Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-6944-z

MINI-REVIEW

Dye-linked D-amino acid dehydrogenases: biochemical characteristics and applications in biotechnology

Takenori Satomura[1] · **Haruhiko Sakuraba**[2] · **Shin-ichiro Suye**[1,3] · **Toshihisa Ohshima**[4]

Received: 21 May 2015 / Revised: 12 August 2015 / Accepted: 17 August 2015

Abstract Dye-linked D-amino acid dehydrogenases (Dye-DADHs) catalyze the dehydrogenation of free D-amino acids in the presence of an artificial electron acceptor. Although Dye-DADHs functioning in catabolism of L-alanine and as primary enzymes in electron transport chains are widely distributed in mesophilic Gram-negative bacteria, biochemical and biotechnological information on these enzymes remains scanty. This is in large part due to their instability after isolation. On the other hand, in the last decade, several novel types of Dye-DADH have been found in thermophilic bacteria and hyperthermophilic archaea, where they contribute not only to L-alanine catabolism but also to the catabolism of other amino acids, including D-arginine and L-hydroxyproline. In this minireview, we summarize recent developments in our understanding of the biochemical characteristics of Dye-DADHs and their specific application to electrochemical biosensors.

✉ Takenori Satomura
satomura@u-fukui.ac.jp

[1] Department of Applied Chemistry and Biotechnology, Graduate School of Engineering, University of Fukui, 3-9-1 Bunkyo, Fukui 910-8507, Japan

[2] Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, 2393 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0795, Japan

[3] Department of Frontier Fiber Technology and Sciences, Graduate School of Engineering, University of Fukui, 3-9-1 Bunkyo, Fukui 910-8507, Japan

[4] Department of Biomedical engineering, Faculty of Engineering, Osaka Institute of Technology, Ohmiya, 5-16-1 Asahi-ku, Ohsaka 535-8585, Japan

Keywords Dye-linked dehydrogenase · D-Amino acid dehydrogenase · Flavoenzyme · Amino acid catabolism · Electrochemical biosensor

Introduction

Dye-linked dehydrogenases (Dye-DHs) are flavoenzymes that use FAD or FMN as a coenzyme and are a group of oxidoreductases that catalyze the dehydrogenation of various biomolecules, including organic acids, amino acids, and alcohols. In vivo, many of these enzymes (e.g., succinate dehydrogenase and NADH dehydrogenase) exist as membrane-bound proteins and contribute to energy generation within cells as the primary enzymes in the electron transfer systems (Davis and Hatefi 1971; Hanstein et al. 1971; Hederstedt et al. 1979; Dancey et al. 1976; Bergsma et al. 1982; Nisimoto et al. 1986). Dye-DHs can utilize artificial dyes such as 2,6-dichloroindophenol (DCIP) or potassium ferricyanide as electron acceptors instead of their natural electron acceptor. For this reason, this type of enzyme is called Dye-DH. Additionally, Dye-DHs show great potential for use in electrochemical processing applications, such as in biosensors. This is because the aforementioned artificial electron acceptors can readily mediate transfer of the electrons abstracted from the substrate during the enzyme reaction to an electrode (Frew and Hill 1987).

Among the Dye-DH subgroups are the dye-linked D-amino acid dehydrogenases (Dye-DADHs), which catalyze the oxidative deamination of D-amino acids to their corresponding 2-oxo acids in the presence of an electron acceptor such as DCIP. The first known Dye-DADHs were found in the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* in the 1980s (Olsiewski et al. 1980; Magor and Venables 1987). The *E. coli* Dye-DADH is reportedly

Published online: 11 September 2015

involved in energy production via an electron transfer system as well as in L-alanine catabolism coupled with alanine racemase (Olsiewski et al. 1980; Lobocka et al. 1994). Over the last decade, however, a number of Dye-DADHs with novel enzymatic properties unlike those of previously identified Dye-DADHs have been identified in both bacteria and archaea, and the existence of at least three types of Dye-DADHs has been suggested. The first type is a membrane-bound enzyme with broad substrate specificity for various D-amino acids. The second type is a membrane-bound enzyme with high substrate specificity, and the last is a soluble enzyme with broad substrate specificity. In this minireview, we will provide an overview of these three types of Dye-DADHs and also discuss recent advances in their application to biosensors for D-amino acid detection.

Membrane-bound Dye-DADH with broad substrate specificity

Membrane-bound Dye-DADHs with broad substrate specificity are reportedly distributed in Gram-negative bacteria and hyperthermophilic archaea. Detailed biochemical analyses have been performed with Dye-DADHs from *E. coli* (Olsiewski et al. 1980; Jones and Venables 1983), *P. aeruginosa* (Magor and Venables 1987), and *Rhodothermus marinus* (Satomura et al. 2015). The Dye-DADH from *E. coli* B is a heterodimeric complex consisting of a "large subunit" (a functionally unknown component) and a "small subunit" (Dye-DADH component) (Olsiewski et al. 1980). It also contains FAD and non-heme iron as a prosthetic group. This enzyme was solubilized from membranes using 0.1 % (*w*/*v*) TritonX-100 and partially purified from the membrane fraction in the presence of 0.02 % (*w*/*v*) Triton X-100 serving as a solubilizer. The solubilized enzyme catalyzes oxidative deamination of several D-amino acids, including D-alanine (relative activity, 100 %), D-phenylalanine (100 %), D-methionine (97 %), D-α-aminobutyrate (89 %), and D-serine (29 %), in a coenzyme Q1 (CoQ1) and DCIP-coupled system. The Km values for D-alanine, D-phenylalanine, and D-methionine are 3, 6, and 11 mM, respectively. This enzyme thus exhibits fairly broad electron donor specificity and can utilize coenzyme Q (CoQ) analogues as a natural electron acceptor in addition to artificial electron acceptors.

Like the *E. coli* B enzyme, the solubilized and partially purified *E. coli* K12 Dye-DADH is fairly labile, that is, 90 % of the initial activity is lost after incubation at 37 °C for 10 min (Jones and Venables 1983). Under both membrane-bound and solubilized conditions, *E. coli* K12 Dye-DADH shows broad electron donor specificity and utilize D-alanine as the most preferred substrate. The general effect of solubilization on *E. coli* K12 Dye-DADH is to increase the specific activity with all substrates. The optimum pH is 8.9 for the membrane-bound enzyme and 7.9 for the solubilized enzyme in an assay system containing D-alanine and phenazine methosulfate (PMS) as the substrates.

P. aeruginosa Dye-DADH is also characterized after solubilization from membranes (Magor and Venables 1987). The molecular mass of this enzyme is estimated to be about 200 kDa using a gel filtration chromatography, and SDS-PAGE shows a single band migrating as a molecular mass of about 49 kDa, indicating the enzyme to be homotetramer. The structure of *P. aeruginosa* Dye-DADH clearly differs from the heterodimeric structure of the *E. coli* B enzyme described above. On the other hand, the electron donor specificity of *P. aeruginosa* Dye-DADH is similar to those of the *E. coli* enzymes: D-alanine is the most preferred substrate, though the enzyme shows broad substrate specificity for D-amino acids. The solubilized enzyme is also labile and loses 95 % of its activity after incubation at 42 °C for 10 min.

