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Co-synthesis of Human δ-Aminolevulinate Dehydratase (ALAD) Mutants with the Wild-type Enzyme in Cell-free System —Critical Importance of Conformation on Enzyme Activity—

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Summary **Properties of mutant δ-aminolevulinate dehydratase (ALAD) found in patients with ALAD porphyria were studied by enzymological and immunological analyses after the synthesis of enzyme complexes using a cell-free system. Enzyme activities of homozygous G133R, K59N/G133R, V153M, and E89K mutants were 11%, 22%, 67%, and 75% of the wild-type ALAD, respectively, whereas that of K59N, a normal variant, was 112%. Enzyme activities of L273R, C132R and F12L were undetectable. Co-synthesis of F12L, L273R, G133R, K59N/G133R, or C132R mutants with the wild-type at various ratios showed that ALAD activity was proportionally decreased in the amount of the wild-type in the complex. In contrast, co-synthesis of V153M, K59N, and E89K with the wild-type did not influence enzyme activity of the wild-type. Surface charge changes in K59N, E89K, C132R and G133R predicted by mutations were also confirmed by native polyacrylamide gel electrophoresis. A compound E89K and C132R complex showed ALAD activity similar to that was found in erythrocytes of the patient. These findings indicate that cell-free synthesis of ALAD proteins reflects enzymatic activities found in patients, and suggest that, in addition to the direct effect of mutations on the catalytic activity, conformational effects play an important role in determining enzyme activity.**

Key Words: **δ-aminolevulinate dehydratase, hepatic porphyria, quaternary structure, cell-free protein synthesis, hetero-oligomer**

Introduction

Heme is the prosthetic group of all hemoproteins, and plays a crucial role in fundamentally important physiological reactions [*1*]. Porphyrias are due to deficiencies of specific enzymes of the heme biosynthetic pathway. ALAD (PBG synthase; E.C.4.2.1.24) porphyria (ADP) is an autosomal recessive disorder caused by a homozygous ALAD deficiency. Six unrelated cases of ADP have been reported to date [*2–7*]. The molecular defects of the first three patients were due to six different mutations (R240W and A274T in German B, G133R and V275M in Swedish boy, and V153M and L273R in German H) which were inherited in a compound heterozygous manner, indicating a highly heterogeneous nature of this disease [*4*, *8*, *9*]. The fourth case was a Belgian adult male who was an exception to the rule as he developed the disease late in his life due to heterozygous G133R mutation [*10*]. G133R had also been found previously in a Swedish boy with ADP [*4*]. The fifth case was the third ADP in Germany, who carried two novel base changes in intron 3 at –11 bp upstream of the exon 3

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start site [*6*]. The sixth case carried two novel mutations within exon 5 of the ALAD gene, resulting in amino acid substitutions, E89K and C132R, respectively [*7*]. Both mutants in this patient influence the binding of the enzyme to zinc, which is essential for enzyme activity. In addition, an asymptomatic healthy Swedish girl was found to have a unique mutant ALAD (F12L) in a heterozygous manner, with 12% enzyme activity in her erythrocytes [*11*]. The F12L mutant, which has also been found in an unrelated patient [*12*], has recently been shown that it displays an altered quaternary structure [*13*].

ALAD catalyzes an asymmetric condensation of two molecules of ALA to form PBG [*14*]. The wild-type ALAD has been shown to be a homo-octamer composed of four hugging dimers [*15*], however, size exclusion studies suggested that there may also be other quaternary assemblies in ALAD mutants [*16*]. For example, though F12L mutation did not occur at the enzyme's active site, F12L mutant protein had a decreased enzyme activity, due to its inability to form an octameric enzyme [*13*]. As there is a critical change in the configuration of an N-terminal arm domain (residue 1-24) of the wild-type in F12L, it leads to the formation of a detached dimer, resulting in an inactive hexamer, instead of an active octamer [*13*]. Other mutants in ADP have also been shown to have an increased propensity to form a low activity hexamer in *E. coli* expression system [*17*]. Based on these findings, Jaffe *et al.* [*18*] proposed that low ALAD activity in some ADP may be due to a disequilibrium of quaternary structure assemblies, and that ADP may be a conformational disease.

Although homozygous or compound heterozygous deficiency of ALAD is very rare, heterozygous enzyme deficiency with 50% normal enzyme activity may not be so rare, as the prevalence of such condition was reported to be 2% in a normal healthy population [*3*]. These individuals with low ALAD activity are clinically unaffected, but may potentially be more vulnerable than normal subjects to toxic effects of chemicals or compounds which inhibit ALAD activity [*19*]. Thus it may be important, from an environmental and prophylactic point of view, to detect heterozygous carriers of ALAD deficiency and to define the nature of their enzymatic defect. In fact, ALAD in the heterozygous carriers of an ADP mutation, namely parents of an ADP patient, showed a decreased sensitivity to lead [*7*].

In order to study the nature of decreased ALAD activities of various ADP mutants, we expressed a mixture of the wild-type and mutant ALAD mRNAs in this study using a cell-free protein synthesizing system to produce an enzyme complex as it occurs in patients' cells. Cell-free system was prepared from insect cells, with pTD1 plasmid as an expression vector, which included the translational enhancer sequence derived from *Malacosma neustria* nucleopolyhedrovirus polyhedrin gene [*20*]. Various ratios of mRNA mixture transcribed from pTD1 plasmid, which encoded the wild-type human ALAD or ALAD mutant cDNAs, were used. Characterization of the synthesized proteins was performed by colorimetric enzyme assays of ALAD activity, and by immunoblot analysis of the proteins separated by PAGE with SDS, followed by detection with a polyclonal antibody against human ALAD. Oligomeric features of the synthesized protein complex were examined using PAGE without SDS (native-PAGE).

