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# Coordination of a histidine residue of the protein-component S to the cobalt atom in coenzyme $B_{12}$-dependent glutamate mutase from *Clostridium cochlearium*

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**Abstract** **Electron paramagnetic resonance (EPR) spectroscopy of glutamate mutase from *Clostridium cochlearium* was performed in order to test the idea, that a histidine residue of component S replaces the dimethylbenzimidazole ligand of the Co-atom during binding of coenzyme $B_{12}$ to the enzyme. The shapes and the superhyperfine splitting of the $g_z$-lines of the Co(II) EPR spectra were used as indicators of the interaction of the axial base nitrogen with the Co-atom. A mixture of completely $^{15}N$-labelled component S, unlabelled component E, coenzyme $B_{12}$ and glutamate gave slightly sharper $g_z$-lines than that with unlabelled component S. A more dramatic change was observed in the Co(II) spectrum of the inactivated enzyme containing tightly bound cob(II)alamin, in which unlabelled component S caused a threefold superhyperfine-splitting of the $g_z$-line, whereas the $^{15}N$-labelled protein only caused a twofold splitting, as expected for a direct interaction of a nitrogen of the enzyme with the Co-atom. By using a sample of $^{15}N$-labelled component S, in which only the histidines were $^{14}N$-labelled, the EPR spectra showed no difference to those with unlabelled component S. The experiments indeed demonstrate a replacement of the dimethylbenzimidazole ligand in coenzyme $B_{12}$ by a histidine when bound to glutamate mutase. The most likely candidate is H16, which is conserved among the carbon skeleton rearranging mutases and methionine synthase.**

*Key words:* Coenzyme $B_{12}$; Glutamate mutase; *Clostridium cochlearium*; Electron paramagnetic resonance; [$^{15}N$]Histidine

## 1. Introduction

The coenzyme $B_{12}$ (adenosylcobalamin)-dependent glutamate mutase catalyzes the reversible carbon skeleton rearrangement of (*S*)-glutamate to (2*S*,3*S*)-3-methylaspartate [1]. The enzyme from *Clostridium cochlearium* consists of two protein components, E ($\varepsilon_2$, 107 kDa) and S ($\sigma$, 14.8 kDa). The active enzyme complex was characterized as a heterotetramer ($\varepsilon_2\sigma_2$) containing one coenzyme $B_{12}$ [2]. The glutamate mutase genes from *C. cochlearium*, *glmE* [2] and *glmS* [3], as well as from *C. tetanomorphum*, *mutE* [4–6] and *mut S* [6,7], have been cloned, sequenced and overexpressed in *Escherichia coli*. The deduced amino acid sequences of the components S revealed significant similarities to a number of other cobamide-dependent enzymes [7,8] suggesting a cobamide-binding motif. Component S from *C. cochlearium* was shown to be indeed able to bind coenzyme $B_{12}$ [2]. Electron paramagnetic resonance (EPR)-measurements performed with the recombinant protein components of glutamate mutase [2] confirmed a reaction mechanism suggesting the participation of radical intermediates [9,10]. Two different EPR signals were observed. Short incubation (20 s at 25°C) of the substrate or of a competitive inhibitor with the enzyme induced a signal ($g_{x,y} = 2.1$, $g_z = 1.985$), which was interpreted as that of a biradical generated by interaction of cob(II)alamin with an organic radical. It was shown to represent a catalytically active state. The other EPR signal ($g_{x,y} = 2.25$, $g_z = 1.989$) was typical for cob(II)alamin alone when bound to the hydrophobic environment of the enzyme. This signal was formed during inactivation of glutamate mutase as a result of extended incubation of the enzyme (15 h at 37°C) in the presence of coenzyme and substrate or inhibitor under anaerobic conditions.

A comparable inactive glutamate mutase fraction was previously purified from *C. cochlearium*. In contrast to the active components E and S, which were obtained separately, an inactive enzyme fraction was identified as a complex of both protein components and tightly bound cob(II)amide and aquocobamide [11]. All attempts to completely separate these cobamide species from the protein failed. The coenzyme $B_{12}$-dependent 2-methyleneglutarate mutase from *C. barkeri* also contains very tightly bound cobalamin, of which only 50% could be removed by the treatment of the enzyme with 8 M urea [12,13]. Since there was an example for the coordination of the cobalt atom of a *p*-cresolylcobamide from *Sporomusa ovata* to a histidine residue of the peptide chain [14], a direct protein–cobalt interaction was also assumed to be the cause for the tight binding of the cobamides to the mutases [12].

