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Topology of 4-Pyridoxic acid dehydrogenase in transformed *Escherichia coli* cells

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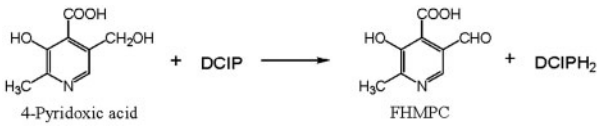
The topology of 4-pyridoxic acid dehydrogenase in the *Escherichia coli* cell membrane was examined with transformed *E. coli* cells overexpressing the enzyme from *Mesorhizobium loti*. The recombinant enzymes with a His_6-tag either in the N-terminal region or at the C-terminus were localized on the *E. coli* cell membrane like the wild-type enzyme without a His_6-tag. The His_6-tags were labelled with Ni–NTA AP conjugate only when the *E. coli* protoplast cells were broken. The membrane-bound enzyme in the intact protoplast cells was not digested by trypsin, although the one in the gently broken protoplast cells was almost totally digested. Thus, 4-pyridoxic acid dehydrogenase was an integral monotopic protein, protruding into a cytoplasm side from the bacterial membrane. The deletion or mutation of a deduced transmembrane segment in 4-pyridoxic acid dehydrogenase made it an inclusion body, and the enzyme protein was not found in the *E. coli* cell membrane. Thus, it was suggested that the intact deduced transmembrane segment was necessary for 4-pyridoxic acid dehydrogenase to be localized on the bacterial cell membrane.

Keywords: membrane topology/4-pyridoxic acid dehydrogenase/pyridoxine (vitamin B_6)-degradation/*Mesorhizobium loti*.

Abbreviations: DCIP, 2, 6-dichlorolindophenol; FHMPC, 5-formyl-3-hydroxy-2-methylpyridine-4-carboxylic acid; KPB, potassium phosphate buffer; PAD-Gln543His_6, recombinant 4-pyridoxic acid dehydrogenase with a His_6-tag at the C-terminus; PAD(Δ355-373)-Gln543His_6, PAD-Gln543His_6 without 355-373 residues; PAD(FDM)-Gln543His_6, PAD-Gln543His_6 in which three amino acid residues (LAA) at 367–369 were changed to FDM; PAD-Met1His_6, PAD with His_6-tag between Met1 and Phe2; PAD-Glu5His_6, PAD with His_6-tag between Glu5 and Ser6.

4-Pyridoxic acid dehydrogenase is the fourth enzyme in the degradation pathway for pyridoxine (vitamin B_6) (*1*), and was purified from *Pseudomonas* MA-1 and characterized (*2*). Its gene has been cloned from *Mesorhizobium loti*, a nitrogen-fixing symbiotic bacterium (*3*). It is a membrane-bound FAD-dependent enzyme and catalyses oxidation of 4-pyridoxic acid to 5-formyl-3-hydroxy-2-methylpyridine-4-carboxylic acid (FHMPC), coupling with reduction of 2,6-dichloroindophenol (DCIP), as follows.



The enzyme can use CoQ, which is a plausible electron acceptor in the bacterial cells (membranes), as an electron acceptor (*2*). It is only membrane bound among the enzymes in the degradation pathway and could donate electrons to produce ATP through an electron transport system coupled with ATP synthase. Thus, its topology in the bacterial membranes should be examined to elucidate the mechanism of interaction of the pyridoxine-degradation with the ATP synthesis in the bacterial cells.

Membrane proteins can be categorized as integral membrane proteins, integral monotopic proteins or peripheral membrane proteins (*4*). 4-Pyridoxic acid dehydrogenase requires a detergent to be solubilized (*2*). The enzyme contains a probable transmembrane segment (19 residues, 355–373) in its amino acid sequence (total 543 residues) (*3*). The results suggest that 4-pyridoxic acid dehydrogenase is an integral protein, especially an integral monotopic protein, although there are two possibilities that it protrude to periplasmic or cytosolic side. So far no study has been done on topology of the enzyme in bacterial membranes.

