Eficient production of active form of vitamin D_3 by microbial conversion

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0.5µm

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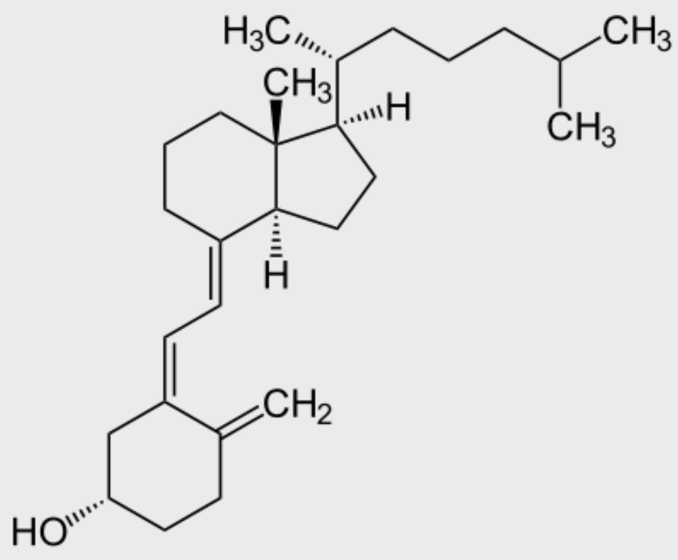
Vitamin D_3

-Vitamin D is a group of fat-soluble secosteroides, the two major forms of which are vitamin D_2 (or ergocalciferol), and vitamin D_3 (or cholecalsiferol).

-Vitamin D_3 (VD_3) has important functions in modulating calcium metabolism in mammalians.

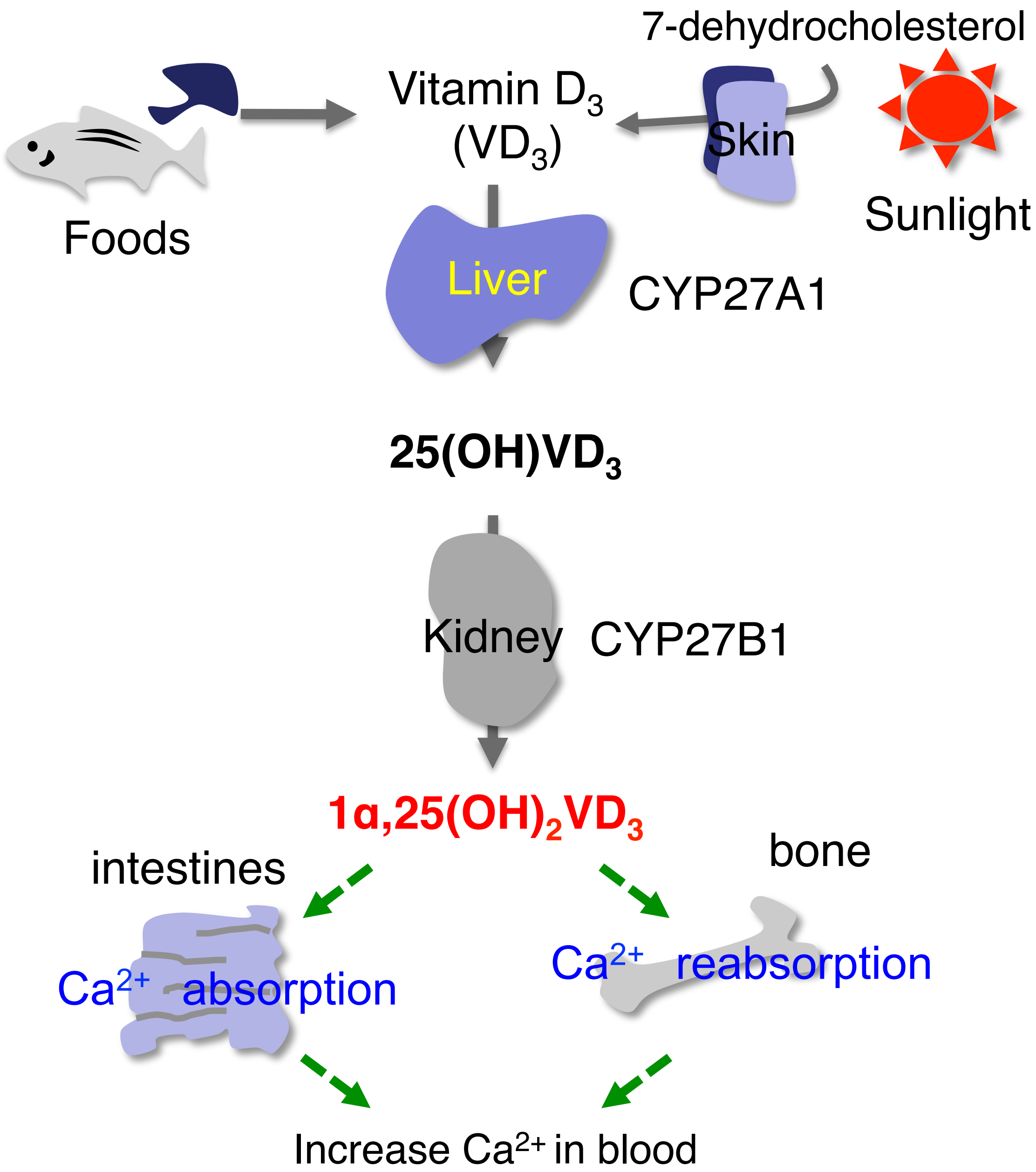
-the most active form, 1α, $25(OH)_2VD_3$ has been used in treatment of chronic renal failure, hyperparathyroidism, osteoporosis, and psoriasis.



Vitamin D_3

・osteoporosis is present in 15% of those 50–59 years of age, but these figures increase quickly to 70% of those over 80 years of age (WHO2015)

Mechanism of action



One of the most important roles of vitamin D_3 is

- maintaining skeletal calcium balance by promoting calcium absorption in the intestines, promoting bone resorption by increasing osteoclast number.
- maintaining calcium and phosphate levels for bone formation, and allowing proper functioning of parathyroid hormone to maintain serum calcium levels.

- Half-life

  $25(OH)VD_3$ 1/2t = 15 day

  $1\alpha,25(OH)_2VD_3$ 1/2t = 15hr

- Blood concentration

  $25(OH)VD_3$ 30～80 nmol/L (12 ～ 32 ng / ml)

- The required daily intake

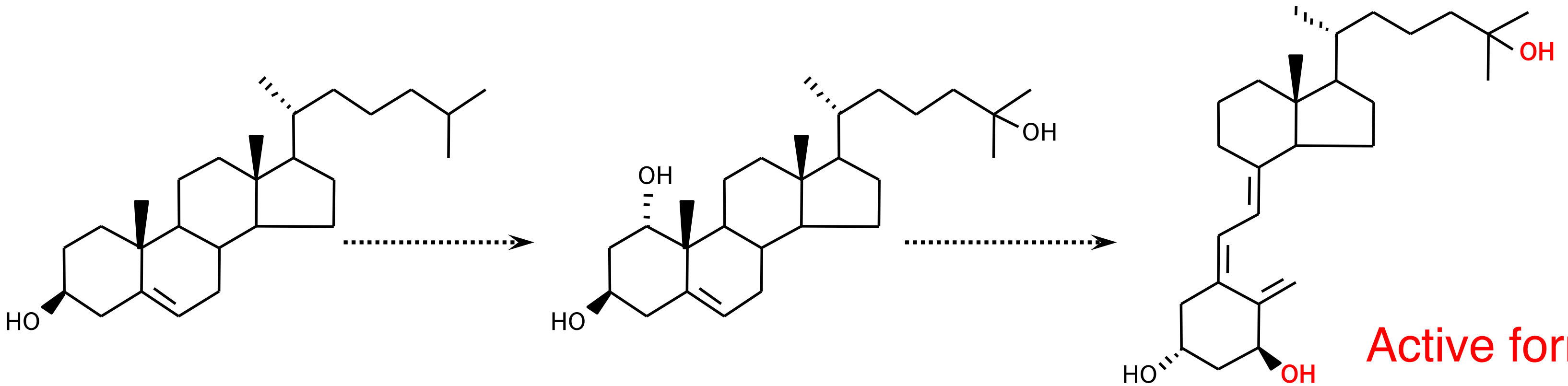
  5 μg / day

- Tolerable upper intake level

  50μg / day for adult

Synthesis of active form of vitamin D_3

Chemical processes



Cholesterol

Active form of VD_3

$1\alpha,25(OH)_2D_3$

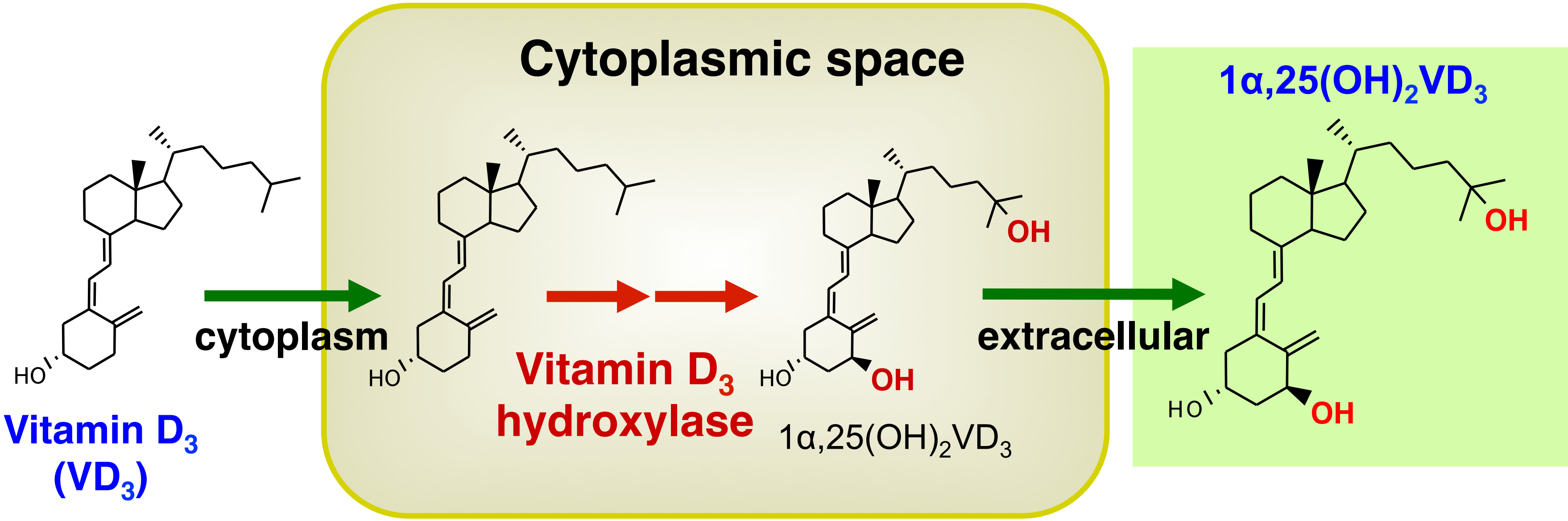
- about 20 processes
- yield, 1% of start material
- site-specific hydroxylation is difficult

Synthesis of active form of vitamin D_3

Bacterial conversion

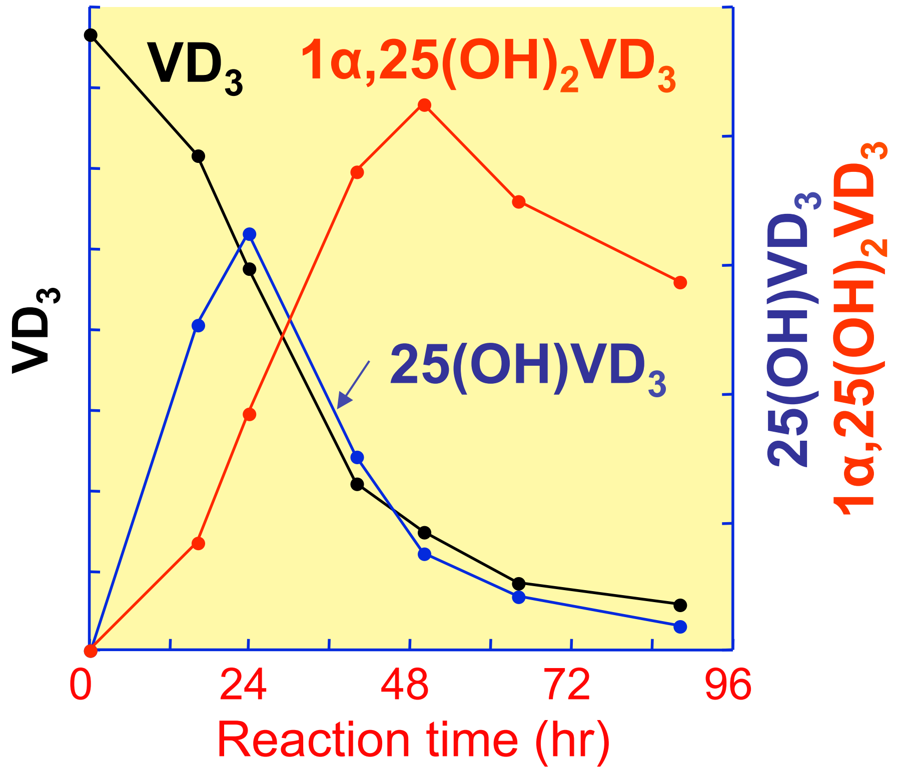
Bacterial and fungal strains producing $1\alpha,25(OH)_2VD_3$ from VD_3 were screened and the actinomycete *Pseudonocardia autotrophica* was identified in 1992.

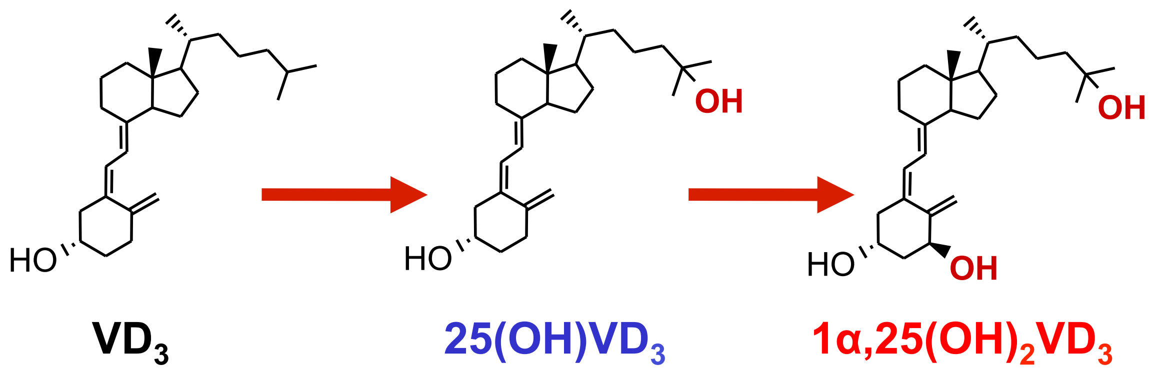
Pseudonocardia autotrophica



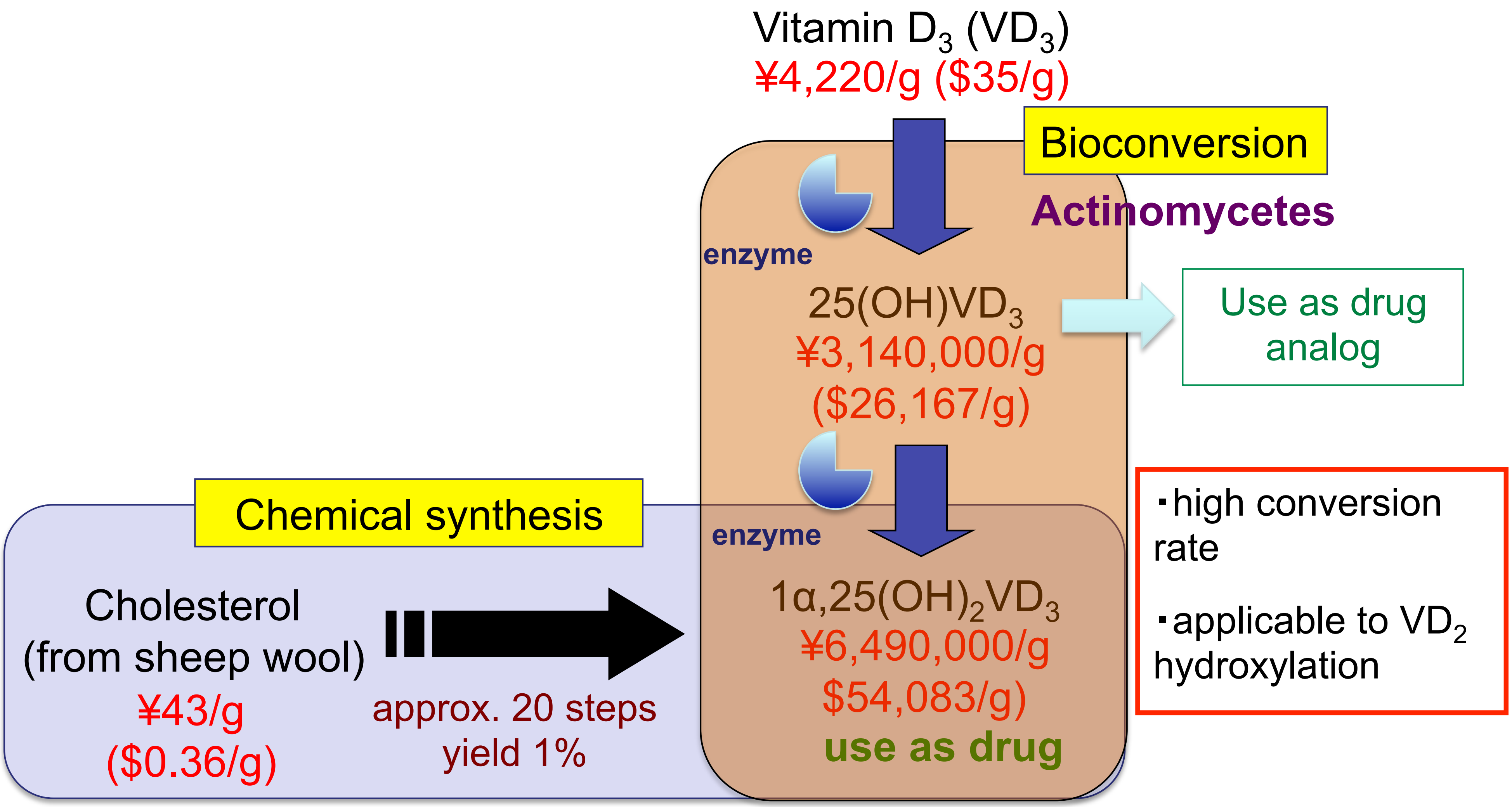
- one step process
- yield is low
- side reactions can not control (eg, 26-hydroxylation)