The solution of the crystal structure of the $B_{12}$-binding domain of methionine synthase from *E. coli* revealed this enzyme as the second example for direct cobalt–protein interaction. The cobalt atom of cobalamin is coordinated to a conserved histidine residue (H759 in methionine synthase, H16 in component S of glutamate mutase and H485 in 2-methyleneglutarate mutase) [8,15].

For the first time in a carbon-skeleton rearranging mutase, we here present evidence that a histidine residue of the protein rather than dimethylbenzimidazole coordinates to the Co-atom of coenzyme $B_{12}$.

## 2. Materials and methods

### 2.1. Bacterial strains and plasmids and growth media

*E. coli* strain MC 4100 [16] containing pOZ3 [3] and *E. coli* strain DH5α [17] containing pOZ5 [2] were used for overproduction of glutamate mutase components S and E [2,3], respectively. *E. coli* strain MC 4100 was grown on M9 minimal medium [18]. *E. coli* DH5α was culti-

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vated on Standard I nutrient broth (Merck, Darmstadt). For labelling of component S with $^{15}N$, the *E. coli* strain MC4100 containing pOZ3 was grown on M9 minimal medium containing $(^{15}NH_4)_2SO_4$ (1 g/l). The his$^-$ *E. coli* strain K12 Hfr G6 [19] was grown in M9 medium containing $(^{15}NH_4)_2SO_4$ (1 g/l) and 1 mM [$^{14}$N]histidine.

*2.2. Purification of the components of glutamate mutase and EPR spectroscopy*

The protein components E and S of glutamate mutase were purified according to the method of Zelder et al. [2]. For EPR experiments the assay contained component E (200–500 $\mu$M), component S (200–500 $\mu$M), 2.3 mM coenzyme $B_{12}$, 20 mM Tris-HCl pH 8.3, 10 mM 2-mercaptoethanol. The mixture was incubated at 25°C and the formation of the catalytically relevant EPR signals [2] was induced by addition of 10 mM (*S*)-glutamate or 2-methyleneglutarate followed by further 20 s incubation before freezing the sample in liquid nitrogen. In order to achieve the formation of the 'classical' cob(II)alamin EPR signal, which represents an inactive form of glutamate mutase [2], the mixture was incubated prior to freezing for 15 h under anaerobic conditions at 37°C in the presence of (*S*)-glutamate (10 mM) or the inhibitor 2-methyleneglutarate (10 mM).

EPR spectra were recorded with a Varian E-3 EPR spectrometer at a temperature of 77 K, microwave frequency 9100 MHz, microwave power 25 mW, modulation frequency 100 kHz and modulation amplitude 0.4 mT.

## 3. Results and discussion

For labelling recombinant component S with $^{15}N$, pOZ3, the expression vector containing *glmS*, was transformed into *E. coli* MC 4100. Cultivation of the bacteria on M9 minimal medium containing $(^{15}NH_4)_2SO_4$ as sole nitrogen source followed by induction with isopropyl $\beta$-D-thiogalactopyranoside (IPTG) at $A_{578}$ = 1.0 resulted in overproduction of component S, in which all the nitrogens were $^{15}N$-labelled ([$^{15}$N]component S). The protein was purified to homogeneity using the standard procedure [2,3]. The specific activity of [$^{15}$N]component S was in the range of 600 nkat/mg protein and thus did not differ from that of [$^{14}$N]component S. Unlabelled recombinant component E was overproduced and purified as described earlier [2].

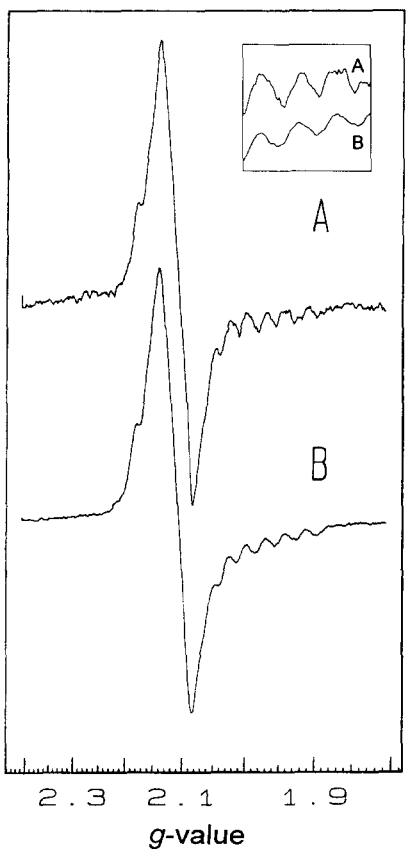


Fig. 1. EPR spectra of active glutamate mutase. The samples containing component E, component S and coenzyme $B_{12}$ were incubated with (*S*)-glutamate for 20 s at 25°C followed by freezing in liquid nitrogen. (A) All nitrogens of component S were $^{15}N$-labelled. (B) All nitrogens of component S except those of the histidines were $^{15}N$-labelled. The insert shows details of the $g_z$-lines.

