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Identification of a paramagnetic species as an early intermediate in the coenzyme B_{12}-dependent glutamate mutase reaction

A cob(II)amide?*

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Highly active and cobamide-free glutamate mutase was obtained from *Clostridium cochlearium* by a modification of the original purification procedure. After incubation of the enzyme with dithiothreitol, adenosylcobalamin (coenzyme B_{12}) and the substrate (*S*)-glutamate, a paramagnetic species was observed by EPR-spectroscopy. The signal was maximal within 15 ms after mixing with glutamate. Different signals were detected after incubating the system with the competitive inhibitors (2*S*,4*S*)-4-fluoroglutamate or 2-methyleneglutarate instead of the substrate. The former developed with an at least 100-fold lower rate then the signal with glutamate. All three signals are probably due to low-spin cob(II)amide species with an extraordinary low g_{xy} value as compared with cob(II)alamin.

Adenosylcobalamin; Coenzyme B_{12}; Glutamate mutase; *Clostridium cochlearium*; Electron paramagnetic resonance

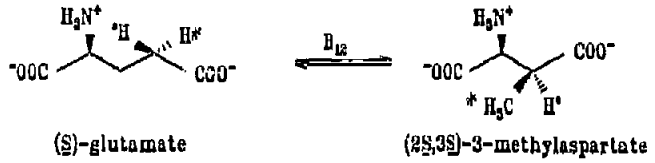
1. INTRODUCTION

Glutamate mutase catalyzes the reversible coenzyme B_{12}-dependent rearrangement of the carbon skeleton of (*S*)-glutamate to (2*S*,3*S*-3-methylspartate. The enzyme from *Clostridium tetanomorphum* was purified and characterised (for a review see [1]). The first pure preparation of glutamate mutase was recently obtained from the related *C. cochlearium* [2]. The enzyme consists of two components: E, a homodimer with a md.wt. of 100 kDa, and S, a monomer of 16 kDa. A cob(II)amide species present in component E was detected by EPR spectroscopy, but its relevance for the catalytic process was not clear.

Coenzyme B_{12}-dependent enzymes catalyze the migration of a group, X, from one corban to an adjacent carbon, while a hydrogen atom migrates in the opposite direction. The mechanism of this rearrangement has not been fully understood until now, especieally when an organic residue, like the glycyl residue in the glutamate mutase reaction, is the migrating X group (Scheme 1). It is generally accepted that the homolytic cleavage of the cobalt–carbon bound in the coenzyme is the first step of catalysis, followed by the abstraction of a hydrogen atom from the substrate by the deoxyadenosyl radical. Radical species were detected in the B_{12}-dependent ribonucleotide reductase [3], diol dehydrase [4] and ethanol-amine-ammonia-lyase [5]. Only in the latter case could a substrate-derived radical be identified by EPR-spectroscopy.

Recently novel paramagnetic species were reported for the carbon skeleton-rearranging methyleneglutarate mutase [6] and methylmalonyl- CoA-mutase [7], which were formed during the course of the reaction. These species could not be characterised and show some similarities to the rapid reaction intermediate found in the coenzyme B_{12}-dependent ribonucleotide reductase from *Lactobacillus leichmannii* [8]. The latter has been interpreted by Coffman et al. [9] as a mixture of two cob(II)amide signals, while a coupling of cobalt(II) to an organic radical was suggested by Pilbrow [10].

A comparable paramagnetic species has now been found in glutamate mutase as well. Here we present EPR-spectra obtained from the purified glutamate mutase from *C. cochlearium* in the presence of adenosylcobalamin and (*S*)-glutamate, (2*S*,3*S*)-4-fluoroglutamate



(S)-glutamate (2S,3S)-3-methylaspartate

Scheme 1: The glutamate mutase reactin.

*Dedicated to Professor Dr. Hermann Eggerer on the occasion of his 65th birthday.

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of 2-methyleneglutarate. They are tentatively interpreted as due to low-spin Co(II) with either 5 or 6 ligands.

2. MATERIAL AND METHODS

2-Methyleneglutarate was synthesised as described in [11]. (2*S*,4*S*)-4-Fluoroglutamate was purchased from Prof. V. Tolman (Czechoslovak Academy of Sciences, Praha, CSFR).