Vitamin D_3 hydroxylation by *Pseudonocardia autotrophica*





Production of vitamin D_3 hydroxylated forms



prices are from Sigma catalog (1$ = ¥120)

Objective

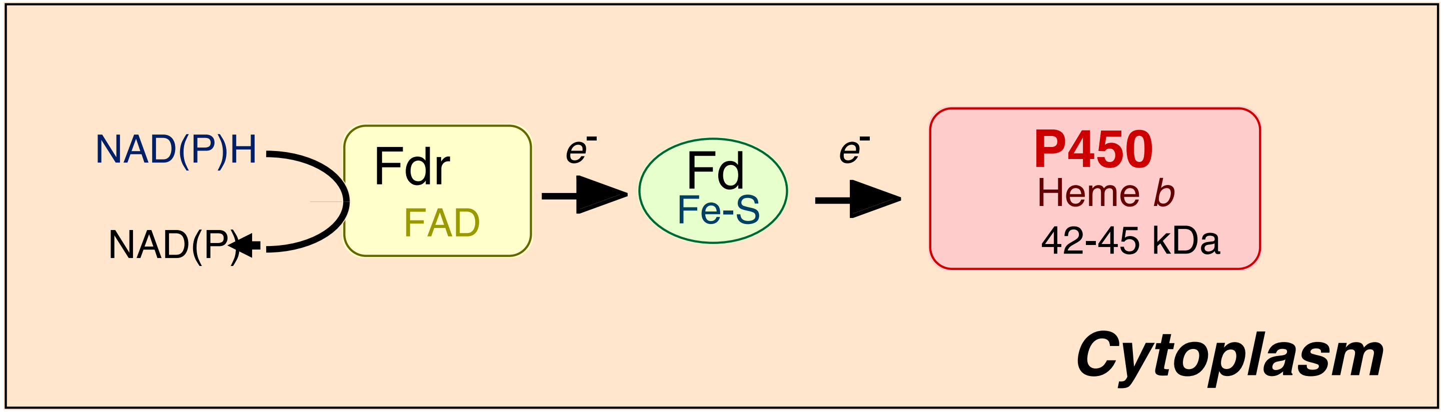
Identify and characterization of VD_3 hydroxylase and development of bacterial platform for the effect production of hydroxylated forms of vitamin D_3

Vitamin D_3 hydroxylase (Vdh)

The actinomycete *Pseudonocardia autotrophica* was identified in 1992 as a bacterial strain producing 1α,25$(OH)_2VD_3$ from VD_3.

- vitamin D_3 hydroxylase was purified from *Pseudonocardia autotrophica*.
- the vdh gene encodes a protein containing 403 amino acids with a molecular weight of 44,368 Da.
- this hydroxylase was found to be homologous with the P450 belonging to CYP107 family.

Properties of Bacterial P450



Fdr; Ferredoxin reductase
Fd; Ferredoxin
FAD; Flavin adenine dinucleotide
Fe-S; Fe-S cluster

- Versatile biological catalyst (steroid biosynthesis, xenobiotics metabolism, secondary metabolites biosynthesis etc.)
- Catalyze hydroxylation, dealkylation, epoxidation, etc.
- Bacterial P450s exist as soluble enzymes
  (Eukaryotic P450s exist as membrane-bound enzymes)

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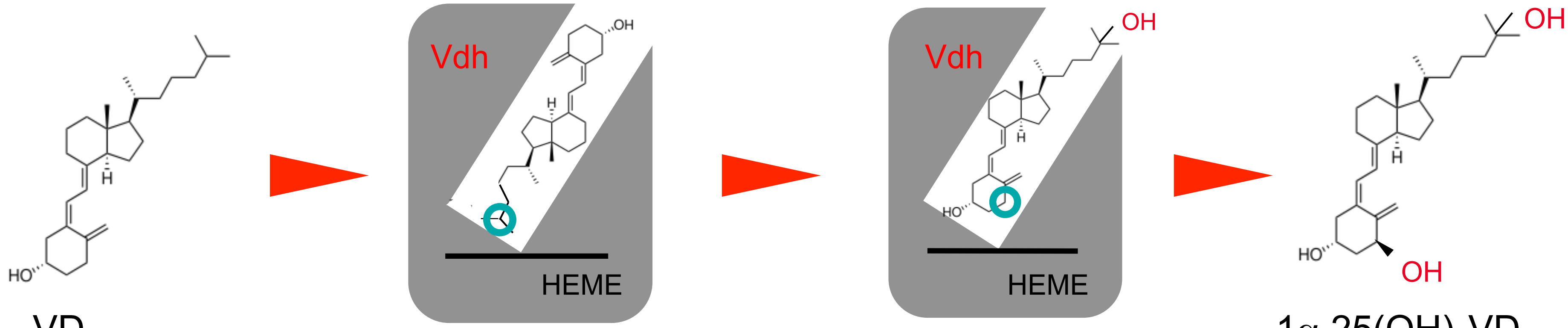
Kinetic parameters of Vdh and other VD3 hydroxylating enzymes



VD_3 — Hydroxylation at C-25 — Hydroxylation at C-1α — $1\alpha,25(OH)_2VD_3$

	Vdh	*Streptomyces* CYP105A1	Human CYP27A1	Mouse CYP27B1	Human CYP2R1
25-hydroxylase activity against VD_3					
K_m (μM)	9.1	0.54	3.2	ND	4.4
V_{max} (mmol/min/mol of P450)	244	16	270	ND	480
V_{max}/K_m	27	30	84	ND	109
1α-hydroxylase activity against $25(OH)VD_3$					
K_m (μM)	3.7	0.91	3.5	0.05	ND
V_{max} (mmol/min/mol of P450)	588	3.6	21	2730	ND
V_{max}/K_m	159	3.9	6	54600	ND

Rhodococcus erythropolis as a host cell

1. Growth at a wide temperature range

   4 - 35 ˚C (*R. erythropolis*)

2. Organic solvent resistance

   Ethanol, Acetone, Propanol, DMSO, Ethylacetate, etc

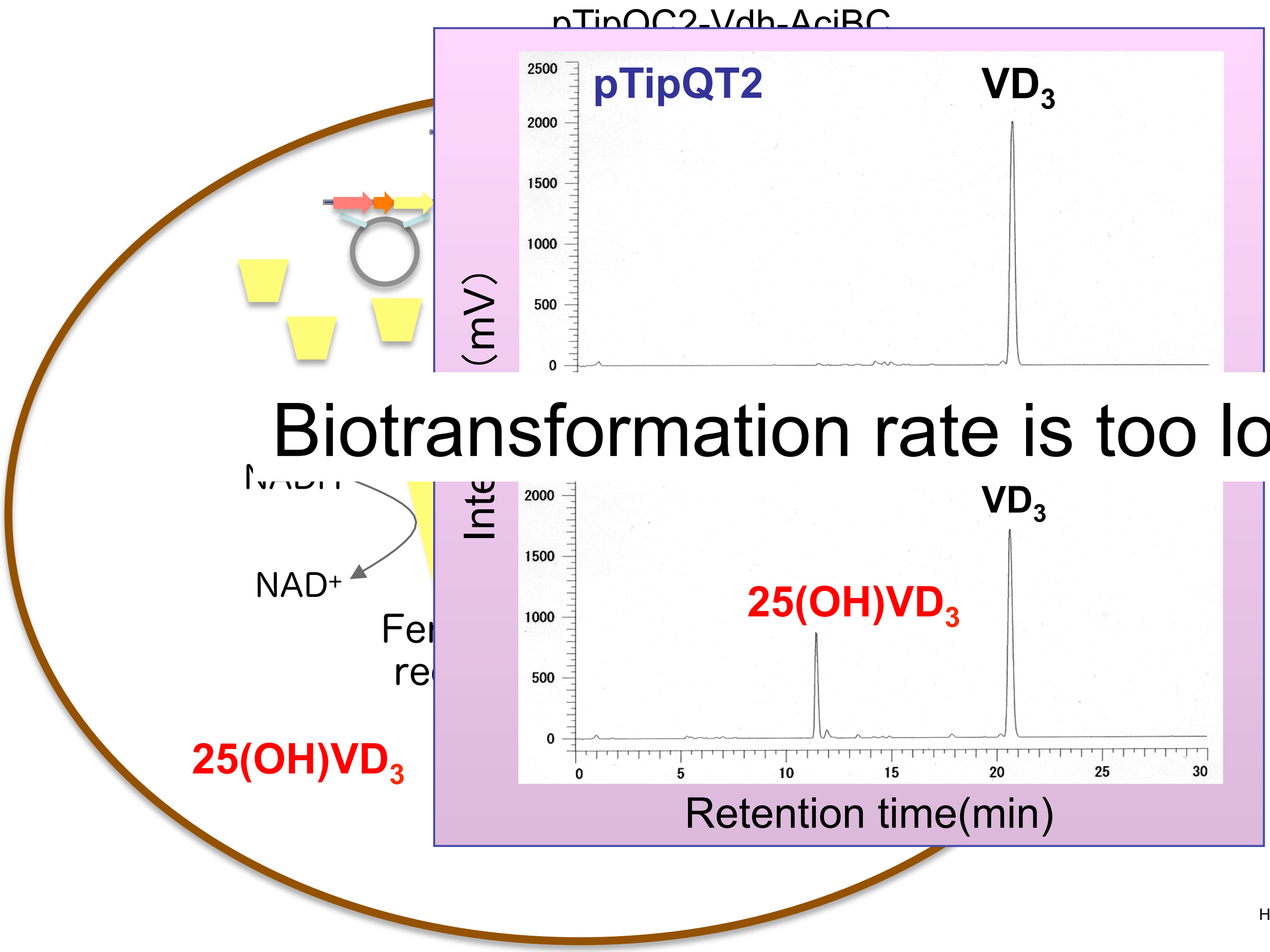
   ⇒ cells can grow under a variety of conditions

3. Broad catabolic diversity

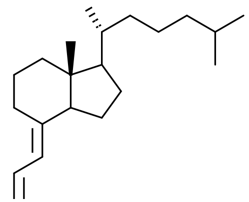
   toxic compound degradation, PCB degradation, steroid compound production, desulfurization, antibiotics production etc.

The genus *Rhodococcus* may uses as platform to apply for a wide range of processes

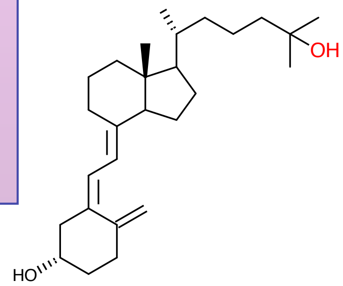
Development of a VD_3 hydroxylation system by using *Rhodococus erythropolis* as a host cell



Biotransformation rate is too low !!!



VD_3



$25(OH)VD_3$

Problems to be solved

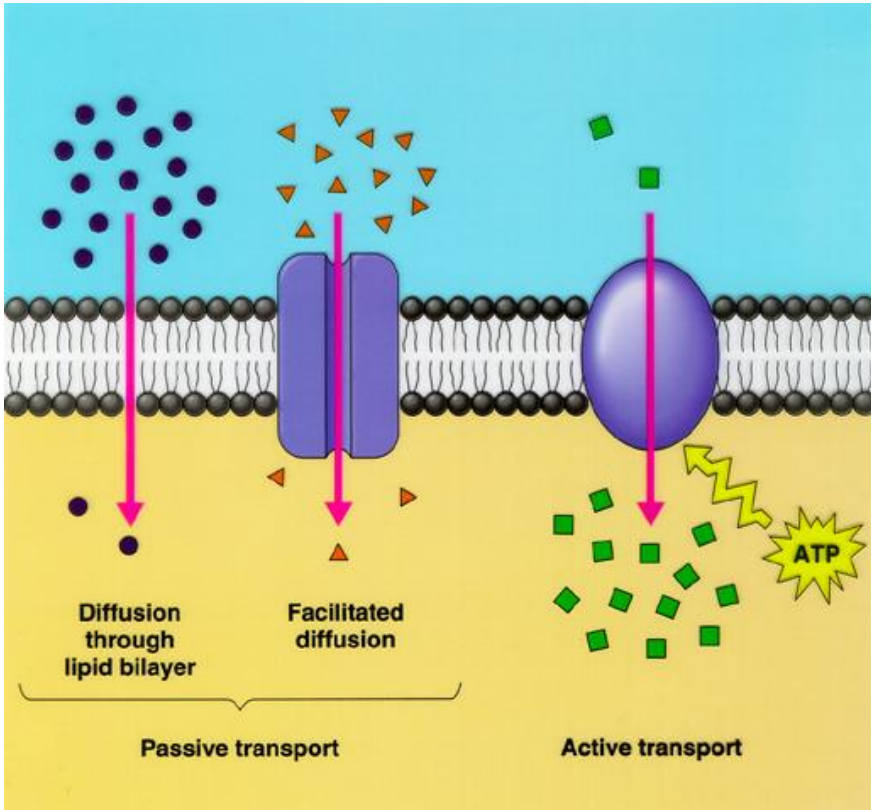
1. Solubility of vitamin D_3
2. Permeability of vitamin D_3
3. P450vdh and its redox partner

Poor solubility and low transport rates of the substrates and products to and from cells are the rate limiting steps in the biotransformation process.