The Dye-DADHs from mesophiles are thus not sufficiently stable, once solubilized, to enable a high degree of purification. This prevented the biochemical characterization of Dye-DADHs for many years.

To overcome the limitations imposed by the instability of mesophilic Dye-DADHs, we have been screening for highly stable Dye-DADHs and recently found two in the thermophilic bacterium *R. marinus* (Satomura et al. 2015) and the hyperthermophilic archaeon, *Pyrobaculum islandicum* (Satomura et al 2002). Their greater stability enabled both enzymes to be successfully purified and characterized. The gene encoding the *R. marinus* Dye-DADH was identified based on its genome information and expressed in *E. coli*. The recombinant protein purified from the *E. coli* cell extract was highly stable, even at high temperatures and under both high and low pH conditions. The recombinant *R. marinus* Dye-DADH retained more than 80 % of its full activity after incubation at 70 °C for 10 min and lost 80 % of its full activity after incubation at 90 °C for 10 min. It retained full activity after incubation for 10 min at pHs ranging from 4.0 to 11.0 (50 °C). Using a gel filtration chromatography, the recombinant protein was found to have a molecular mass of about 115 kDa in the presence of 0.1 % (*w*/*v*) Tween-20, and the subunit molecular mass was estimated to be about 46 kDa using SDS-PAGE, indicating the enzyme is a homodimer. D-Phenylalanine is the most preferred substrate of *R. marinus* Dye-DADH, but L-phenylalanine is inert. Several other D-amino acids, including D-proline (relative activity 93 %), *cis*-4-hydroxy-D-proline (63 %), D-histidine (49 %), D-tryptophan (41 %), D-isoleucine (37 %), D-leucine (36 %), and D-arginine (22 %), are also able to serve as the electron donor, as was the case with *E. coli* and *P. aeruginosa* Dye-DADHs. The most preferred electron acceptor for *R. marinus* Dye-DADH is DCIP, though a PMS and *p*-iodonitrotetrazolium violet (INT)-coupled system also exhibits 17 % relative electron acceptor activity. Ferricyanide and O_2 are inert as electron acceptors.

A dye-linked D-proline dehydrogenase (D-ProDH) was first identified in *P. islandicum* JCM 9189 cells (Satomura et al. 2002). This enzyme was tightly bound to the membrane and was solubilized from membrane fractions by treatment with 1 % (*w*/*v*) Tween-20. The solubilized enzyme purified to homogeneity in 0.1 % (*w*/*v*) Tween-20 has a molecular mass of about 145 kDa and exists as a homotetramer composed of subunits with a molecular mass of about 42 kDa. The enzyme's prosthetic group is FAD. The most preferred electron acceptor for D-ProDH was DCIP. This enzyme could also utilize PMS as the electron acceptor, but not O_2, NAD, NADP, ferricyanide, menadion, benzyl viologen, horse heart cytochrome c, or methylene blue. D-ProDH is extremely stable, even under solubilized conditions, and full activity was observed after incubation for 10 min at 80 °C or at pHs ranging from 4.0 to 10.0 (50 °C). In addition, this enzyme lost no activity when stored for more than 1 month at around 4 °C. Indeed, D-ProDH is the most stable Dye-DADH so far reported. The enzyme catalyzes the dehydrogenation of D-proline as its most preferred substrate to Δ^1-pyrroline-2-carboxylate. The substrate specificity of *Py. islandicum* D-ProDH is broad and similar to the previously discussed Dye-DADHs: *cis*-4-hydroxy-D-proline (relative activity: 89 %), D-isoleucine (49 %), D-valine (41 %), D-leucine (39 %), D-histidine (30 %), and D-phenylalanine (28 %) were all able to function as the electron donor. On the other hand, *Py. islandicum* D-ProDH exhibits no activity toward L-amino acid isomers.

Enzymes from thermophiles are generally much more stable than their counterparts from mesophiles under a variety of conditions (Ohshima and Soda 1989; Adams 1993). This stability makes biochemical analysis of Dye-DADHs from *R. marinus* and *Py. islandicum* possible. The primary structures of several Dye-DADHs have been determined, and comparison of their amino acid sequences showed that the sequences of mesophilic Dye-DADHs are highly homologous with one another (60~90 % identity). By contrast, the amino acid sequence of the thermostable *R. marinus* enzyme shows only 32–34 % identity with the sequences of mesophilic Dye-DADHs, while D-ProDH from the hyperthermophilic archaeon *Py. islandicum* shows only 25–26 % identity with the mesophilic Dye-DADHs. Thus, the sequences of the *R. marinus* and *Py. islandicum* enzymes largely differ from those of mesophilic Dye-DADHs. The thermostable *R. marinus* and *Py. islandicum* enzymes share only 28 % amino acid sequence identity. Nonetheless, high sequence homology was detected among the N-terminal and C-terminal regions of all these Dye-DADHs (Fig.1). A FAD-binding motif (GXGXXG) is present in their N-terminal regions, which also contains a highly hydrophobic region postulated to be one of the membrane anchoring sites (Wierenga et al. 1986; Lobocka et al. 1994). It is therefore predicted that electrons obtained through oxidation of D-amino acids are received by FAD in the N-terminal region of Dye-DADHs and then transferred to as yet unidentified physiological electron acceptors in the membrane fraction. The role of the conserved C-terminal region of Dye-DADH remains obscure. In recent future, when the 3D structures of these enzymes are revealed, their structures may give us further information about the role of C-terminal region.

Membrane-bound Dye-DADH with high substrate specificity

The presence of two different Dye-DADHs with high specificity for *cis*-4-hydroxy-D-proline (D-HypDH) has been reported in the genus *Pseudomonas* (Watanabe et al. 2012). The genes encoding D-HypDHs were identified in the genomes of *P. aeruginosa* and *Pseudomonas putida* and were expressed in *P. putida* cells, after which they were purified to homogeneity in the presence of Tween-20 and characterized. Although the enzymological properties of the two D-HypDHs

