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Purification and some properties of wild-type and N-terminal-truncated ethanolamine ammonia-lyase of *Escherichia coli*

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The methods of homologous high-level expression and simple large-scale purification for coenzyme B_{12}-dependent ethanolamine ammonia-lyase of *Escherichia coli* were developed. The *eutB* and *eutC* genes in the *eut* operon encoded the large and small subunits of the enzyme, respectively. The enzyme existed as the hetero-dodecamer $\alpha_6\beta_6$. Upon active-site titration with adeninylpentylcobalamin, a strong competitive inhibitor for coenzyme B_{12}, the binding of 1 mol of the inhibitor per mol of the αβ unit caused complete inhibition of enzyme, in consistent with its subunit structure. EPR spectra indicated the formation of substrate-derived radicals during catalysis and the binding of cobalamin in the base-on mode, *i.e.* with 5,6-dimethylbenzimidazole coordinating to the cobalt atom. The purified wild-type enzyme underwent aggregation and inactivation at high concentrations. Limited proteolysis with trypsin indicated that the N-terminal region is not essential for catalysis. His-tagged truncated enzymes were similar to the wild-type enzyme in catalytic properties, but more resistant to *p*-chloromercuribenzoate than the wild-type enzyme. A truncated enzyme was highly soluble even in the absence of detergent and resistant to aggregation and oxidative inactivation at high concentrations, indicating that a short N-terminal sequence is sufficient to change the solubility and stability of the enzyme.

Keywords: adenosylcobalamin/coenzyme B_{12}/ethanolamine ammonia-lyase/radical enzyme/truncated enzyme.

Abbreviations: AdoCbl, adenosylcobalamin or coenzyme B_{12}; AdePeCbl, 5-adeninylpentylcobalamin; AdoCbiP-ImPr, adenosylcobinamide 3-imidazolylpropyl phosphate; Buffer A, 50 mM potassium phosphate buffer (pH 8.0); Buffer B, Buffer A containing 0.3 M KCl, 10 mM ethanolamine, and 1 mM PMSF; EAL, ethanolamine ammonia-lyase; His_6, hexahistidine; IPTG, isopropyl-β-D-thiogalactopyranoside; *p*CMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride.

Ethanolamine ammonia-lyase (EAL) (EC 4.3.1.7) or ethanolamine deaminase is an adenosylcobalamin (AdoCbl)-dependent enzyme that catalyses the conversion of ethanolamine to acetaldehyde and ammonia in (eq 1) (*1*).

$$H_3^+NCH_2CH_2OH \longrightarrow CH_3CHO + NH_4^+ \quad \text{(eq 1)}$$

The enzyme was first discovered by Bradbeer in choline-fermenting *Clostridium* sp. (*2*) and shown later to be present in many bacteria in which exogenous vitamin B_{12} is required for growth on ethanolamine (*3*). They include *Escherichia coli* (*4*), *Klebsiella aerogenes* (*4*), *Bacillus megaterium* (*5*) and *Salmonella typhimurium* (*6*). EAL activity was detected in a triethanolamine-fermenting *Acetobacterium* sp. (*7*) as well. EAL was purified to homogeneity from *Clostridium* sp. (8) and *E. coli* (*9*). It is composed of two kinds of subunits—large (α) and small (β) subunits, and the subunit structures of both clostridial (*10*) and *S. typhimurium* (*11*) EALs are $\alpha_6\beta_6$. Although the cobamide bound to clostridial EAL was identified as pseudo-B_{12} (adeninylcobamide) (*12*), AdoCbl serves as coenzyme for all EALs. The α- and β-subunits of EAL are encoded by the *eutB* and *eutC* genes, respectively, in the *eut* operon (*13*), together with other enzymes involved in ethanolamine degradation, regulatory proteins, bacterial microcompartment shell proteins and proteins with unknown functions (*14*). Overexpression and purification of recombinant EAL of *S. typhimurium* have been achieved (*11*).

The mechanism of action of EAL has been extensively studied using clostridial and *Salmonella* enzymes (*15–17*). To date, no X-ray structures of EAL are available yet, although the structure of EutB homohexamer (α_6) has been analyzed recently (PDB ID, 2qez). The 3D structure is awaited for understanding the refined mechanism of action of this enzyme on a molecular level. In order to get crystals for X-ray analysis, we established the methods of high-level homologous expression and of simple purification of *E. coli* wild-type EAL. Since it turned out that purified wild-type EAL undergoes aggregation (precipitation) and inactivation at high concentrations, we attempted to overcome this difficulty by preparing His-tagged truncated EALs with and without mutations. Some properties of *E. coli* wild-type EAL as well as the effects of N-terminal truncations on enzymological properties are also reported here.

Experimental procedures

Materials

AdoCbl was a gift from Eisai Co. Ltd, Tokyo, Japan. 5-Adeninylpentylcobalamin (AdePeCbl) was synthesized by the published procedure (*18*). Adenosylcobinamide 3-imidazolylpropyl phosphate (AdoCbiP-ImPr) and AdoCbiP-[$^{15}N_2$]ImPr were synthesized as described previously (*19*, *20*) using unlabeled and ^{15}N-labeled imidazoles, respectively. Trypsin (*N*-*p*-tosyl-L-phenylalanine chloromethyl ketone-treated) was purchased from Worthington Biochemical Corp. (Lakewood, NJ, USA). Alcohol dehydrogenase and soybean trypsin inhibitor were obtained from Sigma-Aldrich (St Louis, MO, USA). Ni-NTA agarose and polyvinylidene difluoride membrane were purchased from Qiagen GmbH (Hilden, Germany) and Applied Biosystems Inc. (Foster City, CA, USA), respectively. All other chemicals were analytical grade reagents.

DNA manipulations

Standard recombinant DNA techniques were performed as described by Sambrook *et al.* (*21*). Amplification of DNA segments was carried out by PCR with recombinant *Taq* (Invitrogen and Toyobo, Japan) or KOD Dash (Toyobo, Japan) DNA polymerases. Cloning of PCR products was performed by TA-cloning using the Original TA Cloning kit (Invitrogen) or TOPO-XL PCR Cloning kit (Invitrogen), according to the manufacturer's instructions.