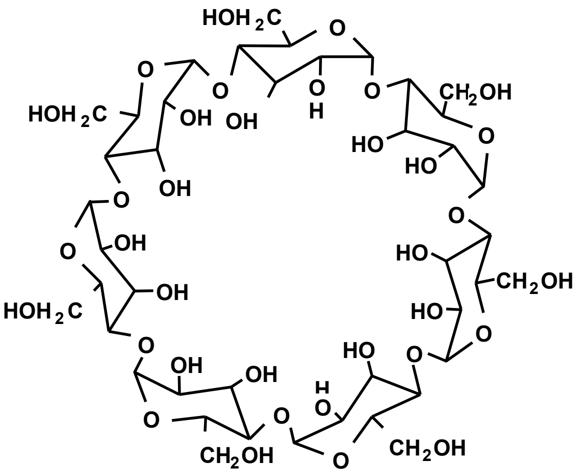
Vitamin D solubility in water

< 0.1g / L (20˚C)

Movement Across Membranes



Cyclodextrins and Bioconversion



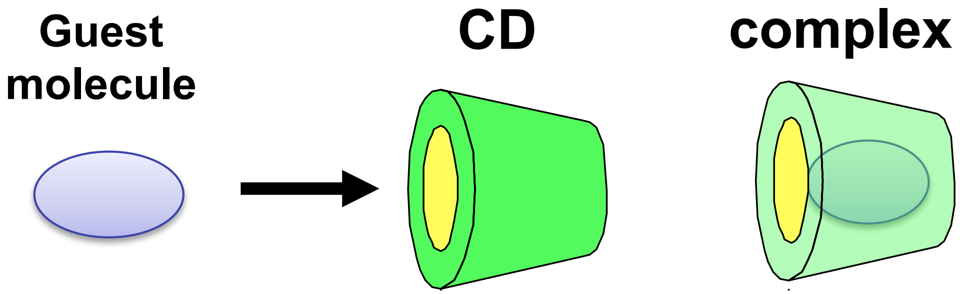
β-Cyclodextrin

CD is circularized oligo saccharide:

Taking guest molecule to inner cavity

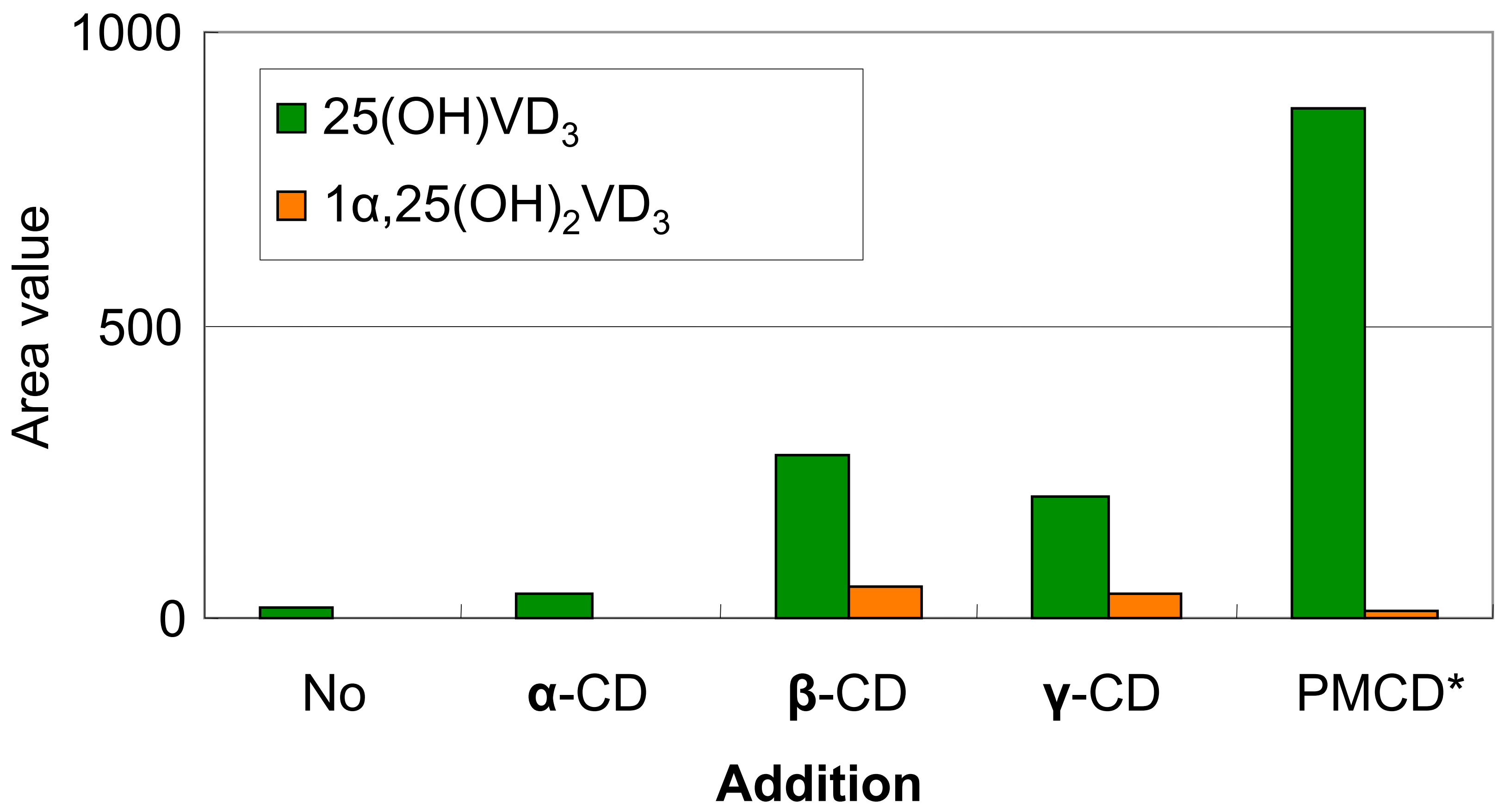
solubilization of hydrophobic compounds

stabilization of the taken compounds



CD	Mass	Outer diameter (nm)	Cavity diameter (Inner ring/Outer ring, nm)	Solubility (g/kg H_2O)
α	972	1.52	0.45/0.53	129.5
β	1134	1.66	0.60/0.65	18.4
γ	1296	1.77	0.75/0.85	249.2

Effect of cyclodextrins on vitamin D_3 hydroxylation

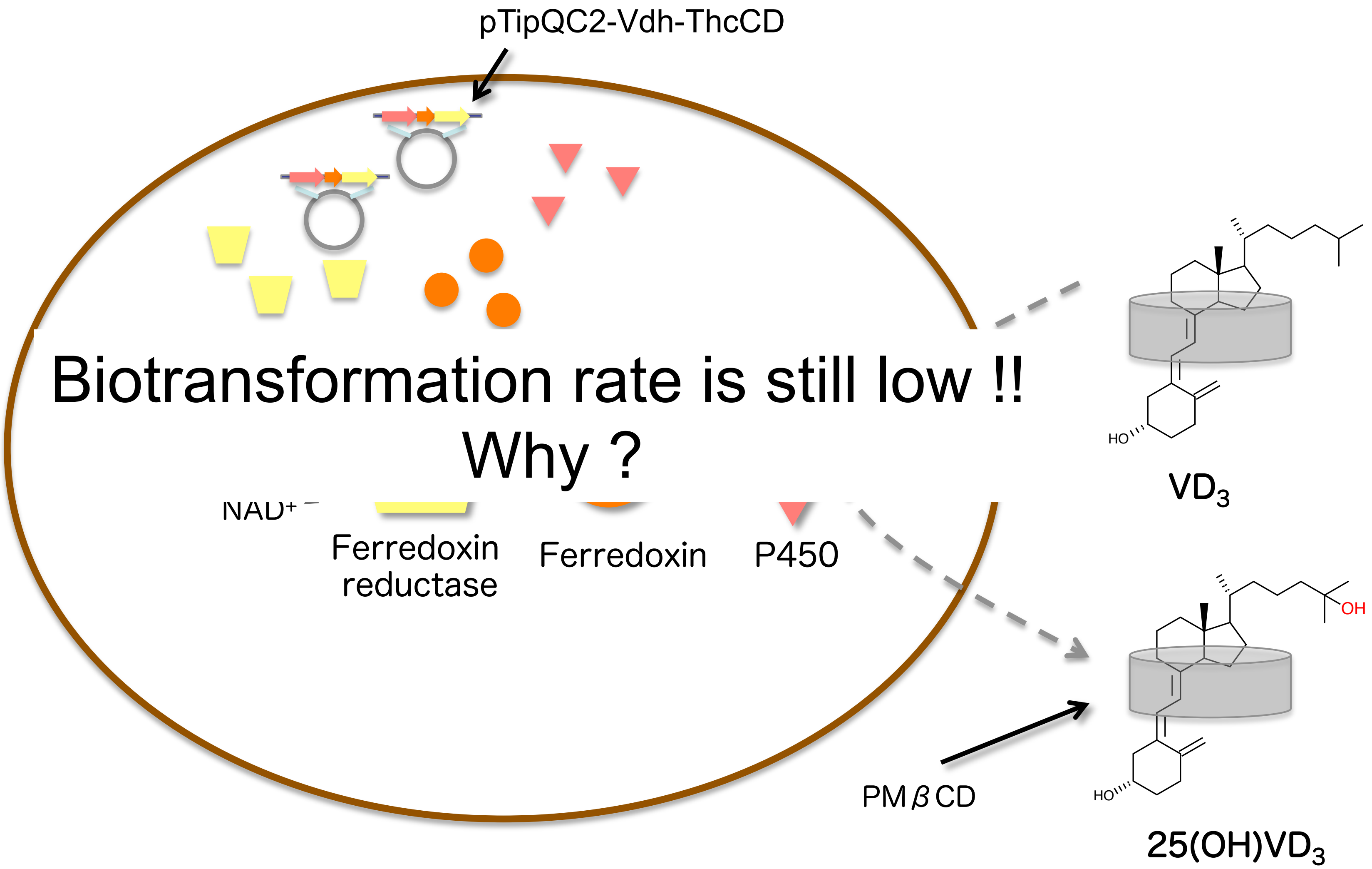


*PMSD: partially methylated β-cyclodextrin

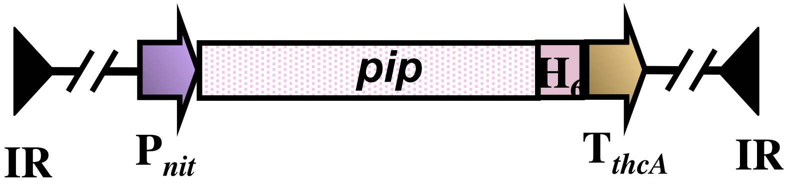
K. Takeda, *et. al, J. Ferment. Bioeng.*, 78, 380-382 (1994)

Development of a VD_3 hydroxylation system by using *Rhodococus erythropolis* as a host cell