Materials and Methods

Construction of expression plasmid

The cDNAs encoding the wild-type and mutant ALAD (F12L, K59N, G133R, K59N/G133R, V153M, L273R, E89K, C132R), which have been found in patients with ADP [*7*, *9–11*], were cloned into pGEM-T Easy vector, and used as the template for the second PCR. Primers used were as follows: a sense primer corresponding to 5'-untranslated region of ALAD cDNA containing the initiation codon; 5'-GGGATATCATGCAGCCCCAGT-3', which was designed to have *Eco* RV site at the 5' end, and an antisense primer corresponding to 3'-untranslated region; 5'-GTTCTAGAG-GGCCTGGCACTGTCCTCCA-3', which was designed to have *Eco* RI site at the 5' end. PCR products were purified by phenol-chloroform extraction, and were inserted into pGEM-T Easy vector (Promega, Madison, WI). Nucleotide sequencing was performed by the dideoxy chain-termination method (SequiTherm Long-Read Cycle Sequencing Kits LC., Epicetre-Technologies, Madison, WI) [*21*]. Subcloned ALAD cDNAs were digested with *Eco* RV and *Eco* RI, followed by purification with QIAEX II Gel Extraction Kit (Qiagen,Tokyo, Japan), and then cDNA fragments were ligated into the pTD1 vector (Shimazu, Kyoto, Japan), which was also digested with *Eco* RV and *Eco* RI.

Synthesis of the wild-type and mutant ALADs in cell-free protein synthesis system

Plasmid pTD1, which encoded ALAD cDNAs, were digested with *Hind* III, and used as the template for mRNA production using CUGA 7 *in vitro* Transcription Kit (NIPPON GENETECH, Tokyo, Japan), as described in the manufacture's protocol. Messenger RNAs were collected and purified by ethanol precipitation and used for the protein synthesis. ALAD proteins were synthesized by addition of prepared mRNA(s) with Transdirect *insect cell* (Shimazu co.), according to the manufacture's protocol.

Immunoblot analysis of proteins synthesized by cell-free system

Human ALAD was specifically detected by immunoblot analysis using rabbit IgG versus human ALAD [*22*]. Synthesized proteins were heated at 100°C for 3 min in SDS

gel-loading buffer (50 mM Tris-Cl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). The samples were loaded on to a 10% polyacrylamide gel containing 0.01% SDS, and electrophoresed according to the method described previously (SDS-PAGE) [*23*]. In order to assess the quaternary structure of synthesized protein, PAGE under a non-denaturing condition (native-PAGE) was carried out, by loading untreated proteins onto the gel without SDS and electrophoresed in the absence of SDS [*11*]. Samples were then transferred to a sheet of nitrocellulose membrane and incubated overnight at 4°C with 10% skim milk in Tris-buffered saline. The membrane was incubated with the primary antibody for 1 h at room temperature. Goat anti-rabbit IgG, conjugated with horseradish peroxidase was used as a secondary antibody. Protein bands to which antibodies were bound were visualized with the enhanced chemiluminescence (ECL) immunoblotting system (Amersham Life Science, Buckinghamshire, England), which was performed according to manufacturer's protocol.

ALAD activity assay

ALAD activity of synthesized proteins was determined colorimetrically as described previously [*14*]. ALAD activity was expressed as the percent of that of the wild-type ALAD, and the specific activity was calculated as ALAD activity per ALAD protein estimated by SDS-PAGE.

Results

Cell-free synthesis of ALAD enzyme complex

In order to determine the effect of ALAD mutations on enzyme activity and composition of multimeric enzyme complexes, the wild-type or mutant ALAD mRNA was translated in a cell-free protein synthesis system. The wild-type and mutant ALADs were detected as a single band at the predicted size of Mr. 36 kDa by SDS-PAGE followed by immunoblot analysis, with the exception of L273R (Fig. 1A). L273R mutant was a deletion mutant, with 2 base deletions at 818 and 819, resulting in a frame shift with a premature stop codon at amino acid residue 294 [*9*]. The predicted size of L273R monomer is 32 kDa, which was also confirmed by SDS-PAGE. In contrast to SDS-PAGE, ALAD proteins applied to native-PAGE resulted in various patterns, reflecting differences in the surface charge of various oligomers (Fig. 1B). The wild-type and V153M mutant ALADs were detected as three bands, with an identical mobility pattern for both isoforms. The three bands presumably reflected an octamer, a hexamer and a dimer in the enzyme complex, as judged from their migration. F12L mutant showed two bands, which corresponded to the hexamer and the dimer of the wild-type complex, as reported previously [*13*]. K59N/G133R mutant, which was found in an adult Belgian ADP patient [*10*], showed a similar pattern as F12L, suggesting that this mutant may also assemble as a low activity hexamer. K59N has one less positive charge per unit than the wild-type enzyme, and, as expected, the mobility of its three bands was slightly faster than those of the wild-type. In contrast to K59N, G133R and C132R have one more positive charge per unit than the wild-type, and both mutants resulted in a drastic change in the proportion of the octamer to the dimer. E89K, which has two more positive charges per unit than the wild-type, resulted in a similar pattern as the wild-type but with a significantly slower mobility. L273R, the only deletion mutant identified in ADP, did not show any detectable band in native-PAGE. ALAD activities of the synthesized proteins (Fig. 1C) were all correlated with the amount of the octamer formed in the enzyme complex (Fig. 1B).

