separated by chromatography on a 300 Å C_3 reverse-phase column (Spherisorb S5xC3) (Figure 2). Samples (100−500 μL) were injected onto the column which was preequilibrated in 0.1% TFA. Compounds were eluted with a linear gradient of acetonitrile containing 0.1% TFA. The flow rate was 1 mL/min, and compounds were detected by monitoring absorbance at 260 nm.

Analysis of Radiolabeled AdoCbl. Radiolabeled AdoCbl was recovered from reaction mixtures by HPLC using a 300 Å C3 column and the solvent system described above. Typically 150 μL of each quenched time point was injected onto the column. The AdoCbl-containing peak was collected by hand into a preweighed tube and made up to a standard volume by adding water. Portions of the peak were then scintillation counted to determine the ^{3}H content of the sample. The area of the AdoCbl-containing peak (in arbitrary units) was determined by direct integration of the output from the detector. The specific activity of the AdoCbl (in arbitrary units) was calculated by dividing the activity of the sample (in dpm) by the peak area and normalized so that specific activity at $t = 0$ was 100%.

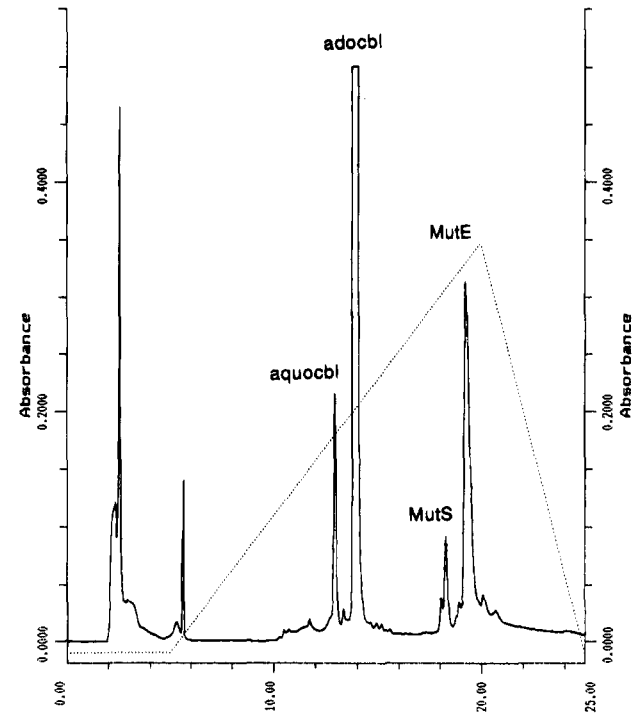

FIGURE 2: Purification of AdoCbl, MutS, and MutE by reverse-phase HPLC. Compounds were eluted a flow rate of 1 mL/min with a gradient of acetonitrile, as indicated by the dotted line. The gradient was as follows: 0−5 min, 0% acetonitrile; 5−20 min, 0−70% acetonitrile; 20−25 min, 70−0% acetonitrile. AquoCbl is a degradation product of AdoCbl in which the 5′-deoxyadenosyl group is replaced by water.

Analysis of Radiolabeled Mesaconate. Radiolabeled mesaconate was recovered from reaction mixtures by HPLC on a 3 μm C_{18} reverse-phase column (Spherisorb ODS-2). The column was equilibrated in 10% methanol and 0.1% TFA, and samples were eluted isocratically at a flow rate of 1 mL/min. Mesaconate eluted after 4 min under these conditions and was detected by monitoring the absorption at 260 nm. Before injection onto the column, unlabeled mesaconate (final concentration ≈ 100 μM) was added to samples as a carrier. The mesaconate-containing peak was collected by hand into a preweighed tube and made up to a standard volume by adding water. Portions of the peak were then counted in a dual channel scintillation counter to determine the ^{14}C and ^{3}H content of the sample. The recovery of mesaconate from the column was determined as 90 ± 2% by chromatography of samples of known specific activity.