Here, we have studied topology of 4-pyridoxic acid dehydrogenase with *Escherichia coli* cells overexpressing the enzyme from *M. loti* cells, because the enzyme activity in the *M. loti* cells were so low, even when they were grown in a pyridoxine synthetic medium, that detailed analyses of its location were difficult (*3*). We have found that 4-pyridoxic acid dehydrogenase is an integral monotopic protein protruding to cytosolic space of *E. coli* cells.

Materials and methods

Preparation of E. coli cells expressing recombinant 4-pyridoxic acid dehydrogenase with a His_6-tag in an N-terminal region or at the C-terminus

The transformed *E. coli* B834/pET21a-*pad*-Gln543His_6 cells expressing recombinant 4-pyridoxic acid dehydrogenase with a His_6-tag at the C-terminus (PAD-Gln543His_6) were prepared as described earlier (*3*). The *E. coli* cells expressing recombinant 4-pyridoxic acid dehydrogenase with a His_6-tag between Met1 and Phe2 (PAD-Met1His_6) or between Glu5 and Ser6 (PAD-Glu5His_6) were prepared as follows. The gene *pad*-Met1His_6 encoding 4-pyridoxic acid dehydrogenase with a His_6-tag between Met1 and Phe2 was amplified by PCR with N-6792F and 6792R (Table 1) as primers, and the chromosomal DNA prepared from *M. loti* MAFF303099 as

Table 1. Oligonucleotide primers used for construction of plasmids.

Primer[a]	Sequence[b]
6792F	5′-CATATGCCGCACGCGGAAAGTTACGAC TAT-3′
6792R	5′-GAATTCTCATTGCACTGCCCTGCCTTCTT TTG-3′
N-6792F	5′-CATATGcatcatcatcatcatcatCCGCACGCGGAAA GTTACGAC-3′
N5-6792F	5′-catcatcatcatcatcatAGTTACGACTATATCATCG TTGGGG-3′
N5-6792R	5′-TTCCGCGTGCGGCATATGTATATCTC-3′
TM6792F	5′-AAGCCGTTCAAGGCGCCGTTTGCGG-3′
TM6792R	5′-GGGTCGCGCCGGACCGCTCTTGAGG-3′
LAA-F	5′-TGGCCATACTTCAAGCCGTTCAAGGCGCC-3′
LAA-R	5′-*CATGTCGAA*CGGCGCCGCCGTGAAGAG-3′

[a]F and R indicate forward and reverse primers, respectively.
[b]Underlined parts show the restriction site of *Nde* I (6792F and N-6792F) or *Eco*R I (6792R); lower cases show the His_6-tag sequence; italic letters show variation from the wild-type sequence.

Table 2. Localization of 4-pyridoxic acid dehydrogenase with His_6-tag in the transformed *E. coli* cells.

Transformed *E. coli* cells (fractions)	Total activity (U)	Specific activity (U/mg)	Relative activity (%)
B834/pET21a-*pad*-Gln543His_6			
Crude extract	0.45 ± 0.07	0.30 ± 0.09	100
Membrane	0.40 ± 0.04	2.90 ± 0.50	88.9
Soluble	0.04 ± 0.02	0.02 ± 0.01	8.9
B834/pET21a-*pad*-Met1His_6			
Crude extract	0.32 ± 0.05	0.30 ± 0.07	100
Membrane	0.26 ± 0.05	1.00 ± 0.21	81.3
Soluble	0.05 ± 0.01	0.07 ± 0.02	15.6
B834/pET21a-*pad*-Glu5His_6			
Crude extract	0.11 ± 0.03	0.09 ± 0.04	100
Membrane	0.09 ± 0.02	0.36 ± 0.11	81.8
Soluble	0.02 ± 0.01	0.02 ± 0.01	18.2

The transformed cells (180 mg) were disrupted by sonication to prepare crude extracts. Membrane and cytosolic fractions were prepared as described earlier (*3*).