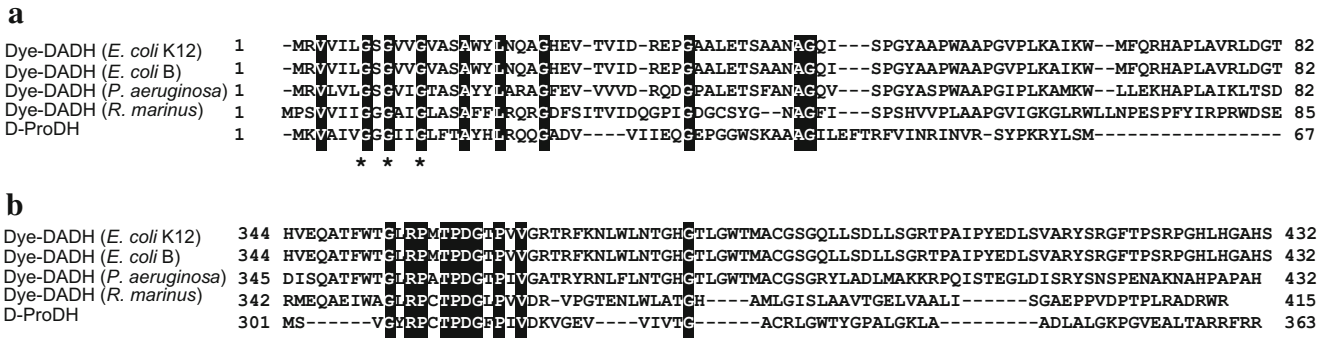


Fig. 1 Multiple sequence alignments of N-terminal region (**a**) and C-terminal region (**b**) of membrane-bound Dye-DADHs with broad substrate specificity. Highly conserved residues are shown depicted in *black boxes*. The *asterisks* below the amino acid sequences indicate the FAD-binding motif. The sequences used were from *E. coli* K12 Dye-DADH, KEGG database ORF ID: b1189; *E. coli* B Dye-DADH ECB_01164; *P. aeruginosa* DyeDADH, PA5304; *Py. islandicum* D-ProDH, Pisl_1862; and *R. marinus* Dye-DADH, GenBank accession number: LC004287

are similar, their subunit structures differ substantially. In the *P. aeruginosa* genome, the genes *lphE* and *lphF* respectively encode a protein of unknown function and ferredoxin and are located in the vicinity of the gene encoding D-HypDH (*lphB*), where they are tandemly arranged as a gene cluster in the order *lphB*, *lphF*, and *lphE*, with the 3′-end of the upstream gene overlapping the 5′-end of the next gene (Fig. 2a). This *lphBFE* gene cluster with a hexahistidine tag sequence attached at the N-terminus of *lphB* was expressed using the constitutive *ahpC* promoter in *P. putida* KT2442-oxyR1 cells, after which the recombinant *P. aeruginosa* D-HypDH was successfully purified to homogeneity. The molecular masses of *lphB*, *lphF*, and *lphE* gene products are 48, 10, and 42 kDa, respectively. The native molecular mass of *P. aeruginosa* D-HypDH is 360 kDa, which means the enzyme has a heterododemaric $\alpha_4\beta_4\gamma_4$ structure.

Because the gene encoding D-HypDH from *P. putida* is not part of a gene cluster like the *P. aeruginosa* D-HypDH gene is, *P. putida* D-HypDH is thought to be homomeric. After expression of the gene in *P. putida* cells, the product was purified to homogeneity in the presence of 0.1 % (*v*/*v*) Tween-20, and the subunit molecular mass was estimated to be about 47 kDa using SDS-PAGE. But because this enzyme was eluted in the void volume of the gel filtration column, its native molecular mass has not yet been determined, and the molecular structure of *P. putida* D-HypDH remains unclear. Both of these D-HypDHs are tightly bound to the cytoplasmic membrane (Yoneya and Adams 1961; Bater et al. 1997; Watanabe et al. 2012), and both show maximum activities around pH 9.5 and retain more than 70 % of their activity after incubation for 2 h at pHs ranging from 8 to 10.5 (30 °C). The prosthetic group of the *P. aeruginosa* D-HypDH $\alpha\beta\gamma$ heteromeric unit contains two molecules of FAD, one molecule of FMN and one [2Fe-2S]-iron cluster, whereas *P. putida* D-HypDH contains only FAD. The amino acid sequence of the *P. aeruginosa* D-HypDH active component has only 23 % identity with the sequence of the *P. putida* enzyme, which means the primary and quaternary structures of *P. aeruginosa* D-HypDH are totally different from those of the *P. putida* enzyme.

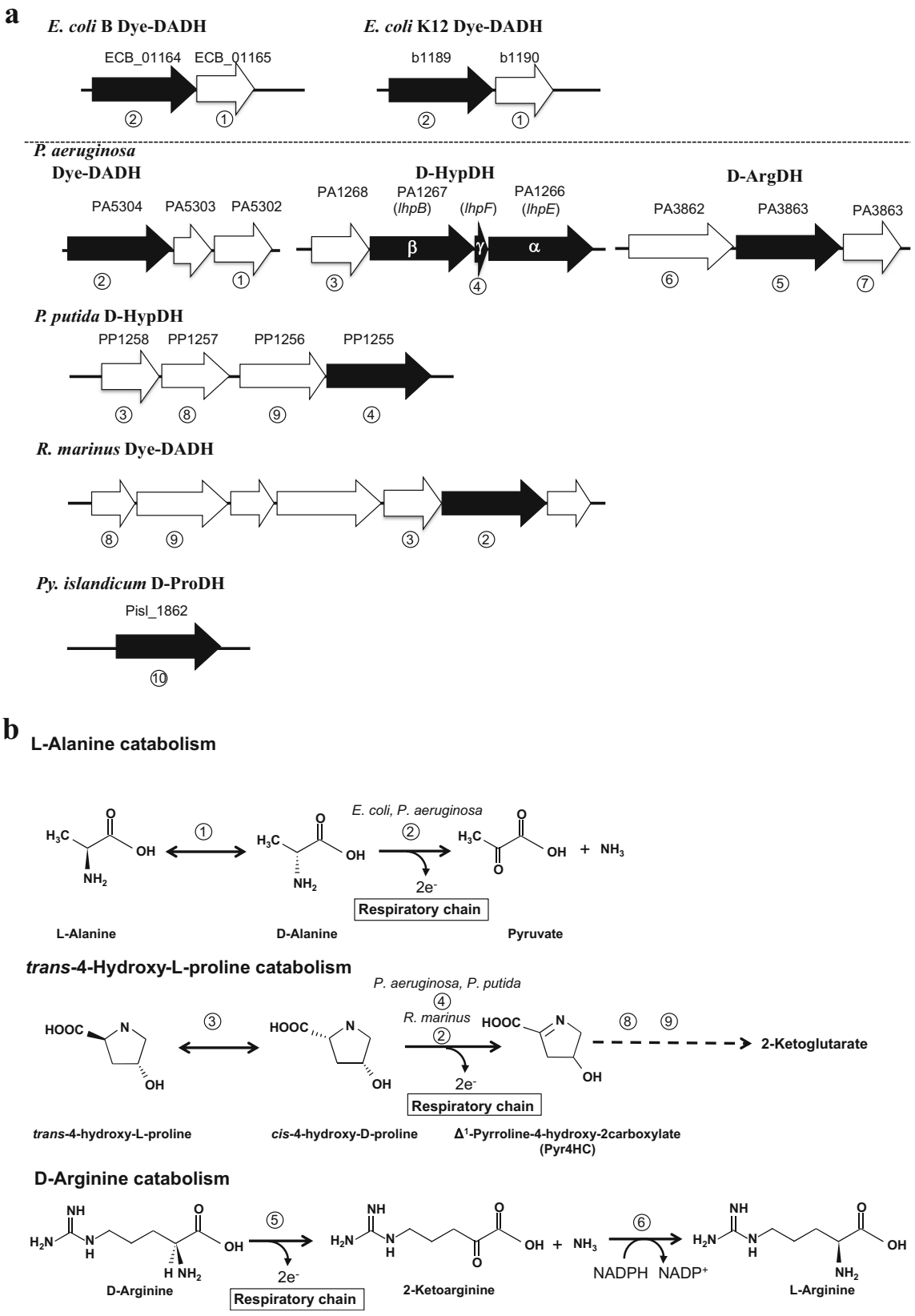
On the other hand, both enzymes utilize D-proline as well as *cis*-4-hydroxy-D-proline as substrates, and the relative activities of *P. aeruginosa* and *P. putida* D-HypDH toward D-proline are 33 and 20 %, respectively, as compared with those toward *cis*-4-hydroxy-D-proline (100 %). These D-HypDHs thus exhibit greater substrate specificity than the Dye-DADHs described above. Both enzymes are able to utilize a PMS and INT-coupled system or a PMS and nitroblue tetrazolium (NTB)-coupled system as the electron acceptor. Neither enzyme utilized DCIP, though DCIP is one of the most preferred electron acceptors for Dye-DADHs. Collectively, these findings suggest the D-HypDHs do not belong to the same enzyme group as the Dye-DADHs.