Constructions of plasmids

The 3.1 kb *Pvu*II fragment containing entire EAL genes (*eutB* and *eutC*) was excised from DNA prepared from clone #421 of the Kohara *E. coli* genomic λ phage library (*22*), treated with *Taq* DNA polymerase, and cloned into pCR2.1 (Invitrogen). The 2.2 kb *Afl*II-*Eco*RI fragment was obtained from the resulting plasmid. The 630 bp DNA fragment containing the 5′-terminal region of *eutB* with preceding *Bam*HI and *Nde*I sites and following *Eco*RI site was amplified by PCR with the same DNA from Kohara clone #421 as template using *Taq* DNA polymerase and eutB-1f and eutB-2r primers (Table 1) and cloned into pCR2.1. The 610 bp *Nde*I-*Afl*II fragment was obtained from the resulting plasmid. The *Nde*I–*Afl*II and the *Afl*II–*Eco*RI fragments were inserted into the *Nde*I–*Eco*RI region of pUSI2ENd (*23*). Unexpectedly, resulting plasmid pUSI2ENd(EAL) contained extra 0.6 kb sequence corresponding to the 5′-terminal region of *eutB* in the upstream of *eutBC*. It was confirmed by sequencing that it was due to co-ligation of the contaminating 620 bp *Afl*II fragment which contains pCR2.1-derived ~10-bp sequence adjacent to the 610 bp *Nde*I-*Afl*II segment. The removal of extra *eutB* 5′-terminal sequence from pUSI2ENd(EAL) by *Nde*I digestion, followed by self-ligation, generated pUSI2ENd(EAL)2. The 171 bp DNA fragment containing the 3′-terminal region of *eutB* with following *Sma*I site was amplified by PCR using *Taq* DNA polymerase and eutB-3f and eutB-4r primers (Table 1) and cloned into pCR2.1. The 140 bp *Bsm*I–*Ava*I fragment from the resulting plasmid and the 1.3 kb *Hin*dIII–*Bsm*I fragment from pSTV(eutBC)2 (*24*) were inserted into the *Hin*dIII–*Ava*I region of pSTV29 (Takara Bio, Japan) to construct pSTV (eutB). The 1.4-kb *Nde*I–*Eco*RI fragment from pSTV(eutB) was inserted into the *Nde*I–*Eco*RI region of pUSI2ENd to produce pUSI2ENd(eutB). The 813 bp DNA fragment containing the *eutC* lacking coding sequence corresponding to amino acid residues 2–29 (referred to as *eutCΔ*) with preceding *Nde*I site and following *Bgl*II site was amplified by PCR using KOD Dash and eutCtr-1f and eutC-2r primers (Table 1), and cloned into pCR2.1 to generate pCR2.1(eutCΔ). Likewise, pCR2.1(eutCΔCS) that replaces the codon for Cys34 of *eutC* on pCR2.1(eutCΔ) with the codon for Ser was constructed using eutCtrCS-1f primer. The 820-bp *Nde*I-*Eco*RI fragments from pCR2.1(eutCΔ) and pCR2.1(eutCΔCS) were inserted into the *Nde*I-*Eco*RI region of pUSI2ENd to generate pUSI2ENd(eutCΔ) and pUSI2ENd(eutCΔCS), respectively. A pair of synthetic oligonucleotides his6-f and his6-r (Table 1) carrying the sequence for the N-terminal hexahistidine (His_6)-tag was hybridized, phospholylated by T4-polynucleotide kinase and inserted to the *Nde*I site of pUSI2ENd(eutB) to produce pUSI2ENd(His_6eutB). The 1.5 kb *Bam*HI–*Bgl*II fragment from pUSI2ENd(His_6eutB) was inserted into the *Bam*HI site of pUSI2ENd (eutCΔ) and pUSI2ENd(eutCΔCS) to generate pUSI2ENd(His_6eutB-eutCΔ) and pUSI2ENd (His_6eutB-eutCΔCS), respectively. The 810-bp DNA fragment containing the entire *eutCΔ* lacking termination codon with preceding *Nde*I site and following *Xho*I site was amplified by PCR using KOD Dash and eutCtr-1f and eutCXho-r primers (Table 1), and cloned into pCR-XL-TOPO (Invitrogen) to generate pCR-XL-TOPO(eutCΔ). The 220 bp *Nde*I–*Csp*45I fragment from pUSI2ENd(eutCΔCS) and the 580-bp *Csp*45I–*Xho*I fragment from pCR-XL(eutCΔ) were inserted into the *Nde*I–*Xho*I region of pET-21b (Merck, Germany) to generate pET-21b-(eutCΔCSHis_6). The 846-bp DNA fragment containing entire *eutCΔ* with C34S mutation, C-terminal His_6-tag sequence, and preceding *Nde*I site and following *Bam*HI site was amplified by PCR using KOD Dash and eutCtrCS-1f and his6Bam-r primers (Table 1) with pET-21b(eutCΔCSHis_6) as template. The resulting DNA fragment was cloned into pCR-XL-TOPO to generate pCR-XL-TOPO (eutCΔCSHis_6). The 830-bp *Nde*I–*Bam*HI fragment from it and the 1.4-kb *Bam*HI-*Sal*I fragment from pUSI2ENd(eutB) were inserted into the *Nde*I–*SalI* region of pET-21b to obtain pET-21b-(eutCΔCSHis_6-eutB).

Cultivation of E. coli *expressing wild-type and truncated EALs*

Recombinant *E. coli* JM109 and BL21(DE3) carrying expression plasmids were aerobically grown at

Table 1. Oligonucleotides used for cloning and construction of plasmids in this study.

Name	Sequence[a]	Location[b] or comments
eutB-1f	AAGGATCCCAT*ATG*AAACTAAAGACCACATTGTTCGGC	*eutB* 1–27 with *Bam*HI and *Nde*I sites
eutB-2r	TTGAATTCGGCTTAAGTTTTCCACGTCGTCAG	*eutB* 587–611 with *Eco*RI site
eutB-3f	TGCTCAACTACCAGACCACC	*eutB* 1202–1221
eutB-4r	TTTACCCGGG*TCA*GAAGAACAGTGACGG	*eutB* 1345–1362 with *Sma*I site
eutCtr-1f	CAT*ATG*GCCACCACCAACTGTGC	*eutC* 88–104 with *Nde*I site
eutC-2r	AGATCT*TTA*TCGGGTCATGTTGATGCCG	*eutC* 867–868 with *Bgl*II site
eutCtrCS-1f	CAT*ATG*GCCACCACCAACagtGC	*eutC* 88–104 with Cβ34S mutation and *Nde*I site
eutCXho-r	CTCGAGTCGGGTCATGTTGATGCCGGATG	*eutC* 863–865 with *Xho*I site
his6-f	T*ATG*GGCCATCATCATCATCATCACAGCAGCG GCCTGGTGCCGCGCGGCAG	N-terminal His_6-tag coding sequence
his6-r	TACTGCCGCGCGGCACCAGGCCGCTGCTGTGAT GATGATGATGATGGCC*CA*	N-terminal His_6-tag coding sequence
his6-Bam-r	GTTAGCAGCGGATCC*TCA*GTGGTG	3′-region of pET-21b His_6-tag coding sequence with *Bam*HI site

[a]Restriction sites added and the initiation/termination codons are indicated by underlines and italics, respectively. The codon corresponding to the Cβ34S mutation is indicated by lower cases. [b]Numbers from the start sites of genes.

30°C or 37°C in LB medium containing ampicillin (50 μg/ml). When the culture reached an OD_{600} of ~0.9, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM for induction. After cultivation for additional 5–6 h, cells were harvested, washed twice with 50 mM potassium phosphate buffer (pH 8.0) (Buffer A), and stored at −80°C.