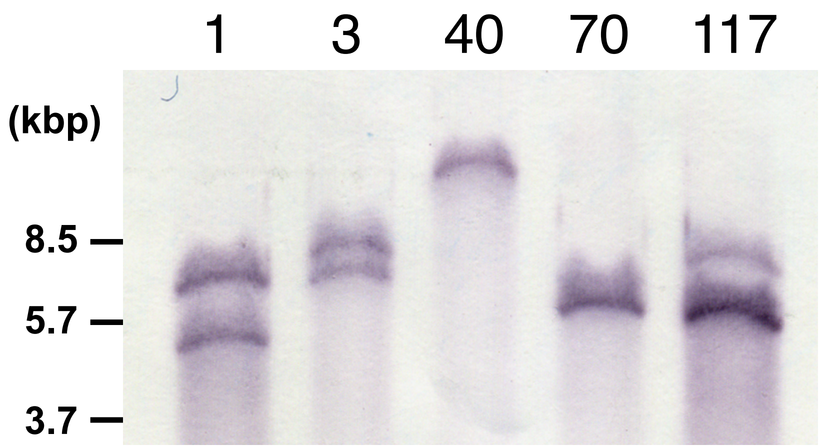
1. Solubilizer

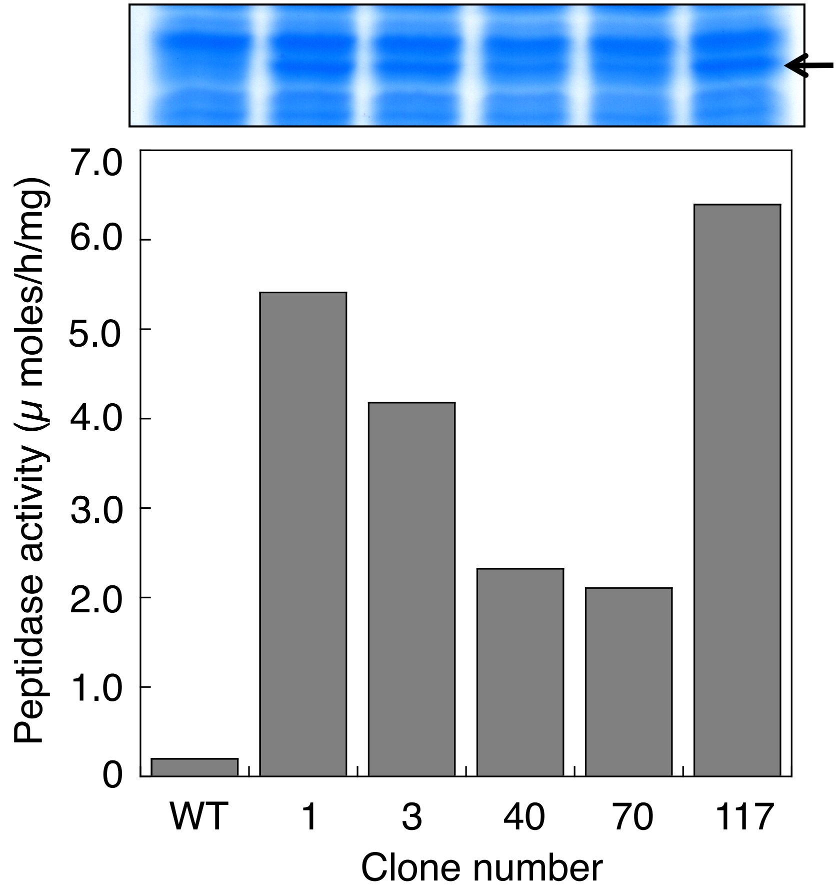


Multiple insertions of *pip* expression cassette into *Rhodococcus* genome

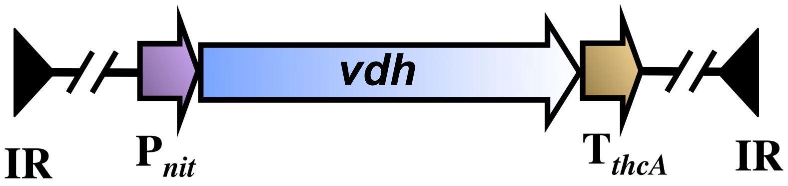


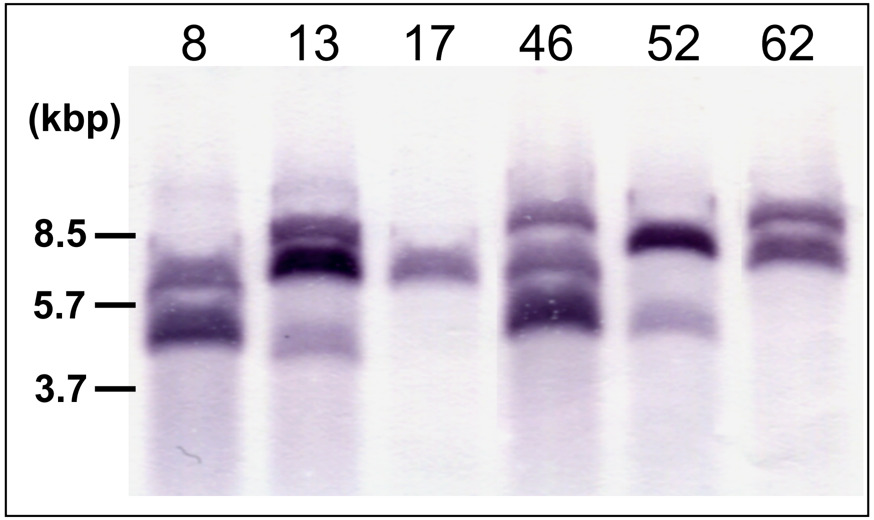
pip: proline iminopeptidase

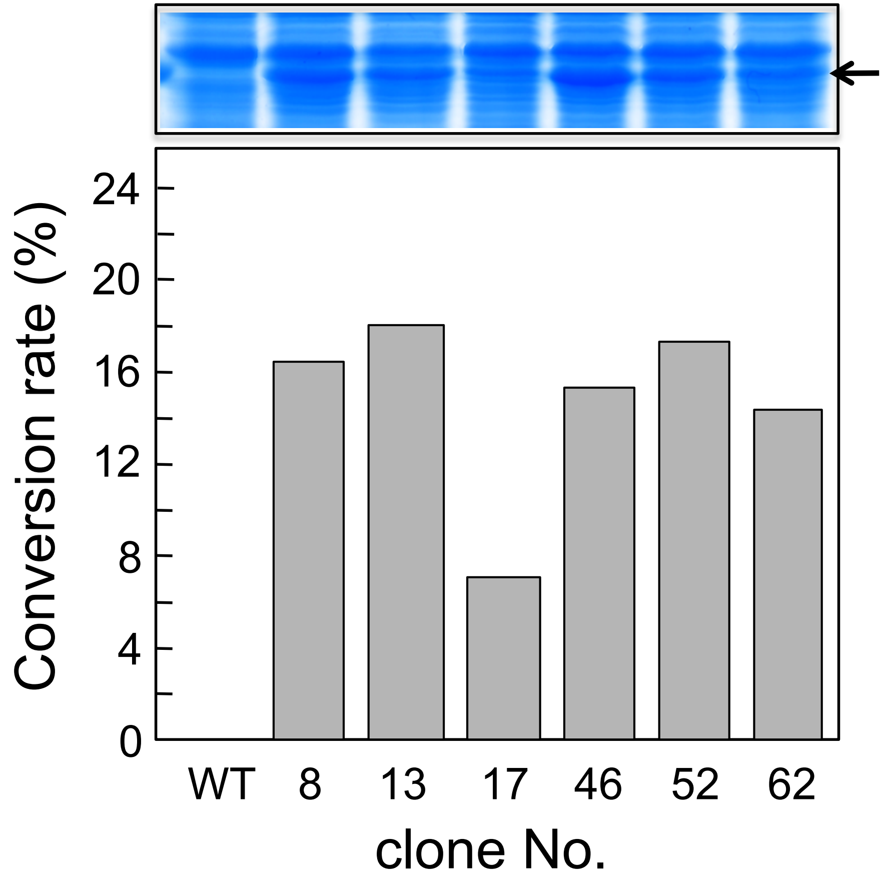




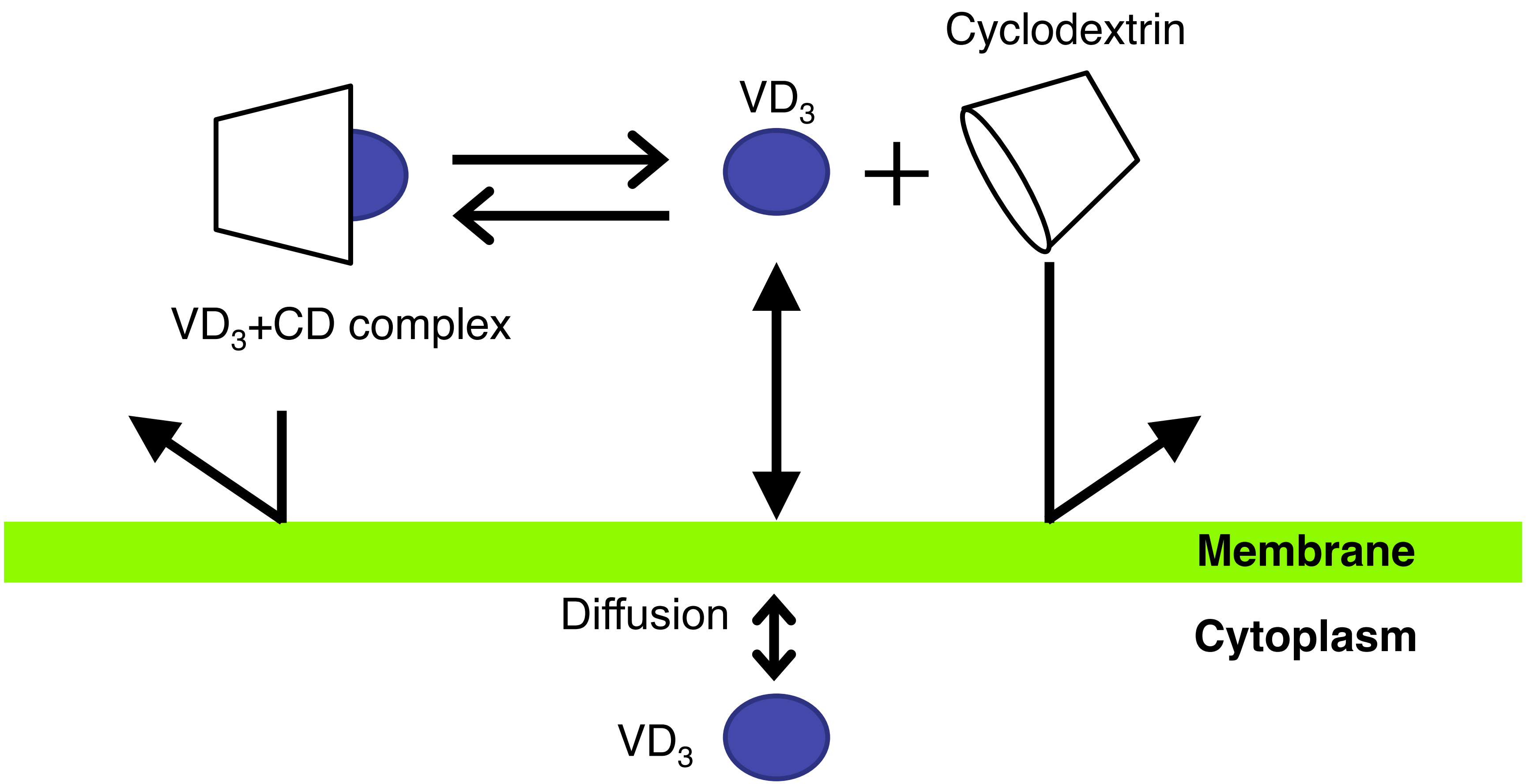
Multiple integration of Vdh expression cassette and the efficiency of VD_3 hydroxylation







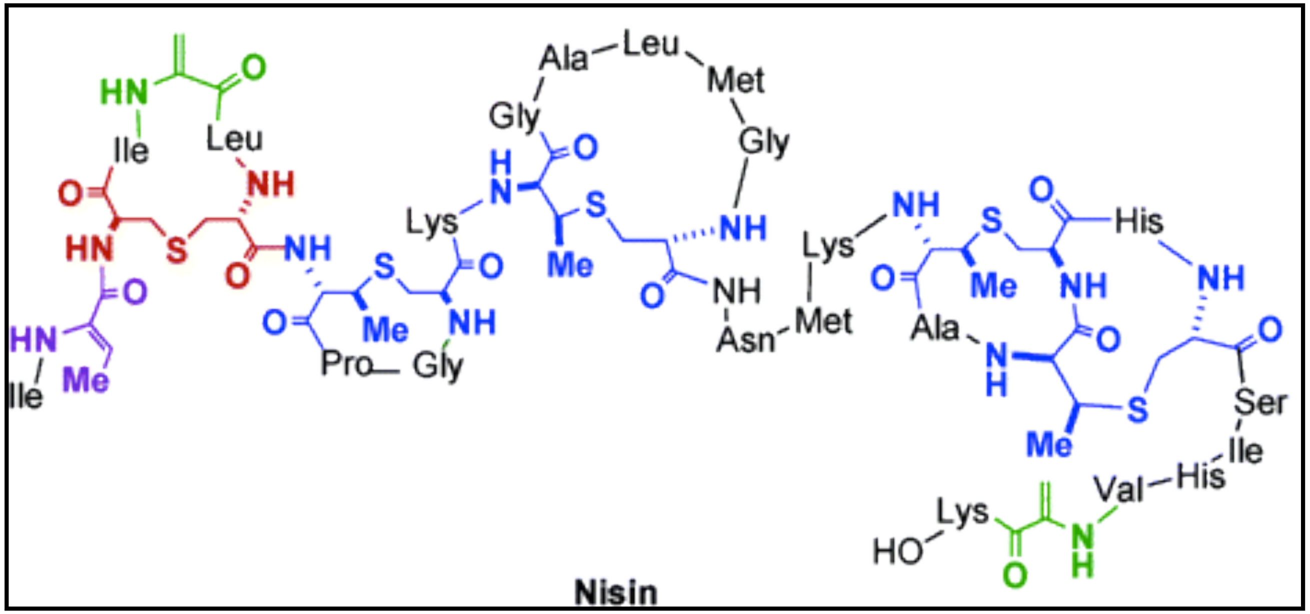
Permeability of vitamin D_3 to cytoplasm is low



Problems to be solved

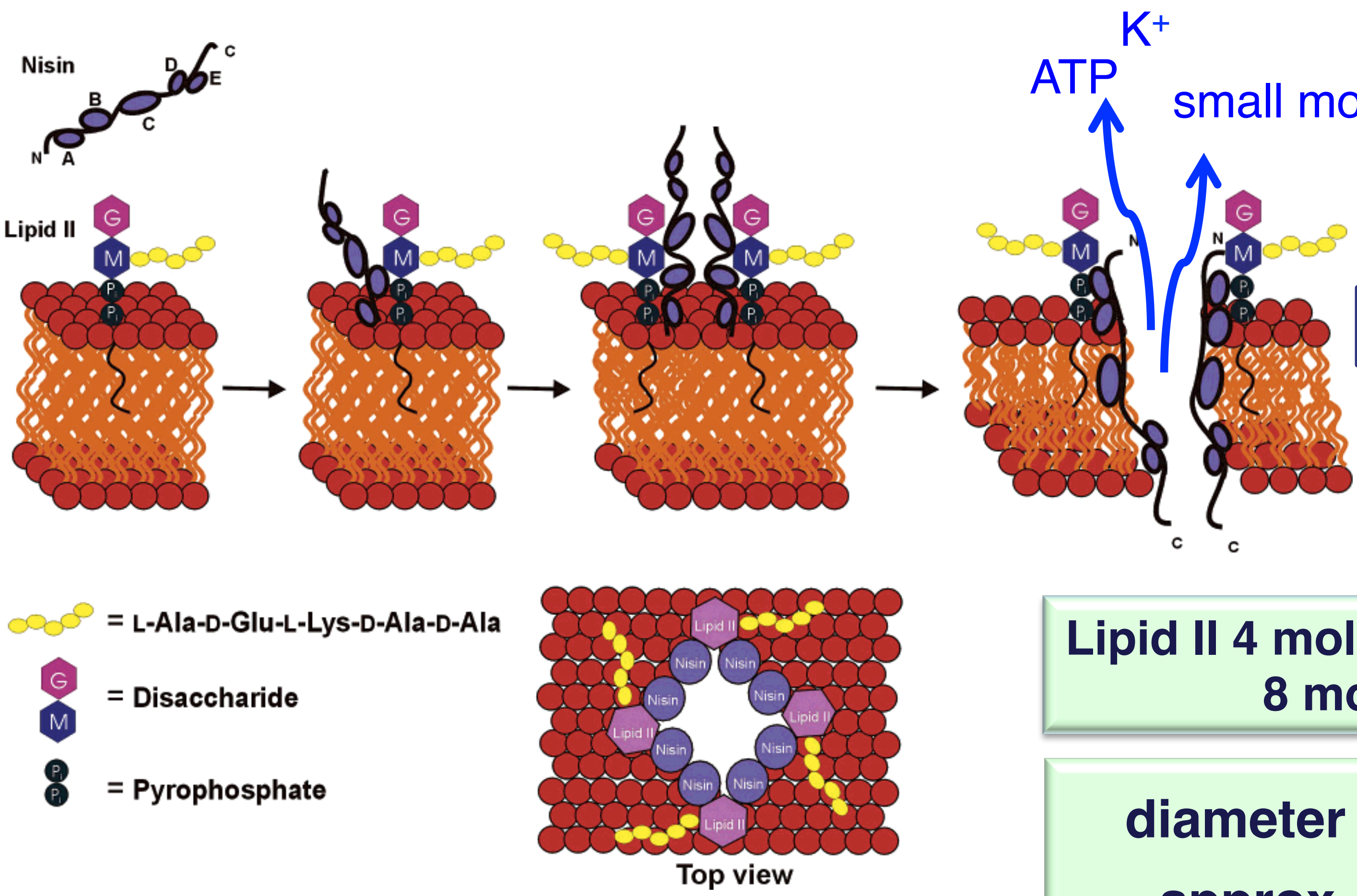
1. Solubility of vitamin D_3
2. Permeability of vitamin D_3
3. P450vdh and its redox partner

Nisin



- a natural antimicrobial agent
- 34 amino acids
- produced by *Lactococcus lactis*
- "broad-spectrum" bacteriocin effective against many gram-positive bacteria
- used as a food preservation in over 50 countries (used in processed cheese, meats, beverages etc.); since 2009 in Japan.

Nisin pore complex



bacteria are killed

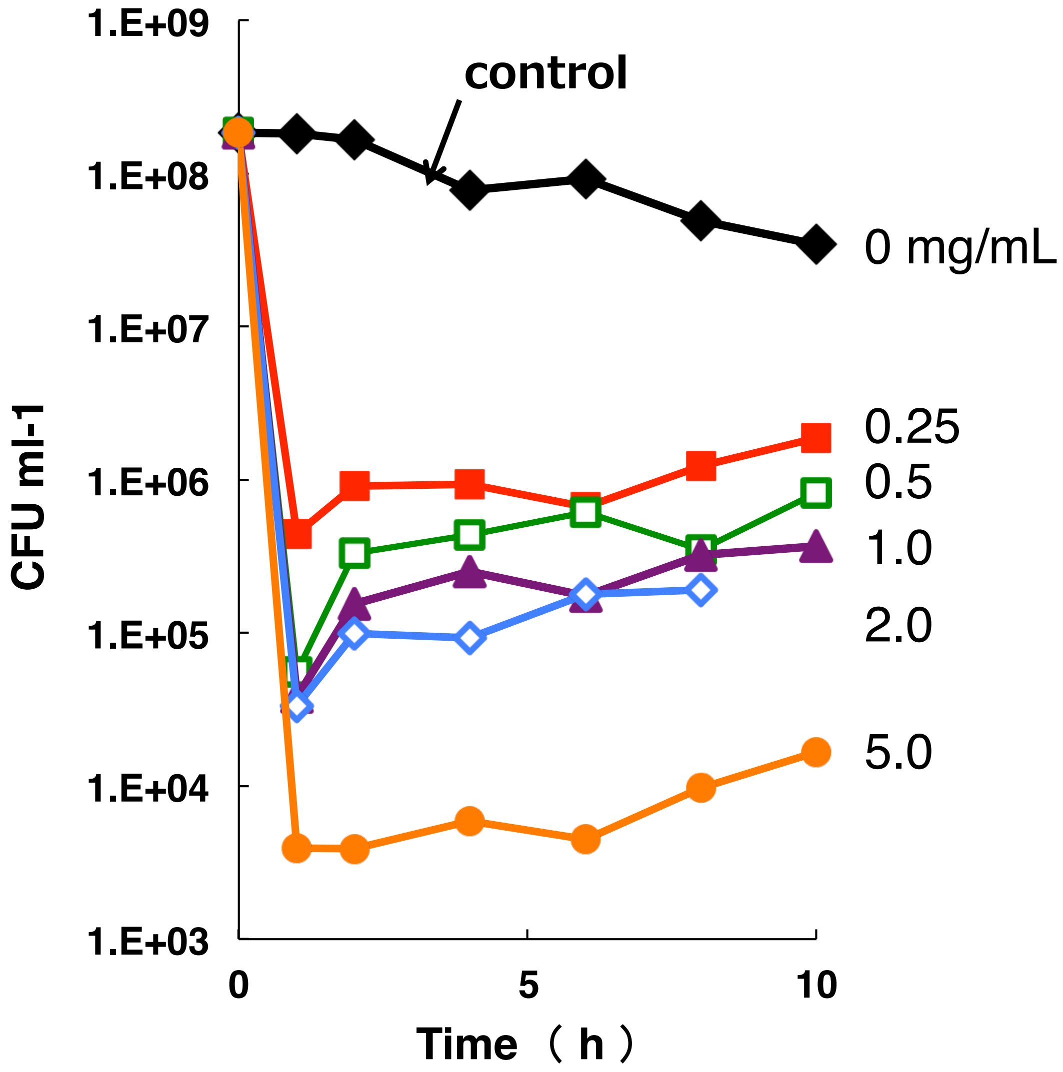
Lipid II 4 molecules and Nisin 8 molecules

diameter of nisin pore approx. 2.0～2.5nm

* Lipid II = peptideglycan precursor

Chatter *et al.*, *Chem Rev* (2005) 105: 633-683.