Fig. 2 **a** Comparison of the genetic organization of Dye-DADH gene regions. The locations and orientations of the genes are indicated by *arrows*. KEGG ORF IDs are shown *above each arrow*. Genes encoding Dye-DADHs are indicated by the *black arrows*. **b** The functions of Dye-DADHs in amino acid catabolism. The *circled numbers* correspond to the following enzymes: 1, alanine racemase; 2, Dye-DADH; 3, 4-hydroxyproline-2-epimerase; 4, D-HypDH; 5, D-ArgDH; 6, NAD-dependent L-arginine dehydrogenase; 7, transcriptional regulator, DauR; 8, Δ1-pyrroline-4R-hydroxy-2-carboxylate deaminase; 9, α-ketoglutaric semialdehyde dehydrogenase; and 10, D-ProDH

Soluble Dye-DADH with broad substrate specificity

A novel type of Dye-DADH that differs greatly from the abovementioned Dye-DADHs was identified in *P. aeruginosa* (Li and Lu 2009; Li et al. 2010) and overexpressed in *E. coli* as a recombinant dye-linked D-arginine dehydrogenase (D-ArgDH) with a hexahistidine tag at the N-terminus. The recombinant D-ArgDH was purified to homogeneity from *E. coli* cell extracts using a nickel chelating column and then biochemically characterized. This enzyme is considered to be soluble because its activity is retained in the soluble fraction after ultracentrifugation. This is in contrast to all of the Dye-DADHs described above, which are tightly bound to the cytoplasmic membrane, and suggests D-ArgDH is inherently different from membrane-bound enzymes and may be a novel type of Dye-DADH. The histidine-tagged recombinant protein is monomeric in its native form, with a subunit molecular mass of 40 kDa (calculated from the primary structure). The purified D-ArgDH is yellow, which is typical of flavoproteins. D-ArgDH activity could be detected using a PMS and INT-coupled system, whereas oxygen was inert as an electron acceptor. D-ArgDH exhibits broad substrate specificity: several different D-amino acids could serve as the electron donor, though the most preferable was D-lysine. Marked substrate inhibition was detected when D-lysine or D-arginine was the electron donor, but that was not the case with other D-amino acids. Similar substrate inhibition has not been reported for other Dye-DADHs.

Among the characterized Dye-DADHs, only D-ArgDH has been successfully crystallized. Fu et al. (2010) reported the X-ray crystal structures of D-ArgDH at 1.06 Å resolution and its complex with iminoarginine and iminohistigine at 1.30 Å resolution. The structure indicates that D-ArgDH contains both a FAD-binding domain and a substrate-binding domain (Fig. 3a) and non-covalently binds one molecule of FAD per subunit. The isoalloxazine ring of FAD is situated at the interface between the FAD-binding domain and the substrate-binding domain. The internal cavity of the substrate-binding site has a triangular cross-section with a narrow entrance at the top. The crystal structure of the enzyme in complex with iminoarginine shows that the Cα, imino group, and carboxylate group of iminoarginine are oriented approximately parallel to the *re* face of FAD (Fig. 3b) and form polar interactions



with several residues in D-ArgDH (Fig. 3b). The carboxylate group of iminoarginine forms hydrogen bonds with the side chain hydroxyl of Tyr^{53}, the guanidium side chain of Arg^{305}, the carbonyl oxygen of Gly^{332}, the side chain nitrogen of

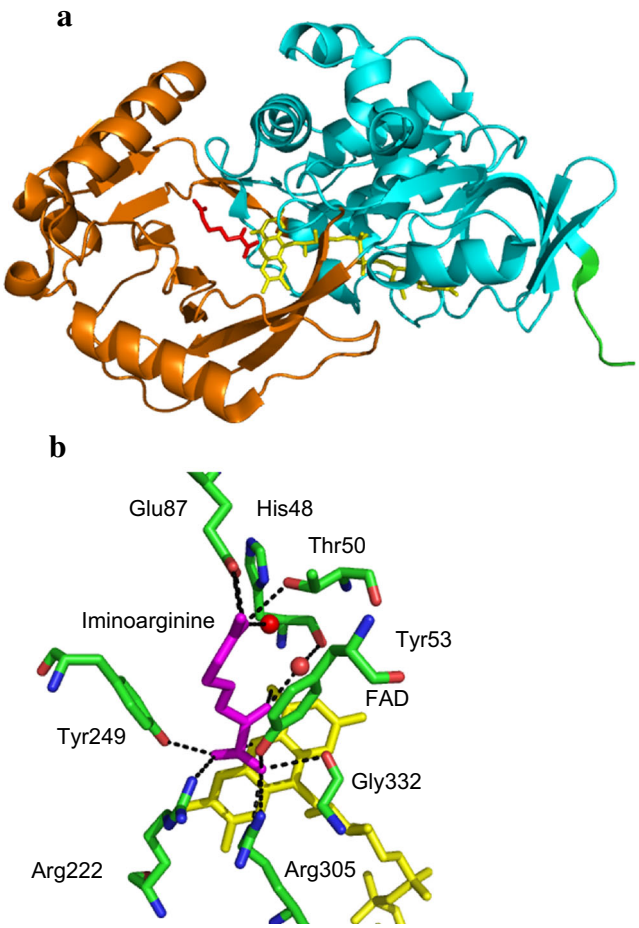


Fig. 3 **a** Overall structure of the D-ArgDH (PDB ID: 3NYE). The FAD- and substrate-binding domains are shown in *cyan* and *orange*, respectively. The N-terminal His-tag is in *green*. FAD (*yellow*) and iminoarginine (*red*) are shown in *stick*. **b** Interactions between D-ArgDH and iminoarginine. Carbons are colored *green* for D-ArgDH active site residues and *magenta* for the iminoarginine. FAD is represented in *yellow*. Figure modifued from Fu et al. (2010)

Arg^{222}, and the side chain hydroxyl of Tyr^{249}. The imino group of iminoarginine, which is situated within hydrogen-bonding distance of the O4 atom of FAD, forms a hydrogen bond interaction with the main chain carboxyl oxygen atom of His^{48} via a water molecule. The guanidinium group of iminoarginine forms a hydrogen interaction with the side chain of Thr^{50} and an ionic interaction with the carboxylate group of Glu^{87} (Fig. 3b). The side chain of Glu^{87} is thought to be the major determinant of the specificity of D-ArgDH for positively charged substrates such as D-arginine and D-lysine. On the other hand, within the structure of iminohistidine-bound D-ArgDH, the side chain orientation of the ligand substantially differs from that in the iminoarginine-bound enzyme; the side chain of iminohistidine lies almost parallel to the isoalloxazine ring of FAD, while that of iminoarginine is nearly perpendicular to the isoalloxazine ring. In contrast, the carboxylate and imino groups of iminohistidine are fixed at a position similar to those of iminoarginine via conserved hydrogen bond interactions between the two structures. The binding pocket of D-ArgDH has a small entrance but expands at the bottom near the FAD, and a flexible loop that may control substrate accessibility is situated near the active site. The shape and flexibility of active site, as well as the conserved interactions around the substrate Cα, are thought to enable D-ArgDH to accommodate different substrates.