Co-synthesis of F12L with the wild-type ALAD

To reproduce the ALAD protein found in a healthy subject with a 12% ALAD activity due to heterozygous F12L mutation, ALAD mixture was synthesized in cell-free protein synthesis system by using mRNAs of the wild-type and F12L at different ratios (Fig. 2). These enzyme complexes analyzed in native-PAGE showed two bands, one corresponding to the octamer reflecting principally the wild-type, and the other corresponding to the hexamer reflecting F12L, consistent with the findings reported previously in *E. coli* expression system [*13*]. An increase in F12L in the mixture led to a decrease in the octamer formation, and decreased enzyme activity. ALAD protein synthesized from a mixture of an equal amount of the wild-type and F12L mRNAs was predominantly represented by the low active hexamer, with significant reduction in the amount of the active octamer. In addition, the amount of the fastest moving isoform, i.e., the dimer, increased significantly when both ALAD forms were synthesized at an equal ratio of their mRNA. These findings may also account for a 12%, rather than an expected 50%, ALAD activity found in erythrocytes of the subject who was heterozygous for F12L mutation [*11*].

Effect of ALAD mutants on the wild-type ALAD conformation

L273R and V153M, found in a German ADP patient [*9*], were co-synthesized with the wild-type ALAD by cell-free system (Fig. 3). Proteins synthesized showed a mixture of two different sizes by SDS-PAGE, one corresponding to the wild-type (36 kDa), while the other corresponding to L273R (32 kDa) (Fig. 3A). Native-PAGE results showed not only a decreased amount of the octamer but also that of a hexamer and a dimer, when the wild-type-mutant ratio was decreased. Moreover, the amount of octamer detected by native-PAGE was correlated with that of the wild-type ALAD detected by SDS-PAGE (Fig. 3A, B), suggesting that L273R mutant

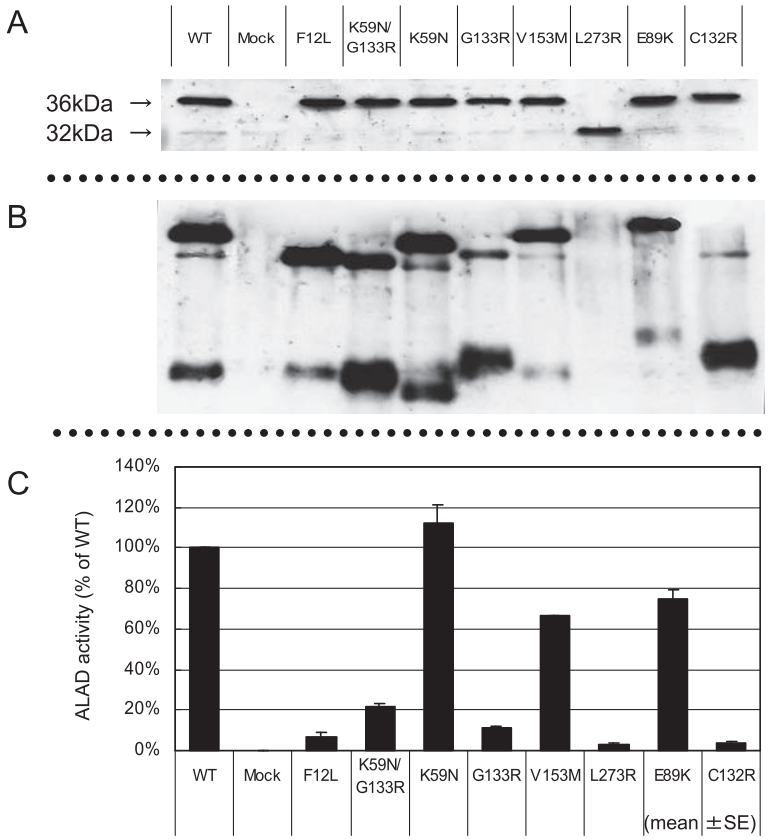

Fig. 1. ALAD mutants synthesized in cell-free system. ALAD protein synthesis followed by the immunoblot analysis and enzyme assay was carried out as described in "Materials and methods." (A) Immunoblot analysis after SDS-PAGE. The molecular size of the wild-type and mutants were 36 kDa, except for L273R, which was 32 kDa. (B) Immunoblot analysis after native-PAGE. (C) ALAD activities in synthesized proteins. Data are the mean ± SE of triplicate assays.

formed no oligomer. ALAD activities found in these proteins were well correlated with the amount of the octamer (Fig. 3B, C, D). Although the other ALAD mutant, V153M, produced as an immunologically positive low activity protein in CHO cells [*9*], V153M mutant itself had significant enzyme activity, accompanied by heteromeric octamer formation as judged from native-PAGE analysis. The enzyme activities found in the complex were also correlated with the amount of the octamer in the complex (Fig. 3B, C).