Effect of nisin on actively growing cell

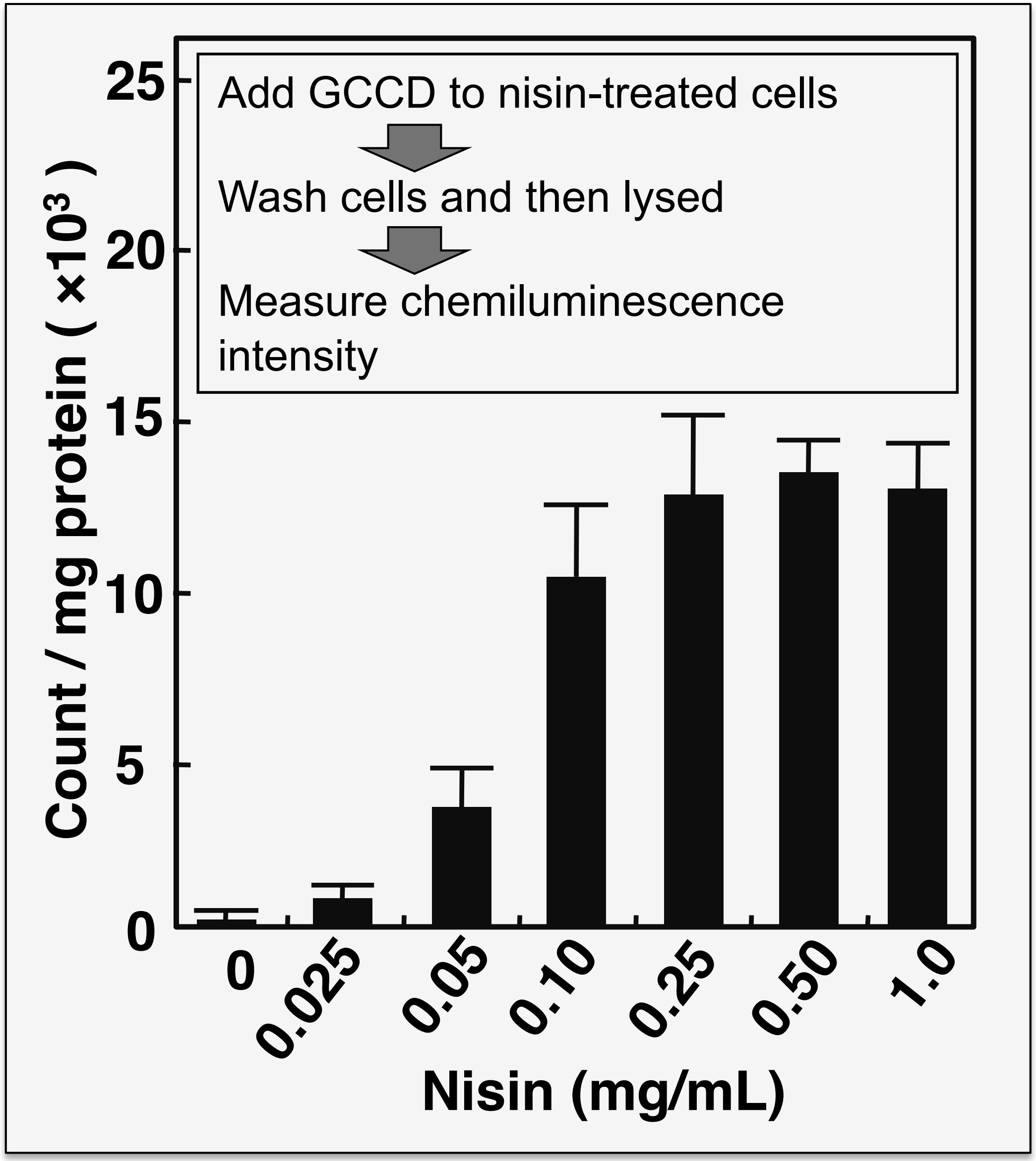


Effect of nisin on translocation of Green Chemiluminescent Cycrodextrin (GCCD)

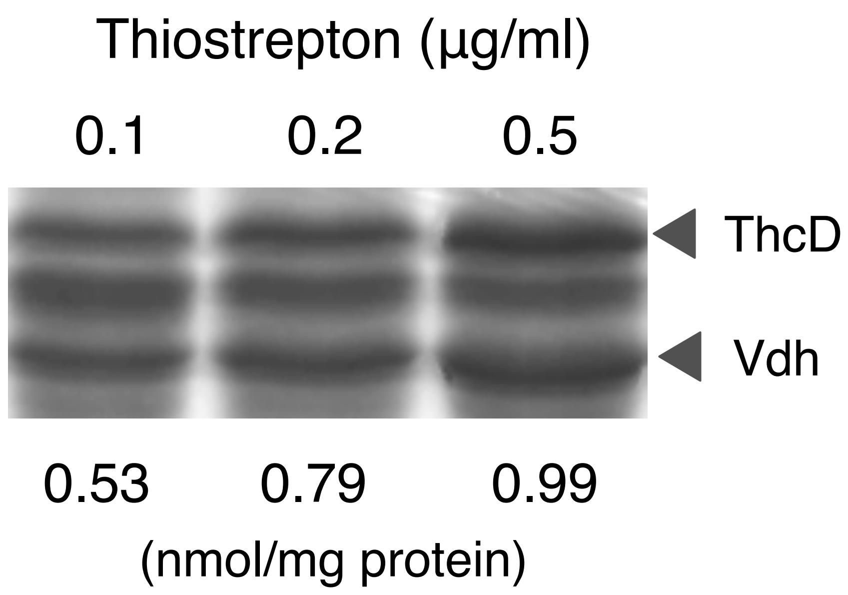
Model substrate : Green Chemiluminescent γ-Cycrodextrin (GCCD)

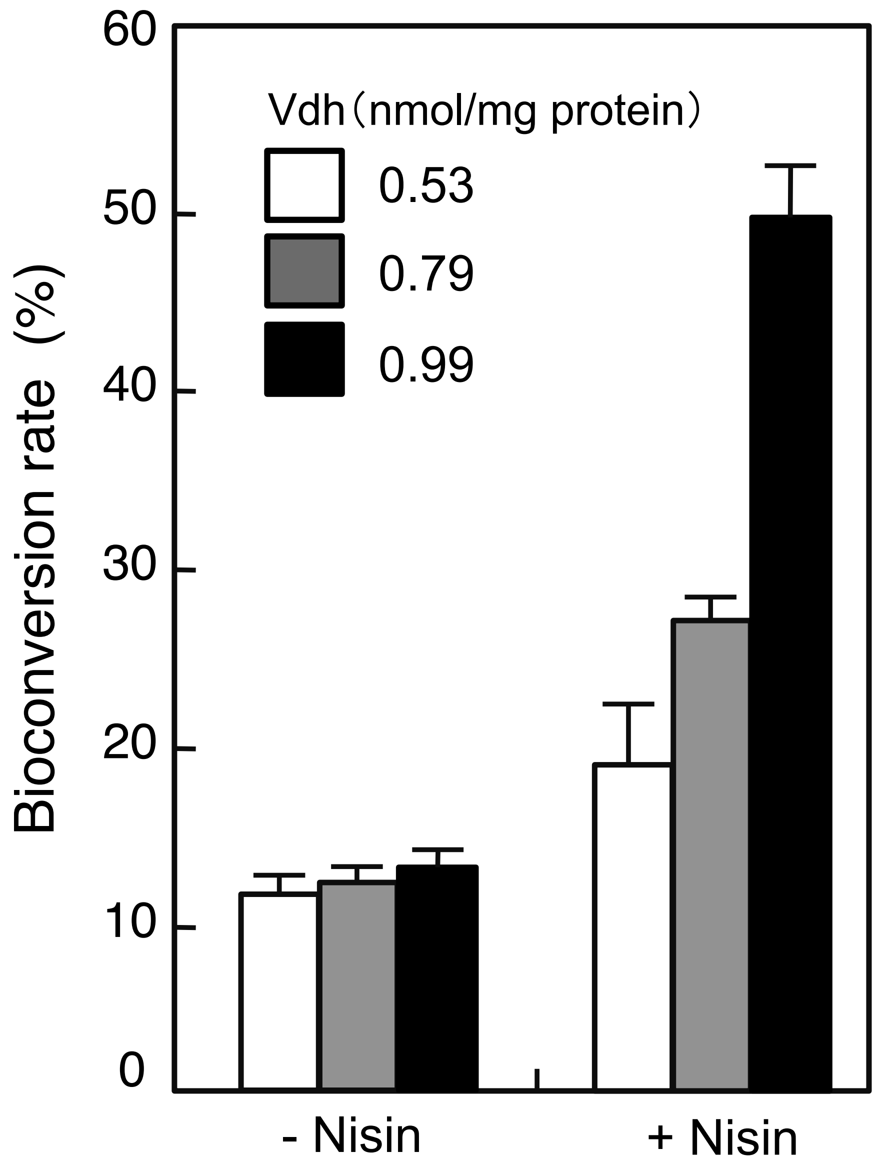
Using the hypoxanthine-xanthine oxidase system for the generation of the superoxide anions, the chemiluminescent probes showed higher superoxide-induced chemiluminescence intensity (530nm)

γ-Cyclodextrin
glucose : 8
diameter : 17.5Å
depth : 7.9Å

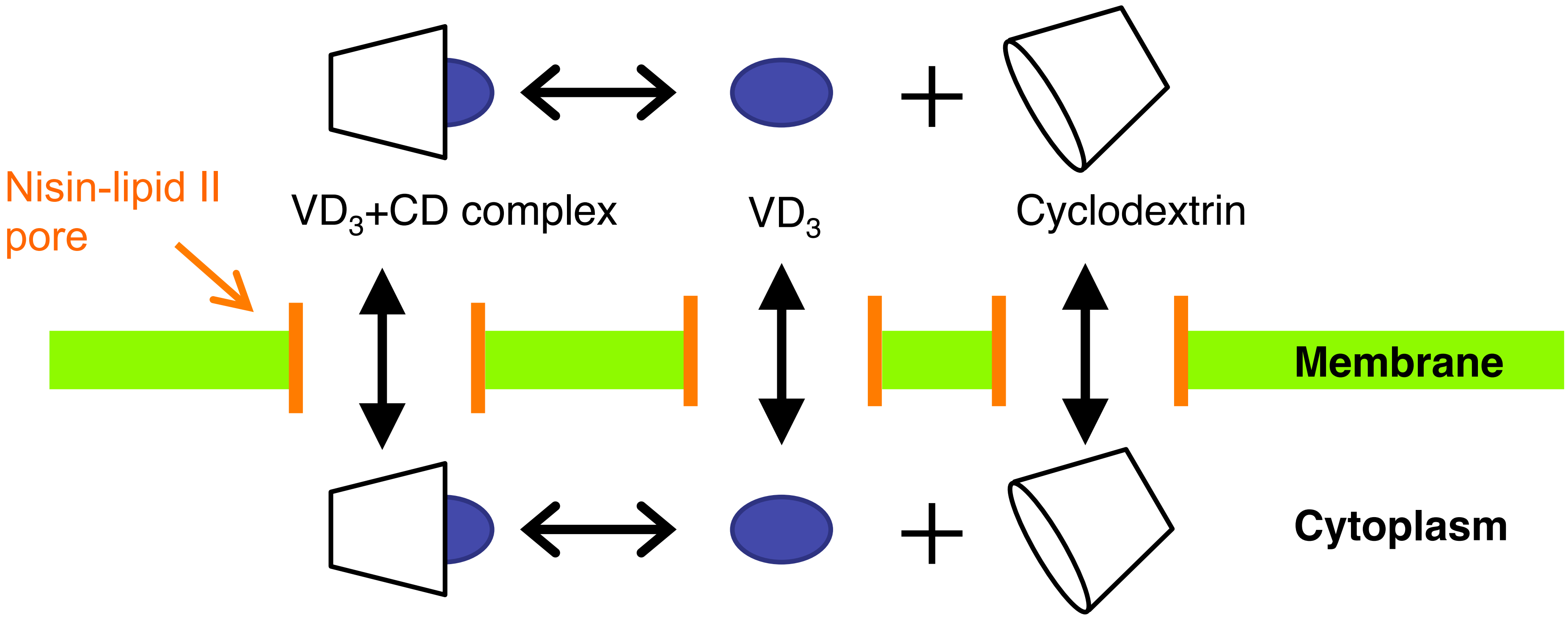


VD_3 hydroxylation in nisin treated cells





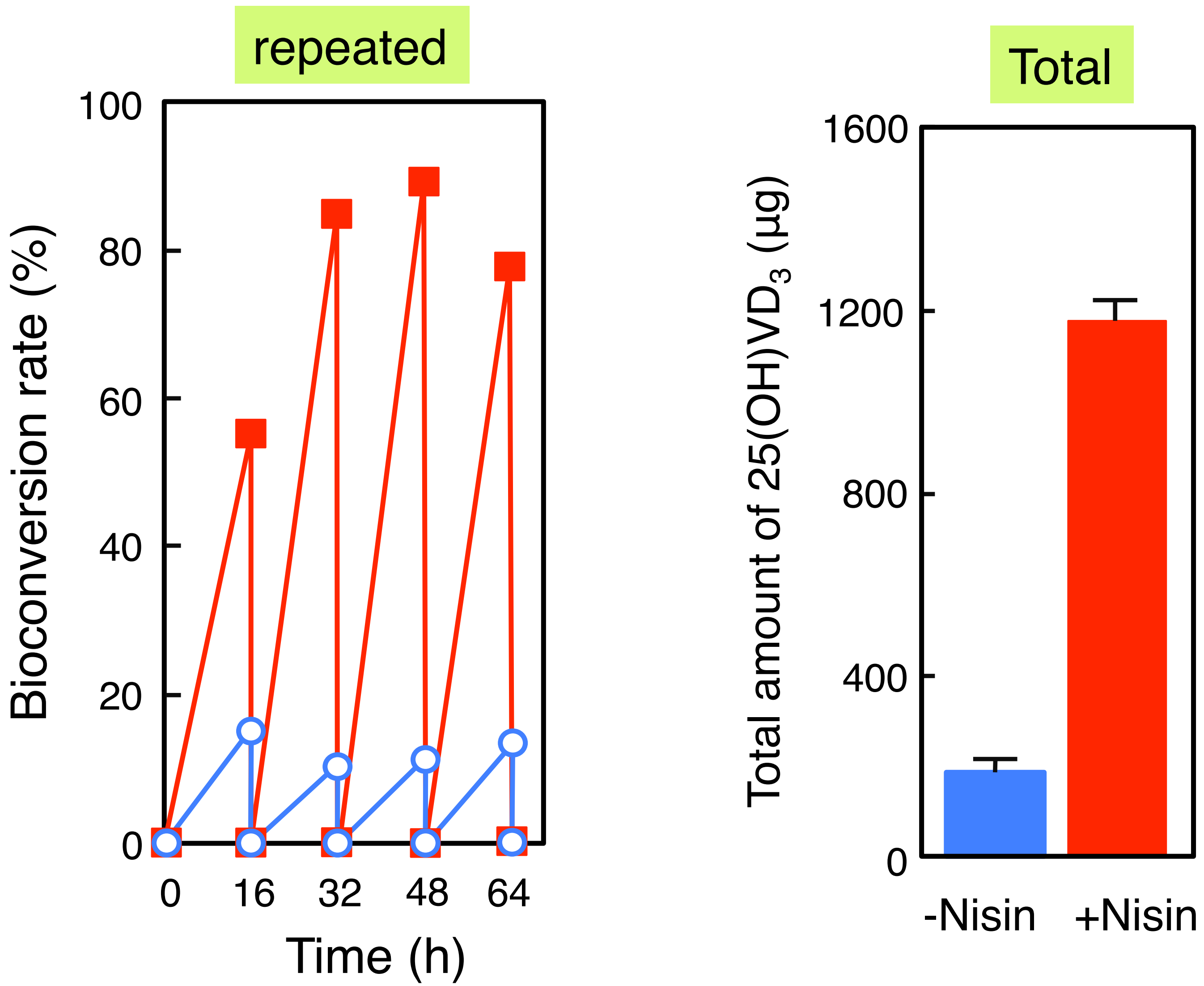
Permeability is induced by nisin



Intracellular substrate concentration is increased.