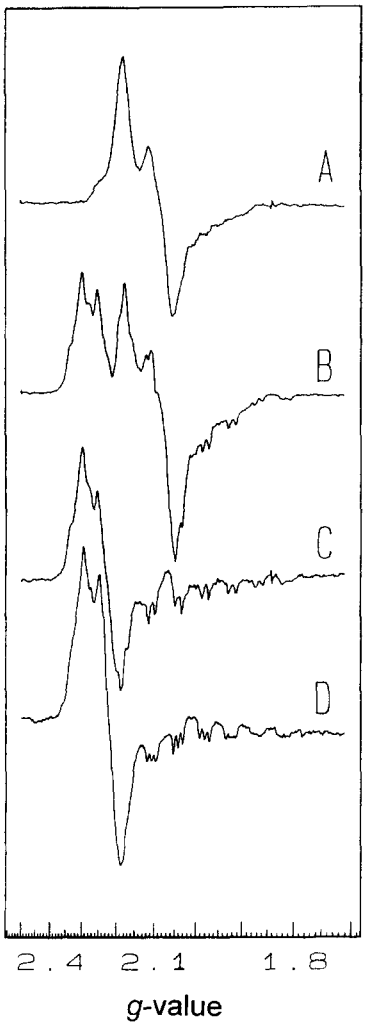


Fig. 2. EPR spectra of inhibited and inactivated glutamate mutase. (A) Conditions as in Fig. 1A, but glutamate was replaced by 2-methyleneglutarate. (B) As in A, except the anaerobic incubation prior to freezing was extended to 15 h at 37°C. (C) is the difference spectrum B − A. (D) Conditions as in Fig. 1B, except the anaerobic incubation prior to freezing was extended to 15 h at 37°C.

After addition of (*S*)-glutamate (Fig. 1A) or the competitive inhibitor 2-methyleneglutarate (Fig. 2A) to samples containing [$^{15}$N]component S, [$^{14}$N]component E and [$^{14}$N]coenzyme $B_{12}$, the 'catalytically relevant' EPR signals were induced. The (*S*)-glutamate dependent signal differed slightly but significantly from the EPR signals previously observed with [$^{14}$N]component S [2]. The values $g_{x,y}$ = 2.1 and $g_z$ = 1.985 were not altered, but the superhyperfine-splitting of the $g_z$-line was less, resulting in

sharper lines. This result suggests that the coenzyme is in the base-on state during catalysis [20] and that the cobalt is coordinated to a nitrogen atom which belongs to component S. In addition the data support the idea that the unresolved superhyperfine-splitting in the $z$-direction of the substrate induced EPR signal (Fig. 1A and B) is not only due to a base-on form of cobalamin, but additionally to the interaction of the cobalt atom with another ligand during catalysis. By replacing (*S*)-glutamate by (*S*)-(2,3,3,4,4-$^2H_5$)-glutamate this second interaction was previously attributed to a hydrogen atom of the substrate or of the 5′-methyl group of 5′-desoxyadenosine [2].

In contrast to the glutamate induced signal, that induced by the inhibitor 2-methyleneglutarate (Fig. 2A) obtained in the presence of [$^{15}$N]component S did not show significant alterations as compared to earlier obtained spectra using [$^{14}$N]component S [2]. After extended (15 h) anaerobic incubation of the sample at 37°C, the inhibitor induced EPR signal was partly converted to the cob(II)alamin signal with a $g_{x,y}$-value of 2.25 (Fig. 2B), which is typically formed after long incubation of glutamate mutase or other $B_{12}$-dependent enzymes in the presence of substrate or competitive inhibitors [2]. The triplet structure in the individual Co-hyperfine lines in the $z$-direction, due to the coupling of the unpaired spin of Co(II) with the nuclear spin of the axial [$^{14}$N]ligand ($I = 1$), was changed into a doublet (Fig. 2B) indicating a replacement of $^{14}$N by $^{15}$N ($I = 0.5$). This is better resolved in the difference spectrum (Fig. 2C = Fig. 2 B − Fig. 2A), which was necessary because of the incomplete conversion of the catalytically relevant 'inhibitor signal' to the cob(II)alamin EPR signal of the inactive enzyme. The twofold splitting clearly shows that the cobalt is coordinated to a nitrogen atom of [$^{15}$N]component S in axial position.