Purification of recombinant wild-type EAL

All operations were carried out at 0–4°C. About 15.4 g of wet cells were suspended in 31 ml of Buffer A containing 10 mM ethanolamine, 2 mM EDTA and 2 mM phenylmethanesulfonyl fluoride (PMSF), and disrupted by sonication for 10 min at 240 W with a Kaijo TA-5287 ultrasonic destruction system (Japan) after adjusting pH to 8.2. After centrifugation at 23,000*g* for 30 min, the crude membrane fraction containing EAL was washed twice with 50 ml of the same buffer. The precipitate was further washed three times with 31 ml of Buffer A containing 10 mM ethanolamine, 1 mM PMSF and 0.2% Brij35. EAL was then extracted from the precipitate with 31 ml of the same buffer by increasing the concentration of Brij35 to 1%. The suspension was sonicated and centrifuged at 23,000*g* for 20 min, and the supernatant containing EAL was collected. This extraction procedure was repeated twice. The supernatant pooled was further centrifuged at 100,000*g* for 20 min. The extracted EAL was almost homogeneous (Fig. 1). The enzyme solution was then diluted into four volumes of ice-cold 10 mM ethanolamine containing 1 mM PMSF, 1% Brij35 and 5 mM 2-mercaptoethanol and applied to a DEAE-cellulose column (bed volume, 50 ml) which had been equilibrated with 10 mM potassium phosphate buffer (pH 8.0) containing 10 mM ethanolamine, 1% Brij35 and 5 mM 2-mercaptoethanol. The column was washed successively with 250 ml of the same buffer and 250 ml of the same buffer without Brij35. EAL was then eluted with Buffer A containing 80 mM KCl, 10 mM ethanolamine, 1 mM PMSF, 20 mM sucrose monocaprate and 5 mM 2-mercaptoethanol. About 10 ml-fractions were collected. Purified enzyme was stored at −80°C until use.

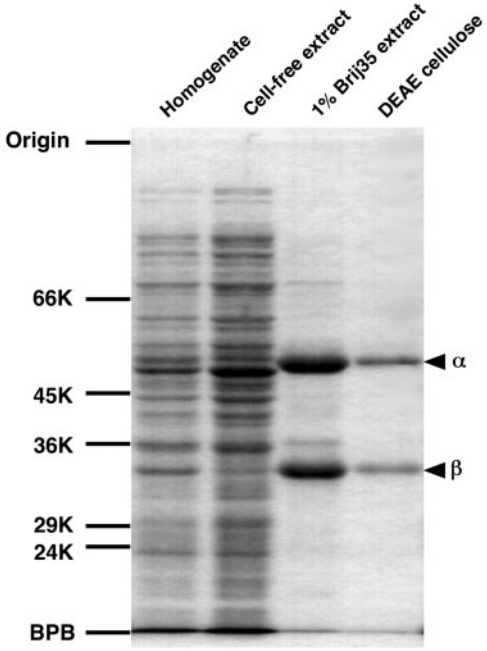

Fig. 1 SDS-PAGE analysis of the wild-type EAL at each purification step. Fractions at each purification step were subjected to SDS–PAGE on 10% polyacrylamide gel. Molecular weight markers, SDS-7 (Sigma). Positions of the α- and β-subunits are indicated with *arrowheads* in the right of the gel.

Purification of His_6-tagged EALs

About 2–3.2 g of wet *E. coli* cells expressing each His_6-tagged EAL were suspended in 5–6.4 ml of Buffer A containing 10 mM ethanolamine and 1 mM PMSF and disrupted by sonication. After centrifugation at 23,000*g* for 30 min, to supernatants were added 1/2 volume of Buffer A containing KCl, ethanolamine, imidazole and PMSF to final concentrations of 0.3 M, 10 mM, 10 mM and 1mM, respectively. The mixtures were then loaded on to a Ni-NTA agarose column (bed volume, 4 ml) that had been equilibrated with Buffer A containing 0.3 M KCl, 10 mM ethanolamine and 1 mM PMSF (Buffer B) plus 10 mM imidazole. The effluents were passed through the column twice.

After washing the columns successively with 40 ml of Buffer B containing 10 mM imidazole and then 40–80 ml of Buffer B containing 20–50 mM imidazole, a His_6-tagged EAL was eluted with 40–70 ml of Buffer B containing 100 mM imidazole. EAL-containing fractions were pooled, dialyzed against Buffer A containing 10 mM ethanolamine and 5 mM 2-mercaptoethanol, and loaded on to a Superose 6 column (10/300GL) attached to the FPLC system (GE Healthcare, UK) that had been equilibrated with the same buffer. The column was developed with the same buffer at a flow rate of 0.4 ml/min, and the EAL-containing fractions were pooled.

Enzyme assays

EAL activity was routinely measured by the 3-methyl-2-benzothiazolinone hydrazone method (*24*, *25*). The standard assay mixture contained an appropriate amount of apoenzyme, 15 μM AdoCbl, 10 mM ethanolamine, 50 mM KCl and 35 mM potassium phosphate buffer (pH 8.0), in a total volume of 1.0 ml. After incubation at 37°C for 10 min, reaction was terminated by adding 1 ml of 0.1 M potassium citrate buffer (pH 3.6). 3-Methyl-2-benzothiazolinone hydrazone hydrochloride was then added to a final concentration of 0.9 mM, and the mixture was incubated again at 37°C for 15 min. The concentration of acetaldehyde formed was determined by measuring the absorbance at 305 nm. One unit is defined as the amount of enzyme activity that catalyses the formation of 1 μmol of acetaldehyde per minute at 37°C under the standard assay conditions. K_m for ethanolamine was determined by the alcohol dehydrogenase–NADH coupled method. In this method, assay mixture contained an appropriate amount of apoenzyme, 15 μM AdoCbl, 0.005–2.5 mM ethanolamine, 0.2 mM NADH, 0.05 mg/ml yeast alcohol dehydrogenase and 40 mM potassium phosphate buffer (pH 8.0), in a total volume of 1.0 ml. Reaction was started by the addition of AdoCbl. The oxidation of NADH to NAD^+ with acetaldehyde at 37°C was monitored by measuring ΔA_{340}.

Protein assays

Protein concentrations in crude extracts were determined by the method of Lowry *et al.* (*26*) with crystalline bovine serum albumin as standard. The concentration of purified EALs was estimated from A_{280}. Molar absorption coefficients at 280 nm ($\varepsilon_{M,280}$) for purified wild-type and truncated enzymes were calculated by the method of Gill and von Hippel (*27*).

SDS–PAGE

SDS–PAGE was carried out as described by Laemmli (*28*). Protein was stained with Coomassie Brilliant Blue R-250. Molecular weight markers used were SDS-7 (Sigma). Densitometric analysis of gels was performed with a Printgraph AE-6911CX system (ATTO, Tokyo, Japan) and the NIH Image program version 1.63 (National Institutes of Health, USA).