Assay of Glutamate Mutase Activity. In experiments to examine the transfer of tritium from AdoCbl to 3-methylaspartate, the activity of glutamate mutase was determined radiometrically. [^{14}C]Glutamate was used as the substrate, and the reaction was coupled to the production of mesaconate

by including 3-methylaspartase in the assay. The reaction was stopped at various time points by quenching with TFA, and the radiolabeled mesaconate formed was analyzed by HPLC. The concentrations of MutE and MutS were determined by measuring their absorbance at 280 nm and using the following values for their absorption coefficients: MutE $\epsilon_{280} = 56\,300\ M^{-1}\ cm^{-1}$; MutS $\epsilon_{280} = 9380\ M^{-1}\ cm^{-1}$ (Holloway & Marsh, 1994).

Kinetics of Tritium Loss from AdoCbl. To study the slow phase of tritium loss from AdoCbl, the following experimental setup was used. Reactions were conducted at room temperature, under argon, in a light-proofed 2.5 mL glass vial equipped with a microstirring flea and a septum. The reaction mixture contained, in a total volume of 1 mL, the following: potassium phosphate, pH 8.0, 50 mM; magnesium sulfate, 1 mM; MutE protein, 3 μM; MutS protein, 3 μM; 3-methylaspartase, 30 U; tritiated AdoCbl, 2 μM. The mixture was allowed to equilibrate under argon for 15 min with rapid stirring. Immediately before the reaction was started, 100 μL was withdrawn by syringe and quenched in 150 μL of 0.5% TFA in a light-proof tube on ice. This sample served as the $t = 0$ time point. The reaction was started by adding, through a syringe, 18 μL of 0.5 M sodium L-[^{14}C]glutamate of specific activity approximately 10^5 dpm/μmol. At various times, 100 μL portions were withdrawn and quenched with 0.5% TFA as described above. Samples were stored at -20 °C until the tritium and ^{14}C content of AdoCbl and mesaconate could be determined by HPLC and scintillation counting.

The rapid phase of tritium loss was examined using quenched stopped-flow techniques. The measurements were made on a Bio Logic quench flow machine. Three stock solutions were made up: the 'enzyme' solution contained 6 μM MutE protein, 30 μM MutS protein, 5 μM tritiated AdoCbl, 64 U/mL 3-methylaspartase, 50 mM potassium phosphate, pH 8.0, and 1 mM magnesium sulfate; the 'substrate' solution contained 0.1 M sodium L-[^{14}C]glutamate of specific activity 3.7×10^5 dpm/μmol; the 'quench' solution contained 0.5% TFA. Reactions were started by mixing 200 μL of enzyme solution and 20 μL of substrate over a time of 80 ms. The solution was allowed to age for a variable period of time (420 - 9920 ms), and then 187 μL of the aged solution was quenched with 187 μL of quench solution over 68 ms and delivered to the collection syringe. Samples were transfered to light-proof tubes and stored at -20 °C before analysis of the radiolabeled AdoCbl and mesaconate.

Data Analysis. The data were plotted and curves fitted using the KaleidaGraph program (Abelbeck Software).

RESULTS

Transfer of Tritium from L-Glutamate to AdoCbl. The glutamate mutase-catalyzed exchange of tritium from glutamate to AdoCbl proved a simple and efficient method for the synthesis of tritiated coenzyme. Reverse-phase HPLC (Figure 2) allowed radiolabeled AdoCbl of high purity to be recovered rapidly in good yield, usually around 75%. The labeling experiments were performed at low concentrations of substrate and in the absence of coupling enzyme so that the reaction was reversible. Under these conditions, at equilibrium the specific activity of the coenzyme should exceed that of the substrate since there are two exchangeable hydrogens in the coenzyme. In practice, this was generally not observed, in part because the AdoCbl, which was in 5−10-fold molar excess over enzyme, exchanges very slowly with enzyme-bound coenzyme (see later discussion). There is also likely to be an equilibrium isotope effect on the exchange of tritium that may operate to reduce the specific activity of AdoCbl relative to that calculated simply from the specific activity of glutamate. Typically, 45−55% of the theoretical maximum number of tritium atoms were incorporated into AdoCbl. Experiments with deuterated glutamate confirmed that only the 5′-hydrogens of AdoCbl exchange with the substrate: analysis of the 1-D proton NMR spectrum showed that only the resonances due to the 5′-hydrogens were reduced in intensity (data not shown).

