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Directed evolution of an aminoalcohol dehydrogenase for efficient production of double chiral aminoalcohols

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The aminoalcohol dehydrogenase (AADH) of *Rhodococcus erythropolis* MAK154, which can be used as a catalyst for the stereoselective reduction of (*S*)-1-phenyl-1-keto-2-methylaminopropane to *d*-pseudoephedrine (dPE), is inhibited by the accumulation of dPE in the reaction mixture, limiting the yield of dPE. To improve this weak point of the enzyme, random mutations were introduced into *aadh*, and a mutant enzyme library was constructed. The mutant library was screened with a color detectable high-throughput screening method to obtain the evolved enzymes showing the activity in the presence of a high concentration of dPE. Two mutant enzymes showed higher tolerability to dPE than the wild type enzyme. Each of these enzymes had a single amino acid substitution in a different position (G73S and S214R), and a third mutant enzyme carrying both of these amino acid substitutions was constructed. *Escherichia coli* transformant cells, which express mutant AADHs, showed activity in the presence of 100 mg/ml dPE. A kinetic parameter analysis of the wild type and mutant enzymes was carried out. As compared with the wild type enzyme, the mutant enzymes carrying the S214R amino acid substitution or both the S214R and G73S substitutions showed higher k_{cat} values, and the mutant enzymes carrying the G73S amino acid substitution or both the G73S and S214R substitutions showed higher K_m values. These results suggest that the Ser214 residue plays an important role in enzyme activity, and that the Gly73 residue participates in enzyme–substrate binding.

[**Key words:** Aminoalcohol dehydrogenase; Short-chain alcohol dehydrogenase; Directed enzyme evolution; Amino acid substitution; Enzyme screening]

Optically active aminoalcohols are valuable compounds that have been applied mainly to the production of pharmaceuticals, agrochemicals and chiral building blocks. Ephedrine is an aminoalcohol used in decongestant or anti-asthmatic compounds. Ephedrine has two asymmetric carbon atoms, so that there are four stereoisomers. Among these four isomers, *d*-pseudo enantiomer (*d*-pseudoephedrine: dPE) shows the best combination of high biological activity and low risk, and thus there is need of an efficient and stereospecific production system for supplying dPE.

Recently, we found that $NADP^+$-dependent L-1-amino-2-propanol dehydrogenase (hereafter referred to as aminoalcohol dehydrogenase: AADH) from *Rhodococcus erythropolis* MAK154 catalyzed the stereospecific reduction of (*S*)-1-phenyl-1-keto-2-methylaminopropane ((*S*)-MAK) to dPE (1). In this reaction, only the (*S*)-enantiomer of racemic MAK is reduced by the enzyme; therefore, dPE is specifically formed. The remaining (*R*)-MAK can be spontaneously racemized under weak alkaline conditions, so it is expected that all of the substrate racemic MAK can be specifically converted to dPE using this enzyme. In our previous study, a recombinant *Escherichia coli* strain coexpressing the AADH gene from *R. erythropolis* MAK154 and the glucose dehydrogenase (GDH) gene from *Bacillus megaterium* was constructed, and using cells of the recombinant *E. coli* as catalyst, 40 mg/ml (200 mM) of racemic MAK·HCl was converted to 36 mg/ml (178 mM) of dPE·HCl with a molar reaction yield of 89.1% and optical purity >99% *e.e.* (2). However, the concentration of dPE production was limited to about 40–50 mg/ml, because AADH was inhibited by the accumulated dPE in the reaction mixture. Therefore, in order to put this reaction system to practical use, there is a need for enzymes that are not inhibited by the product accumulation.