Physiological function of Dye-DADH

Dye-DADH reportedly functions in both energy generation, as the primary enzyme in the electron transport chain, and the catabolism of amino acids. For example, Dye-DADHs from *E. coli* and *P. aeruginosa* utilizing D-alanine as the most preferable substrate are involved in alanine catabolism (Lobocka et al. 1994; Boulette et al. 2009; He et al. 2011). The gene encoding Dye-DADH is located adjacent to the gene encoding alanine racemase and is arranged as an operon (*dad* operon) inducible by L-alanine (Fig. 2a). In both cell types, Dye-DADH is thought to function in the conversion of L-alanine to pyruvate and ammonia via D-alanine formation catalyzed by alanine racemase (Fig. 2b). Therefore, Dye-DADH is necessary for L-alanine metabolism. On the other hand, the alanine racemase gene is not situated adjacent to the gene encoding *R. marinus* Dye-DADH, which belongs to the group of membrane-bound Dye-DADHs with broad substrate specificity. Instead, a gene encoding 4-hydroxyproline 2-epimerase is located in the immediate vicinity of the *R. marinus* Dye-DADH gene (Fig. 2a), and these genes are arranged in an operon (*hyp* operon). The *hyp* operon consists of genes encoding enzymes involved in *trans*-4-hydroxy-L-proline catabolism (Fig. 2b), and marked enhancement of this operon is observed in the presence of *trans*-4-hydroxy-L-proline (Satomura et al. 2015). The *R. marinus* Dye-DADH may thus be involved in *trans*-4-hydroxy-L-proline catabolism. *Pseudomonas* D-HypDH not only belongs to a different group than *R. marinus* Dye-DADH but is also thought to be involved in *trans*-4-hydroxy-L-proline catabolism (Watanabe et al. 2012). Like the *R. marinus* Dye-DADH gene, the D-HypDH genes form a cluster encoding enzymes involved in *trans*-4-hydroxy-L-proline catabolism (Fig. 2a). In addition, D-HypDH activity was found to be markedly elevated in cell-free extracts of *P. aeruginosa* and *P. putida* grown on minimal medium containing *trans*-4-hydroxy-L-proline (Watanabe et al. 2012). This suggests D-HypDH, which exhibits strong reactivity toward *cis*-4-hydroxy-D-proline, also participates in *trans*-4-hydroxy-L-proline catabolism.

Notably, however, the amino acid sequences of the Dye-DHs involved in *trans*-4-hydroxy-L-proline catabolism share only about 30 % identity, and D-HypDH clearly differs from *R. marinus* Dye-DADH, though these enzymes likely share a

similar physiological functions. In addition, D-ProDH from the hyperthermophilic archaeon *Py. islandicum* shows strong activity toward *cis*-4-hydroxy-D-proline (Satomura et al. 2002), though the amino acid sequence of D-ProDH shares less than 29 % identity with the sequences of the Dye-DHs involved in *trans*-4-hydroxy-L-proline catabolism, and a 4-hydroxyproline 2-epimerase homologue gene has not been detected in the vicinity of the *Py. islandicum* D-ProDH gene. The physiological function of *Py. islandicum* D-ProDH and the catabolic pathway of D-amino acids in the hyperthermophilic archaeon remain to be defined.

The gene encoding *P. aeruginosa* D-ArgDH is located in the immediate vicinity of the two genes encoding NAD-dependent L-arginine dehydrogenase and transcriptional repressor (Fig. 2a), and the three genes are arranged in an operon (*dauBAR* operon) (Li and Lu 2009; Li et al. 2010). When *P. aeruginosa* is grown in minimal medium containing D-arginine or D-lysine, the *dauBAR* operon is induced, and D-ArgDH is responsible for a unique racemization coupled with NAD-dependent L-arginine dehydrogenation (Fig. 2b). The conversion of D-arginine to L-arginine through the conjugated reactions of D-ArgDH and NAD-dependent L-arginine dehydrogenase makes the utilization of D-arginine as a sole source of carbon and nitrogen possible.

Application for Dye-DADH to D-amino acid biosensors

Advances in analytical methods and other techniques have confirmed that a variety of D- and L-amino acids are present in many foods and biological tissues. Furthermore, the specific biological and physiological functions of the D-amino acids in several types of organisms have been determined. For example, D-serine is known to act as a co-agonist for the N-methyl-D-aspartate (NMDA) receptor which is widely distributed in the central nervous system. NMDA receptor is the cation channel that plays an important role in memory and learning (Mondadori et al. 1989; Rison and Stanton, 1995). D-Aspartic acid is distributed in the nervous and endocrine tissues of mammals, where it functions to control the production of some hormones (Furuchi and Homma 2005). D-Proline has been detected in the gastric juice, brain, and human saliva, and it specifically accumulates in stomach cancer and reportedly exhibits neural toxicity (Hamase et al. 2001; Nagata et al 2006, 2007). In addition, D-proline has also been found in foods such as vinegar and maple syrup (Erbe and Brückner 2000; Pätzold and Brückner 2005), and its concentration in vinegars reportedly increases with their aging. The analytical methods currently being employed to determine D-amino acid levels in organ tissues and foods include gas chromatography, high-performance liquid chromatography, and use of automated amino acid analyzers (Hamase et al. 2002). However, these instruments are expensive and require mastery of sophisticated techniques. As an alternative approach to D-amino acid determination, electrochemical methods using Dye-DADHs are being developed. These Dye-DADH-based biosensors enable electrochemical detection of D-amino acids and show great potential for easy and sensitive detection of D-amino acids in biological tissues and foodstuffs and for monitoring procedures for D-amino acid production. Although mesophilic Dye-DADHs are not suitable for use due to their instability, stable enzymes from thermophiles can be applicable for use in biosensors under various conditions.