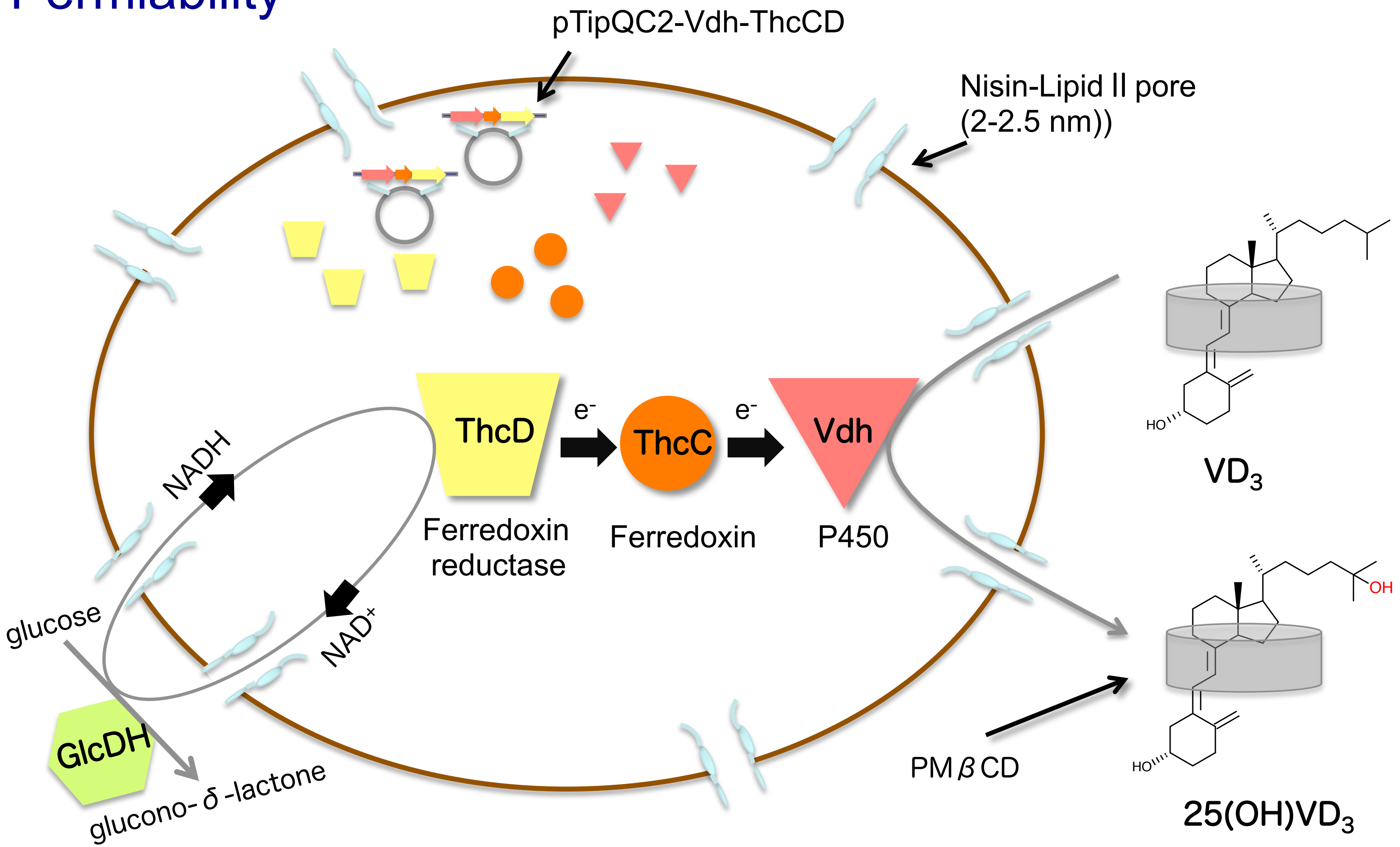
VD3 hydroxylation will be improved.

Production of 25(OH)VD_3 by nisin treated cells



Development of a VD_3 hydroxylation system by using *Rhodococus erythropolis* as a host cell

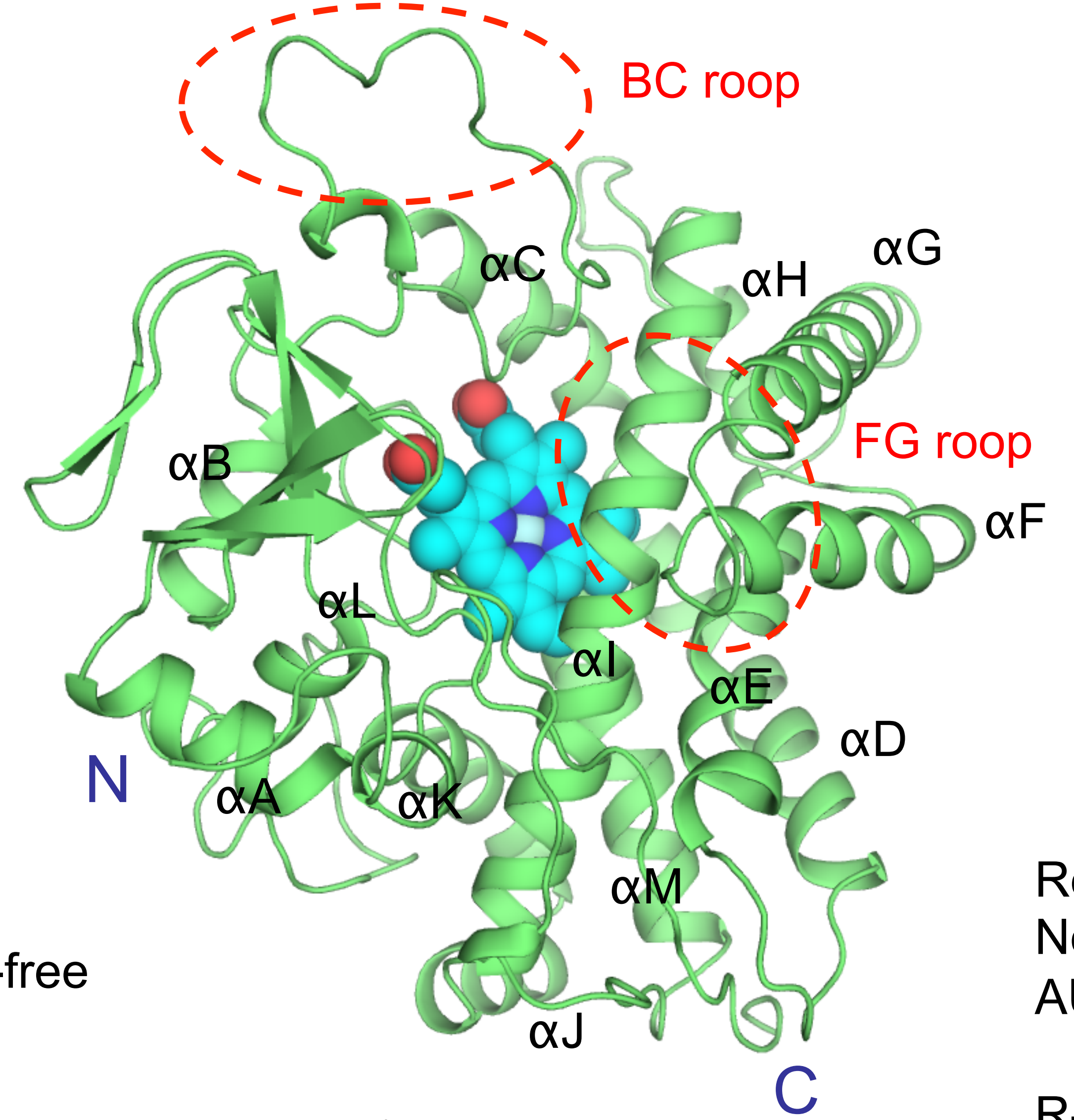
2. Permiability



Problems to be solved

1. Solubility of vitamin D_3
2. Permeability of vitamin D_3
3. P450vdh and its redox partner

Structure of wild-type Vdh



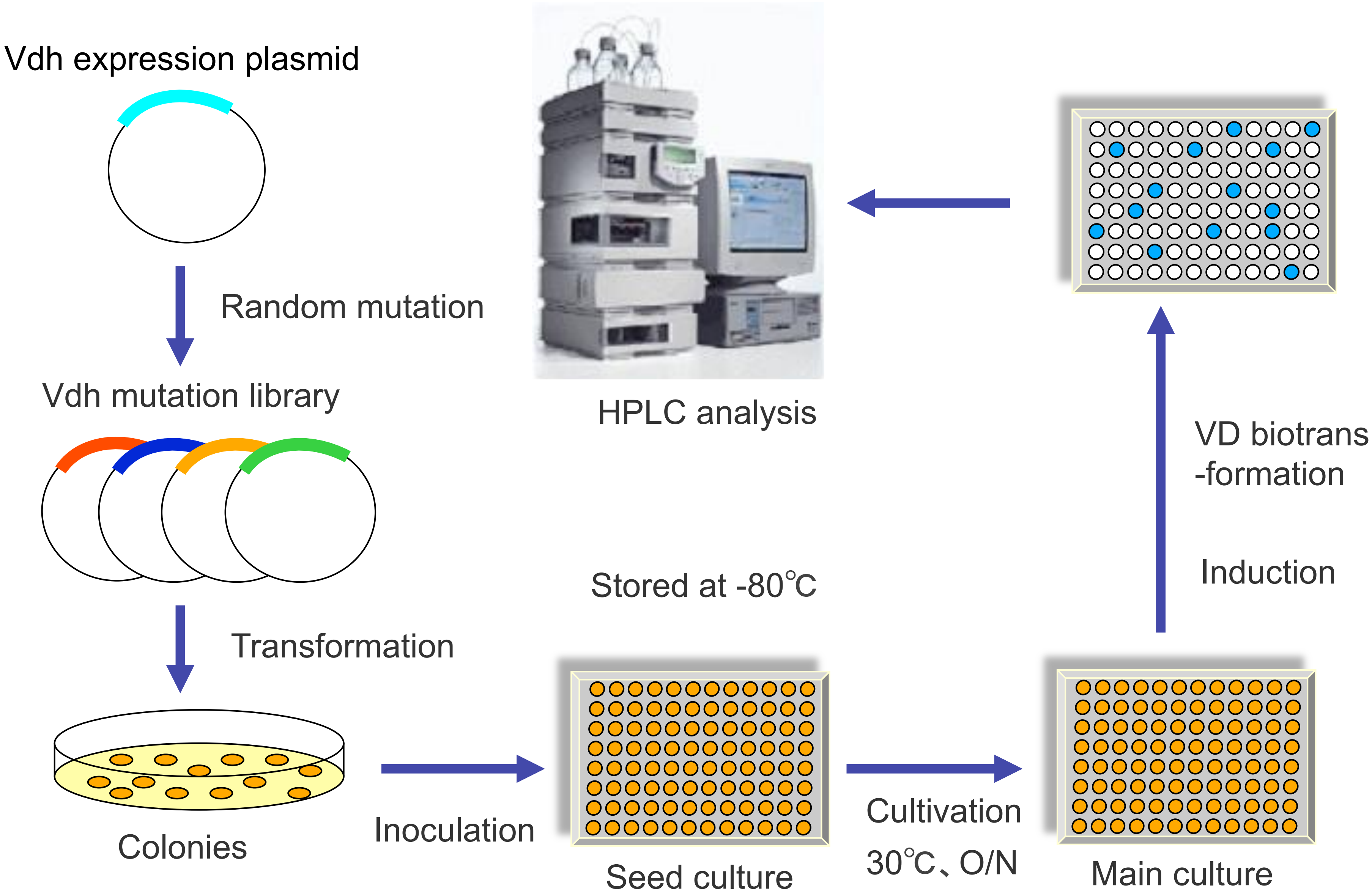
Vdh-WT substrate-free

Space group：$P3_1$
Unit cell parameters：*a*=*b*=61, *c*=99 Å

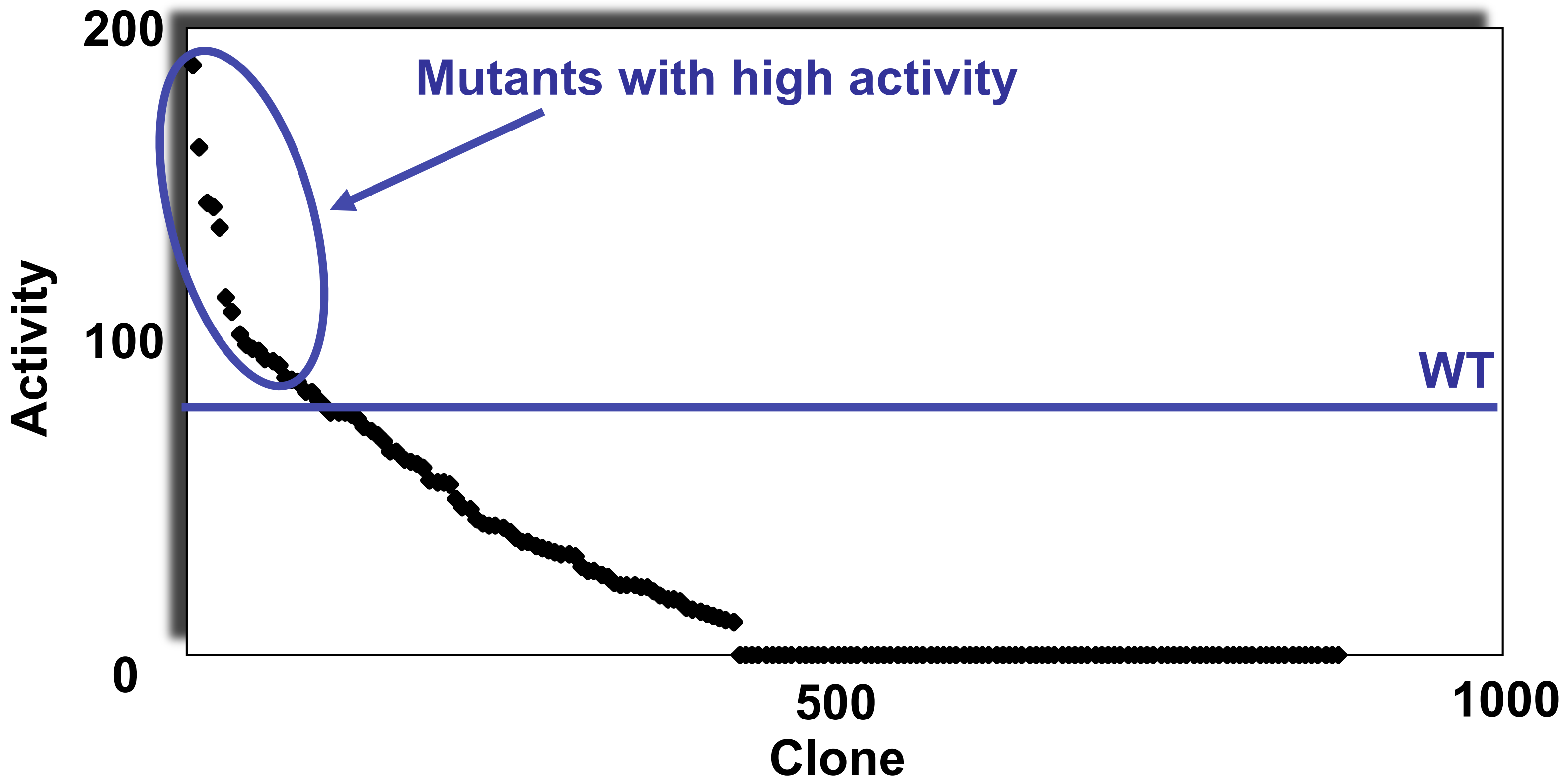
Resolution：1.75 Å
No. monomer in the AU : 1