AADH belongs to the short-chain dehydrogenase/reductase (SDR) superfamily (3,4), but little is known about the relationships between the structure of this protein and its enzymatic functions, including the mechanisms which inhibit its activity. AADH mutants, which show higher activity in the presence of a high concentration of dPE, should be helpful in future investigations of the structure–function relationships of SDR family proteins. Rational design based on the known three-dimensional structure of a protein can be highly efficient and quite effective in such cases (5–8), but the three-dimensional structure of AADH has not yet been elucidated. More recently, random mutagenesis approaches have become popular (9). Directed enzyme evolution (10–12), the most frequently used strategy, has been

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applied to improve the substrate specificity, activity, enantioselectivity or thermostability of enzymes (13–20). In this paper, to improve the function of AADH, its gene was randomly mutagenized by error-prone PCR, and the mutant enzymes showing higher activity in the presence of dPE were screened. Two mutant enzymes were obtained by the screening, and their mutation sites were identified. The mutated enzymes were then expressed in *E. coli*, purified and characterized.

MATERIALS AND METHODS

Enzymes and chemicals All restriction enzymes and DNA modification enzymes were purchased from Takara Bio (Shiga, Japan), and GDH (75.3 U/mg) was from Amano Enzyme (Aichi, Japan). (*RS*)-1-phenyl-1-keto-2-ethylaminopropane ((*RS*)-EAM), *d*-2-ethylamino-1-phenyl-1-propanol (EPE) and dPE were donated by Daiichi Fine Chemical Co., Ltd. (Toyama, Japan). All other chemicals used in this study were of analytical grade, commercially available and used without further purification.

General DNA technique and DNA sequence analysis All basic DNA procedures, such as plasmid DNA preparation, restriction enzyme digestion, ligation of DNA and transformation of *E. coli*, were performed by following standard protocols (21). The nucleotide sequence was determined by the dideoxy chain termination method (22) using a CEQ dye terminator cycle sequencing kit (Beckman Coulter, Fullerton, CA, USA) with a CEQ2000XL automated sequencer DNA analysis system (Beckman Coulter). Sequence data were analyzed with the program GENETYX (Software Development, Tokyo, Japan).

Analysis of (*RS*)-EAM, EPE and dPE (*RS*)-EAM, EPE and dPE were analyzed by HPLC on an Inertsil Ph-3 column (4.6×75 mm; GL Science, Tokyo, Japan) at 40°C. For this analysis, 50 mM NaH_2PO_4 containing 3% (v/v) acetonitrile was used as a mobile phase and the flow rate was 1.2 ml/min. The absorbance of the eluent was monitored at 220 nm.

Random mutagenesis and library construction Random mutagenesis was carried out by error-prone PCR. Plasmid pKKAADH (2) containing the wild type AADH gene was used as the template. Error-prone PCR was performed using primer-N (5′-CGGAATTCATGTTCAACTCCATTGAAGGTCG-3′; the *Eco*RI restriction site is underlined) and primer-C (5′-CGCCTGCAGTTACAGTTCGCCGAGCGC-3′; the *Pst*I restriction site is underlined) to amplify the gene. To obtain the desired base substitutions (1–3 bp/*aadh*), the conditions used for PCR were optimized as follows. A 100 µl reaction mixture contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 400 pmol of each primer, 50 µM $MnCl_2$, 0.1 ng of template DNA, and 2 U of *Taq* DNA polymerase (Takara Bio). PCR was performed with a PTC-200 automatic thermal cycler (MJ-research, Watertown, MA) for 40 cycles consisting of 94°C for 30 s, 57°C for 30 s, and 72°C for 90 s. The amplified PCR product was digested with *Eco*RI and *Pst*I, and then ligated with plasmid pKK223-3 (GE Healthcare Bio-Sciences, Buckinghamshire, UK) digested with the same two restriction enzymes. The resulting plasmids were introduced into *E. coli* JM109, and then the cells were spread on Luria–Bertani (LB) agar plates containing 50 µg/ml ampicillin. The ampicillin-resistant colonies were used as a mutant AADH library.

Screening method Each colony of the library was inoculated into 100 µl of LB medium containing 50 µg/ml ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in a 96-well plate. After overnight cultivation, cells in each well were harvested by centrifugation and suspended in 50 µl of reaction mixture containing 25 mM Tris–HCl buffer (pH 8.0), 15 mg/ml glucose, 7.5 units/ml GDH, 180 µg/ml $NADP^+$ and 80 mg/ml dPE·HCl. After pre-incubation at 37°C for 30 min with shaking, (*RS*)-EAM·HCl was added for a final concentration of 5 mg/ml, and further incubation was done at 37°C. These reaction conditions were used for both the first and second screening. The first screening was executed with color detection by adding 2 µl of 2 mg/ml phenol red solution after 3 h of incubation. For the second screening, (*RS*)-EAM, EPE and dPE were determined by HPLC after 7 h of incubation.