Edman sequencing of the subunits

Purified EAL was separated into the α- and β-subunits by SDS–PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane. Protein bands were visualized with Coomassie Brilliant Blue R-250, excised, and analyzed for NH_2-terminal amino acid sequences on an Applied Biosystems 491 protein sequencer.

Trypsin treatment of wild-type EAL

To purified wild-type EAL (3.8 mg/ml) in Buffer A containing 0.07 M KCl, 8 mM ethanolamine and 0.8% Brij35 were added 0, 1, 5, 10, 50 and 100 μg/ml of trypsin. After incubation at 15°C for 15 h, reaction was terminated by the addition of excess soybean trypsin inhibitor (86 μg/ml). The resulting mixtures were subjected to residual activity measurements and SDS–PAGE on 13% polyacrylamide gel.

EPR measurements

Substrate-free apoenzyme was obtained by dialysis against 33 volumes of Buffer A containing 1% Brij35 for 45 h with two buffer changes. About 80 U of the apoenzyme were mixed at 0°C with 50 nmol of AdoCbl in 0.6 ml of 30 mM potassium phosphate buffer (pH 8.0) containing 1% Brij35 in a quartz EPR tube (OD 5 mm) stoppered with a rubber septum. After replacement of the air in the tube with nitrogen by repeated evacuation and flushing, holoenzyme was formed by incubation at 25°C for 3 min. After reaction with 100 μmol of substrate or its analogs at 4°C for 1 min, the mixture was rapidly frozen in an isopentane bath (cooled to ca. −160°C) and then in a liquid nitrogen bath. The sample was transferred to the EPR cavity that had been cooled with a cold nitrogen gas flow controlled by a JEOL JES-VT 3A temperature controller. EPR spectra were taken at −130°C on JEOL JES-RE 3X spectrometer modified with a Gunn diode X-band microwave unit. The mixture was then incubated at 25°C for additional 3, 5 and 25 min and frozen for the second, third and fourth measurements. EPR conditions are as follows: microwave frequency, 9.174–9.176 GHz; modulation amplitude, 1 mT; modulation frequency, 100 kHz; microwave power, 10 mW.

To determine the mode of cobalamin binding, wild-type apoenzyme (ca. 100 U) was incubated at 25°C for 33 min with 50 nmol of AdoCbiP-ImPr or AdoCbiP-[$^{15}N_2$]ImPr in the presence of 0.3 M ethanolamine under a nitrogen atmosphere. The mixtures were rapidly frozen, and EPR spectra were taken at −130°C as described above, except for the microwave power of 20 mW.

Susceptibility to SH inhibitors

Ethanolamine and 2-mercaptoethanol were removed from EAL by dialysis at 4°C against 800 volumes of Buffer A with one buffer change. To 0.1 mg/ml of apoenzymes in Buffer A were added 0–1 mM iodoacetamide. After incubation at 37°C for 30 min, residual iodoacetamide was destroyed with excess mercaptoethanol (10 mM). In the case of *p*-chloromercuribenzoate (*p*CMB) inhibition, 0.1 mg/ml of

apoenzymes were incubated at 37°C for 30 min with 0–5 μM *p*CMB in the presence of 10 mM ethanolamine. Remaining activities of SH-inhibitor-treated EALs were measured with added AdoCbl and ethanolamine after appropriate dilution.

Results and discussion

High-level expression and purification of recombinant E. coli *EAL*

The expression plasmid pUSI2ENd(EAL) contained an extra DNA segment corresponding to the 5′-terminal coding sequence of *eutB* (~0.6-kb) upstream of full-length *eutB* (see Experimental procedures section). This extra sequence encodes a polypeptide with M_r of 24,000 that is not fused to full-length EutB protein and hence does not alter its primary structure. We constructed another expression plasmid pUSI2ENd(EAL)2 as well by removing this extra sequence. Although *E. coli* JM109 harboring pUSI2ENd(EAL)2 expressed EAL in unbalanced subunit ratio ($\alpha \gg \beta$) (data not shown), EAL was produced in well balanced ratio ($\alpha/\beta \sim 1$) by *E. coli* cells carrying pUSI2ENd(EAL) (Fig. 1). Since an upstream gene closer to a promoter region tends to be expressed more efficiently than a downstream gene in general, it seems likely that the presence of the extra region upstream of *eutBC* genes results in the balanced expression of the α- and β-subunits.

Although the EAL polypeptides were expressed mainly in the precipitant fraction, active enzyme was easily solubilized with 1% Brij35-containing buffer (Fig. 1 and Table 2). This is due to the low solubility of the enzyme, rather than the formation of inactive inclusion body. On the basis of this property, we developed a novel, simplified method for purifying *E. coli* wild-type EAL from *E. coli* JM109 carrying pUSI2ENd(EAL). By the purification procedures described above, ~15-fold purification was achieved in an overall yield of 31%. Specific activity of purified enzyme was 55–67 U/mg, which was considerably higher than those reported for purified EALs from *E. coli* (13.9 U/mg) (*9*) and from *Clostridium* sp. (13–21 U/mg) (8) and purified recombinant EAL of *S. typhimurium* (25.3 U/mg) (*11*). Table 2 summarizes a typical result of purification of recombinant *E. coli* EAL.

Purity, molecular weight and subunit structure of purified wild-type EAL

Upon IPTG induction, *E. coli* JM109 carrying pUSI2ENd(EAL) overproduced two polypeptides with M_r of 49,000 and 32,000, which agreed well with the predicted molecular weights of the α (EutB; 49,402) and β (EutC; 31,781) subunits of *E. coli* EAL, respectively. Upon purification, these polypeptides were progressively enriched to the molar ratio of 1 : 0.97, as judged by the densitometric analysis. The N-terminal 6-amino acid sequences of them, determined by Edman sequencing, were MKLKTT and MDQKQI, which agreed completely with those deduced from the nucleotide sequences of *eutB* and *eutC* genes, respectively. These results indicated that the purified enzyme was homogeneous. M_r of EAL determined by gel filtration on a calibrated Superose 6 column was ~470,000 (data not shown). It was thus concluded that the subunit structure of *E. coli* EAL is most likely $\alpha_6\beta_6$. $\varepsilon_{M,280}$ for *E. coli* EAL, calculated by the method of Gill and von Hippel (*27*) from the predicted amino acid compositions and subunit structure, was 302,400 $M^{-1}\,cm^{-1}$. $\varepsilon_{1\%,280}$ was calculated to be 6.21 on the basis of the predicted molecular weight of 487,102.