R-factor: 20.6%
Free R-factor: 23.7%

Protein engineering of Vitamin D_3 hydroxylase

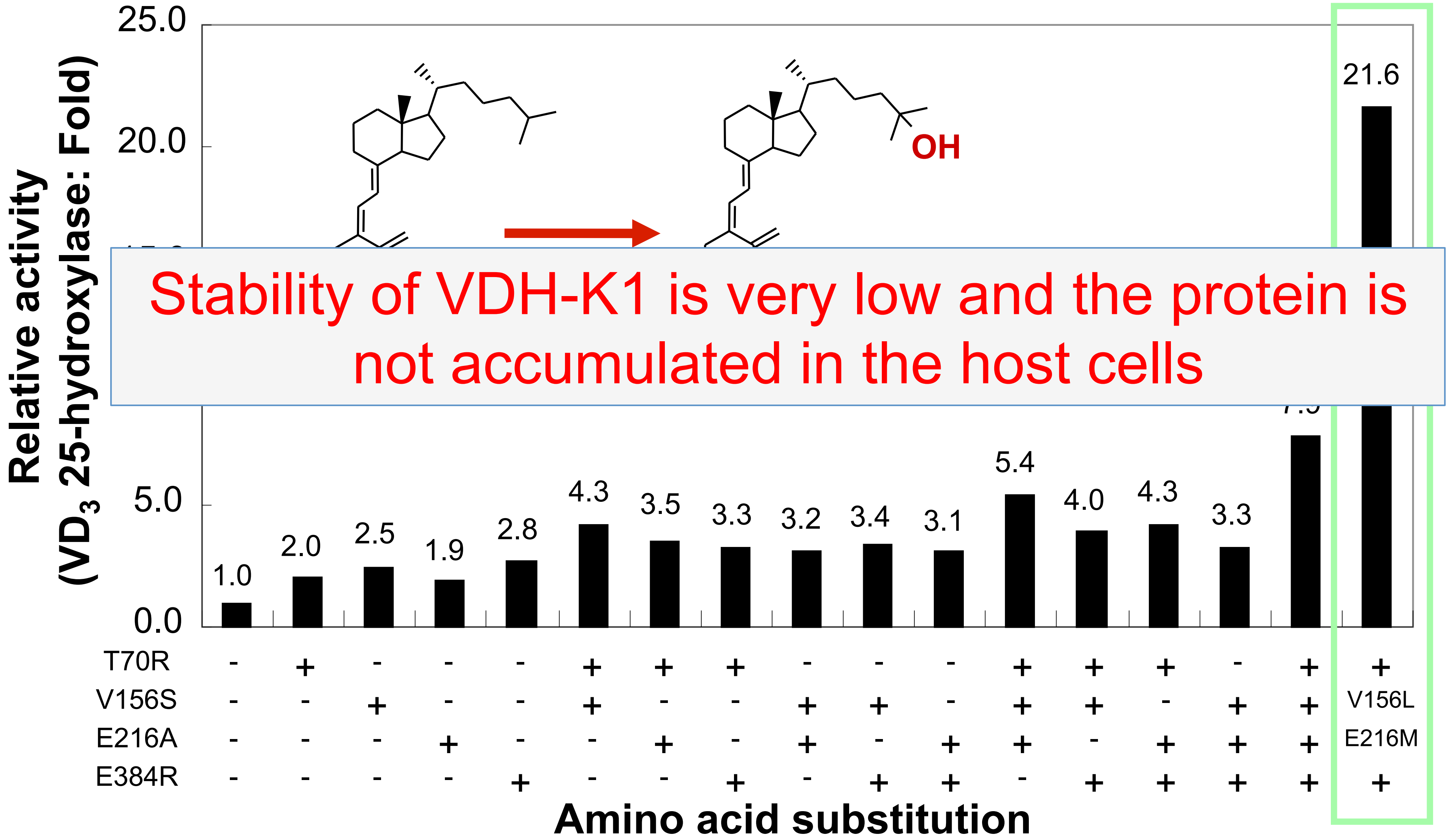


Screening for Vdh mutant with high activity



8 clones with high activity were identified

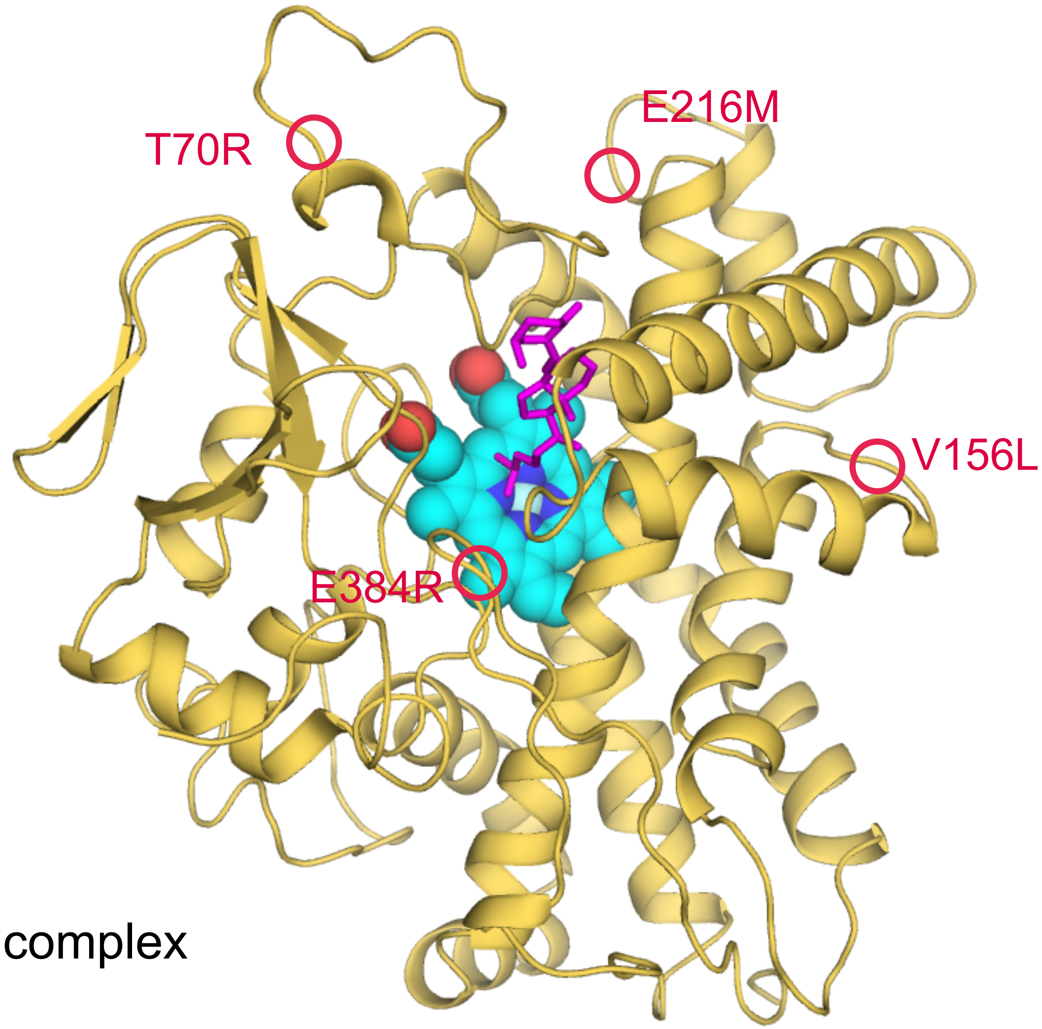
Vdh K1mutant



Stability of VDH-K1 is very low and the protein is not accumulated in the host cells

Y. Fujii, *et al.*, *Biochem. Biophys. Res. Commun.*, **385(2)**, 170-175 (2009).

Structure of Vdh K1 mutant



Vdh-K1 substrate complex

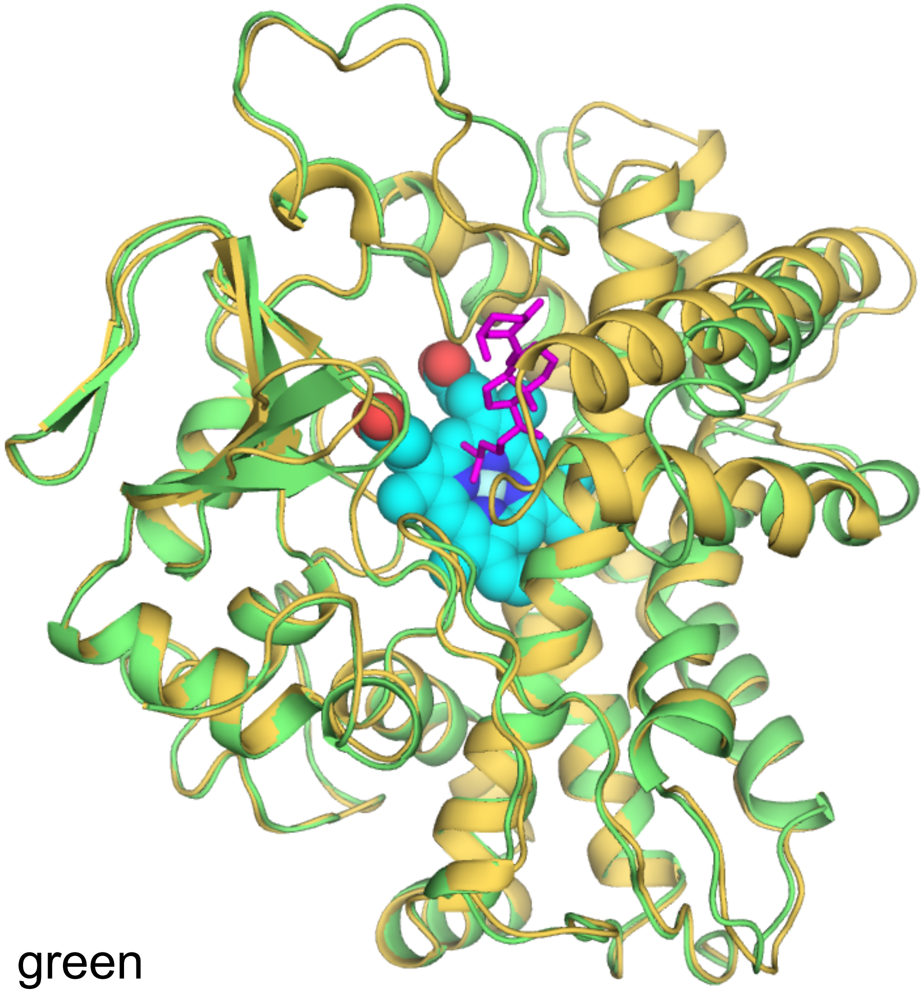
Space group : $P2_12_12_1$
Unit cell parameters : a=77, b=172, c=189 Å

Resolution : 2.0-2.1 Å
No. monomers in the AU : 5

VD3 complex
R-factor: 21%
Free R-factor: 25%

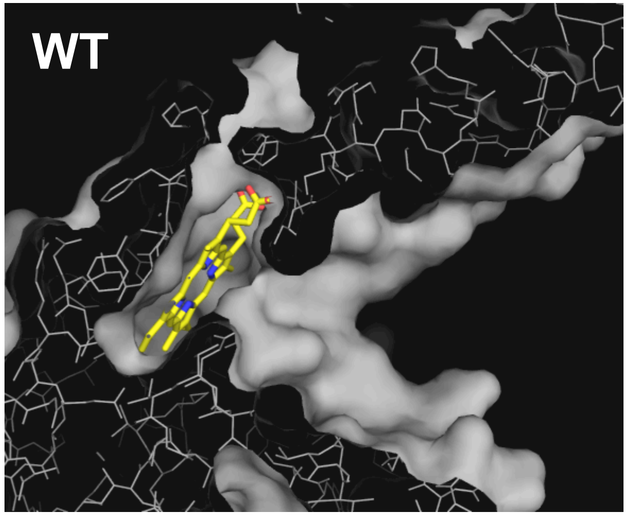
25OHVD3 complex
R-factor: 19%
Free R-factor: 24%

Structural changes between Vdh-wt and Vdh-K1

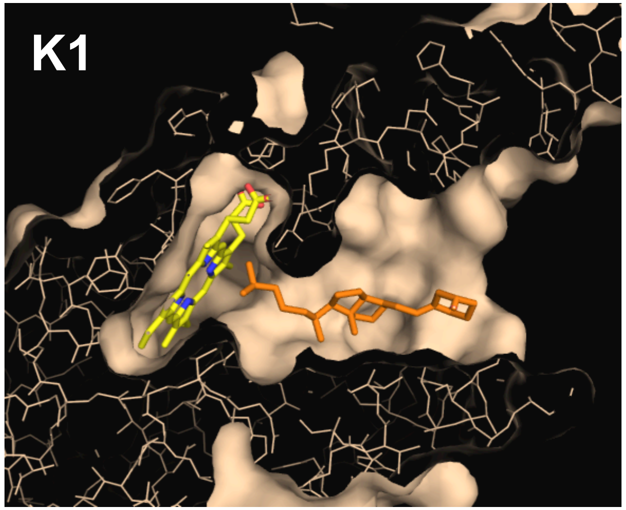


WT : green
K1 : yellow

Open form



Closed form



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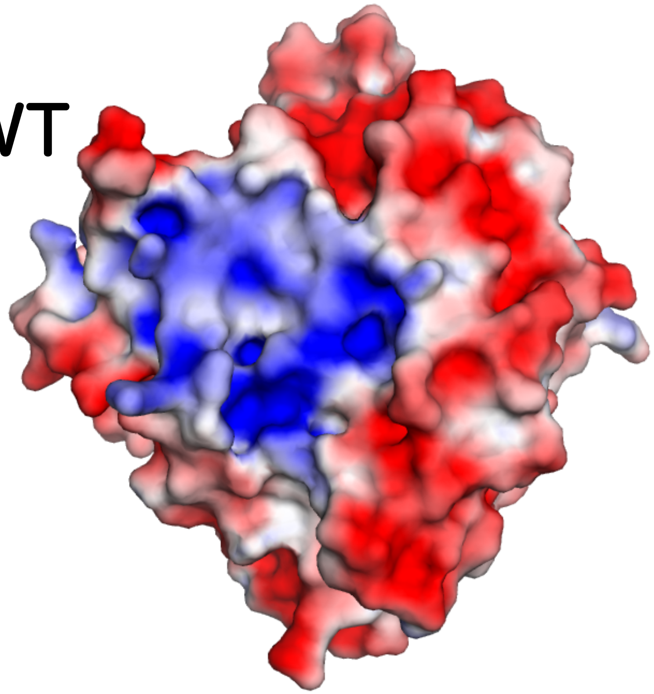
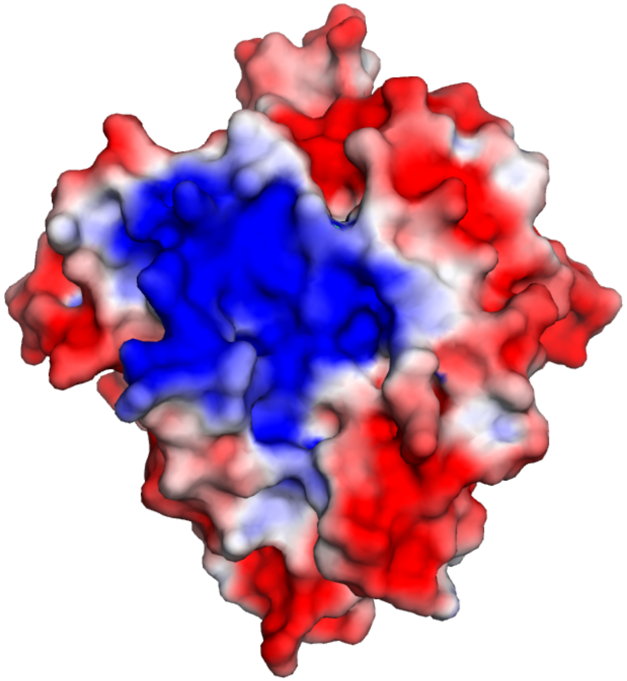
Electrostatic potentials of Fdx-binding surface of Vdh-WT and Vdh-K1

WT



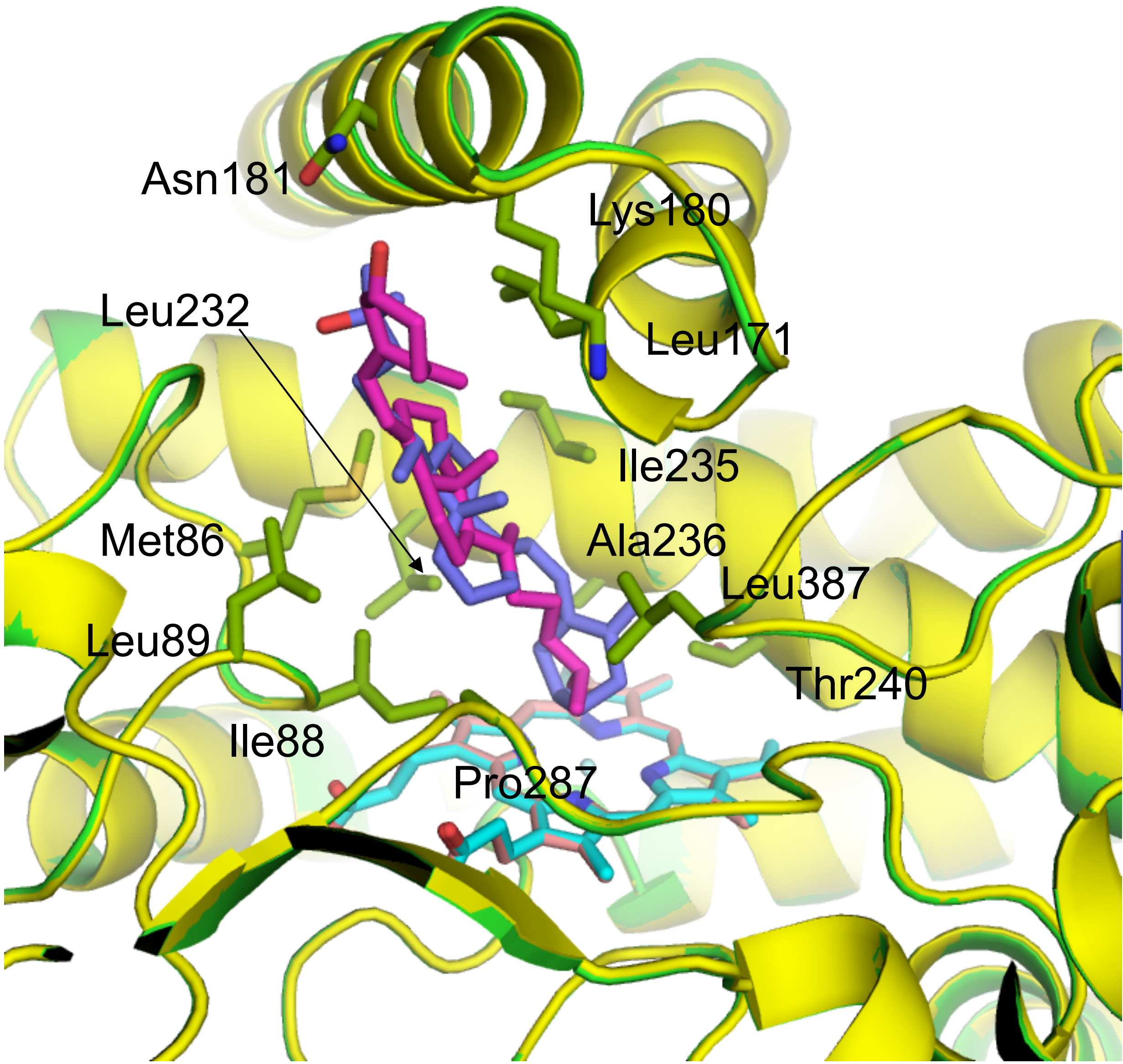
K1



It is known that the P450-binding surface of Fdx is negatively charged and the Fdx-binding surface of P450 is positively charged