Construction of $AADH_{M1}$ To construct the G73S/S214R double amino acid substitution enzyme ($AADH_{M1}$), both T642A and G217A base substitutions were combined together using an *Sph*I restriction enzyme site (at positions 377 to 382 on *aadh* (GeneBank accession no. AB084906) as follows. Plasmid $pKKAADH_{W1}$ (carrying T642A) and $pKKAADH_{W2}$ (carrying G217A) were digested with *Sph*I. The larger fragment of $pKKAADH_{W1}$ and the smaller fragment of $pKKAADH_{W2}$ were collected and then ligated. The resulting plasmid ($pKKAADH_{M1}$) encodes the mutant enzyme $AADH_{M1}$.

AADH activity assay in the presence of dPE For coexpression of both *gdh* and the wild type or respective mutant *aadh* in *E. coli* cells, two compatible recombinant plasmids, pACGD (23) and a series of pKKAADH vectors, were simultaneously introduced into *E. coli* JM109. These recombinant *E. coli* strains were cultured in LB medium containing 50 µg/ml ampicillin and 25 µg/ml kanamycin at 37°C. When OD_{660} reached 0.6, 0.2 mM IPTG was added to the medium and further cultivation was performed for 5 h. Then cells were harvested and lyophilized. The reaction was carried out in a 500 µl mixture containing 2 mg/ml of each lyophilized cells, 200 mM Tris–HCl buffer (pH 8.0), 0.3 mg/ml $NADP^+$, 200 mM glucose, 20 mg/ml (*RS*)-EAM·HCl, and 0–100 mg/ml dPE·HCl, and incubation was performed at 37°C with shaking for 20 h. After centrifugation, the resulting supernatant was analyzed by HPLC.

Bioconversion of EAM to EPE A reaction mixture comprising 500 mM sodium phosphate buffer (pH 8.0), 4 mg/ml lyophilized recombinant *E. coli* cells, 100 mg/ml (556 mM) glucose, and 0.8 mM $NADP^+$ in a final volume of 40 ml was used, and 10 mg/ml (46.8 mM) (*RS*)-EAM·HCl was added every 1 h up to a total amount of 80 mg/ml (374 mM). The mixture was vigorously stirred at 37°C, and the pH was adjusted to 8.0 by adding 1 M Na_2CO_3 automatically.

Overexpression and purification of recombinant AADH The genes encoding the wild type or either of the mutant AADH proteins were amplified by PCR using the primers ex-N (5′-GGTAAACAGAACATATGTTCAACTCC-3′; the *Nde*I restriction site is underlined) and ex-C (5′-GCCAAGCTTGGCTGCTCGAGCAGTTCGCC-3′; the *Xho*I restriction site is underlined), and a series of pKKAADH vectors as template. The PCR-amplified products were digested with *Nde*I and *Xho*I, and ligated with pET-21a(+) (Novagen, Madison, WI) digested with the same two restriction enzymes. The resulting plasmids containing the wild type or either of the mutant AADH genes were introduced into *E. coli* BL21(DE3).

These recombinant *E. coli* strains were cultured in LB medium containing 50 µg/ml ampicillin at 37°C. When the OD_{660} reached 0.8, 0.2 mM IPTG was added to the medium, and the culturing was continued at 18°C. After cultivation for 16–20 h, the cells were harvested by centrifugation, suspended in binding buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0), and then disrupted with an ultrasonic oscillator (Insonator 201M; Kubota, Tokyo, Japan). After centrifugation, the resulting supernatant containing AADH (with His-tag) was loaded onto Profinity IMAC Resins (Bio-Rad Laboratories, Hercules, CA) pre-equilibrated with the binding buffer. The resin was washed with 5 volumes of washing buffer (20 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8.0), and then the binding protein was eluted with elution buffer (500 mM imidazole, 50 mM sodium phosphate, 300 mM NaCl, pH 8.0). Active fractions were concentrated and replaced with 100 mM Tris–HCl (pH 8.0) using a Microcon YM-10 filter device (Millipore, Billerica, MA). Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories) using bovine serum albumin as a standard, as described by Bradford (24).