Kinetic parameters of wild-type EAL

K_m values of recombinant wild-type EAL for the co-enzyme and substrate were determined (Table 3). K_m for AdoCbl was 0.055 μM, which was significantly smaller than those reported previously for purified EALs from *E. coli* (0.42 μM) (*9*) and *Clostridium* sp. (1.5 μM) (8) and purified recombinant EAL of *S. typhimurium* (0.51 μM) (*11*). K_m for ethanolamine (8.2 μM) was also smaller than those of purified EALs of *E. coli* (44 μM) (*9*) and *Clostridium* sp. (22 μM) (8). In clear contrast to the clostridial enzyme (8), monovalent metal ions, such as K^+, were not required for activity (data not shown).

Table 2. Purification of recombinant wild-type and His-tagged truncated EALs.

EAL	Purification step	Total activity (U)	Total protein (mg)	Yield (%)	Specific activity (U/mg)	Purification (fold)
Wild-type	Homogenate	13,120	3,453	(100)	3.8	1
	1% Brij35 extract	7,326	180	56	40.7	10.7
	DEAE cellulose	4,100	74	31	55.1	14.5
$H_6\alpha\bullet\beta\Delta$	Crude extract	755	484	(100)	1.55	1
	Ni-NTA	249	33	33	7.55	4.9
	Superose 6	50.4	4.1	6.7	12.5	8.1
$H_6\alpha\bullet\beta\Delta CS$	Crude extract	708	290	(100)	2.44	1
	Ni-NTA	442	56	62	7.89	3.2
	Superose 6	65.9	4.3	9.3	15.3	6.3
$\beta\Delta CSH_6\bullet\alpha$	Crude extract	10,488	550	(100)	19.1	1
	Ni-NTA	5,540	97.5	53	56.8	2.98
	Superose 6	5,698	90.3	54	63.1	3.31

Table 3. K_m values of wild-type and truncated EALs for coenzyme and substrate.

EAL	K_m for AdoCbl (μM)	K_m for ethanolamine (μM)
Wild-type (α•β)	0.055	8.2
H_6α•βΔ	0.041	7.1
H_6α•βΔCS	0.045	5.9
βΔCSH_6•α	0.078	7.6

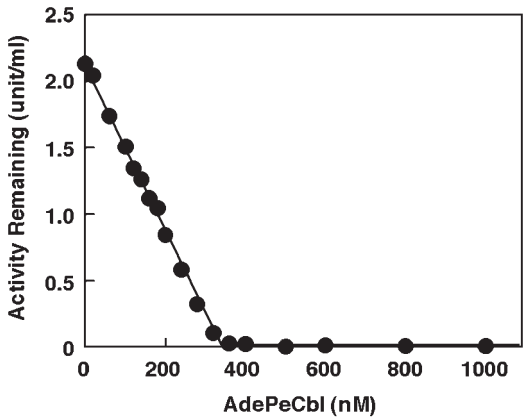

Fig. 2 Active-site titration of the wild-type EAL with AdePeCbl. Apoenzyme (2 U, 61.2 pmol) of the wild-type EAL was incubated with 0–1 μM AdePeCbl at 37°C for 2 h in 1 ml of 35 mM potassium phosphate buffer (pH 7.5) containing 7 mM ethanolamine, 0.7% Brij35 and 1.4 mM 2-mercaptoethanol. After appropriate dilution, remaining activity was measured with added AdoCbl and ethanolamine under the standard conditions.

Active-site titration of wild-type EAL with AdePeCbl

The number of active sites per enzyme molecule (*n*) was reported to be originally two (*29*) and then six (*30*) with clostridial EAL and originally two (*11*) and recently six (*31*) with recombinant EAL of *S. typhimurium*. To determine the number of functional active site of *E. coli* EAL, the active site was titrated with AdePeCbl. AdePeCbl is an inactive analog of AdoCbl that has an adenine ring in the upper axial ligand and is bound very tightly to EAL (*32*) and other AdoCbl-dependent enzymes (*18*, *25*). The incubation of 61.2 pM apoenzyme with 343 pM AdePeCbl resulted in complete inhibition of the enzyme (Fig. 2), indicating that the binding of 1 mol of the inhibitor per mol of the αβ unit leads to complete loss of enzyme activity. It was thus concluded that *n* is six for *E. coli* EAL. This is consistent with its subunit structure.

Optical and EPR spectra of the reacting EAL holoenzyme

Upon incubation of EAL with AdoCbl in the presence of ethanolamine, AdoCbl was converted to cob(II)alamin (B_{12r}) within 1 min of incubation, indicating that the Co–C bond is homolytically cleaved during catalysis. When the apoenzyme was incubated with AdoCbl in the absence of substrate, the enzyme underwent inactivation, irrespective of the presence

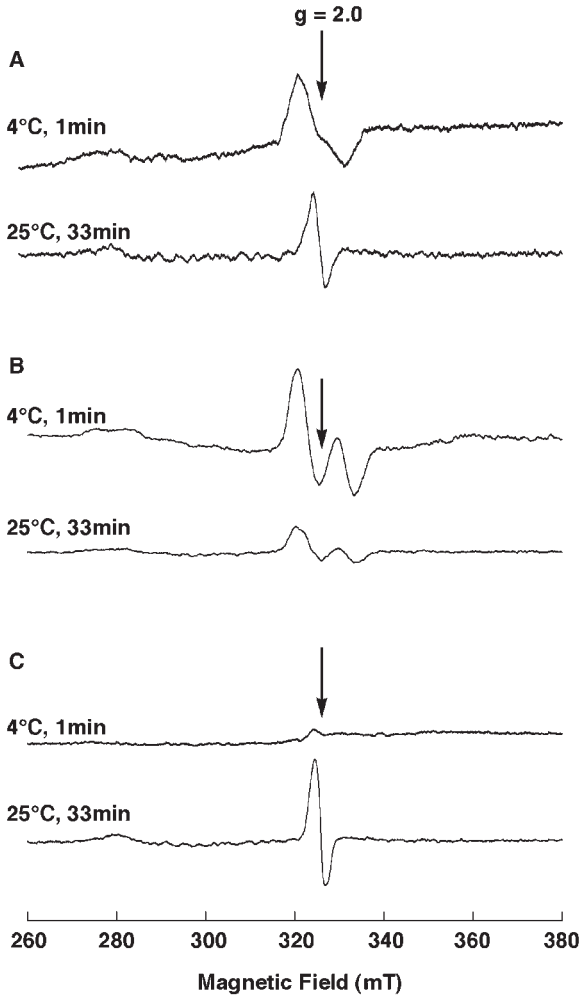

Fig. 3 EPR spectra of the wild-type holoenzyme reacting with substrate and its analogs. Holoenzyme of the wild-type EAL was formed from 80 U of substrate-free apoenzyme and reacted with 100 μmol of ethanolamine (A), 2-amino-1-propanol (B) or 1-amino-2-propanol (C), and EPR spectra were taken as described in the text. The position of $g = 2.0$ is indicated by *arrow*.