described earlier (*5*) as the template. The PCR conditions were those described in the KOD-Plus-protocol (TOYOBO, Osaka, Japan). The amplified DNA fragment was inserted into a pTA2 vector according to the TArget Clone-Plus-protocol (TOYOBO), and then the sequence of the amplified gene was confirmed with an ABI PRISM 31000-Avant Genetic Analyser (Applied Biosystems, Tokyo, Japan). The plasmid pTA2-*pad*-Met1His_6 obtained was digested with *Nde*I and *Eco*RI, and then the *Nde*I/*Eco*RI fragment was inserted into pET-21a (TaKaRa, Ohtsu, Japan) to obtain an expression plasmid pET21a-*pad*-Met1His_6. The *E. coli* B834(DE3) cells were co-transformed with this plasmid and plasmid pKY206 to express the recombinant 4-pyridoxic acid dehydrogenase with a His_6-tag between Met1 and Phe2. The co-transformation of pKY206 carrying *groES* and *groEL* (*6*) was necessary to express the active membrane-bound PAD-Met1His_6 (*7*).

The gene *pad*-Glu5His_6 encoding 4-pyridoxic acid dehydrogenase with a His_6-tag between Glu5 and Ser6 was prepared with pET21a-*pad* as the template, which had been prepared by PCR with 6792F and 6792R (Table 1) as primers and the chromosomal DNA from *M. loti* cells as a template as described earlier (*3*). The N5-6792F and N5-6792R (Table 2) were used as primers according to the instruction of the KOD-Plus-Mutagenesis Kit (TOYOBO). The *E. coli* B834 (DE3) cells were co-transformed with the plasmids pET21a-*pad*-Glu5His_6 prepared and pKY206.

Preparation of mutated 4-pyridoxic acid dehydrogenases

The gene *pad(Δ355–373)*, in which 19 residues corresponding to the predicted transmembrane segment (355–373) was deleted, was then prepared with the KOD-Plus-Mutagenesis Kit. The plasmid pET21a-*pad*-Gln543His_6 prepared previously to express 4-pyridoxic acid dehydrogenase with a His_6-tag at the C-terminus (*3*) was used as a template while TM6792F and TM6792R (Table 1) as primers. The *E. coli* B834 (DE3) cells were co-transformed with pET21a-*pad(Δ35–373)*-Gln543His6 obtained and pKY206. The gene *pad(FDM)*, in which L387-A368-A369 was changed to F387-D368-M369, was also prepared with the KOD-Plus-Mutagenesis Kit. The plasmid pET21a-*pad*-Gln543His_6 was used as a template while LAA-F and LAA-R (Table 1) as primers. The *E. coli* B834 (DE3) cells were co-transformed with pET21a-*pad(FDM)*-Gln543His6 obtained and pKY206.

Preparation of protoplast and treatment with Ni–NTA AP conjugate or trypsin

Protoplasts of *E. coli* cells were prepared as described by Miura *et al.* (*8*) or Osborn *et al.* (*9*). *Escherichia coli* B834/pET21a-*pad*-Gln543His_6 cells were grown in an LB medium containing 50 μg/ml ampicillin at 23°C. *Escherichia coli* B834/pKY/pET21a-*pad*-Met1His_6 cells were grown in the medium containing 50 μg/ml ampicillin and 12.5 μg/ml tetracycline. The cells (200 mg) were washed with 0.9% NaCl twice, and then suspended in 4.2 ml of water. To the suspension, 2.3 ml of 0.1M Tris–HCl (pH 8.3), 2.1 ml of 2M sucrose, 0.4 ml of 1% Na–EDTA (pH 7.0) and 0.4 ml of 0.5% lysozyme were added in this order. The cell suspension was incubated at 40°C for 3 min and then at 30°C for 1 h. Then, the cells were collected by centrifugation and washed with an isotonic buffer [0.05M potassium phosphate buffer (KPB), pH 8.0, 0.44 M sucrose]. The washed cells were suspended in 4 ml of the isotonic buffer and used as protoplasts.