Enzyme kinetics Initial rates were determined by monitoring the decrease in absorbance at 340 nm, corresponding to the conversion of NADPH to $NADP^+$ in a spectrophotometer (UV1650PC; Shimadzu, Kyoto, Japan) at 37°C. To determine the k_{cat} and K_m values for (*RS*)-EAM·HCl of each AADH, the reaction mixture contained 100 mM Tris–HCl (pH 8.0), 0.32 mM NADPH and six different (*RS*)-EAM·HCl concentrations over the range of 0.005–2.0 mM, and K_i values were determined under the same conditions as described above with addition of dPE·HCl in four different concentrations from 0 to 25 mM. The data were fitted to the Michaelis–Menten equation, and represent an average of all statistically relevant data.

RESULTS

Construction of the screening method Because it was difficult to measure MAK-reducing activity or dPE-producing activity in the presence of a high concentration of dPE by HPLC analysis, (*RS*)-EAM was selected as an alternative substrate. EAM is an analogue compound of MAK containing an ethylamino moiety instead of a methylamino moiety of MAK (Fig. 1). (*S*)-EAM was reduced and converted to EPE by AADH. The characteristics and behaviors of EAM or EPE against AADH were quite similar to those of MAK and dPE, respectively (data not shown). The EAM-reducing activity of AADH was also inhibited in the presence of dPE (Fig. 2). The mutant AADHs showing EAM-reducing activity in the presence of high concentration of dPE were expected to show also MAK-reducing activity in the presence of dPE. Therefore, we tried to obtain the evolved AADH which catalyzed the conversion of EAM to EPE in the presence of high concentrations of dPE.

In order to improve AADH, random mutations were introduced into *aadh* by an error-prone PCR. After examining various error-prone PCR conditions, including different Mn^{2+}, dNTP and template concentrations and different polymerase types and cycle numbers, the base substitution rate was set to 1–3 bp per *aadh* to yield an average of one amino acid substitution. This low mutation rate made it possible to identify the contribution of individual amino acid substitutions on enzyme function. DNA sequencing of randomly selected clones showed that 0–2 amino acid substitutions occurred under the conditions chosen, as described in Materials and methods.

Because most amino acid substitutions have either no or deleterious effects on enzyme function, most clones of the mutant library will not be suitable for the desired applications. An efficient high-throughput screening or selection system is, therefore, an absolute prerequisite for identifying the evolved enzyme variants

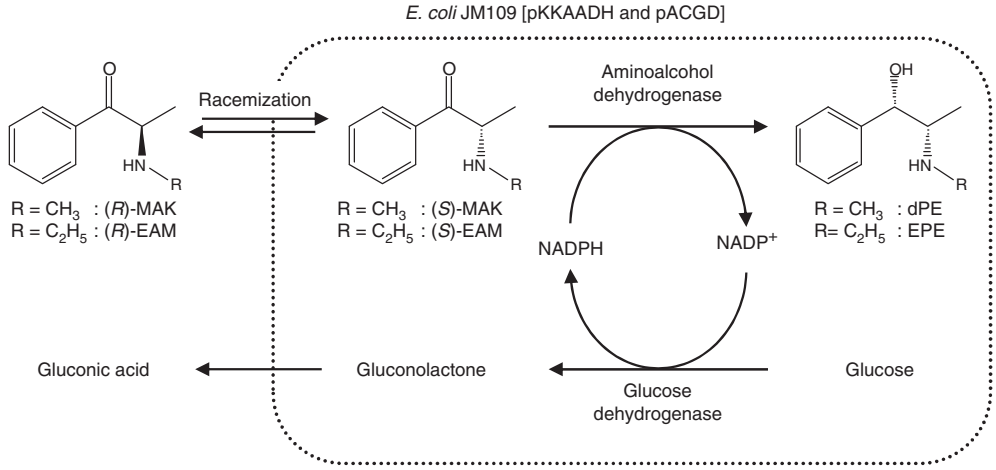

FIG. 1. Asymmetric reduction of the racemic substrate to a chiral product, and the NADPH regeneration system using recombinant *E. coli* cells.