G133R was found both in a Swedish child [*4*], and in a Belgian adult with ADP [*10*]. Using CHO cells transfected with G133R cDNA, it was demonstrated that this mutation was indeed responsible for the enzyme defect, which is due to a decreased binding of the enzyme to zinc. G133R synthesized *in vitro* in this study showed a serial decrease in the amount of the octamer as well as its enzyme activity, in proportion to a serial increase in the amount of the mutant mRNA in cell-free system (Fig. 4B, C, D). Hetero-oligomers of K59N, a normal variant previously also reported as ALAD2 [*24–26*] with the wild-type were examined using the same cell-free system (Fig. 4). ALAD activity was not affected at all by changing the ratio of the mutant in the mixture to the wild-type (Fig. 4C). Since the surface charge of K59N was altered, hetero-oligomers composed of the wild-type and K59N were expected to show different mobility in native-PAGE. Proteins synthesized *in vitro* by the wild-type and K59N mRNAs showed indeed sequential differences in mobility of oligomers in native-PAGE in parallel to an increase in K59N in the enzyme complex (Fig. 4B), suggesting that K59 and N59 were freely composed to form a hetero-dimer and a hetero-octamer, without affecting enzyme activity (Fig. 4C). Since the Belgian patient carried G133R and K59N on the same allele, we examined the nature of K59N/G133R complex synthesized by cell-free system (Fig. 4). Since K59N resulted in the loss of a positive charge but G133R gained a positive charge, the mobility of this mutant-wild-type complex in native-PAGE was expected to be similar to that of the wild-type, and the results also confirmed this view (Fig. 4). The property of hetero-oligomers composed of the wild-type and K59N/G133R was quite similar to that of the wild-type and G133R hetero-oligomers (Fig. 4).

The first ADP patient in North America carried two novel point mutations, i.e. E89K and C132R, both affecting

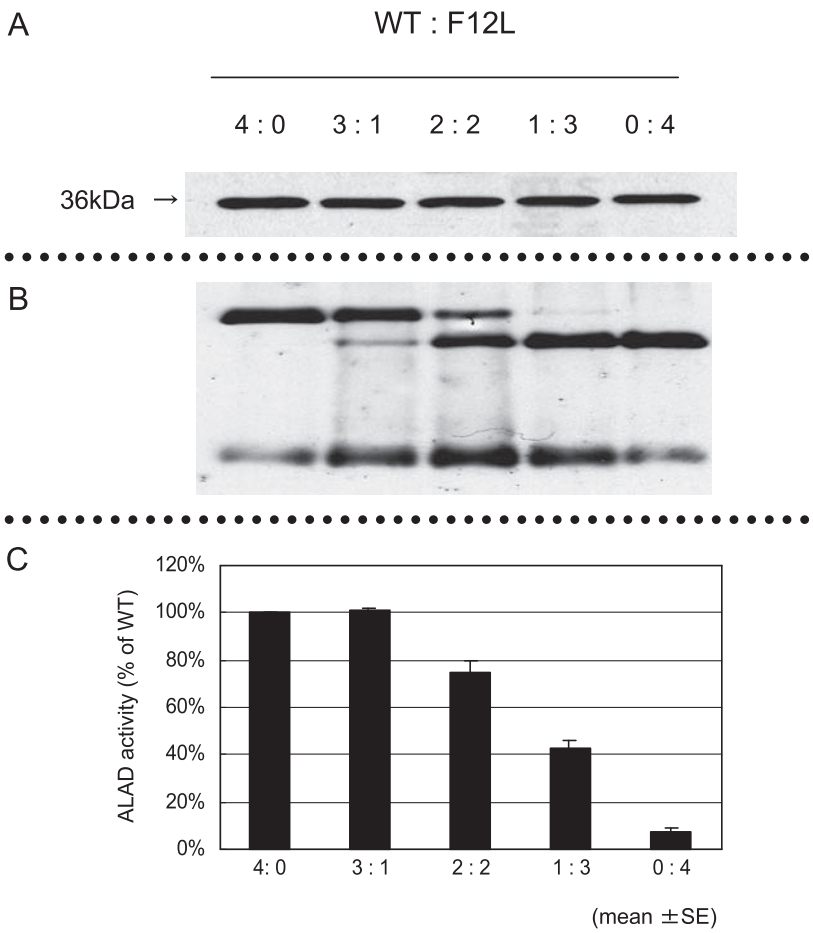

Fig. 2. Co-synthesis of F12L with the wild-type isoform. Messenger RNA encoding F12L ALAD variant is mixed with that of the wild-type in various ratios. ALAD protein synthesis followed by the immunoblot analysis and enzyme assay was carried out as described in "Materials and Methods." (A) Immunoblot analysis after SDS-PAGE. (B) Immunoblot analysis after native-PAGE. (C) ALAD activities in synthesized proteins. Data are the mean ± SE of triplicate assays.

enzyme's sensitivity to lead [*7*]. Since the surface charge of E89K has been altered from negative to positive by the mutation, mobility in native-PAGE was expected to be significantly different. In fact, both the octamer (the main form) and the hexamer (the minor form) moved slower than those in the wild-type, when the amount of E89K was increased in the complex (Fig. 5B). It should be noted that the dimer was detected as three different bands, and each band presumably represented a homozygous wild-type, a heterozygous wild-type/E89K, and a homozygous E89K (Fig. 5B). Enzyme activity decreased only slightly with an increase in the amount of the mutant in the complex (Fig. 5C). In contrast, C132R in the complex decreased ALAD activity more significantly than did E89K (Fig. 5C). Results of native-PAGE suggested that C132R may also form three forms of the dimer, as did E89K (Fig. 5B). ALAD activity of these complexes was well correlated with the amount of the hetero-octamer of the wild-type and C132R in the complex (Fig. 5D).