The protoplast suspension (1 ml) was centrifuged, and then 1 ml of 50 mM KPB (pH 8.0) was added to the precipitated protoplast cells to gently burst them. To the burst cell suspension, 20 μl of Ni–NTA AP conjugate (QIAGEN, Tokyo, Japan) was added and then the suspension was incubated at 37°C for 2 h to label His_6-tag with the conjugate. The protoplast suspension in the isotonic buffer was also incubated at 37°C for 2 h with 20 μl of Ni–NTA AP conjugates to label the intact protoplast cells. The labelled burst and intact protoplast cells were collected by centrifugation and then washed with 50 mM KPB (pH 8.0). The membrane fraction of these protoplast cells was prepared as described earlier (*3*). Protoplast integrity was checked by monitoring malate dehydrogenase activity (*10*) leaked from the cells.

Protoplasts of *E. coli* B834/pET21a-*pad*-Gln543His_6 cells (120 mg) prepared by essentially the same procedure as described above were suspended in 4 ml of the isotonic buffer. Half of the suspension (2 ml) was gently sonicated to disrupt the protoplasts in the isotonic buffer. Trypsin (5 mg/ml) was added to the gently disrupted protoplast and intact protoplast suspensions and then the suspensions were incubated at 37°C for 3 h. The gently pre-disrupted and intact protoplasts were washed thoroughly to remove trypsin with 50 mM KPB (pH 8.0), and then disrupted sufficiently in 50 mM KPB (pH 8.0) by sonication to make crude extracts in which 4-pyridoxic acid dehydrogenase-binding membrane fragments were suspended. The membrane fragments were collected by ultra-centrifugation as described earlier (*3*).

Assay for 4-pyridoxic acid dehydrogenase and protein

The oxidation of 4-pyridoxic acid was measured at 25°C by monitoring the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm in the presence of phenazine methosulphate. A reaction mixture (1 ml) consisted of 0.1M KPB (pH 8.0), 2.5 mM 4-pyridoxic acid, 48 μM DCIP, 0.1 mM phenazine methosulphate and the enzyme. The reaction was initiated by addition of 4-pyridoxic acid. One unit of enzyme activity was defined as the amount that catalysed the reduction of 1 μmol of DCIP per min. A molar extinction coefficient for DCIP of 21 000 $M^{-1}cm^{-1}$ at 600 nm (*2*) was used for activity calculations. The protein concentration was determined by the dye-binding method with BSA as the standard (*11*).

Purification of PAD(Δ355–373)-Gln543His$_6$ and PAD(FDM)-Gln543His$_6$ in the inclusion body

Escherichia coli cells (1 g) expressing PAD(Δ355–373)-Gln543His$_6$ or PAD(FDM)-Gln543His$_6$ suspended in 15 ml of KPB (pH 8.0) were sonicated thoroughly and then the mixture was centrifuged at 10 000*g* for 20 min at 4°C. The precipitate was solubilized with a lysis buffer [100 mM Na_2HPO_4, 10 mM Tris–HCl (pH 8.0) and 8 M urea] overnight. The solubilized protein solution (2 ml) was gently mixed with Ni–NTA-affinity resin (2 ml) equilibrated with the lysis buffer. The mixture was poured into a column and then the column was consecutively washed with 3 ml (each) of the lysis buffers (pH 8.0, 6.3 and 5.9). The mutated enzymes were eluted with the lysis buffer (pH 5.9).

Other analytical methods

Alkaline phosphatase (*12*) and malate dehydrogenase (*10*) activities were determined as described earlier. Proteins with a His$_6$-tag were stained with the Ni–NTA AP conjugate according to the company's manual (QIAGEN). The tertiary structure of 4-pyridoxic acid dehydrogenase was predicted by Geno3D (*13*)

Results and discussion

Expression and localization of 4-pyridoxic acid dehydrogenases with His$_6$-tag inttransformed E. coli cells

Escherichia coli cells transformed with the plasmids, in which the genes encoding 4-pyridoxic acid dehydrogenase with His$_6$-tag were inserted, expressed the correspondent enzymes. Activities in the crude extracts and the membrane and cytosolic fractions are shown in Table 2. The enzymes with His$_6$-tag were localized on the membrane of the transformed cells like the wild-type enzyme without His$_6$-tag (*3*). Thus, the presence of a His$_6$-tag in the C- or N-terminal region did not affect the location of 4-pyridoxic acid dehydrogenase in the bacterial cells.