2.1. *Improved purification of component E*

Component E was purified as previously described [2] with some modifications. Charcoal treatment was omitted, as well as the MonoQ chromatographic step. Instead the pooled fractions from the Superdex 200 column (66 ml, 0.8 M ammonium sulfate) were directly applied to a phenyl-Sepharose, high-loaded column (instead of phenyl-Superose, both from Pharmacia, Freiburg). Buffer A contained 5 mM potassium phosphate, pH 7.4, and 1 mM EDTA. Buffer B contained 50 mM potassium phosphate, pH 7.4 1 mM EDTA and 1 M ammonium sulfate. Gradient: 100–20% B (400 ml); 20–0% B (200 ml); 0% B (200 ml). Colourless and highly active component E fractions were pooled and concentrated with Centricon 30 microconcentrators.

2.2. *EPR Spectroscopy*

Spectra were recorded at 77 K with an ECS-106 EPR spectrometer (Bruker). The 200 μM component E and 150 μM component S were preincubated with 5 mM dithiothreitol and 500 μM adenosylcobalamin at ambient temperature in a total volume of 300 μl in an EPR tube before the substrate or the inhibitor was added. The tubes were then immersed in cold isopentane (130 K) and stored in liquid nitrogen. Rapid-mixing, rapid freezing experiments were conducted as described in [12], one syringe containing enzyme, adenosylcobalamin, and dithiothreitol, and the other containing substrate or inhibitors. The reaction was performed at ambient temperature (295 K) and quenched in cold isopentane (130 K). The other methods applied were used as previously described [2].

3. RESULTS AND DISCUSSION

Purity and yield of component E were improved by using phenyl-Sepharose instead of phenyl-Superose hydrophobic interaction chromatography. At the end of a decreasing ammonium sulfate gradient highly active component E (217 nk at mg^{-1}) was eluted from the column with 5 mM potassium phosphate, pH 7.4, showing only the 50 kDa band, as analysed by SDS-PAGE. In contrast to earlier preparations the enzyme was now colourless, indicating the absence of any cobamide. The cob(II)amide signal observed by EPR-spectroscopy, as described earlier [2] (g_{xy}= 2.24; g_z = 2.005), was only found in a red, inactive preparation from the phenyl-Sepharose step. Earlier active preparation were contaminated by this red enzyme. A mixture of pure E and S components incubated with 5 mM dithiothreitol and 250 μM adenosylcobalamin was nearly EPR-silent. After the addition of the substrate or some inhibitors a new paramagnetic species could be observed in the active enzyme.

In the presence of 25 mM (*S*)-glutamate the signal displayed in Fig. 1, trace A was observed. The signal is tentatively ascribed to low-spin Co(II) with the g_{xy} line in the g = 2.1 region and the g_z line in the g = 2.0 region.

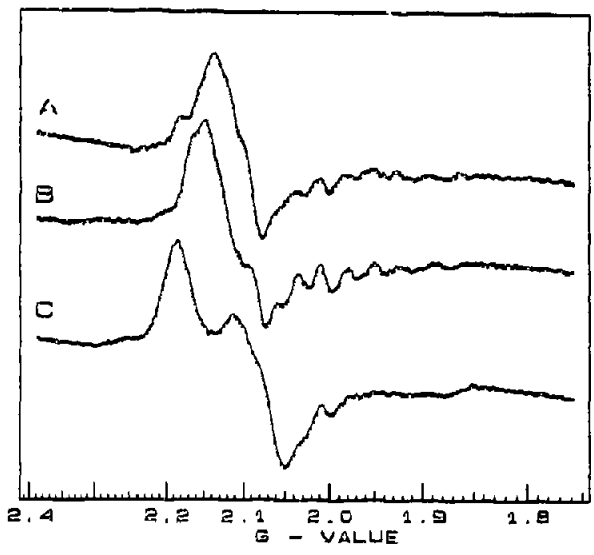

Fig. 1. EPR-spectra of glutamate mutase in the presence of substrate or competitive inhibitors. 200 μM of component E and 150 μM of component S were pre-incubated with 5 mM dithiothreitol, 500 μM adenosylcobalamin before 25 mM (*S*)-glutamate (A), 6 mM (2*S*,4*S*)-4-fluoroglutamate (B) or 6 mM 2-methyleneglutarate (C), respectively, were added. The gain for traces A and B was 1.3 times that of C. EPR conditions: microwave frequency, 9.63 GHz; microwave power, 2.5 mW; modulation amplitude, 0.63 mT; temperature, 77 K.