Compound heterozygous expression of E89K and C132R by cell-free synthesis to mimic the condition in the patient

Finally compound heterozygous expression of E89K and C132R was examined that would have mimicked the condition in the patient [*7*]. These molecular defects were extensively studied by enzymological, immunological as well as by molecular analysis in blood samples from the proband and his family members (Table 1). Since C132R mutant is more stable than E89K by approximately two-fold [*7*], mRNAs encoding E89K and C132R were mixed at a ratio of 1 to 2. Results of three independent experiments all showed that the amount of the active octamer decreased to 22 ± 2% (mean ± SE) of the wild-type (Fig. 6B). Enzyme activity of the complex was also 20% ± 1% (mean ± SE) of the wild-type. Since the level of ALAD protein in the patient was about 44% of the normal subjects [*7*] (Table 1), ALAD activity brought about by E89K-C132R hetero-octamer in the patient was estimated to be about 9% of the wild-type, which was roughly similar to that actually observed in the

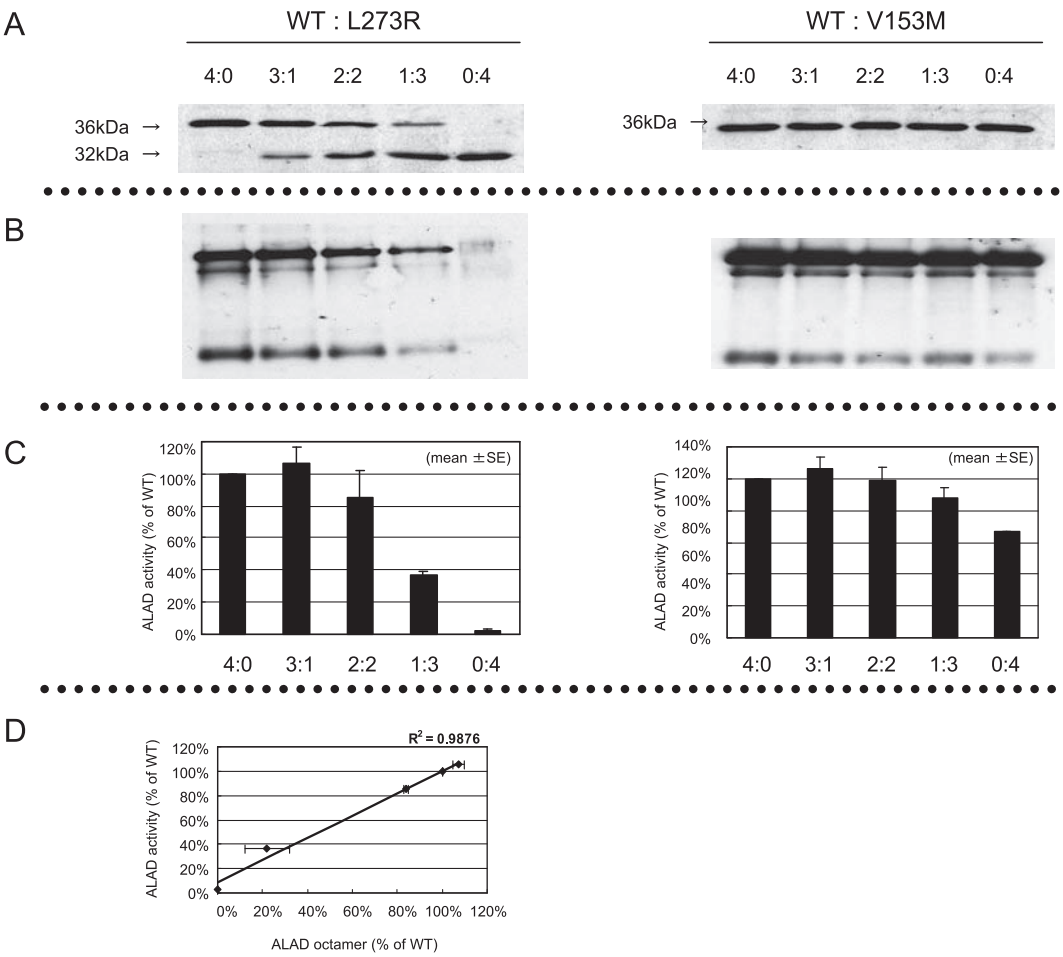

Fig. 3. Co-synthesis of L273R or V153M with the wild-type isoform. Messenger RNA encoding L273R ALAD variant is mixed with that of the wild-type in various ratios (left side). The same experiment was carried out using V153M instead of L273R (right side). ALAD protein synthesis followed by the immunoblot analysis and enzyme assay was carried out as described in "Materials and Methods." (A) Immunoblot analysis after SDS-PAGE. (B) Immunoblot analysis after native-PAGE. (C) ALAD activities in synthesized proteins. Data are the mean ± SE of triplicate assays. (D) The relationship between the enzyme activity and octamer, which was calculated as % of the wild-type. Data are the mean ± SE of triplicate assays.

patient's erythrocytes (1% of normal) (Table 1).