Possibility of Tritium Transfer to a Protein-Based Intermediate. Tritium transfer to a labile site on the protein has been demonstrated for AdoCbl-dependent ribonucleotide reductase (Hogenkamp et al., 1968) and ethanolamine ammonia-lyase (O'Brien et al., 1985). It was therefore of interest to determine whether tritium is transferred to the protein during the course of the glutamate mutase reaction. In the tritium-labeling experiments, the reactions were allowed to approach equilibrium, and therefore tritium should have populated all exchangable sites, whether on the coenzyme or on the protein.

The HPLC method employed to purify AdoCbl also allowed MutS and MutE to be recovered (Figure 2), albeit in a denatured state. MutS eluted at around 60% and MutE eluted at 65% acetonitrile; both proteins were recovered in approximately 50% yield. Neither protein contained any significant amount of radioactivity. Compared with the counts associated with the AdoCbl peak, less than 0.1% of the radioactivity was associated with the protein. This suggests that tritium does not become stably attached to the protein during transfer between substrate and coenzyme.

MutE and MutS could also be separated from AdoCbl as the native proteins by FPLC gel filtration on a Superose 12 column. Reactions were set up in which tritiated AdoCbl was allowed to exchange with unlabeled substrate. After 2 min, the reaction mixture was injected onto the column, and the components separated. Again, negligible amounts of radioactivity were associated with the protein. In a separate experiment, portions of the reaction mixture were rapidly frozen, and water was removed by small-scale bulb to bulb distillation under vacuum. The water was collected and counted for tritium. Less than 1% of the radioactivity was found in the volatile fraction; similar amounts of volatile tritium were found in controls in which either substrate or enzyme was omitted. This result confirms previous observations that tritium from in the coenzyme does not exchange with solvent during the reaction (Switzer et al., 1969).

Kinetics of Tritium Transfer. Initially, measurements were made with equimolar concentrations of MutE and MutS (3 μM of each), which were present in excess over AdoCbl (2 μM). The reaction was started by the addition of glutamate to 10 mM and made irreversible by including a large excess of 3-methylaspartase in the reaction mixture so that the product, 3-methylaspartate, was removed by conversion to mesaconate. At intervals between 15 and 300 s, portions of the reaction mixture were removed and quenched with TFA. Subsequently, the tritium content of AdoCbl and the ^{14}C and tritium content of mesaconate were determined.