that display improved properties. In this report, the use of a 96-well plate assay for the first screening as described in Materials and methods proved to be a very suitable method. In this screening system, as the reduction of EAM proceeds, glucose is oxidized to gluconolactone for NADPH regeneration, and then gluconolactone is hydrolyzed nonenzymatically and converted to gluconic acid (Fig. 1). Accumulation of gluconic acid causes a drop in the pH of the reaction mixture. Therefore, the color of phenol red, a pH indicator, turns from red to yellow as the reduction of EAM proceeds (Fig. 3).

Directed evolution of AADH Approximately 5000 clones were screened for mutants showing AADH activity in the presence of 80 mg/ml dPE·HCl. About 100 clones that caused the reaction mixture to appear orange or yellow by color detection were screened again by measuring the bioconversion rate of (*RS*)-EAM to EPE with HPLC analysis. Consequently, two clones producing significant amounts of EPE in the presence of 80 mg/ml dPE·HCl were found. From these two clones, the plasmid DNAs (designated pKKAADH$_{W1}$ and pKKAADH$_{W2}$) were isolated and the nucleotide sequences of each *aadh* were determined. Three base substitutions were observed in pKKAADH$_{W1}$ at C111T, T591C and T642A, and two were observed in pKKAADH$_{W2}$ at G217A and A408G. The mutations of C111T, T591C and A408 were silent mutations, and therefore each mutant enzyme had only one amino acid substitution at S214R (pKKAADH$_{W1}$, with a mutation at T642A) or G73S (pKKAADH$_{W2}$, with a mutation at G217A) and these mutant enzymes were named AADH$_{W1}$ and AADH$_{W2}$, respectively.

Considering that a mutant enzyme that contained both amino acid residue substitutions (G73S and S214R) might be able to show higher capability than AADH$_{W1}$ or AADH$_{W2}$, a plasmid pKKAADH$_{M1}$ encoding the double mutant enzyme AADH$_{M1}$ was also constructed as described in Materials and methods.

To construct an *in situ* NADPH regeneration system, pACGD, an expression plasmid of *gdh* from *B. megaterium* (23), was introduced into *E. coli* bearing pKKAADH$_{XX}$. The AADH activities (EAM-reducing activities) of *E. coli* transformants, which coexpressed *gdh* and the wild type or mutant *aadh*, were determined using lyophilized cells in the presence of 0–100 mg/ml of dPE·HCl (Fig. 4). The reactions of all transformants were inhibited at higher dPE concentrations. However, when the dPE·HCl concentration was 80 mg/ml, about 2–7 mg/ml of products was detected in the reaction mixtures with transformants expressing mutant *aadh*s, whereas only very small amounts of products were produced in the reaction mixtures with the wild type enzyme. Even in the presence of 100 mg/ml dPE·HCl, the recombinant *E. coli* expressing the AADH$_{M1}$ gene showed high activity and produced about 6 mg/ml EPE·HCl. In all reactions, none of the other stereoisomers were detected.

Bioconversion of (*RS*)-EAM to EPE Bioconversion of (*RS*)-EAM to EPE using lyophilized *E. coli* cells possessing pACGD, and pKKAADH or pKKAADH$_{M1}$ was also carried out (Fig. 5) as described in Materials and methods. This reaction conditions was suitable for bioconversion considering substrate stability, reaction rate, stereoselectivity of product and racemization rate of substrate. Using the wild type AADH, after the concentration of produced EPE·HCl reached about

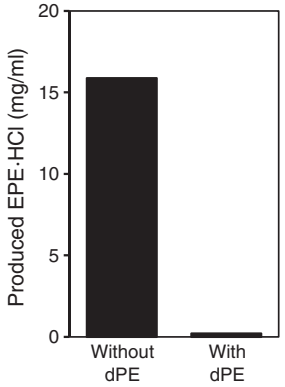

FIG. 2. Effects of dPE on the EAM-reducing activity of wild type AADH. (*RS*)-EAM·HCl (20 mg/ml) was used as a substrate, and the amount of corresponding product (EPE) was determined after the reaction with or without 80 mg/ml dPE·HCl.