Discussion

Eleven different molecular defects of ALAD have been reported to date in patients with ADP. In this study, we examined the properties of seven mutants as well as those of the wild-type and K59N, a normal variant, by synthesizing them either as a homo- or a hetero-oligomer, using cell-free system. The wild-type ALAD is known to occur as a homo-octamer, consisting of four hugging dimers, and the homo-octameric configuration is thought to be essential for its enzyme activity [*13*]. The enzyme activity is also critically dependent on the availability of zinc, the essential cofactor. For example, displacement of zinc by lead inhibits enzyme activity, while supplementation of zinc relieves the enzyme from the lead-mediated inhibition [*27*]. ALAD mutations such as C132R, G133R and E89K have been shown to occur at or in the vicinity of the active site, i.e., the zinc binding site of the enzyme, and are thought to account for the observed loss of enzyme activity in cells in these patients [*4*, *7*, *10*]. In contrast, F12L mutation which resulted in a major decrease in enzyme activity in a healthy subject heterozygous for this mutation does not involve the active site, suggesting that other aspects may also be important in affecting enzyme activity. Further analysis of F12L mutation demonstrated that there is a critical change in the configuration of an N-terminal arm domain (residue 1-24) of the wild-type in F12L, resulting in the formation of a detached dimer and a low active hexamer, rather than a hugging dimmer and a high active octamer. This finding indicated that conformational alteration may significantly influence enzyme activity. When mutants in ADP such as E89K, C132R, G133R, R240W and A274T were studied by *E. coli*

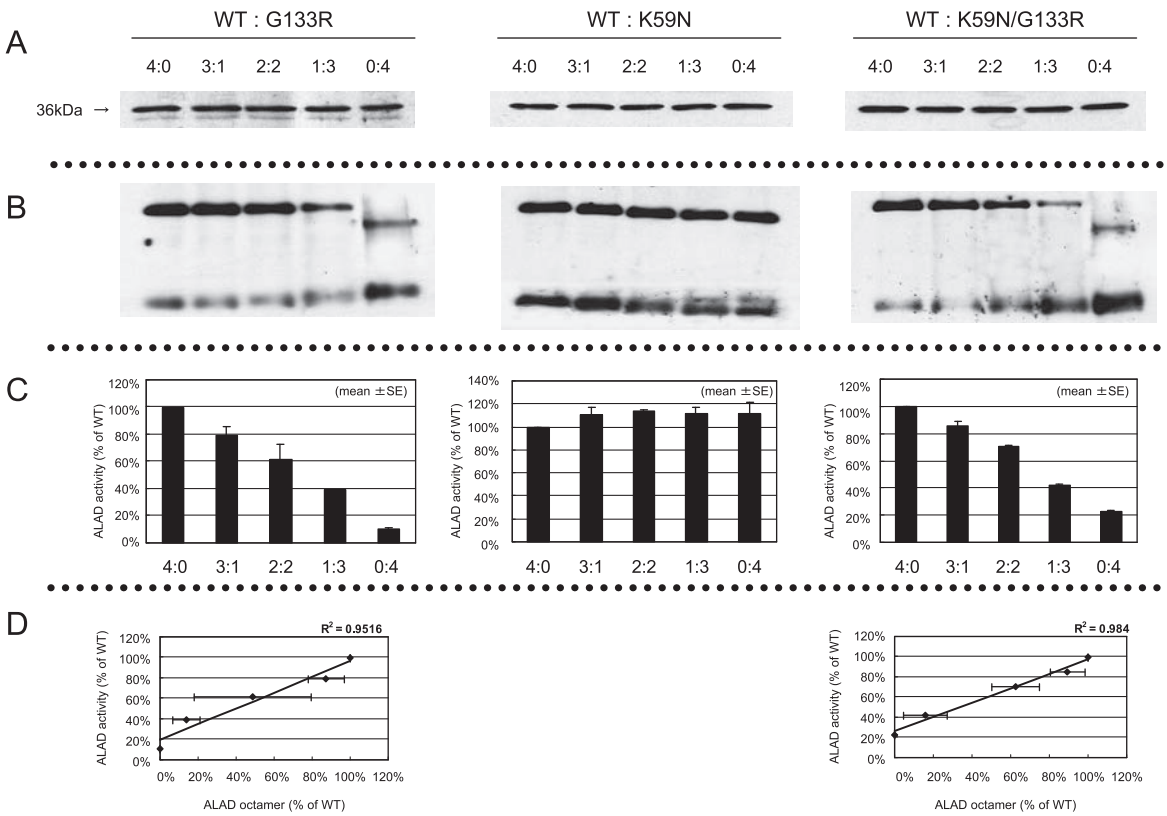

Fig. 4. Co-synthesis of G133R, K59N or K59/G133R with the wild-type isoform. Messenger RNA encoding G133R ALAD variant is mixed with that of the wild-type in various ratios (left side). The same experiment was carried out using K59N (middle) or K59/G133R (right side), instead of G133R. ALAD protein synthesis followed by the immunoblot analysis and enzyme assay was carried out as described in "Materials and Methods." (A) Immunoblot analysis after SDS-PAGE. (B) Immunoblot analysis after native-PAGE. (C) ALAD activities in synthesized proteins. Data are the mean ± SE of triplicate assays. (D) The relationship between the enzyme activity and octamer, which was calculated as % of the wild-type. Data are the mean ± SE of triplicate assays.

expression system, evidence also suggested that these mutants have an increased propensity to form a low activity hexamer. Based on these findings, Jaffe *et al.* [*18*] suggested that ADP may be a conformational disease.

In order to clarify the question whether an alteration in the enzyme assembly may influence its activity, we synthesized ALAD mutants found in ADP by using an insect-cell based cell-free system, together with the wild-type and a normal variant K59N, and studied their properties by enzymological and immunological analyses. Our studies demonstrated that ALAD proteins were produced as respective homo- and hetero-oligomeric complexes, and ALAD activities of enzyme complexes were well correlated with the amount of octamers.