of O_2. The B_{12r}-like spectrum was obtained (data not shown), suggesting that the homolytic Co–C cleavage takes place even in the absence of substrate. To get more information about reaction intermediates, EPR spectra of reacting holoenzymes were measured (Fig. 3). Upon incubation of holoenzyme with ethanolamine and 2-amino-1-propanol at 4°C for 1 min under anaerobic conditions, EPR spectra shown in Fig. 3A and B were obtained, respectively. In the similar spectra obtained previously with EALs from other bacteria (*33*, *34*), the high-field signals with *g*-value of ~2 were identified as the ethanolamine-1-yl and 2-amino-1--propanol-1-yl radicals (substrate-derived radicals), and the low-field broad signals were assigned to low-spin Co(II) of cob(II)alamin (*35*, *36*). Such spectra arise from the weak coupling in the Co(II)-organic radical pair (*37–39*). In contrast, almost no signals were observed upon incubation of holoenzyme with 1-amino-2-propanol (Fig. 3C). Upon prolonged incubation with this substrate analog, a singlet signal with *g* ~2 appeared that is similar to that observed with

ethanolamine after substrate consumption. Thus, this might be due to the inactivated holoenzyme although not yet identified.

Mode of cobalamin binding

Cobalamin enzymes bind cobalamin either in the base-on mode (with 5,6-dimethylbenzimidazole coordinating to the cobalt atom) (*20*, *40*, *41*) or in the base-off/His-on mode (with the imidazole group of His residue coordinating to the cobalt atom instead of 5,6-dimethylbenzimidazole) (*42*). To determine the mode of cobalamin binding to *E. coli* EAL, the complex of EAL with a coenzyme analog AdoCbiP-ImPr was incubated with ethanolamine, and EPR spectra were measured. AdoCbiP-ImPr is a coenzyme analog in which the D-ribose moiety and 5,6-dimethylbenzimidazole are replaced by the trimethylene group and imidazole, respectively, and serves as a suicide coenzyme for diol dehydratase (*19*). As shown in Fig. 4, the coenzyme analog was converted to cob(II)alamin upon suicide inactivation, and the superhyperfine splitting of the hyperfine lines in EPR spectra reflected a nuclear spin of the nitrogen atom in the lower axial position. When unlabeled AdoCbiP-ImPr was used, octet hyperfine lines showed superhyperfine splitting into triplets (Fig. 4A), due to the interaction with the ^{14}N nucleus ($I = 1$) of imidazole. In contrast, the octet lines showed superhyperfine splitting into doublets when AdoCbiP-[$^{15}N_2$]ImPr was used (Fig. 4B), suggesting the interaction with the ^{15}N nucleus ($I = 1/2$). These results indicated that Co(II) formed from AdoCbiP-ImPr is coordinated by the coenzyme's imidazole group, but not by the apoenzyme, and hence offered evidence for the base-on mode of cobalamin binding in *E. coli* EAL. This is consistent with the fact that the enzyme does not have the DxHxxG motif, a characteristic motif for proteins that bind cobalamin in the base-off/His-on mode (*42*). The same conclusion was reached by other investigators with the *S. typhimurium* enzyme (*43*, *44*).

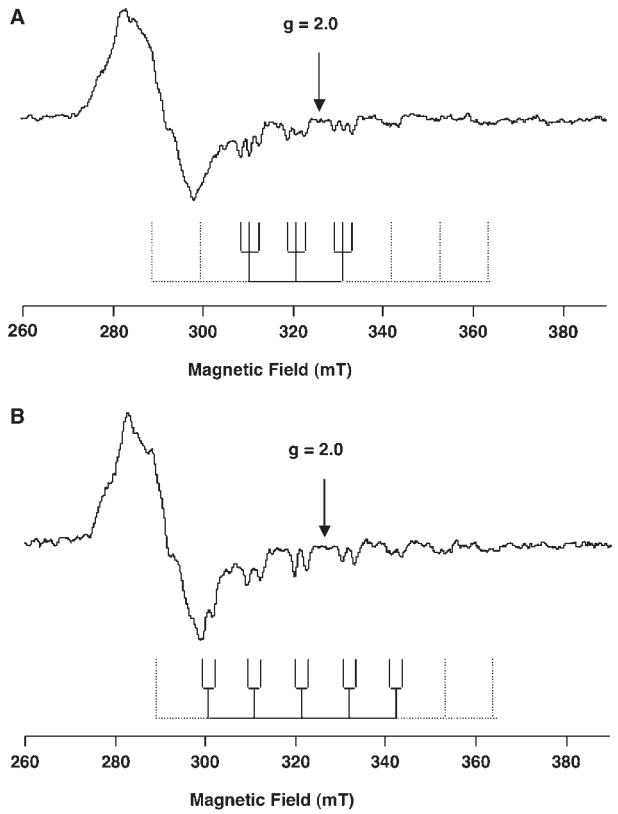

Fig. 4 EPR spectra of the ethanolamine-inactivated complexes of EAL with [$^{14}N_2$]- and [$^{15}N_2$]imidazolyl trimethylene coenzyme analog. Apoenzyme (100 U) was incubated at 25°C for 33 min with AdoCbiP-ImPr (A) or AdoCbiP-[$^{15}N_2$]ImPr (B) in the presence of 0.3 M ethanolamine, and EPR spectra were taken as described in the text. The position of $g = 2.0$ is indicated by *arrow*.

Stability of wild-type EAL

Purified wild-type EAL in sucrose monocaprate-containing buffer showed a tendency to aggregate to form precipitates at high concentrations (>4 mg/ml) with the loss of enzyme activity. Precipitates formed could not be re-solubilized with gentle detergents, such as Brij35, but they underwent solubilization and 5- to 6-fold activation upon incubation at 37°C for 2 h with 10 mM dithiothreitol in Buffer A containing 1% Brij35 (data not shown). The precipitate formation was not observed in Brij35-containing buffer. These results suggested that the oxidation of enzyme SH groups is involved in the aggregation and resulting loss of activity. To crystallize EAL, we attempted to overcome this difficulty and get highly soluble, stable EAL by protein engineering.

Limited proteolysis of wild-type EAL with trypsin

We have reported that short N-terminal regions of subunits determine the solubility of diol dehydratase (*23*). On the analogy of this, we examined the possibility that EALs with truncated subunits might not undergo aggregation and/or inactivation at high concentrations. To determine which terminal can be removed without affecting the EAL activity, the effect of limited proteolysis with trypsin on enzyme activity was investigated. When 3.8 mg/ml of wild-type EAL was digested with 1 μg/ml of trypsin, enzyme activity was almost completely retained (Fig. 5A), but digestion with higher concentrations of trypsin resulted in a severe loss of activity. Upon digestion with 1 μg/ml of trypsin, most (>97%) of the β-subunit underwent proteolysis to a M_r 30,000 polypeptide although the α-subunit was not cleaved at all (Fig. 5B). With >50 μg/ml of trypsin, the M_r 30,000 polypeptide underwent further cleavage, and a M_r 40,000 polypeptide was also formed from the α-subunit. These results indicated that partial digestion of the β-subunit to the M_r 30,000 polypeptide does not affect the enzyme activity, but further proteolysis of subunits leads to significant loss of activity. Edman sequencing indicated that the N-terminal amino acid sequence of the M_r 30,000 polypeptide was CATTNC (Fig. 5C). This sequence corresponds to the amino acid residues from Cysβ29 to Cysβ34 of the β-subunit. The predicted molecular weight of the truncated β-subunit (βΔ) is 28,796. It was thus likely that the M_r 30,000 polypeptide was βΔ that lacks the N-terminal 28 residues.