An electrochemical D-amino acid detection system using highly stable *Py. islandicum* D-ProDH has been constructed (Tani et al. 2008). With this system, D-ProDH is immobilized on a glassy carbon electrode along with an agar gel, which is a heat reversible polymer. This electrode is easily prepared by spin-coating with a 1.5 % agar solution containing the enzyme (agar/immobilized-enzyme electrode) (Fig. 4a). The great advantage of this system is that the agar/immobilized-enzyme electrode can be easily prepared by mixing the enzyme in hot agar solution at 70 °C. Using the resultant agar/immobilized-enzyme electrode, D-proline elicited a current at temperatures up to 70 °C, and the response was maintained for 80 days. A limitation of this system is that the D-amino acid selectivity of the electrode was not high, reflecting the broad substrate specificity of D-ProDH. In addition to D-proline (relative activity 100 %), the immobilized electrode was sensitive to D-isoleucine (46 %), D-histidine (29 %), D-leucine (19 %), D-valine (18 %), D-alanine (9 %), and D-aspartate (7 %), but was insensitive to many L-amino acids, including L-proline. Linear regression showed that the relation between the D-proline concentration and the response of the agar/immobilized-enzyme electrode was linear from 0.5 to 5.0 mM. In addition, the electrode was successfully used to detect D-amino acids in human urine sample.

To improve sensitivity for analysis of D-amino acids, a novel type of electrode composed of a mixture of single-wall carbon nanotubes (CNT), electrolyte, and D-ProDH has been constructed (CNT/immobilized-enzyme electrode) (Fig. 4b) (Tani et al. 2009). The carbon nanotubes have great potential for use in electrodes owing to their large surface area and superior electric conductivity. The CNT/immobilized-enzyme electrode has a much smaller Km value for D-proline (0.83 mM) than the CNT-free immobilized-D-ProDH-only electrode (7.9 mM), and it was demonstrated that this CNT/immobilized-enzyme electrode could be applicable for highly sensitive detection of D-amino acids in food samples such as rice wine (0.0210 mM) and vinegar (0.55 mM). This sensor system would be useful for simple electrochemical sensing of D-amino acids in samples containing mainly the corresponding D-amino acid, but it would not always be useful for sensing a

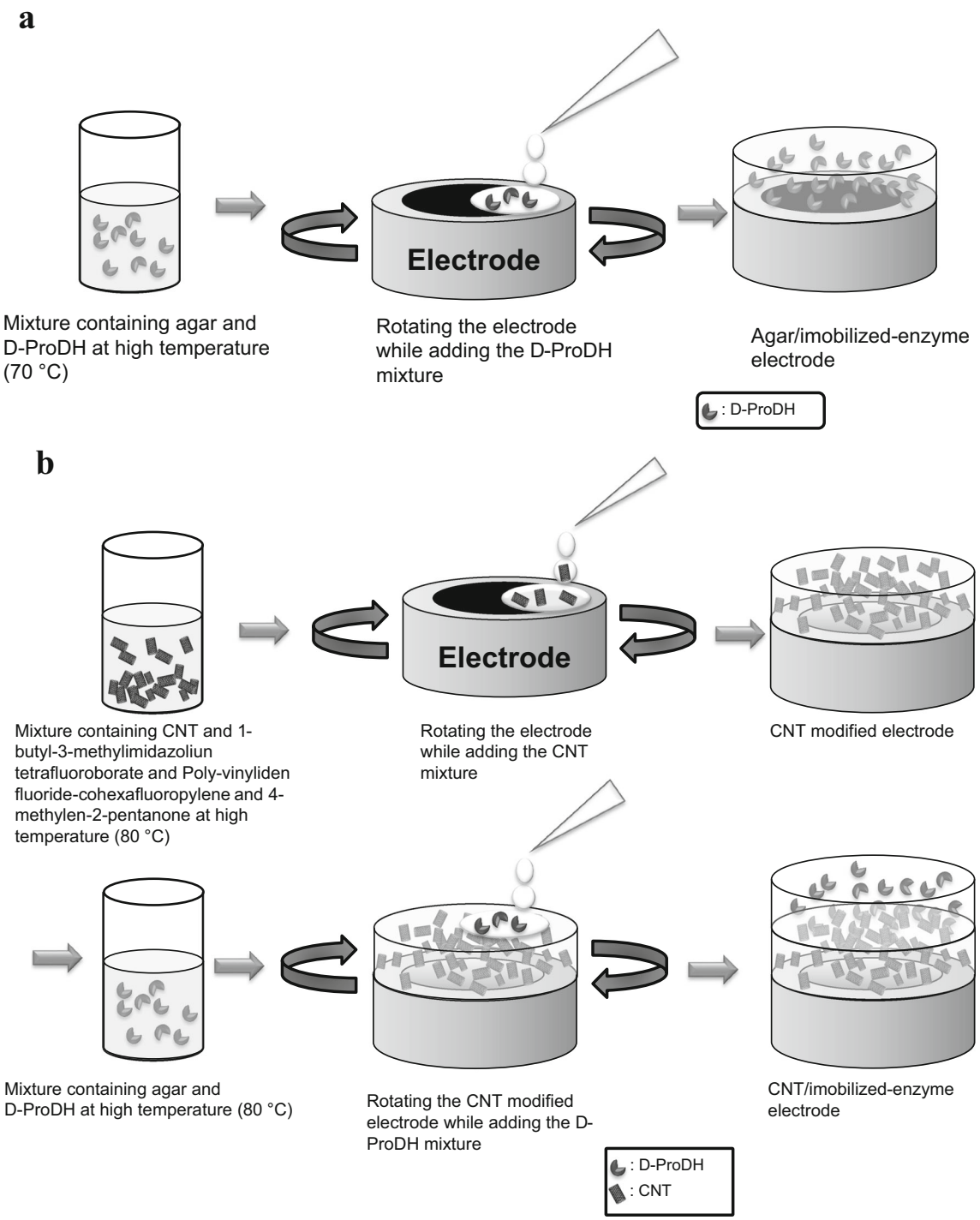


Fig. 4 Scheme for the preparation of an agar/immobilized-enzyme electrode (**a**) and a CNT/immobilized-enzyme electrode (**b**) using the spin-coating method

specific D-amino acid in biological samples—for example, D-proline in the cell extract containing other D-amino acids. This again reflects the broad substrate specificity of D-ProDH. The next step will be to identify other novel Dye-DADHs with potentially greater substrate specificity for application to improved biosensor electrodes.