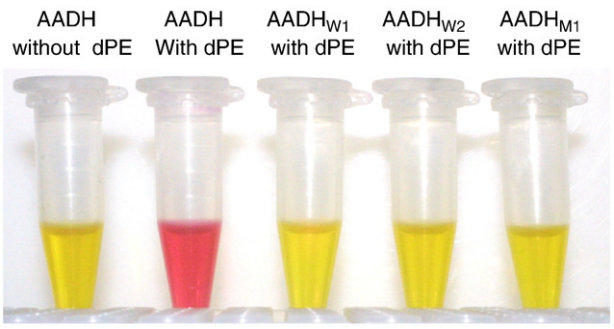

FIG. 3. Color detection of AADH activity of the *E. coli* strain expressing the wild type or mutant *aadh*. After incubation with a reaction mixture containing 0 or 80 mg/ml of dPE·HCl, phenol red was added.

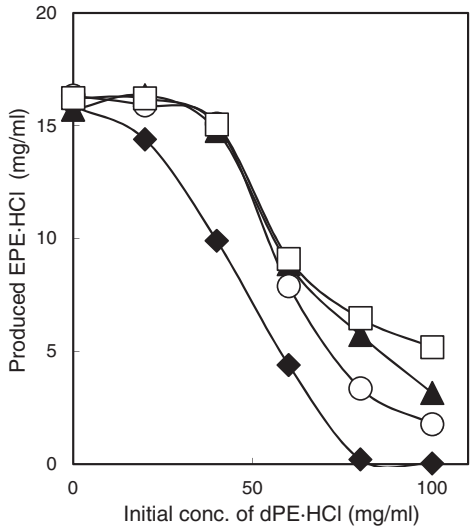

FIG. 4. AADH activity of *E. coli* transformant cells coexpressing *aadh* and *gdh* in the presence of dPE. The lyophilized *E. coli* transformant cells coexpressing the GDH and wild type (closed diamond) or mutant AADH ($AADH_{W1}$, closed triangle; $AADH_{W2}$, open circle; $AADH_{M1}$, open square) genes were used for the EAM reduction reaction as described in the text.

40 mg/ml, no further EPE was produced, even when 80 mg/ml (*RS*)-EAM·HCl was added. In contrast, when using $AADH_{M1}$, the amount of EPE·HCl produced not only exceeded 40 mg/ml, but reached as high as 70.7 mg/ml (328.0 mM) with a molar reaction yield of 87.6%.

Enzyme kinetics The kinetic parameters of the purified His-tagged wild type and mutant AADHs were determined. Table 1 shows the K_m and k_{cat} values of these enzymes for (*RS*)-EAM, and the K_i values for dPE under 0.32 mM NADPH. As compared with the wild type enzyme, $AADH_{W1}$ showed similar K_m and about 3-fold higher k_{cat} values, and $AADH_{W2}$ showed about 1.3-fold higher K_m and similar k_{cat} values. $AADH_{M1}$ showed almost the same K_m value as $AADH_{W2}$ and almost the same k_{cat} value as $AADH_{W1}$. All evolved AADHs showed 2–3 fold higher K_i values than the wild type enzyme.

TABLE 1. Kinetic parameters of the wild type and mutant AADHs.

Enzyme	Mutation point(s)	k_{cat} (min^{-1})	K_m for (*RS*)-EAM (μM)	k_{cat}/K_m (μM^{-1} min^{-1})	K_i (mM)
AADH	None	264	295	0.90	5.7
$AADH_{W1}$	S214R	865	290	2.99	11.8
$AADH_{W2}$	G73S	278	390	0.71	15.2
$AADH_{M1}$	G73S/S214R	874	406	2.16	17.3

All data were determined with 0.32 mM NADPH.