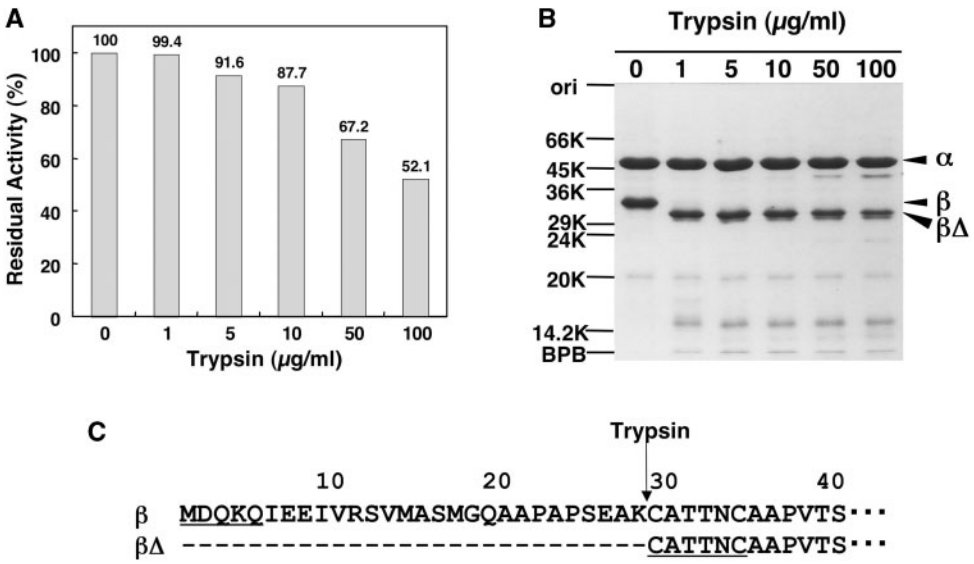

Fig. 5 Tryptic digestion of the wild-type EAL. (A) Enzyme activity remaining after trypsin treatment. (B) SDS–PAGE analysis of digested EAL. Molecular weight markers, SDS-7 (Sigma). Positions of the α- and β-subunits and M_r 30,000 polypeptide are indicated with *arrowheads* in the right of the gel. The band with M_r 20,000 may be the trypsin inhibitor. (C) The site of limited proteolysis with trypsin. The N-terminal amino acid sequences of the β-subunit and M_r 30,000 polypeptide obtained by Edman sequencing are underlined.

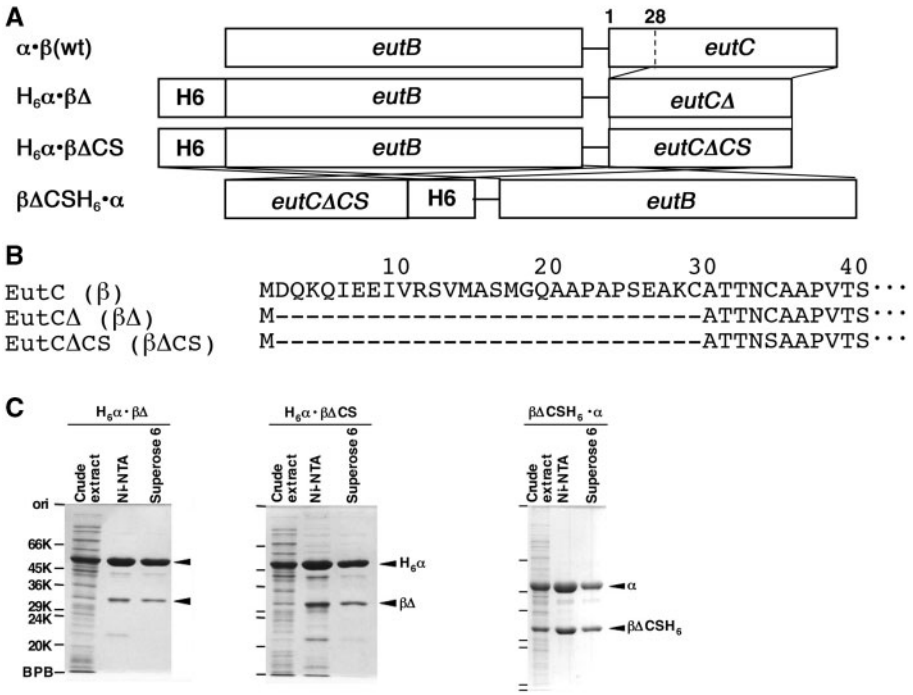

Fig. 6 Expression and purification of His_6-tagged truncated EALs. (A) Schematic representation of the wild-type and truncated EAL genes. *eutB* and *eutBΔ*, genes for the wild-type and truncated β-subunits, respectively. *eutBΔCS*, gene for the truncated β-subunit with the Cβ34S mutation. H6, gene for the His_6-tag peptide. (B) Alignment of N-terminal amino acid sequences of the wild-type and truncated β-subunits. (C) SDS–PAGE analysis of the expression and purification of His_6-tagged truncated EALs. Positions of the α- and β-derived subunits are indicated with *arrowheads* in the right of the gels.

Expression and purification of His-tagged truncated EALs

Since the N-terminal region of the β-subunit does not affect the enzyme activity, we attempted to construct expression plasmids for His-tagged truncated EALs (Fig. 6A and B). H_6α•βΔ EAL consists of the His_6-tagged α-subunit and the β-subunit lacking residues 2-29. H_6α•βΔCS EAL is the same as H_6α•βΔ, but bears the Cβ34S mutation. βΔCSH_6•α EAL is the same as H_6α•βΔCS, but His_6-tag is attached to the C-terminus of the β-subunit with the reversed order of the genes. The α- and β-subunits of *E. coli* EAL contain 9 and 8 Cys residues, respectively. Since Cysβ34 is in the N-terminal region that might determine the solubility and stability of EAL, the Cβ34S mutation was introduced in expectation of increased

resistance to oxidative inactivation of the enzyme. When expressed in *E. coli*, both the $H_6\alpha\bullet\beta\Delta$ and $H_6\alpha\bullet\beta\Delta CS$ enzymes were produced in soluble fractions, but in unbalanced subunit ratios – relatively small amounts of βΔ and βΔCS subunits. In contrast, the $\beta\Delta CSH_6\bullet\alpha$ enzyme was expressed in soluble form in apparently balanced ratio of subunits (Fig. 6C).