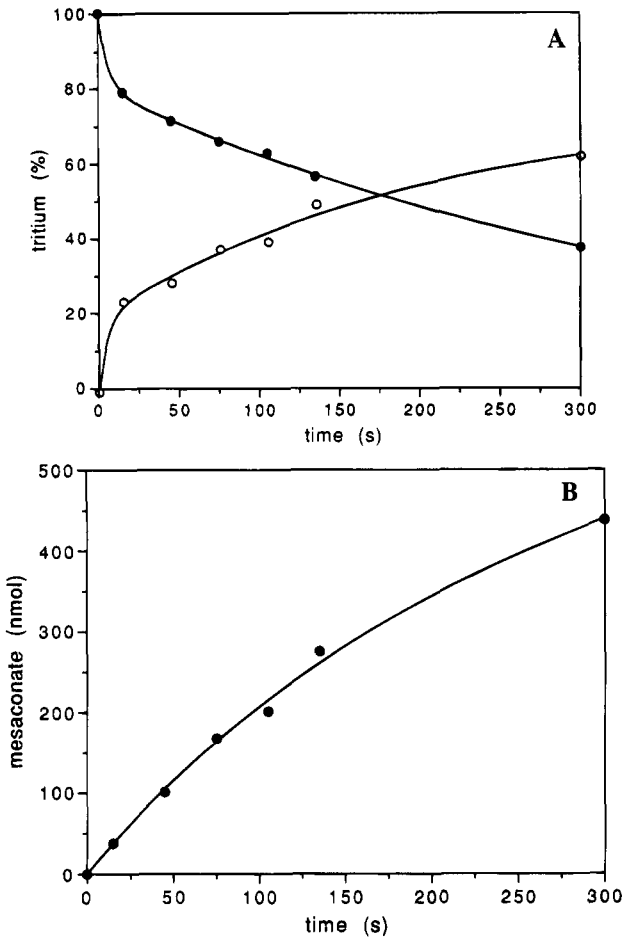

FIGURE 3: Kinetics of the slow phase of tritium loss from AdoCbl. (A) Percentage of total tritium remaining in AdoCbl (●) and appearing in mesaconate (○). (B) Formation of mesaconate during the time course of tritium transfer. The reaction remained essentially linear for the first 130 s.

The loss of tritium from AdoCbl was clearly biphasic (Figure 3). There was a rapid fall in the specific activity of AdoCbl upon the addition of substrate, which was essentially complete within 15 s. The loss of tritium then followed a much slower phase, which was well fitted by an exponential decay. Tritium loss from AdoCbl was mirrored by its appearance in mesaconate. The total amount of tritium in product and coenzyme remained roughly constant throughout the course of the reaction. A slight fall in the velocity of the reaction was evident after 300 s, which may be due to substrate depletion and/or some loss of enzyme activity.

It was found that the proportion of tritium released from AdoCbl in the rapid phase of the reaction could be increased to a limiting value of about 80% by increasing the concentration of MutS in the reaction (Figure 4). Previously, it has been shown that increasing the concentration of MutS relative to MutE lowers the apparent dissociation constant for AdoCbl binding to glutamate mutase (Holloway & Marsh, 1994). This suggests that the rapid phase of tritium loss occurs from AdoCbl which is bound to the enzyme at the start of the reaction, whereas the slower phase represents exchange of enzyme-bound coenzyme with free coenzyme.

Off Rate for AdoCbl. The pseudo-first-order rate constant for the exchange of AdoCbl, k_{ex}, was calculated from data points between 15 and 135 s where product formation was essentially linear (Figure 3). Under these conditions (3 μM each of MutE and MutS), $k_{ex} = 2.5 \times 10^{-3} \pm 1.0 \times 10^{-4}$ s^{-1}. The off rate, k_{off}, for AdoCbl can be calculated from k_{ex} assuming (i) that enzyme-bound and free AdoCbl are at equilibrium during the reaction; (ii) that loss of tritium is much faster than exchange of coenzyme, so that once bound, tritium in AdoCbl is always lost to product before release of coenzyme into solution—this represented by the scheme shown in Figure 5; (iii) binding of substrate does not alter the kinetics of AdoCbl binding [which has been demonstrated previously (Holloway & Marsh, 1994)]. At equilibrium

$$k_{off} = \frac{k_{on}[E][AdoCbl]}{[AdoCbl{\cdot}E]}$$

where [AdoCbl·E] is the concentration of enzyme-bound AdoCbl and [E] is the concentration of free glutamate mutase present in a form capable of binding AdoCbl. Since the association of MutE and MutS is required for glutamate mutase to bind AdoCbl (Holloway & Marsh, 1994), the effective concentration of E is always less than the concentration of either subunit and therefore difficult to determine; however, since $k_{on}[E] = k_{ex}$, k_{off} can be obtained easily. The relative concentrations of enzyme-bound and free AdoCbl are given by the fraction, F, of the total tritium initially lost from coenzyme in the rapid phase of the reaction, so that

$$k_{off} = k_{ex}\frac{(1 - F)}{F}$$

For the data shown in Figure 3, $F = 0.21$, and hence $k_{off} = 0.01 \pm 5 \times 10^{-4}$ s^{-1}. The 'true' dissociation constant for AdoCbl, determined under conditions where MutE is saturated by MutS, is about 2 μM (Holloway & Marsh, 1994), and therefore $k_{on} \approx 5000$ s^{-1} M^{-1}.

