The Reaction of the Substrate Analog 2-Ketoglutarate with Adenosylcobalamin-dependent Glutamate Mutase*

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Glutamate mutase is one of several adenosylcobalamin-dependent enzymes that catalyze unusual rearrangements that proceed through a mechanism involving free radical intermediates. The enzyme exhibits remarkable specificity, and so far no molecules other than L-glutamate and L-threo-3-methylaspartate have been found to be substrates. Here we describe the reaction of glutamate mutase with the substrate analog, 2-ketoglutarate. Binding of 2-ketoglutarate (or its hydrate) to the holoenzyme elicits a change in the UV-visible spectrum consistent with the formation of cob(II)alamin on the enzyme. 2-Ketoglutarate undergoes rapid exchange of tritium between the 5′-position of the coenzyme and C-4 of 2-ketoglutarate, consistent with the formation of a 2-ketoglutaral radical analogous to that formed with glutamate. Under aerobic conditions this leads to the slow inactivation of the enzyme, presumably through reaction of free radical species with oxygen. Despite the formation of a substrate-like radical, no rearrangement of 2-ketoglutarate to 3-methylmalonolactate could be detected. The results indicate that formation of the C-4 radical of 2-ketoglutarate is a facile process but that it does not undergo further reactions, suggesting that this may be a useful substrate analog with which to investigate the mechanism of coenzyme homolysis.

EXPERIMENTAL PROCEDURES

Materials—The purification of glutamate mutase apoenzyme, GlmES, has been described previously (15). 3-Methylaspartase was purified as described by Hsiang and Bright (16). 5′-[3H]AdoCbl was synthesized by using glutamate mutase to catalyze the exchange of tritium from tritiated glutamate to AdoCbl (9). AdoCbl and 2-KG were purchased from Sigma; tritiated glutamic acid was purchased from Amersham PLC. The sources of other materials have been described previously (9, 15) or were purchased from commercial suppliers.

Inactivation of Glutamate Mutase by 2-KG—Reactions were set up in small vials containing 10 mM GlmES, 10 μM AdoCbl, and 10 mM 2-KG in 50 mM potassium phosphate buffer, pH 7.0 at 25 °C in the dark. At various times, portions of the reaction mixture were withdrawn and divided in two; one portion was assayed for enzyme activity in the presence of fresh coenzyme; the other portion was used to determine the amount of AdoCbl remaining in the reaction by HPLC. For anaerobic work the vials were fitted with septa and made anaerobic by repeated evacuation and flushing with argon; transfer of reagents between vials was made by syringe.

UV-visible Spectra of Enzyme-Substrate Complexes—A solution containing 50 μM glutamate mutase and 50 μM AdoCbl in 50 mM potassium phosphate buffer, pH 7.0, was made anaerobic by repeated evacuation and flushing with argon. The solution was introduced by syringe into a cuvette fitted with a septum. The spectrum of the resting enzyme was recorded using a Hewlett-Packard diode array spectrometer. A concen-

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The abbreviations used are: AdoCbl, adenosylcobalamin; Cbl(II) cob(II)alamin; 2-KG, 2-ketoglutarate; GlmES, glutamate mutase apoenzyme; HPLC, high pressure liquid chromatography.
trated anaerobic solution of either 2-KG or L-glutamate was then added (final concentration 5 mM), and after 20 s, the spectrum of the enzyme-substrate complex recorded.

**Tritium Exchange into 2-KG**—Reactions were set up at 25 °C under argon in light proofed 2.5-m1 glass vials fitted with septa. Reaction mixtures (final volume, 0.5 ml) contained 10 μM GlmES protein, 10 μM 5′-[3H]AdoCbl (100,000 dpm/mmol) in 50 mM potassium phosphate buffer, pH 7.0. The reaction was initiated by addition of 50 μl of 100 mM 2-KG to give a final concentration of 10 mM. At various times, 50-μl portions of the mixture were withdrawn, and the reaction was quenched by the addition of an equal volume of 5% trifluoroacetic acid solution. The reaction products were separated by HPLC on a C8 reverse phase column as described previously (9), and the tritium content of the recovered 2-KG and AdoCbl was determined by scintillation counting.