These three kinds of truncated EALs were purified to homogeneity by metal-chelate affinity chromatography on a Ni-NTA agarose column, followed by gel filtration on a Superose 6 column. Results of purification are also summarized in Table 2. Even after Superose 6 column chromatography, specific activities of the $H_6\alpha\bullet\beta\Delta$ and $H_6\alpha\bullet\beta\Delta CS$ enzymes were only 23–28% that of the wild-type EAL. SDS–PAGE analysis indicated that purified $H_6\alpha\bullet\beta\Delta$ and $H_6\alpha\bullet\beta\Delta CS$ enzymes contained much less amounts of the βΔ and βΔCS subunits than expected from the amounts of the α-subunit (Fig. 6C). This suggested that low specific activities of these enzymes must be owing to the deficiency of their β-subunit. In contrast, specific activity of purified $\beta\Delta CSH_6\bullet\alpha$ enzyme was almost the same as that of the wild-type EAL. The densitometric analysis showed that this enzyme contained its α- and β-subunits in a molar ratio of about 1 : 1 (Fig. 6C). M_r of this truncated EAL determined by Superose 6 gel filtration was ~426,000, suggesting that it also exists as $\alpha_6\beta_6$.

Comparison of catalytic properties between wild-type and truncated EALs

K_m values of the truncated EALs for coenzyme and substrate are also summarized in Table 3. Binding affinities of the $H_6\alpha\bullet\beta\Delta$, $H_6\alpha\bullet\beta\Delta CS$ and $\beta\Delta CSH_6\bullet\alpha$ enzymes for AdoCbl and ethanolamine were in reasonable agreement between truncated enzymes and coincided with those of the wild-type EAL as well.

To compare the catalytic properties between the wild-type and truncated EALs, inactivation kinetics of holoenzymes were also studied. The incubation of apoenzyme with AdoCbl in the absence of substrate resulted in irreversible inactivation, irrespective of O_2. Although the detailed mechanism of this inactivation is not known yet, it is believed that the inactivation is accompanied by the irreversible Co–C bond cleavage of enzyme-bound AdoCbl and is closely related with the catalytic efficiency of holoenzyme. The apparent inactivation rate constant (k_{inact}) of the truncated EALs for the inactivation in the absence of substrate at 37°C were in the range of 0.41–0.51 min^{-1}. These values were quite similar to that of the wild-type enzyme (0.43 min^{-1}). EAL undergoes irreversible inactivation as well upon incubation with AdoCbl in the presence of 2-amino-1-propanol, a suicide substrate (*45*). The apparent k_{inact} values of truncated EALs for the inactivation with 2-aminopropanol at 37°C were 0.26–0.28 min^{-1}, which reasonably agreeed with that of wild-type enzyme (0.34 min^{-1}). From these lines of evidence, it was concluded that the catalytic properties of truncated enzymes are similar to those of the wild-type EAL, and that neither N-terminal truncation of the β-subunit, Cβ34S mutation, nor addition of His_6-tag to the β-subunit does not affect the catalytic properties of EAL.

Comparison of susceptibilities to SH-inhibitors between wild-type and truncated EALs

The susceptibilities of the wild-type and truncated apoenzymes to SH-inhibitors were compared, because EAL contains essential SH groups the blockage of which abolishes catalytic activity (*8*, *46*). Although sensitivities of the $H_6\alpha\bullet\beta\Delta$ and $H_6\alpha\bullet\beta\Delta CS$ enzymes to iodoacetamide were similar to that of the wild-type enzyme (IC_{50} ~0.07 mM), the $\beta\Delta CSH_6\bullet\alpha$ enzyme was less susceptible to this inhibitor (IC_{50} ~0.24 mM) (Fig. 7A). When *p*CMB was used as SH-inhibitor, somewhat different results were obtained. The wild-type enzyme was highly susceptible to *p*CMB (IC_{50} 0.3 μM), but the sensitivity was lowered by N-terminal truncation and Cβ34S mutation (Fig. 7B). Again, the $\beta\Delta CSH_6\bullet\alpha$ enzyme showed the lowest sensitivity (IC_{50} ~1.3 μM). From these data, it was concluded that both Cysβ29 and Cysβ34 residues in the N-terminal region of β-subunits are not essential for activity, but at least partly responsible for high susceptibility of the wild-type enzyme to this SH-inhibitor.

Stability of truncated EALs

It turned out that, unlike the wild-type apoenzyme, the $\beta\Delta CSH_6\bullet\alpha$ EAL is highly soluble in the absence of detergent and did not undergo either aggregation

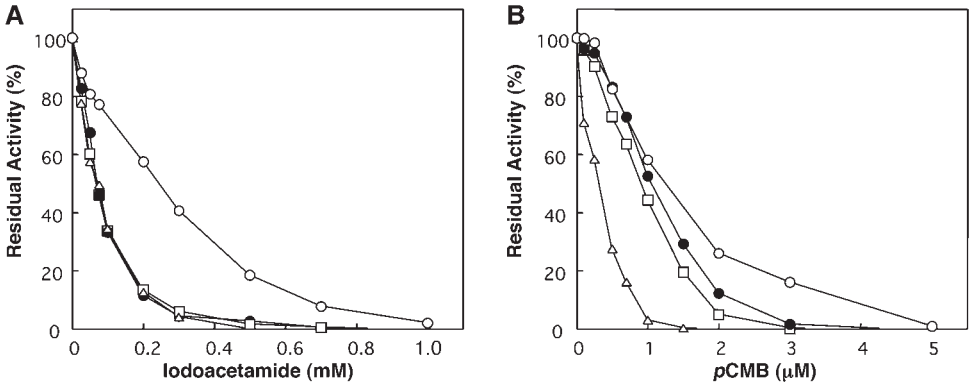

Fig. 7 Susceptibilities of the wild-type and truncated EALs to SH-Inhibitors. (A) Iodoacetamide. (B) *p*CMB. The wild-type (open triangle), $H_6\alpha\bullet\beta\Delta$ (open square), $H_6\alpha\bullet\beta\Delta CS$ (closed circle) and $\beta\Delta CSH_6\bullet\alpha$ (open circle) EALs were incubated with indicated concentrations of SH-inhibitors, and residual activity was measured as described in the text.

(precipitation) or oxidative inactivation even at high concentrations (data not shown). It is therefore evident that a short N-terminal sequence is sufficient to change the solubility and stability of the enzyme. The truncated EAL $\beta\Delta CSH_6{\bullet}\alpha$ might be useful for the crystal structure analysis as well as the mechanistic study of EAL. Very recently, we have succeeded in the crystallization and X-ray analysis of a truncated EAL, in cooperation with Prof. Higuchi's group. These results will be published elsewhere.

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Conflict of interest

None declared.

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