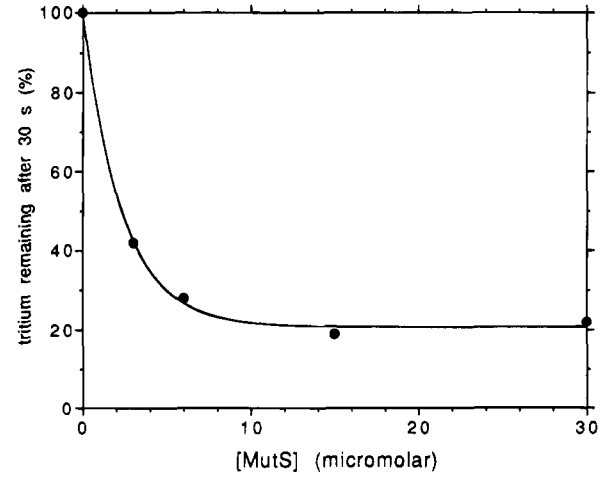

FIGURE 4: Tritium loss from AdoCbl as a function of MutS concentration. The percentage of tritium released from AdoCbl in the rapid phase of the reaction (i.e., after 30 s) was determined in reactions containing fixed concentrations of MutE and AdoCbl (3 and 2 μM, respectively) and varying concentrations of MutS.

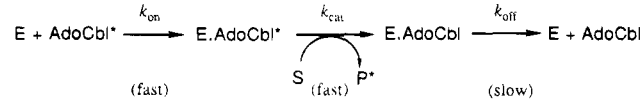

FIGURE 5: Reaction scheme describing the slow phase of tritium loss from AdoCbl in which release of AdoCbl from the protein is the rate-limiting step.

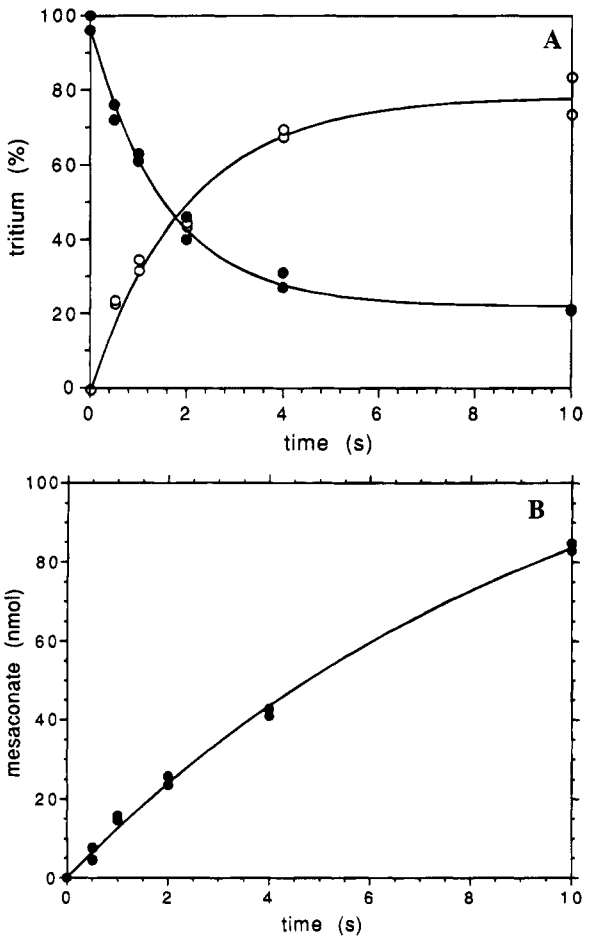

FIGURE 6: Rapid phase kinetics of tritium transfer from AdoCbl to product. (A) Percentage of total tritium remaining in AdoCbl (●) and appearing in mesaconate (○). (B) Formation of mesaconate during the time course of tritium transfer. Some loss of enzyme activity is evident (see text for discussion).