The expressed proteins were detected on an SDS–PAGE gel (Fig. 1A). The PAD-Gln543His$_6$ protein was observed in the membrane fraction (Fig. 1A and lane 1). The dense protein band of chaperonin GroEL coexpressed forced down the bands of PAD-Met1His$_6$ and PAD-Glu5His$_6$ (lanes 2 and 3, respectively) proteins in the crude extracts containing the membrane fragments. Although the protein bands are hardly seen in Fig. 1A, lanes 2 and 3, the dense protein bands could be seen around the lower boundary of the dense GroEL protein band on the original dried SDS–PAGE gel. The presence of a His$_6$-tag on the enzyme proteins was confirmed by the His$_6$-tag staining with the Ni–NTA AP conjugate as shown in Fig. 1B. The co-expression of chaperonin was necessary to express the active membrane-bound 4-pyridoxic acid dehydrogenase with a His$_6$-tag at the N-terminal region. In contrast, the active membrane-bound enzyme with a His$_6$-tag at C-terminal was expressed without coexpression with the chaperonin proteins.

Labelling of His$_6$-tag of membrane-bound 4-pyridoxic acid dehydrogenases

The N-terminal His$_6$-tag of PAD-Met1His$_6$ and C-terminal His$_6$-tag of PAD-Gln543His$_6$ were labelled with Ni–NTA AP conjugate only when the *E. coli* protoplast cells were broken (Table 3). If these His$_6$-tags protrude into periplasmic space from the cell membrane, then the labelling (alkaline phosphatase activity) of the intact and broken protoplast cells should be the same. Thus, the results showed that N- and C-terminal portions of 4-pyridoxic acid dehydrogenase protrude into cytosolic side from the bacterial cell membrane.

Tryptic digestion of membrane-bound 4-pyridoxic acid dehydrogenase

The intact and broken protoplast cells were digested with trypsin. 4-Pyridoxic acid dehydrogenase was not