The multiple lines in the g = 1.9–2.05 region are interpreted as hyperfine splitting due to the nuclear spin of Co (I = 7/2). The linewidth in the z-direction is considerably larger than observed in Co(II)alamin. This could be caused by unresolved hyperfine interaction of the unpaired electron with hydrogen or nitrogen nuclei. A similar, though not identical, signal was detected after addition of 6 mM of the competitive inhibitor (2*S*,4*S*)-4-fluoroglutamate to the glutamate mutase system (Fig. 1, trace B). In the case of the inhibitor, 2-methyleneglutarate (6 mM), a more rhombic-like signal was observed (Fig. 1, trace C). No obvious hyperfine structure was detected in this case. Instead a broad band extending to g = 1.85 was detected. The line shapes and g values of all signals suggest that they are due to low-spin Co(II) with the unpaired electron in an orbital with a predominant d_{z^2} character. The presence of more than one Co(II) species is not ruled out. The g_{xy} values are unusually low, as compared to cob(II)alamin and related cob(II)amides [8]. No signal was observed with the competitive inhibitors, (*S*)-3-methylitaconate or itaconate (5 mM each) [2].

Results from the rapid-mixing, rapid-freezing experiments are given in Table I. In the case of (*S*)-glutamate the signal arose in 15 ms with constant intensity up to 203 ms, indicating that the observed species could likely be an intermediate of the reaction. Earlier experiments showed that this species was unchanged in intensity between 15 s and 15 min, indicating that the reaction was in equilibrium (data not shown). With the competitive inhibitor, (2*S*,4*S*)-4-fluoroglutamate, the signal de-

Table 1

Rapid-mixing, rapid-freezing experiments

Compound	Incubation time (ms)	Relative signal amplitude (cm)
(*S*)-Glutamate (25 mM)	15	14.6
	20	14.3
	30	13.2
	35	15.8
	203	14.4
(2*S*,4*S*)-4-fluoro-glutamate (5 mM)	15	0
	105	0
	>5000*	14.4

*Sample mixed by hand and frozen in cold isopentane (130 K) as described in the text.

veloped in the range of seconds, and thus was at least 100-times slower than the appearance of the glutamate signal. The low rate suggests that the inhibitor not only induces the cleavage of the Co–C bond of the coenzyme but also is itself modified. However, an enzymatic conversion of (2*S*,4*S*)-4-fluoroglutamate is difficult to imagine since the fluorine atom replaces the 4-*si*-hydrogen which is removed from the natural substrate during catalysis.

As yet, determination of the spin concentration of the new species has not been tried. A free radical species (*g* value around 2.002) was not observed in any of the experiments, which would have been detected even in substoichiometric amounts (>1%) with respect to Co(II). The formation of Co(II) after homolytic cleavage of the Co–C bond should be accompanied by the generation of a second paramagnetic species. No other signal could, however, be detected in the field range form 0– 400 mT, even down to a sample temperature of 16 K.

The substrate and two of the inhibitors (Fig. 1) induced different EPR-spectra. This suggests that there might be a close interaction of these agents with the cobalt of the coenzyme and a ligand derived from the added substrate or inhibitors. If so, a change on the line shape of the signal might also be observed with substrates or inhibitors labelled with the stable isotopes, ^{2}H, ^{13}C or ^{15}N. Preliminary experiments, however, showed no significant difference between the line shape of the signal obtained with (*S*)-glutamate and with (*S*)-(2,3,3,4,4-^{2}H)-glutamate.

A possible explanation for the extraordinariiy low g_{xy} value is provided by Coffman et al. [8], who investigated the rapid reaction intermediate observed with the coenzyme B_{12}-dependent ribonucleotide reductase from *Lactobacillus leichmannii*. The authors suggest that the signal is composed of two cobalt(II) species in a ratio of 0.35 to 0.65. From a comparison of the A- and g-values of the simulated signals with model compounds, they concluded that the signals are due to low-spin cobalt(II) with a strongly distorted 6-fold coordination. A carbonyl, amide or sulfhydryl group on the enzyme side chain is proposed to be the sixth ligand. In our case this ligand could be derived from the substrate, inhibitors or possibly deoxyadenosin, indicating that the rearrangement takes place in the coordination sphere of the cobalt(II). Further experiments are required before the nature of the Co(II) complex and of the putative sixth ligand can be established and a more complete picture of the reaction mechanism may be drawn.

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