Conclusion and perspectives

Over the last decade, a variety of Dye-DADHs have been detected and characterized, and it has become apparent that Dye-DADHs are widely distributed in bacteria and archaea and are not always rare. Based on their biochemical characteristics, these enzymes have been grouped into three types

Table 1 Summary of the enzymatic properties of Dye-DADHs

	Dye-DADH[a]	Dye-DADH[b]	Dye-DADH[c]	D-ProDH[d]	Dye-DADH[e]	D-HypDH[f]	D-HypDH[f]	D-ArgDH[g]
Microorganisms	*E. coli* B	*E. coli* K12	*P. aeruginosa*	*Py. islandicum*	*R. marinus*	*P. aeruginosa*	*P. putida*	*P. aeruginosa*
Localization	Membrane	Membrane	Membrane	Membrane	Membrane	Membrane	Membrane	Cytoplasm
Molecular mass								
Native (kDa)	N.D.[h]	N.D.	200	145	115	362	N.D.	N.D.
Subunit (kDa)	55, 42	N.D.	49	42	46	48,42,10	47	40
Prosthetic groups	FAD, Fe	N.D.	FAD	FAD	FAD	FAD, FMN, [2Fe-2S]	FAD	FAD
Electron acceptor	DCIP, CoQ1, CoQ2, CoQ3, CoQ4, CoQ5, CoQ6, CoQ7, CoQ8, Co Q1-DCIP	PMS-DCIP	PMS-DCIP	DCIP, PMS-INT	DCIP, PMS-INT	PMS-INT PMS-NTB	PMS-INT PMS-NTB	PMS-INT
Electron donor[i]	D-Ala D-Phe D-Met D-Amino butyrate	D-Met D-Ala D-α-Amino-*n*-butyrate D-Asn D-Ser	D-Ala D-Cycroserine D-Asp DL-α-Amino-*n*-butyrate D-Met D-Phe D-Gln D-Arg	D-Pro *cis*-4-Hydroxy-D-proline D-Ile D-Val	D-Phe D-Pro *cis*-4-Hydroxy-D-proline	*cis*-4-Hydroxy-D-proline	*cis*-4-Hydroxy-D-proline	D-Lys D-Arg D-Met D-Phe D-Tyr D-His D-Ornithine D-Leu D-Trp
Optimum pH	N.D.	7.9	8.5	7.5	7.5	9.5.	9.5	N.D.
pH stability[j]	N.D.	N.D.	N.D.	4–10	4–11	8–10.5[k]	8–10.5[k]	N.D.
Optimum temp.	N.D.	N.D.	N.D.	70>	75	N.D.	N.D.	N.D.
Thermal stability[l]	N.D.	37	42	100	90	N.D.	N.D.	N.D.
Physiological function	L-Alanine catabolism	L-Alanine catabolism	L-Alanine catabolism		L-Hydroxyproline catabolism	L-Hydroxyproline catabolism	L-Hydroxyproline catabolism	D-Arginine catabolism

[a] Olsiewski et al. (1980)

[b] Lobocka et al. (1994)

[c] Magor and Venables (1987); He et al. (2011)

[d] Satomura et al. (2002)

[e] Satomura et al. (2015)

[f] Watanabe et al. (2012)

[g] Li and Lu (2009); Li et al. (2010)

[h] Not determined

[i] Substrates for which the relative activity of solubilized Dye-DADH is more than 40 % relative to the best substrate

[j] pH at which the enzyme retains its full activity after incubation for 10 min

[k] pH at which more than 70 % of the initial enzyme activity is lost after incubation for 2 h

[l] Temperature at which 80 % of the initial enzyme activity is lost after incubation for 10 min

(Table 1): (1) membrane-bound Dye-DADHs that exhibit broad substrate specificity for electron donor D-amino acids, (2) membrane-bound enzymes with high specificity for *cis*-4-hydroxy-D-proline, and (3) soluble Dye-DADHs with broad substrate specificity for D-amino acids. Comparison of the amino acid sequences of these three groups of Dye-DADHs revealed they share less than 34 % identity, and there are large differences in their enzymological properties and primary structures, even though their physiological functions are often similar. This suggests Dye-DADHs evolved convergently in their organisms.

In addition, Dye-DADHs exhibit great potential for application to electrochemical detection of D-amino acids through transfer of electrons from the D-amino acids to an electrode via a mediator. Mesophilic Dye-DADHs are not suitable for this application due to their comparative instability. Among characterized Dye-DADHs, D-ProDH from the hyperthermophilic archaeon *Py. islandicum* exhibits the greatest stability under different conditions. In particular, it showed stable performance for 80 days on an agar/immobilized-D-ProDH electrode. This is noteworthy, as prolonged stability is crucial for accurate long-term use in a biosensor system. Moreover, the *Py. islandicum* immobilized-D-ProDH electrode was successfully used to detect D-amino acids in biological and food samples. This is the first example of a D-amino acid detection system using Dye-DADHs. However, problems remain with the D-ProDH sensor system. The broad substrate specificity of D-ProDH prevents accurate detection of a specific D-amino acid in a sample containing more than two D-amino acids. No Dye-DADH catalyzing the dehydrogenation of a specific D-amino acid has yet been identified in a hyperthermophile or thermophile, though a D-HypDH-like enzyme with high substrate specificity has been found in a mesophile. A great deal of information on the genomes of hyperthermophiles and thermophiles is currently available in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/). Genes encoding Dye-DADH homologues have been detected in the genomes of several hyperthermophiles and thermophiles, establishing the potential existence of one or more stable Dye-DADHs with high substrate specificity in thermophiles or hyperthermophiles.

Among Dye-DADHs, the 3D structure of only D-ArgDH has been determined. Although the D-ArgDH crystal structure has provided some information about the molecular interaction between Dye-DADH and a substrate D-amino acid, the available information on the Dye-DADH-substrate recognition mechanism remains limited. We anticipate that in the near future, new information on the structure of a Dye-DADH in complex with its substrate will provide crucial insight into the recognition mechanism. Such structural information may provide the basis for detailed analyses of the physiological and catalytic functions of Dye-DADH, while protein engineering techniques may make it possible to design proteins for the interconversion between D-amino acid dehydrogenase and oxidase. Both enzymes are FAD-dependent oxidoreductases; the difference between the two is the ability to utilize oxygen as electron acceptor from reduced FAD. D-Amino acid oxidase is highly potential for various applications such as an optical resolution for racemate mixture of amino acid and the synthesis of pharmaceutical intermediates. In particular, thermostable D-amino acid oxidase may be expected in the longer term use for their applications. Perhaps through protein engineering, a highly thermostable D-amino acid oxidase could be produced from hyperthermophilic Dye-DADHs, which would increase the versatility of Dye-DADHs, broadening their applicability. Almost all membrane-bound dehydrogenases are huge molecule and have less solubility in aqueous solution. For their application, the enzyme must be solubilized from the membrane before the use. However, the preparation of solubilized enzymes from membrane is cumbersome because the high concentration of membrane-bound enzymes in solution trends to cause the aggregation even if detergent is added to the solution. In general, the aggregation makes application of membrane-bound dehydrogenases very difficult and thus solubilized enzymes are needed for the better application in solution. It would be useful to increase the solubility of membrane-bound dehydrogenases, as this would open additional avenues for potential application of these enzymes in biotechnology.

Acknowledgments We thank Profs. Tomoki Yabutani and Junko Motonaka for their crucial contributions to the application of Dye-DADH.

Compliance with ethical standards This work was supported by JSPS KAKENHI Grant Number 25870271.

Conflict of interest The authors declare that they have no competing interests.