There have been a few other studies that examined ALAD activity of the ADP mutants by using different expression systems. These results are summarized in Table 2, together with our findings in this study. The results of our present study based on cell-free synthesis were generally in good agreement with those reported from other systems such as stable expression in CHO cells, *E.coli* expression system and fused with glutathione S-transferase (GST) and expressed in *E.coli* [*7*, *9–11*, *18*, *28*]. Mutant ALADs synthesized by our cell-free system had, however, generally higher enzyme activity than those obtained by other expression systems (Table 2). The reasons for the observed difference remain unclear, but the products by our cell-free system must be more stable than those obtained by cell-based expression systems, since proteins synthesized by our system are completely free from endogenous proteolysis.

It has been shown that the wild-type ALAD assembles as a homo-octamer and is thought to be responsible for a high enzyme activity found in normal tissues such as circulating erythrocytes [*29*], while F12L mutant which fails to form an octamer results in insignificant enzyme activity [*13*]. Decreased amounts of octamers were found in C132R, G133R and K59/G133R, which were also accompanied by low enzyme activity (Fig. 1). As expected, mutants having different surface charges, e.g., K59N, E89K, C132R and G133R, showed different mobilities in native-PAGE.

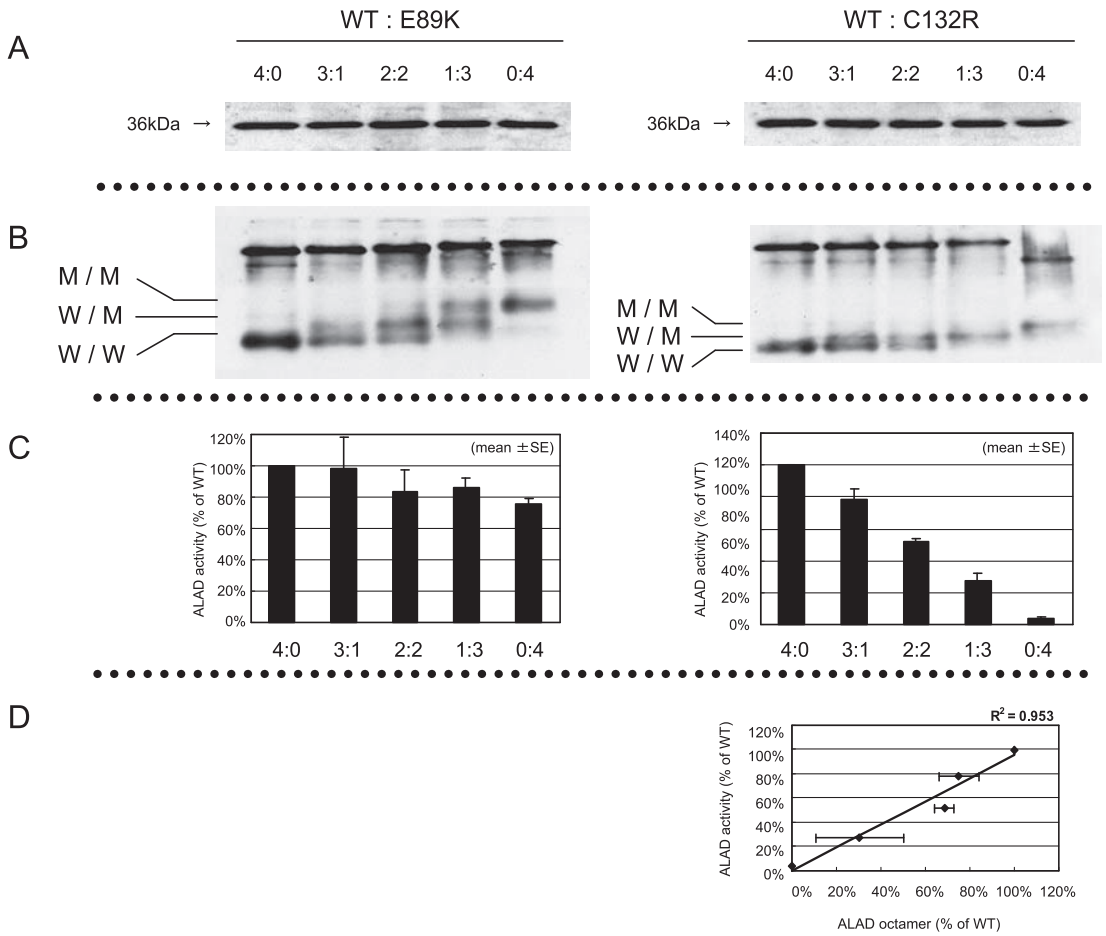

Fig. 5. Co-synthesis of E89K or C132R with the wild-type isoform. Messenger RNA encoding E89K ALAD variant is mixed with that of the wild-type in various ratios (left side). The same experiment was carried out using C132R instead of E89K (right side). ALAD protein synthesis followed by the immunoblot analysis and enzyme assay was carried out as described in "Materials and Methods." (A) Immunoblot analysis after SDS-PAGE. (B) Immunoblot analysis after native-PAGE. (C) ALAD activity in synthesized proteins. ALAD activity was measured as described in "Materials and Methods." Data are the mean ± SE of triplicate assays. (D) The relationship between the enzyme activity and octamer, which was calculated as % of the wild-type. Data are the mean ± SE of triplicate assays.