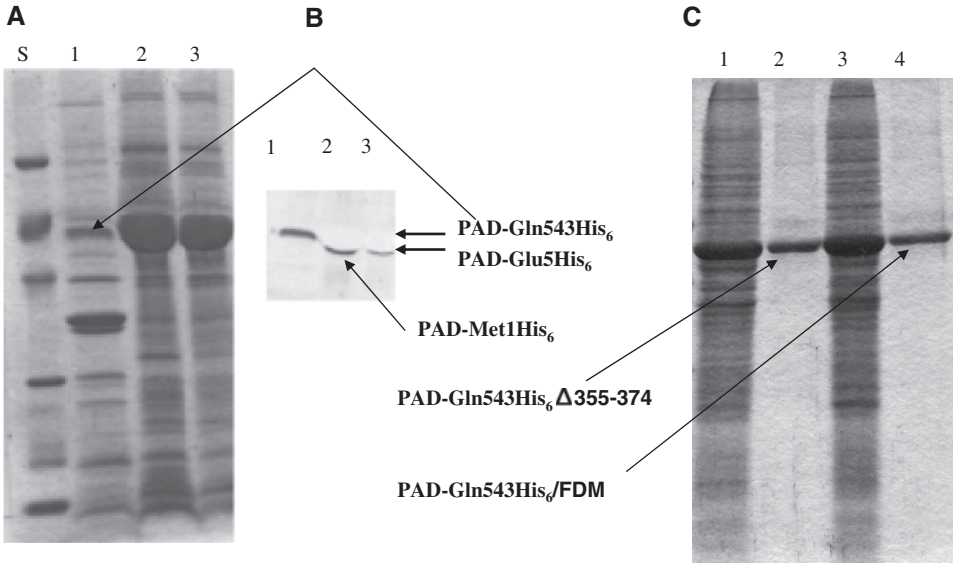


Fig. 1 SDS–PAGE patterns of membrane fractions of *E. coli* cells expressing PAD-Gln543His$_6$, PAD-Met1His$_6$ or PAD-Glu5His$_6$. (**A**) Proteins were stained with Coomassie Brilliant Blue R-250. Lane S, the molecular mass standard markers; lane 1, membrane fraction (10 μg of protein) from *E. coli* cells expressing PAD-Gln543His$_6$; lane 2, crude extract (20 μg) of *E. coli* cells expressing PAD-Met1His$_6$; lane 3, crude extract (20 μg) of *E. coli* cells expressing PAD-Glu5His$_6$. (**B**) Staining of His$_6$-tag with Ni–NTA AP conjugate. The protein bands around the PAD with His$_6$-tag on the SDS–PAGE gel shown in **A** were electro-blotted to a nitrocellulose membrane (Amersham). (**C**) Solubilized inclusion body fractions from *E. coli* cells expressing PAD-Gln543His$_6$/Δ355–373 (lane 1, 20 μg) and PAD-Gln543His$_6$/FDM (lane 3, 20 μg) were applied. Purified PAD-Gln543His$_6$/Δ355–373 (lane 2, 5 μg) and PAD-Gln543His$_6$/FDM (lane 4, 5 μg) were applied.

Table 3. Labelling of His_6-tags and tryptic digestion.

Escherichia coli protoplast cells	Alkaline phosphatase activity (U/mg) of Ni–NTA AP bound to cell membranes	4-Pyridoxic acid dehydrogenase activity (U) found in cell membrane from protoplast cells digested with trypsin
B834/pET21a-*pad*-Met1His_6		
Intact	0.000 ± 0.001[a]	ND
Burst	0.021 ± 0.003[a]	ND
B834/pET21a-*pad*-Gln543His_6		
Intact	0.001 ± 0.001[b]	0.33 ± 0.01[c]
Burst	0.018 ± 0.001[b]	0.01 ± 0.001[c]

[a,b]Ni–NTA AP conjugate bound to the His_6 moiety of PAD-Met1His_6 and PAD-Gln543His_6 on the intact or burst *E. coli* protoplast cell membranes. [c]Tryptic degradation of 4-pyridoxic acid dehydrogenase in the intact or burst *E. coli* protoplast cells. ND, Not determined

degraded by trypsin when the *E. coli* protoplast cells were intact. In contrast, 98% of the enzymes were degraded in broken ones. The results also showed that 4-pyridoxic acid dehydrogenase protrudes into the cytosolic side of the transformed *E. coli* cells from the cell membranes.

Effect of deletion or mutation of deduced transmembrane part of 4-pyridoxic acid dehydrogenase

The deduced transmembrane segment (amino acid residues, 356–373) in the amino acid sequence of 4-pyridoxic acid dehydrogenase was deleted or mutated to examine its involvement in the binding of the enzyme to the membrane. PAD(Δ355–373)-Gln543His_6 was expressed as an inclusion body without enzyme activity (Fig. 1C and lane 1) even when pET21a-*pad(Δ355–373)*-Gln543His6 was co-expressed with pKY206. Several trials to refold and activate the purified PAD(Δ355–373)-Gln543His_6 (Fig. 1C and lane 2) ended unsuccessfully. Thus, it was suggested that the deduced transmembrane segment was necessary for binding of the enzyme to the membrane and making it enzymatically active. To examine the contribution of special amino acid residues in the segment to the membrane binding, only three residues in the segment (L369–A368–A369), which were predicted to locate in an almost central part of the interacting site between the enzyme and the membrane, were changed to FDM. The amino acid residues were selected based on the corresponding amino acid sequence found in a homologous (35% identity) cytosolic enzyme (choline oxidase) from *Arthrobacter globiformis* (PDB code, 2JBV) (*14*). The PAD(FDM)-Gln543His_6 was also expressed as an inclusion body (Fig. 1C and lane 3) even when pET21a-*pad(FDM)*-Gln543His6 was co-expressed with pKY206. The purified PAD(FDM)-Gln543His_6 protein (Fig. 1C and lane 4) could not be refolded and activated. The results suggested that the three residues were essential in making the enzyme protein to be membrane-bound. Although the reason why these mutant enzymes [PAD(Δ355–373)-Gln543His6 and PAD(FDM)-Gln543His6] are expressed only in an inactive inclusion body is unknown, it is likely that the loss of interaction of the protein segment containing these three residues with the bacterial membrane leads to the failure in protein translocation and thereby misfolding of the protein.