For producing a sample of component S, in which all the nitrogens except those of the histidines were $^{15}$N-labelled, the histidine auxothroph *E. coli* strain K12 Hfr G6 transformed with pOZ3 was used. The cells were grown on a mineral medium supplemented with $(^{15}NH_4)_2SO_4$ and [$^{14}$N]histidine. The (*S*)-glutamate induced EPR signal using this special type of component S is shown in Fig. 1B. There are no differences to the EPR spectra obtained previously with [$^{14}$N]component S [2]. After anaerobic incubation of the sample for 15 h at 37°C, the signal was totally converted to the cob(II)alamin form (Fig. 2D) clearly showing a $^{14}$N superhyperfine-splitting. This result strongly suggests that the nitrogen, which coordinates the cobalt atom of the coenzyme, belongs to a histidine residue of component S. This is in good agreement with the sequence data from several coenzyme $B_{12}$-dependent enzymes [7,8]. The highly conserved histidine residue (H16 in component S) belongs to the coenzyme $B_{12}$-binding motif and the corresponding residue in methionine synthase was already shown to coordinate the cobalt atom [15].

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## References

[1] Barker, H.A., Weissbach, H. and Smyth, R.D. (1958) Proc. Natl. Acad. Sci. USA 44, 1993–1997.
[2] Zelder, O., Beatrix, B., Leutbecher, U. and Buckel, W. (1994) Eur. J. Biochem. 226, 577–585.
[3] Zelder, O., Beatrix, B. and Buckel, W. (1994) FEMS Microbiol. Lett. 118, 15–22.
[4] Holloway, D.E. and Marsh, E.N.G. (1993) FEBS Lett. 317, 44–48.
[5] Becht, M., Kellermann, J. and Plückthun, A.(1993) FEBS Lett. 319, 84–89.
[6] Holloway, D.E. and Marsh, E.N.G. (1994) J. Biol. Chem. 269, 20425–20430.
[7] Marsh, E.N.G. and Holloway, D.E. (1992) FEBS Lett. 310, 167–170.
[8] Beatrix, B., Zelder, O. and Buckel, W. (1994) Eur. J. Biochem. 221, 101–109.
[9] Rétey, J. (1982) in: B12 (Dolphin, D., Ed.) Vol. 2, pp. 357–380, Wiley, New York.
[10] Ashwell, S., Davies, A.G., Golding, B.T., Hay-Motherwell, R. and Mwesigye-Kibende, S. (1989) J. Chem. Soc. Chem. Commun. 1989, 1483–1485.
[11] Leutbecher, U., Albracht, S.P.J. and Buckel, W. (1992) FEBS Lett. 307, 144–146.
[12] Michel, C., Albracht, S.P.J. and Buckel, W. (1992) Eur. J. Biochem. 205, 767–773.
[13] Zelder, O. and Buckel, W. (1993) Biol. Chem. Hoppe-Seyler 347, 85–90.
[14] Stupperich, E., Eisinger, H.J. and Albracht, S.P.J. (1990) Eur. J. Biochem. 186, 657–661.
[15] Luschinsky-Drennan, C., Huang, S., Drummond, J.T., Matthews, R.G. and Ludwig, M.L. (1994) Science 266, 1669–1674.
[16] Casadaban, M.J. (1976) J. Mol. Biol. 104, 541–555.
[17] Hanahan, D. (1985) in: DNA Cloning Vol. 1. A Practical Approach (Glover, D.M., Ed.) Technics for Transformation of *E. coli*, Vol.1, pp. 109–136, IRL Press, Oxford, Washington DC.
[18] Miller, J.H. (1992) in: A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria (C.S.H. Laboratories, Eds.) Vol. 1, p. 437, Cold Spring Habor, New York.
[19] Matney, T.S., Goldschmidt, E.P., Erwin, N.S. and Scroggs, R.A. (1964) Biophys. Biochem. Res. Commun. 17, 278–281.
[20] Pratt, J.M. (1982) in: B12 (Dolphin, D., Ed.) Vol. 1, pp. 325–388, Wiley, New York.