DISCUSSION

AADH produces various chiral aminoalcohols, such as dPE, through the asymmetric reduction of the corresponding prochiral aminoketone compound. As previously described (1), AADH can recognize the α-carbon configuration of the (*S*)-enantiomer of the racemic substrate and reduce the carbonyl group stereospecifically. Because of its high and unique stereoselectivity, it might have high potential for practical application to chiral aminoalcohol production. However, this enzyme is inhibited by the accumulation of produced aminoalcohol (dPE), so that productivity is limited. In this study, we introduced random mutations into *aadh*, and tried to create an enzyme that showed high activity even in the presence of high concentrations of product.

SDR proteins, including AADH, exist widely and form a huge protein superfamily. However, because of their distant duplications and early divergence, they have a low residue similarity level, and only a few amino acid residues are conserved among them (3). Therefore, little is known about what role each amino acid plays, with the exception of the following points: (i) in the N-terminal region, there is a coenzyme binding motif T-G-X_3-G-X-G, and (ii) in the middle of the sequence, an active site consisting of S-X_n-Y-X_3-K is conserved. When little is known about the three-dimensional structure or catalysis mechanisms of a protein, as in the present case, directed evolution is a powerful tool to modify the enzyme functions.

The key factor for a successful directed evolution experiment is an effective and easy screening or selection procedure (13,25). AADH requires NADPH as a cofactor, and therefore the simplest method for detecting AADH activity would be measuring the decrease in NADPH

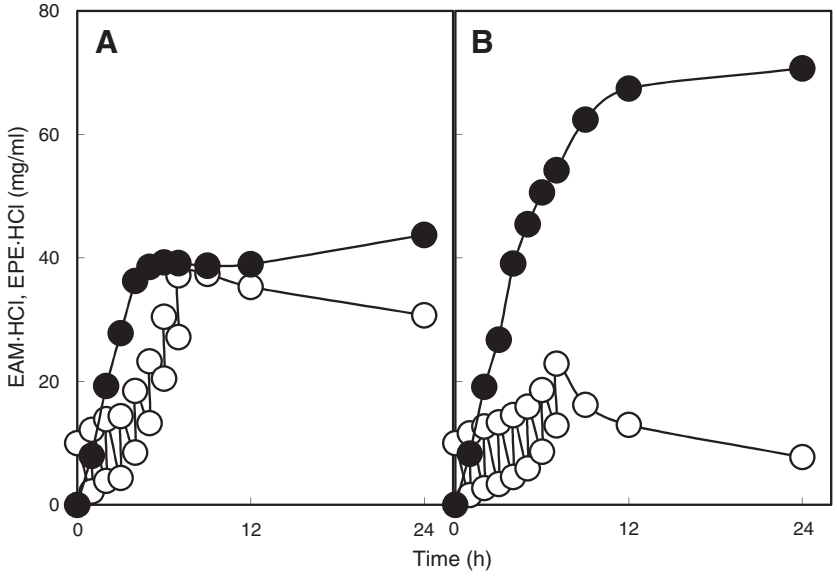

FIG. 5. Bioconversion of (*RS*)-EAM to EPE using lyophilized *E. coli* [pKKAADH and pACGD] (A) or *E. coli* [pKK$AADH_{M1}$ and pACGD] (B) cells. The reactions were carried out as described in the text. Closed circles, EPE·HCl; open circles, EAM·HCl.