Isotope Effect for Tritium Transfer from Coenzyme to Product. The kinetics of the rapid phase of tritium transfer were investigated using quenched stopped-flow apparatus. MutS was present at five times the concentration of MutE to increase the proportion of AdoCbl bound to the enzyme. Under these conditions, tritium loss from AdoCbl exhibited first-order kinetics, as did the appearance of tritium in mesaconate (Figure 6A). The rate constants for the transfer of tritium out of AdoCbl, k_{out}, and into 3-methylaspartate, k_{in}, are 0.67 ± 0.05 and 0.50 ± 0.05 s^{-1}, respectively. Given the statistical errors and possible systematic error inherent in the measurement of these rate constants, the difference between them is not considered significant.

Some loss of enzyme activity was observed during the course of the reaction in these experiments (Figure 6B). This is unlikely to be due to substrate depletion and may reflect the oxygen sensitivity of the enzyme, since it was not possible to perform the stopped-flow measurements anaerobically. The loss of activity is much slower than the transfer of tritium, which was essentially complete after 4 s, and is therefore unlikely to affect significantly the kinetics of transfer.

Assuming that the proportion of tritium lost from AdoCbl after 10 s represents the proportion of active enzyme–coenzyme complex, it is possible to calculate the effective concentration of active holoenzyme and hence the turnover number for the enzyme under these conditions. The turnover number calculated from velocity data between 0 and 2 s (where the reaction was essentially linear) was 18 ± 1 s^{-1}, which is in excellent agreement with the value of 18.1 s^{-1} calculated from the values for k_{cat} and K_m for glutamate mutase determined previously (Holloway & Marsh, 1994). The *V/K* isotope effect for tritium transfer from AdoCbl to 3-methylaspartate is given by the ratio of the turnover number (at the particular substrate concentration used) to the rate constant for tritium transfer (either k_{in} or k_{out}) divided by statistical factor, which accounts for the fact that there are two hydrogens in the methyl group of 5′-deoxyadenosine competing with tritium for transfer. It has been shown previously (Essenberg et al., 1971; Weisblat & Babior, 1971) that for large isotope effects (≥10) the statistical factor is, to a good approximation, 2. The isotope effect, calculated using this statistical factor, is between 18 and 13.5 depending on whether k_{in} or k_{out} is used in the calculation.

DISCUSSION

Protein-based radicals are now implicated in a variety of important reactions that involve the abstraction of hydrogen atom as part of the mechanism (Stubbe, 1989; Marsh, 1995). In particular, the three AdoCbl-dependent enzymes for which a hydrogen transfer to and from the coenzyme has previously been investigated in detail all appear to exchange hydrogen with a site on the protein. This is best demonstrated for AdoCbl-dependent ribonucleotide reductase from *Lactobacillus leichmanni*, in which isotope readily exchanges between coenzyme and water. The protein radical is thought to reside on Cys-408, based on mutagenesis experiments (Booker et al., 1994) and sequence comparisons with the iron-dependent ribonucleotide reductase whose structure has recently been determined (Uhlin & Eklund, 1994). The very large tritium isotope effects measured for ethanolamine ammonia-lyase and diol dehydrase together with the exchange of tritium onto a labile site on the protein in ethanolamine ammonia-lyase (O'Brien et al., 1985) have been interpreted as evidence for a protein radical in these enzymes.