**Position of Tritium within 2-KG**—A sample containing 10 μM glutamate mutase, 10 μM 5′-[3H]AdoCbl (10,000 dpm total activity) and 10 mM 2-KG in 200 μl of 50 mM potassium phosphate buffer, pH 7.0, was allowed to react for 45 min at room temperature, after which time exchange of tritium into 2-KG was essentially complete. AdoCbl was removed from the mixture by adsorption onto activated charcoal, and the sample was divided into two portions: one-half was brought to pH 10.2 by addition of sodium bicarbonate buffer, and as a control, the other half was maintained at pH 7.0. The samples were allowed to equilibrate for 24 h and then the water was removed by bulb-to-bulb distillation under vacuum. The tritium contents of the distilled water and the nonvolatile fractions were determined by scintillation counting.

**NMR Spectroscopy**—A 0.5 ml solution containing 50 μM enzyme, 50 μM AdoCbl, 50 mM potassium phosphate buffer, pH 7.0, in D2O was made anaerobic and introduced into an NMR tube. An anaerobic solution of either 2-KG or L-glutamate was then added (final concentration 5 mM), and after 20 s, the spectrum of the enzyme-substrate complex recorded.

**Results**

Our experiments used an engineered form of glutamate mutase, GlmES, in which the weakly associating E and S subunits of the wild-type enzyme have been genetically fused into one protein chain by introduction of an eleven amino acid linker sequence (15). This enzyme catalyzes the reaction with an efficiency similar to that of wild-type enzyme, but it binds coenzyme stoichiometrically and in a manner that is independent of protein concentration. These properties of the GlmES protein greatly simplify interpretation of mechanistic experiments (10, 15).

**Inactivation of Glutamate Mutase by 2-KG**—Our initial experiments focused on whether 2-KG or the hydrated form that predominates in solution acted as either a competitive inhibitor or an irreversible inhibitor of glutamate mutase. 2-KG did not appear to bind very tightly to the enzyme, as no inhibition of activity was observed at concentrations up to 1 mM; for comparison, the $K_m$ for L-glutamate is 0.5 mM. It was not possible to extend measurements to higher concentrations of 2-KG as the compound absorbs at 240 nm, the wavelength used to assay for glutamate mutase activity.

Despite the apparently weak binding of 2-KG, upon incubation with glutamate mutase and AdoCbl over the course of several hours the compound was found to irreversibly inactivate the enzyme (Fig. 2). The initial loss of activity was well described by pseudo-first order kinetics with $k_{\text{inact}} = 0.058 \pm 0.008 \text{ min}^{-1}$ and was accompanied by degradation of the coenzyme, which occurred with essentially identical kinetics, $k = 0.061 \pm 0.008 \text{ min}^{-1}$, suggesting that the two processes were mechanistically linked. Control experiments established that neither AdoCbl nor 2-KG alone caused significant loss of enzyme activity, indicating that inactivation results from reaction of the coenzyme and substrate on the protein. However, very little loss of activity was observed when the experiments were repeated under rigorously anaerobic conditions (Fig. 2).

Taken together, these observations suggested that, upon binding to the holoenzyme, 2-KG was able to initiate homolysis of AdoCbl and that inactivation resulted from the subsequent reaction of oxygen with free radical species. It was apparent that neither all the coenzyme was degraded nor all the activity lost. This phenomenon may be attributed to the dilution of both active enzyme and AdoCbl that occurs during the reaction, which, in turn, results in dissociation of the enzyme-AdoCbl complex so that at low concentrations the reaction is no longer pseudo-first order.

**UV-visible Spectrum of the Holoenzyme-2-KG Complex**—When glutamate mutase holoenzyme is mixed with either L-glutamate or L-threo-3-methylaspartate, changes in the UV-visible spectrum of AdoCbl are observed that are associated with the formation of Co(II) species on the enzyme (10, 17). We therefore examined whether 2-KG initiated similar spectral changes indicative of Cbl(II) formation, as would be expected if radical species accumulated on the enzyme. Addition of 2-KG (final concentration 5 mM) resulted in a decrease in absorbance at 520 nm and an increase in absorbance at 470 nm (Fig. 3), consistent with the formation of Cbl(II) species on the enzyme.
Adenosylcobalamin-dependent Glutamate Mutase

Increasing the concentration of 2-KG to 25 mM did not result in any further significant changes in the spectrum. Assuming $\Delta_{e,0}$ of 4,000 $\text{cm}^{-1}$, the proportion of enzyme in the Cbl(II) form is 35%, whereas in the presence of 5 mM l-glutamate, 45% of the enzyme is in the Cbl(II) form.