Table 1. ALAD activity and protein levels determined in the blood from individuals carrying ALAD defect(s)

	ALAD mutant	Activity (% of normal)	Protein (% of normal)	Reference
Homozygous	V153M L273R	1.3	11	[*9*, *30*]
	E89K C132R	1	44	[*7*]
Heterozygous	F12L	12–39	91	[*11*, *12*]
	L273R	34	44	[*9*, *30*]
	V153M	53	ND	[*9*, *30*]
	G133R	23–33	22–59	[*3*]
	K59N/G133R	28–32	ND	[*5*]
	E89K	50	67–78	[*7*]
	C132R	45	63	[*7*]

ND; not determined

Table 2. ALAD activities (% of WT) of mutants found in the patients with ADP by using various expression system

Expression system	F12L	G133R	K59N	K59N/ G133R	V153M	L273R	E89K	C132R
CHO cells	≠0	8.1	94.6	16	11	≠0	10	4
E. coli	0.2	12	100	ND	38	ND	64	0.3
E. coli (GST-fusion protein)	1	8	70	8	41	1	ND	ND
cell-free system	7	11	112	22	67	3	75	4

ND; not determined

Enzyme activities of these mutants also well reflected the amount of octamers in the enzyme complex (Fig. 1). Our results thus suggest that, in addition to the lack of enzyme activity due to a mutation directly affecting the active site, the disruption of quaternary structure assemblies leading to a decreased formation of the octamer is another important factor for a decreased enzyme activity observed in certain patients with ADP.

Co-expression of the wild-type ALAD and F12L in *E. coli* resulted in a hetero-oligomer consisting of each isoform [*13*]. Our present study showed that a mixture of an equal amount of the wild-type and F12L mRNA in a cell-free system yielded a dimer, a hexamer and an octamer (Fig. 2B). These results confirm the proposal made by an earlier study that the high active octamer and low active hexamer as well as dimer, may assemble as hetero-oligomers [*13*, *17*].

A wild-type and a mutant ALAD hetero-oligomers are expected to exist in cells of family members of ADP patients, with a decrease of ALAD activity in a half-normal range (Table 1). In this study, when a wild-type and a mutant such as L273R, G133R, K59N/G133R, C132R, were co-synthesized, enzyme complexes showed a decreased amount of octamers in the complex, as well as decreased ALAD activity which was proportional to the decreased amount of the octamer (Fig. 3, 4, 5). K59N synthesized by cell-free system showed a similar ALAD activity as the wild-type, a finding similar that reported by studies using CHO cells and *E, coli* (Table 2). It was also evident that K59N formed an octameric assembly with normal enzyme activity (Fig. 4). The mobility of proteins of their complexes composed of various ratios of the wild-type and a mutant also resulted in a shift of proteins in proportion to a decrease in the surface charge. Similar results were also observed with G133R, E89K or C132R when they were co-synthesized with the wild-type (Fig. 4, 5).

Hetero-dimers as well as homo-dimers formed by the wild-type and E89K, or C132R, were clearly detected by native-PAGE (Fig. 5). As these dimers were derived from the active enzyme complex which moved more slowly than the wild-type when the amount of the mutant was increased in the complex, these findings suggest that the dimeric complexes must have constituted a part of the active octamer. These results also suggest that ALAD hetero-

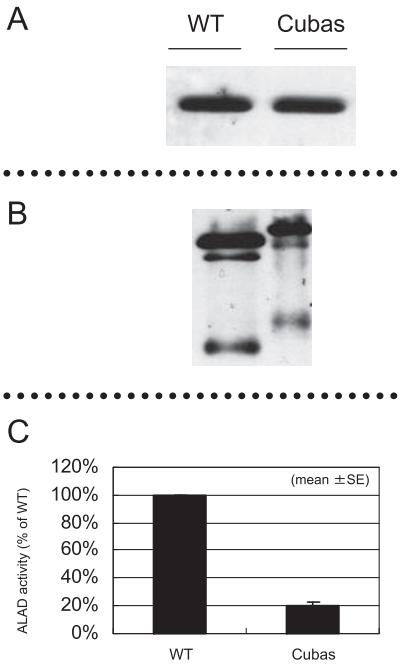

Fig. 6. Compound heterozygous synthesis of ALAD mutants. Mixture of mRNAs encoding E89K and C132R ALAD at the ratio of 1 to 2 and was used for the template of protein synthesis. ALAD protein synthesis followed by the immunoblot analysis and enzyme assay was carried out as described in "Materials and Methods." (A) Immunoblot analysis after SDS-PAGE. (B) Immunoblot analysis after native-PAGE. (C) ALAD activities in synthesized proteins. Data are the mean ± SE of triplicate assays.

dimers may dissociate from the complex, may change conformation, and/or may re-assemble into a different hetero-oligomer with different function. Compound heterozygosity of E89K and C132R in the patient *in vivo* and C132R octamer *in vitro* showed little enzyme activity, while E89K octamer showed a significant enzyme activity *in vitro*, suggesting that C132R exerts a dominant negative effect over E89K (Fig. 6).

The results in the present study establish the fact that, in addition to the effect of mutations directly involving the active site, a disequilibrium in quaternary structure assem-

blies of ALAD also plays a critical role in determining enzyme activity. Use of various ratios of different ALAD mRNAs in cell-free system also demonstrated that significant enzymatic activity is closely correlated with the amount of an active octamer in the enzyme complex. Thus cell-free synthesis of polymeric ALAD enzyme complex is indeed a very useful approach for the investigation into the conformational influences of mutations on enzyme activity, and our study based on such method provided molecular explanation of decreased enzymatic activity in patients with ADP *in vivo*.

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