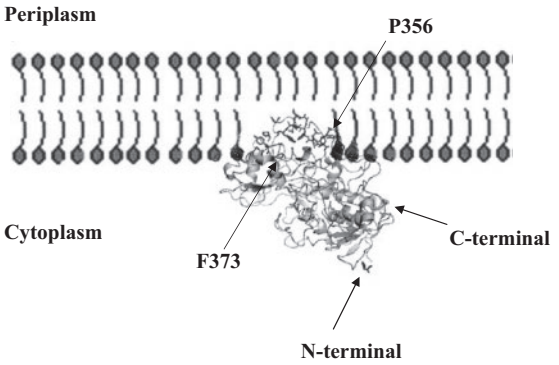


Fig. 2 Proposed model of topology of 4-pyridoxic acid dehydrogenase. The predicted tertiary structure of the enzyme is drawn as a ribbon model with the deduced transmembrane residues (amino acid residues 356–372) shown in a stick model.

Involvement of amino-terminal acyl group in attachment of 4-pyridoxic acid dehydrogenase on the membrane

N-terminal of wild-type 4-pyridoxic acid dehydrogenase without a His_6-tag was acylated (*3*). It was possible that the acyl group was involved in the anchoring of the enzyme to the plasma membrane of *E. coli* cells. Thus, the N-terminal amino acid sequence of PAD-Gln543His_6 purified from the membrane was determined. The N-terminal sequence PHAESYDYII was obtained and it showed that the N-terminal of the membrane-bound PAD-Gln543His_6 was not acylated and the acyl group was not required for binding of 4-pyridoxic acid dehydrogenase to the membrane. The results also showed that the modification of C-terminal end with a His_6-tag prevented the acylation of N-terminal by an unknown reason.

The results obtained here show that 4-pyridoxic acid dehydrogenase is an integral monotopic membrane protein protruding into cytosolic side from the membrane (Fig. 2). The predicted tertiary structure of the enzyme based on a comparison of its primary structure with that of choline oxidase (PDB code, 2JBV) from *A.globiformis* (*14*) showed that the deduced transmembrane segment located the surface area of 4-pyridoxic acid dehydrogenase forming a cluster. The distance between P366 and V358, which may reside at bottom and on the surface of phospholipid bi-layer of the cell membrane, respectively, was 17 Å. Because the thickness of the cell membrane phospholipid bi-layer is about 50 Å, the transmembrane segment may get into the monolayer (Fig. 2). The distance between P356 and Y372, which may reside at the surface of the phospholipid bi-layer was 29 Å, suggesting that the segment

occupies the surface width corresponding to about four phospholipid molecules (Fig. 2).

Here, 4-pyridoxic acid dehydrogenase was shown to be an integral monotopic membrane protein in the transformed *E. coli* cells. The results do not necessarily show that 4-pyridoxic acid dehydrogenase is also localized in the same way in *M. loti* or *Pseudomonas* MA-1 cells. However, it is possible that these bacteria share the localization on the membrane because they are Gram-negative proteobacteria and have a similar cell wall structure including the inner (cytoplasmic) membrane. The fact that the conditions necessary for solubilization of 4-pyridoxic acid dehydrogenase in the bacterial membranes are similar (*2*,*3*) is consistent with the idea that the enzyme is an integral monotopic membrane protein in *M. loti* and *Pseudomonas* MA-1 cells.

We are trying to elucidate a tertiary structure of 4-pyridoxic acid dehydrogenase by the X-ray crystallography to find out a segment, which can interact with co-enzyme Q in the bacterial membrane.

Conflict of interest
None declared.

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