In this context, glutamate mutase is the first AdoCbl-dependent enzyme for which a protein radical appears *not* to be a feature of the mechanism. The evidence for this may be summarized as follows. No incorporation of tritium from either coenzyme or substrate into the protein is seen, neither is tritium lost to water during the reaction. The kinetics of tritium loss are mirrored by its appearance in mesaconate and show no evidence for the formation of an intermediate. The primary isotope effect on *V/K* for the transfer of tritium from AdoCbl to product, which is between 13.5 and 18, is within the range expected for enzymes catalyzing reactions where the isotopically sensitive step is substantially rate limiting. Allowing for this isotope effect, the transfer of hydrogen directly from coenzyme to product is kinetically competent.

Both transfer to product and coenzyme exchange were found to contribute to the kinetics of tritium loss from AdoCbl. This is because the dissociation constant for AdoCbl is sufficiently high that at the concentrations of enzyme and coenzyme used a substantial proportion of the coenzyme remains unbound. The exchange reaction is much slower ($t_{1/2}$ = 2.0 min) than the transfer reaction ($t_{1/2}$ = 0.5 s), and so the two rates could be measured separately. In

particular, the on rate for AdoCbl ($k_{on} \approx 5000\ M^{-1}\ s^{-1}$) is much slower than expected if binding was simply diffusion limited. These results confirm and quantify observations initially made by Switzer et al. (1969); slow exchange of AdoCbl has also be seen for ethanolamine ammonia-lyase (Weisblat & Babior, 1971).

One explanation for the slow kinetics of AdoCbl binding is that a conformational change in the protein and/or the coenzyme is necessary to create the active holoenzyme. Recently, the structure of a cobalamin-binding proteolytic fragment of methylcobalamin-dependent methionine synthase has been determined (Drennan et al., 1994). A surprising and important finding is that the conformation of cobalamin is greatly altered. In the structure, the dimethylbenzimidazole nucleotide loop no longer coordinates cobalt but has been displaced by a hisidine side chain, which is part of a charge relay network. The methionine synthase cobalamin-binding domain shows sequence similarity with MutS (Marsh & Holloway, 1992), including conservation of the histidine residue, and so it is very likely that glutamate mutase binds AdoCbl in a similar manner. The slow kinetics coenzyme exchange could, therefore, be reflecting this process: either the coenzyme may change conformation after it is bound by the protein or the protein may simply select the very small proportion of AdoCbl in the 'base-off' form from solution.

Two mechanistically distinct classes of AdoCbl-dependent enzyme are now apparent: those in which AdoCbl plays a direct role in catalysis, as is the case for glutamate mutase, and those in which it serves as a radical initiator, as in ribonucleotide reductase. One possibility is that this is simply a consequence of the different evolutionary origins of these enzymes, but a consideration of the thermodynamics of hydrogen transfer also provides an explanation. The mechanism of both glutamate mutase and ribonucleotide reductase requires that hydrogen transfer between substrate and coenzyme or protein be reversible. Therefore, the difference in free energies of the enzyme radical:substrate (E•S) complex and the enzyme:substrate radical (ES•) complex should be close to zero. For the transfer of a hydrogen atom, the difference in energies will, to first approximation, correspond to the difference between the bond dissociation energies (BDE) of the bond being made and the bond being broken. For the transfer of hydrogen between the methyl groups (BDE of RCH_2-H $\approx$98 kcal mol^{-1}) of 5′-deoxyadenosine and 3-methylaspartate in the glutamate mutase reaction, this difference in BDE would be expected to be very small. However, the BDE for a C−H bond on a carbon bearing a hydroxyl group is significantly lower, by about 4 kcal mol^{-1} (Bordwell et al., 1992), so that hydrogen atom transfer between the methyl group of 5′-deoxyadenosine and the 3′-carbon of ribose would be disfavored in the ribonucleotide reductase reaction. The BDE of an RS-H bond ($\approx$94 kcal mol^{-1}) on the other hand is well suited to the transfer of hydrogen between the 3′-carbon of ribose and the catalytic cysteine residue of ribonucleotide reductase.