Tritium Exchange between AdoCbl and 2-KG—To seek further evidence that 2-KG was directly participating in the rearrangement analogous to that undergone by the natural substrate, NMR spectroscopy was used to examine whether 2-KG was being converted into 3-methylaspartate or other compounds by the enzyme. No significant changes in the NMR spectrum of the sample were apparent after 24 h. In particular, the rearranged product should contain a methyl group that would give rise to a characteristic doublet in the high field region of the spectrum, but no resonances were evident in this region.

We conservatively estimate that we could detect about 2% conversion of substrate to product under the experimental conditions employed. This would correspond to only four turnovers of the enzyme in 24 h; for comparison, $k_{cat}$ for GlmES is 5 $\text{s}^{-1}$ at 25 °C (15). This result indicates that very little if any of the 2-ketoglutaryl radical generated on the enzyme undergoes rearrangement.

DISCUSSION

The AdoCbl-dependent isomerases in general show very high substrate specificity. This has hampered efforts to gain insight into the mechanism of these enigmatic rearrangements by using substrate analogs that might potentially function as mechanism-based inhibitors of these enzymes. Particularly intriguing...
ing is the ability of these enzymes to stabilize free radical species, formed through homolysis of AdoCbl, by an estimated 25–30 kcal/mol relative to their formation in free solution (18, 19). Our experiments demonstrate that 2-KG participates in a partial catalytic cycle on the enzyme that results in the formation of the C-4 radical of either 2-KG or its hydrazine, however, this radical does not appear to undergo rearrangement to give products.

2-KG is the first molecule, other than the true substrates, for which the reversible transfer of hydrogen between coenzyme and substrate, a crucial step in the mechanism of the enzyme, has been demonstrated. The tritium exchange reaction is rapid, approaching the rate observed previously with methylaspartate (9), indicating that it serves as a proficient substrate for the hydrogen transfer reaction. We have not established the stereochemistry of hydrogen abstraction at C-4 of 2-KG, but it is expected that the pro-S hydrogen is exchanged with AdoCbl by analogy with the stereochemical course of the reaction with L-glutamate. Although incubation with 2-KG under aerobic conditions does lead to inactivation of the enzyme, this appears to be a slow side reaction that is also observed with the natural substrates (13). Oxygen, presumably, reacts with radical species at the enzyme active site, leading to oxidation of the coenzyme and/or the protein that results in inactivation.

It remains unclear whether it is the keto or hydrated form of 2-KG (or both) that is bound by the enzyme. Under the conditions of the reaction the hydrated form predominates, but there is no strong evidence to favor one species over the other, as either can be considered a reasonable mimic of glutamate and could in principle undergo hydrogen abstraction. However, hydration would make a difference in the ability of the substrate radical to undergo rearrangement. In the keto form, the substrate radical could, in principle, rearrange through the intermediary of a cyclopropyl radical in which the unpaired electron is delocalized onto the oxygen: a precedent for such a rearrangement mechanism, analogous to that proposed for the rearrangement of glutamate to methylaspartate (5) would have to apply. This would involve the formation of an oxalyl radical intermediate, as opposed to a glycol radical (as shown in Fig. 1), which may be less energetically favorable.

The UV-visible spectrum (Fig. 3) indicates that about 35% of the holoenzyme exists in the Cbl(II) form when 2-KG is bound, this is similar to the amount of coenzyme cleavage observed with glutamate bound (45%). The identity of the free radical partner to Cbl(II) formed in the reaction of 2-KG with glutamate mutase is of interest. Buckel and co-workers (21) have recently identified the C-4 radical of glutamate as the organic radical that accumulates on the enzyme during catalysis. Furthermore, we have demonstrated that, for both glutamate and methylaspartate, coenzyme homolysis and hydrogen abstraction are kinetically coupled (10). It seems most likely, therefore, that the products of homolysis in the present case are Cbl(II), 5′-deoxyadenosine and 2-ketogluyl radical (or its hydrate), as opposed to 5′-deoxyadenosyl radical and 2-KG.

The mechanism by which AdoCbl-dependent enzymes effect homolysis of the coenzyme, a reaction that is highly endothermic in free solution, to produce relatively stable radical species at the active site remains one of the most challenging aspects of B12 catalysis. Pre-steady state kinetic analysis of the cobalt-carbon bond breaking step is complicated by the fact that the substrate radicals produced go on to rearrange so that multiple equilibria become established on the enzyme (10). In this regard, 2-KG may prove a very useful substrate for studying the coenzyme homolysis/hydrogen atom abstraction reaction because the 2-KG radical, once formed, does not react further. This should simplify the interpretation of kinetic experiments with isotopically labeled substrate and coenzyme that aim to gain insights into the transition state of this reaction.

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