Human and animal rights and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

References

Adams MW (1993) Enzymes and proteins from organisms that grow near and above 100 degrees C. Annu Rev Microbiol 627–658

Bater AJ, Venables WA, Thomas S (1977) Allohydroxy-D-proline dehydrogenase. An inducible membrane-bound enzyme in *Pseudomonas aeruginosa* PAO1. Arch Microbiol 112:287–289

Bergsma J, Van Dongen MB, Konings WN (1982) Purification and characterization of NADH dehydrogenase from *Bacillus subtilis*. Eur J Biochem 128:151–157

Boulette ML, Baynham PJ, Jorth PA, Kukavica-Ibrulj I (2009) Characterization of alanine catabolism in *Pseudomonas aeruginosa* and its importance for proliferation *in vivo*. J Bacteriol 191:6329–6334

Dancey GF, Levine AE, Shapiro BM (1976) The NADH dehydrogenase of the respiratory chain of *Escherichia coli*. I. Properties of the membrane-bound enzyme, its solubilization, and purification to near homogeneity. J Biol Chem 251:5911–5920

Davis KA, Hatefi Y (1971) Succinate dehydrogenase. I. Purification, molecular properties, and substructure. Biochemistry 10:2509–2516

Erbe T, Brückner H (2000) Studies on the optical isomerization of dietary amino acids in vinegar and aqueous acetic acid. Eur Food Res Technol 211:6–12

Frew JE, Hill HA (1987) Electrochemical biosensors. Anal Chem 59: 933A–944A

Fu G, Yuan H, Li C, Lu CD, Gadda G, Weber IT (2010) Conformational changes and substrate recognition in *Pseudomonas aeruginosa* D-arginine dehydrogenase. Biochemistry 49:8535–8545

Furuchi T, Homma H (2005) Free D-aspartate in mammals. Biol Pharm Bull 28:1566–1570

Hamase K, Inoue T, Morikawa A, Konno R, Zaitsu K (2001) Determination of free D-proline and D-leucine in the brains of mutant mice lacking D-amino acid oxidase activity. Anal Biochem 298: 253–258

Hamase K, Morikawa A, Zaitsu K (2002) D-Amino acids in mammals and their diagnostic value. J Chromatogr B Analyt Technol Biomed Life Sci 781:73–91

Hanstein WG, Davis KA, Ghalambor MA, Hatefi Y (1971) Succinate dehydrogenase. II Enzymatic properties. Biochemistry 10:2517–2524

He W, Li C, Lu CD (2011) Regulation and characterization of the *dadRAX* locus for D-amino acid catabolism in *Pseudomonas aeruginosa* PAO1. J Bacteriol 193:2107–2115

Hederstedt L, Holmgren E, Rutberg L (1979) Characterization of a succinate dehydrogenase complex solubilized from the cytoplasmic membrane of Bacillus subtilis with the nonionic detergent Triton X-100. J Bacteriol 138:370–376

Jones H, Venables WA (1983) Effects of solubilisation on some properties of the membrane-bound respiratory enzyme D-amino acid dehydrogenase of *Escherichia coli*. FEBS Lett 151:189–192

Li C, Lu CD (2009) Arginine racemization by coupled catabolic and anabolic dehydrogenases. Proc Natl Acad Sci U S A 106:906–911

Li C, Yao X, Lu CD (2010) Regulation of the *dauBAR* operon and characterization of D-amino acid dehydrogenase DauA in arginine and lysine catabolism of *Pseudomonas aeruginosa* PAO1. Microbiology 156:60–71

Lobocka M, Henning J, Wild J, Klopotowski T (1994) Organization and expression of the *Escherichia coli* K-12 *dad* operon encoding the smaller subunit of D-amino acid dehydrogenase and the catabolic alanine racemase. J Bacteriol 176:1500–1510

Magor AM, Venables WA (1987) Solubilization, purification and characterization of D-alanine dehydrogenase from *Pseudomonas aeruginosa* and effects of solubilization on its properties. Biochimie 69:63–69

Mondadori C, Weiskrantz L, Buerki H, Petschke F, Fagg GE (1989) NMDA receptor antagonists can enhance or impair learning performance in animals. Exp Brain Res 75:449–456

Nagata Y, Higashi M, Ishii Y, Sano H, Tanigawa M, Nagata K, Noguchi K, Urade M (2006) The presence of high concentrations of free D-amino acids in human saliva. Life Sci 78:1677–1681

Nagata Y, Sato T, Enomoto N, Ishii Y, Sasaki K, Yamada T (2007) High concentrations of D-amino acids in human gastric juice. Amino Acids 32:137–140

Nisimoto Y, Wilson E, Heyl BL, Lambeth JD (1986) NADH dehydrogenase from bovine neutrophil membranes. Purification and Properties J Biol Chem 261:285–290

Ohshima T, Soda K (1989) Theremostable amino acid dehydrogenases: applications and gene cloning. Trend Biotechnol 7:210–215

Olsiewski JP, Kaczorowski GJ, Walsh C (1980) Purification and properties of D-amino acid dehydrogenase, an inducible membrane-bound iron-sulfur flavoenzyme from *Escherichia coli* B. J Biol Chem 255: 4487–4494

Rison RA, Stanton PK (1995) Long-term potentiation and N-methyl-D-aspartate receptors: foundations of memory and neurologic disease? Neurosci Biobehav Rev 19:533–552

Pätzold R, Brückner H (2005) Mass spectrometric detection and formation of D-amino acids in processed plant saps, syrups, and fruit juice concentrates. J Agric Food Chem 53:9722–9729

Satomura T, Kawakami R, Sakuraba H, Ohshima T (2002) Dye-linked D-proline dehydrogenase from hyperthermophilic archaeon *Pyrobaculum islandicum* is a novel FAD-dependent amino acid dehydrogenase. J Biol Chem 277:12861–12867

Satomura T, Ishikura M, Koyanagi T, Sakuraba H, Ohshima T, Suye SI (2015) Dye-linked D-amino acid dehydrogenase from the thermophilic bacterium *Rhodothermus marinus* JCM9785: characteristics and role in *trans*-4-hydroxy-L-proline catabolism. Appl Microbiol Biotechnol 99:4265–4275

Tani Y, Tanaka K, Yabutani T, Mishima Y, Sakuraba H, Ohshima T, Motonaka J (2008) Development of a D-amino acids electrochemical sensor based on immobilization of thermostable D-proline dehydrogenase within agar gel membrane. Anal Chim Acta 619:215–220

Tani Y, Itoyama Y, Nishi K, Wada C, Shoda Y, Satomura T, Sakuraba H, Ohshima T, Hayashi Y, Yabutani T, Motonaka J (2009) An amperometric D-amino acid biosensor prepared with a thermostable D-proline dehydrogenase and a carbon nanotube-ionic liquid gel. Anal Sci 25:919–923

Watanabe S, Morimoto D, Fukumori F, Shinomiya H, Nishiwaki H, Kawano-Kawada M, Sasai Y, Tozawa WY (2012) Identification and characterization of D-hydroxyproline dehydrogenase and Δ1-pyrroline-4-hydroxy-2-carboxylate deaminase involved in novel L-hydroxyproline metabolism of bacteria: metabolic convergent evolution. J Biol Chem 287:32674–33288

Wierenga RK, Terpstra P, Hol WG (1986) Prediction of the occurrence of the ADP-binding βαβ-fold in proteins, using an amino acid sequence fingerprint. J Mol Biol 187:101–107

Yoneya T, Adams E (1961) Hydroxyproline metabolism. V. Inducible allohydroxy-D-proline oxidase of *Pseudomonas*. J Biol Chem 236: 3272–3279