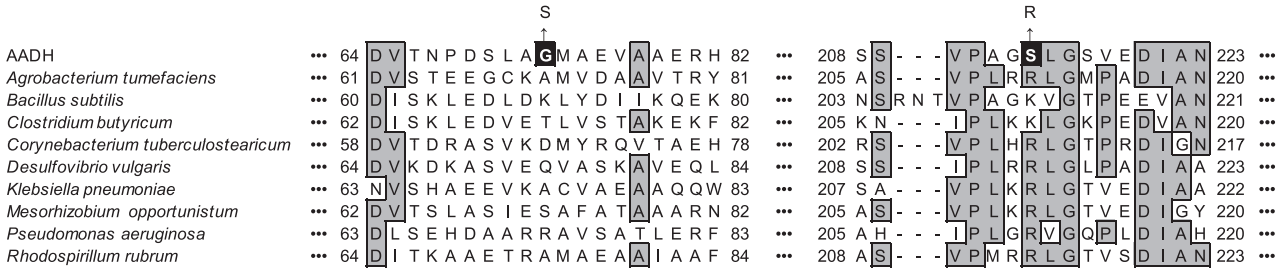

FIG. 6. A partial alignment of the AADH with several putative SDR proteins. The deduced amino acid sequences of AADH and similar proteins are aligned. Gaps in the alignment are indicated by dashes (–). Identical amino acid residues are enclosed in a gray box. The mutation points of AADH are in black boxes. The accession numbers of the listed proteins are as follows: AADH, BAF43657; *Agrobacterium tumefaciens*, AAK90624; *Bacillus subtilis*, CAB13250; *Clostridium butyricum*, EDT74747; *Corynebacterium tuberculostearicum*, ZP_05366588; *Desulfovibrio vulgaris*, AAS97607; *Klebsiella pneumoniae*, ACI08480; *Mesorhizobium opportunistum*, EEW30079; *Pseudomonas aeruginosa*, ABJ13361; *Rhodospirillum rubrum*, ABC23812.

while monitoring the absorbance at 340 nm. However, this is not the best way, because a large amount of expensive NADPH is needed, and the precipitate, which interferes with the process of monitoring the absorbance at 340 nm, must be removed from the reaction mixture. In the bioreduction system using NADPH regeneration with GDH and glucose, as the reduction of EAM proceeds, an equimolar amount of glucose is oxidized to gluconolactone and then immediately and spontaneously turned into gluconic acid (Fig. 1). Therefore, the more the reaction proceeds, the more the pH of the reaction mixture drops, because gluconic acid is an acidic compound ($pK_a = 3.86$). In this study, a very simple screening method was constructed for detecting the progress of the reaction by adding a pH indicator (phenol red) and observing the color change. This method also has the advantage that the threshold of the reaction speed can be adjusted by changing the initial concentration of the reaction buffer.

Using this screening method, two mutant enzymes, $AADH_{W1}$ (S214R) and $AADH_{W2}$ (G73S), and $AADH_{M1}$ containing both of the amino acid substitutions, S214R and G73S, were obtained. Each *E. coli* strain expressing the mutant enzymes could reduce EAM to EPE in the presence of 80–100 mg/ml of dPE·HCl, a condition under which *E. coli* cells expressing the wild type enzyme hardly reduced any EAM at all. Considering that the S214R mutant enzymes ($AADH_{W1}$ and $AADH_{M1}$) showed higher k_{cat} values than the wild type enzyme, the Ser214 residue appears to play an important role and to influence the enzyme activity. The partial amino acid sequence alignment of AADH and various SDR family proteins showing homology with AADH is shown in Fig. 6. It should be noted that all of the amino acids at the position of Ser214 of AADH are highly conserved as basic amino acid residues such as Arg or Lys except for AADH. The $AADH_{W2}$ and $AADH_{M1}$ showed a little higher K_m values than did the wild type enzyme, and the region around the mutation point (Gly73) of these enzymes is not conserved between the other proteins (Fig. 6). This suggests that the Gly73 residue has some involvement in the affinity with substrate. The Lineweaver–Burk plot showed that the dPE interacts with AADH competitively toward EAM (data not shown). Therefore, the affinity of the enzyme to substrate being decreased, the affinity of the enzyme to inhibitor also seems to have been decreased, because the substrate EAM and inhibitor dPE have very similar structures. In addition, a bioconversion experiment (Fig. 5) should be performed using MAK instead of EAM as substrate. Further work is needed for clarifying whether or not this evolved AADH is suitable for dPE production, because the main purpose of this research was to obtain a suitable AADH for producing dPE from (*RS*)-MAK.

In conclusion, the catalytic ability and product inhibition of AADH can be improved by random mutagenesis. It is expected that the mutant enzymes developed herein will be useful in improving the biocatalytic processes of dPE production, and that the obtained insights will assist in further investigations of the structure–function relationships of SDR family proteins.

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