Although a difference in free energy of only 4 kcal mol^{-1} is not sufficient to prevent a reaction being freely reversible, if applied to the activation energy of the rate-limiting step, this could slow the rate of catalysis by several orders of magnitude. The large primary tritium isotope effect for hydrogen transfer seen with glutamate mutase indicates that this step is indeed substantially rate limiting. The adaptation seen in these two enzymes may be viewed as a special case of the more general principle that enzymes evolve so that the internal equilibrium constants for enzyme-bound intermediates are close to unity (Albery & Knowles, 1976).

REFERENCES

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5631−5640.

Babior, B. M., & Krouwer, J. (1979) *CRC Crit. Rev. Biochem. 6*, 35−102.

Barker, H. A., Smyth, R. D., Weissbach, H., Toohey, J. I., Ladd, J. N., & Volcani, B.E. (1960) *J. Biol. Chem. 235*, 480−488.

Barker, H. A., Roove, V., Suzuki, F., & Iodice, A. A. (1964) *J. Biol. Chem. 239*, 3260−3266.

Beatrix, B., Zelder, O., Linder, D., & Buckel, W. (1994) *Eur. J. Biochem. 221*, 101−109.

Booker, S., Licht, S., Broderick, J., & Stubbe, J. A. (1994) *Biochemistry 33*, 12676−12685.

Bordwell, F. G., Zhang, X.-M., & Alnajjar, M. S. (1992) *J. Am. Chem. Soc. 114*, 7623−7629.

Cleland, W. W. (1982) *CRC Crit. Rev. Biochem. 13*, 385−428.

Drennen, C. L., Huang, S., Drummond, J. T., Matthews, R. G., & Ludwig, M. L. (1994) *Science 266*, 1669−1674.

Essenberg, M. K., Frey, P. A., & Abeles, R. H. (1971) *J. Am. Chem. Soc. 93*, 1242−1251.

Frey, P. A. (1993) *FASEB J. 7*, 622−67024.

Halpern, J. (1985) *Science 227*, 869−875.

Hogenkamp, H. P. C., Ghamber, R. K., Brownson, C., Blakely, R. L., & Vitols, E. (1968) *J. Biol. Chem. 243*, 799−806.

Holloway, D. E., & Marsh E. N. G. (1993) *FEBS Lett. 317*, 44−48.

Holloway, D. E., & Marsh, E. N. G. (1994) *J. Biol. Chem. 269*, 20425−20430.

Hsiang, M. W., & Bright H. J. (1967) *J. Biol. Chem. 242*, 5263−5268.

Knappe, J., Elbert, S., Frey, M., & Wagner, A. F. V. (1993) *Biochem. Soc. Trans. 21*, 731−734.

Marsh, E. N. G. (1995) *Bioessays* (in press).

Marsh, E. N. G., & Holloway, D. E. (1992) *FEBS Lett. 310*, 167−170.

O'Brien, R. J., Fox, J. A., Kopczynski, M. G., & Babior, B. M. (1985) *J. Biol. Chem. 260*, 16131−16136.

Ochiai, E.-I. (1994) *Metal Ions Biol. Syst. 30*, 255−278.

Reichard, P. (1993) *Science 260*, 1773−1777.

Stubbe, J. (1989) *Annu. Rev. Biochem. 58*, 257−285.

Stubbe, J. (1990) *Adv. Enzymol. Relat. Areas Mol. Biol. 63*, 349−419.

Switzer, R. L., Baltimore, B. G., & Barker, H. A. (1969) *J. Biol. Chem. 244*, 5263−5268.

Tan, S. L., Kopczynski, M. G., Bachovchin, W. W., Orme-Johnson, W. H., & Babior, B. M. (1986) *J. Biol. Chem. 261*, 3483−3485.

Weisblat, D. A., & Babior, B. M. (1971) *J. Biol. Chem. 246*, 6064−6071.

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