Blue：positive
Red：negative

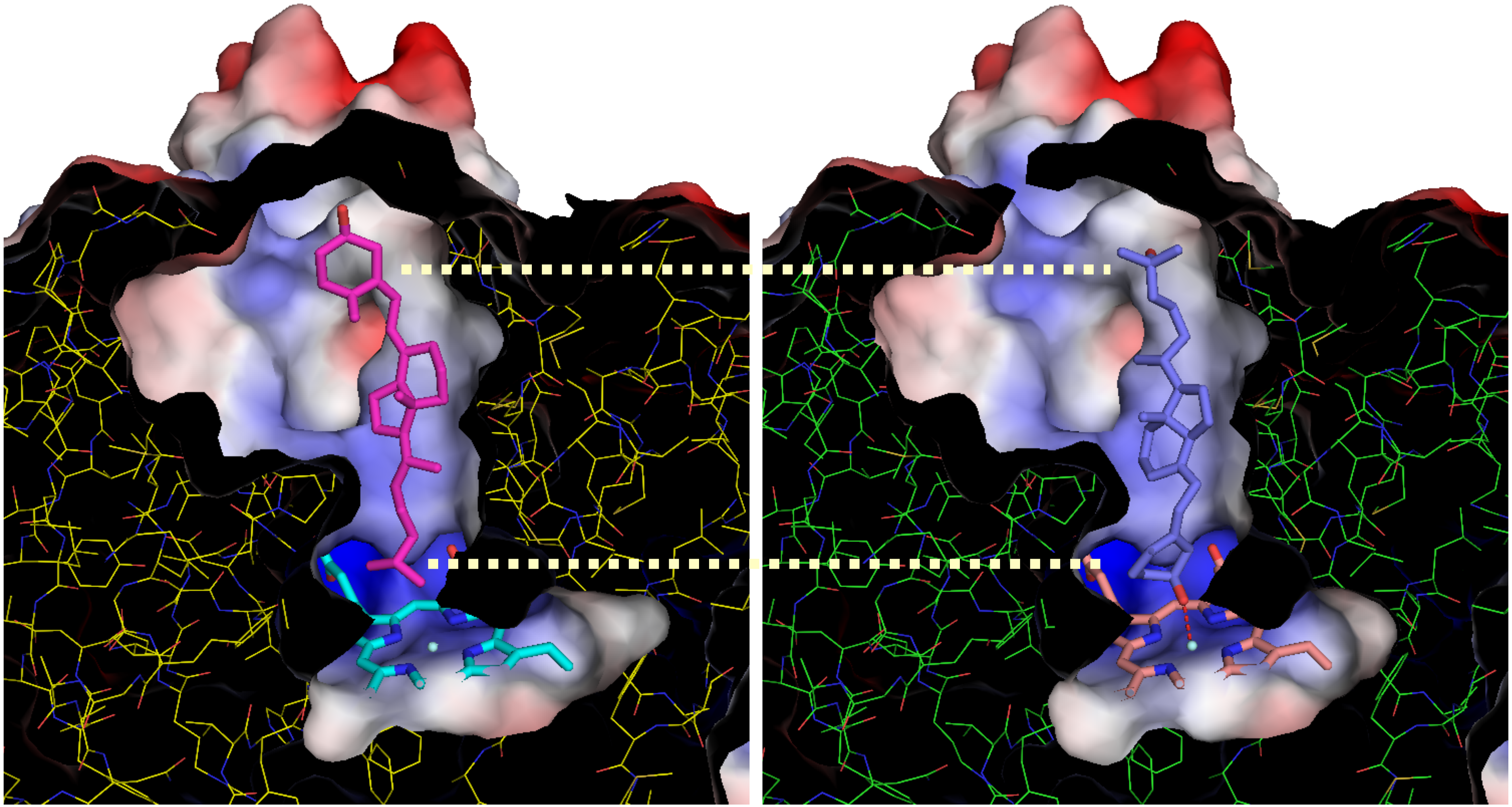
Structural analysis of substrate-VDH complex



mutation of amino acids interacting with substrate

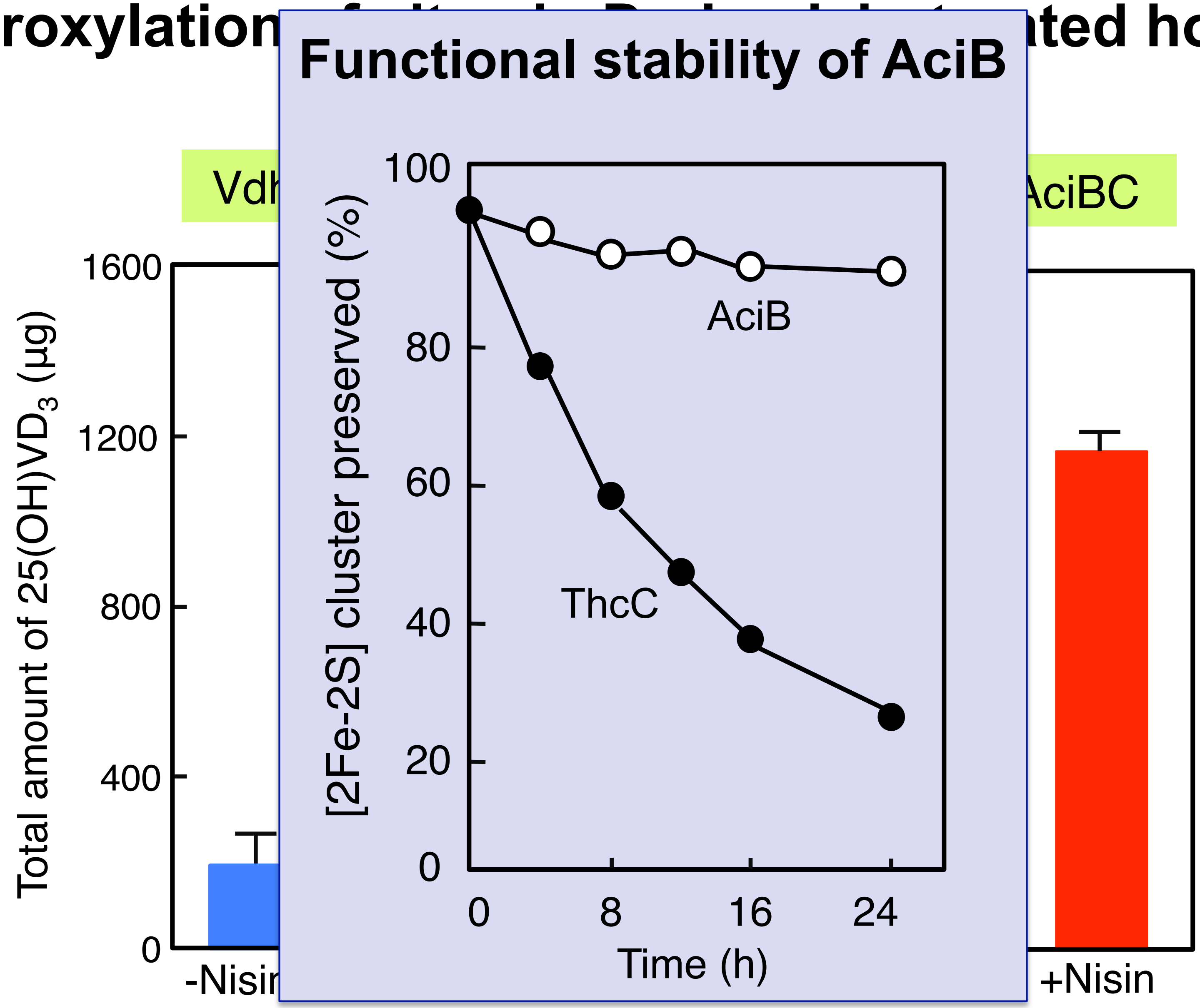
mutants change
- Substrate specificity
- Regiospecificity

Substrate binding pocket of Vdh-K1

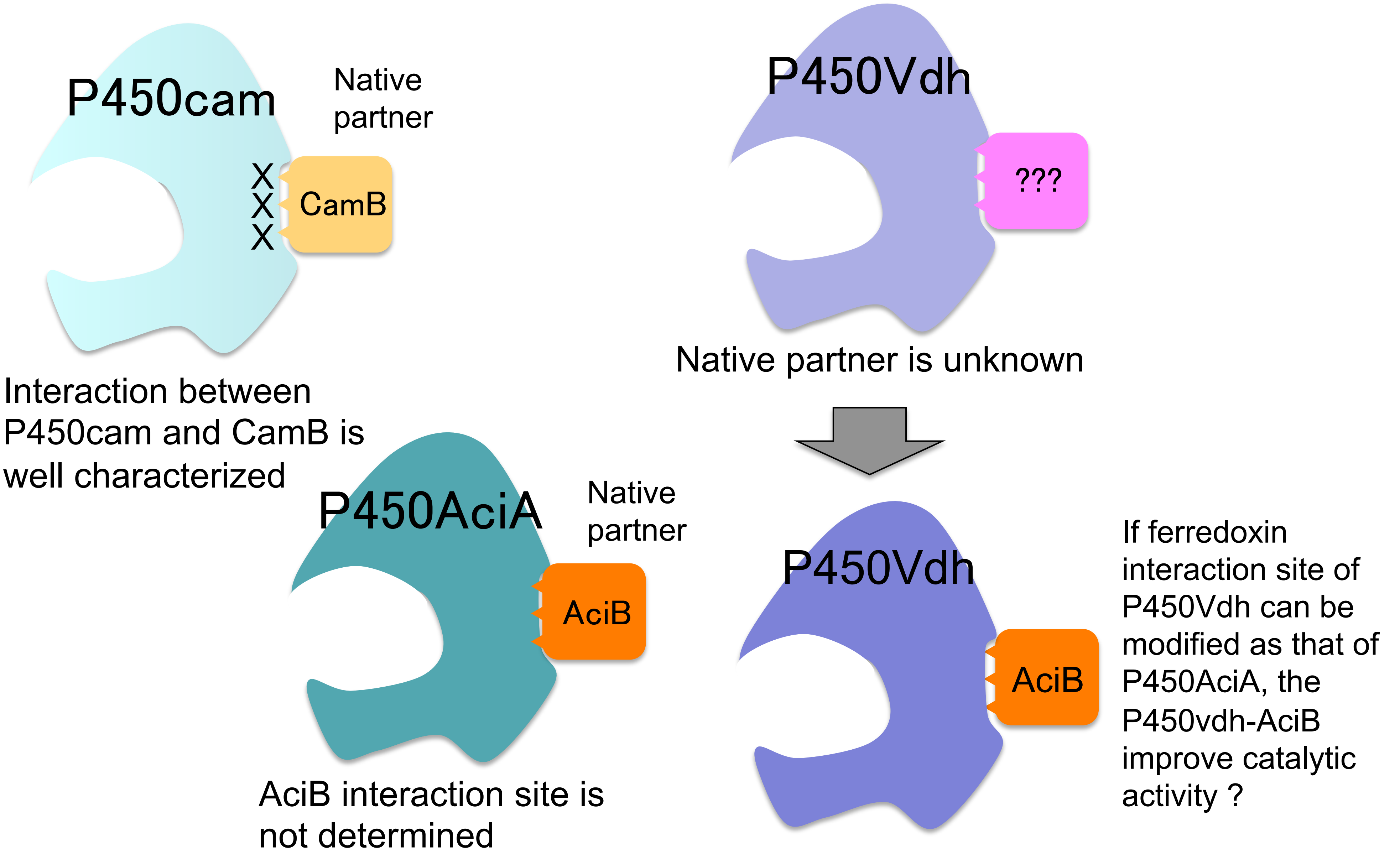


VD$_3$ complex　　　25OHVD$_3$ complex

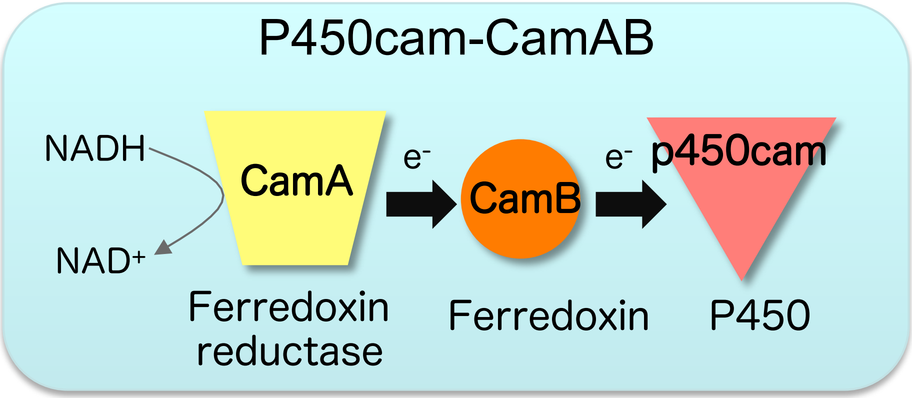
Stability of redox partner is importanat for efficient hydroxylation … ated host cells



Can P450Vdh-AciB interaction improve?



Mutational study on Vdh activity



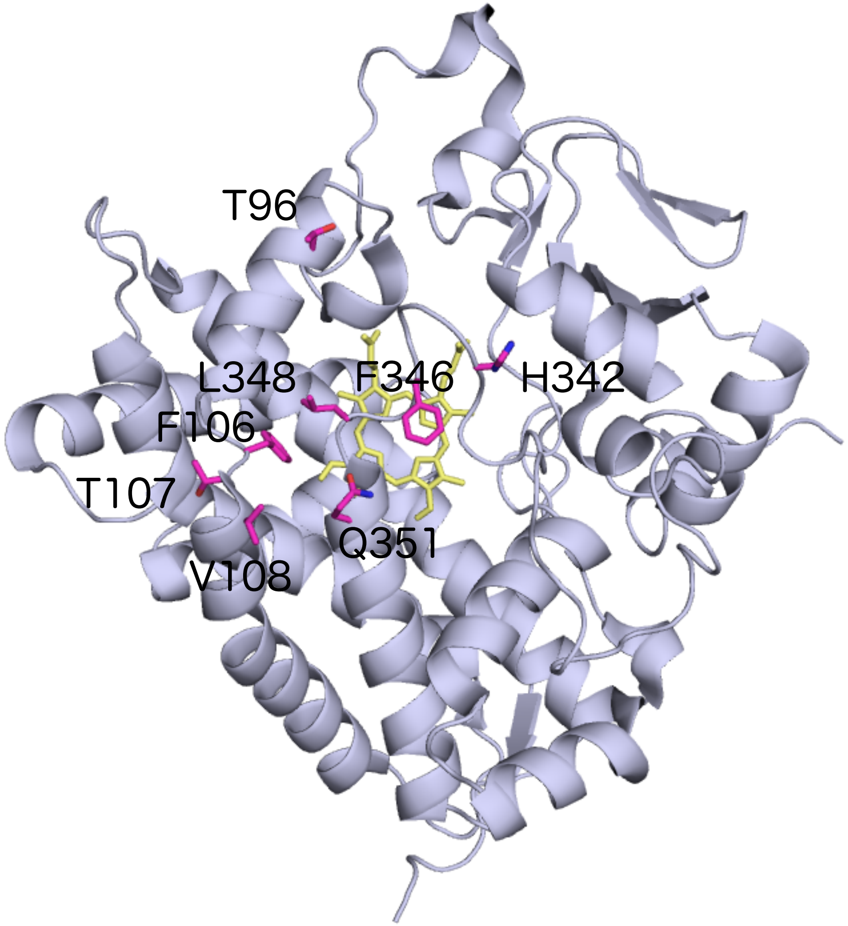
Sequence analysis

```
P450cam     xxxxxxxxxx-xxxxxxx…
p450AciA    xxxxxx-xxxxxxxxxxx…
P450Vdh     xxxxxxxxxx—xxxxxxx……
```

Amino acid residues where interact with ferredoxin

P450cam	P450 AciA	P450 Vdh		
R110	D180	T96	→	T96D
V120	V190	F106	→	F106V
G121	A191	T107	→	T107A
M122	P192	V108	→	V108P
H352	F440	H342	→	H342F
L357	R444	F346	→	F346R
L359	M446	L348	→	L348M
H362	R449	Q351	→	Q351R

Mutational study on Vdh activity

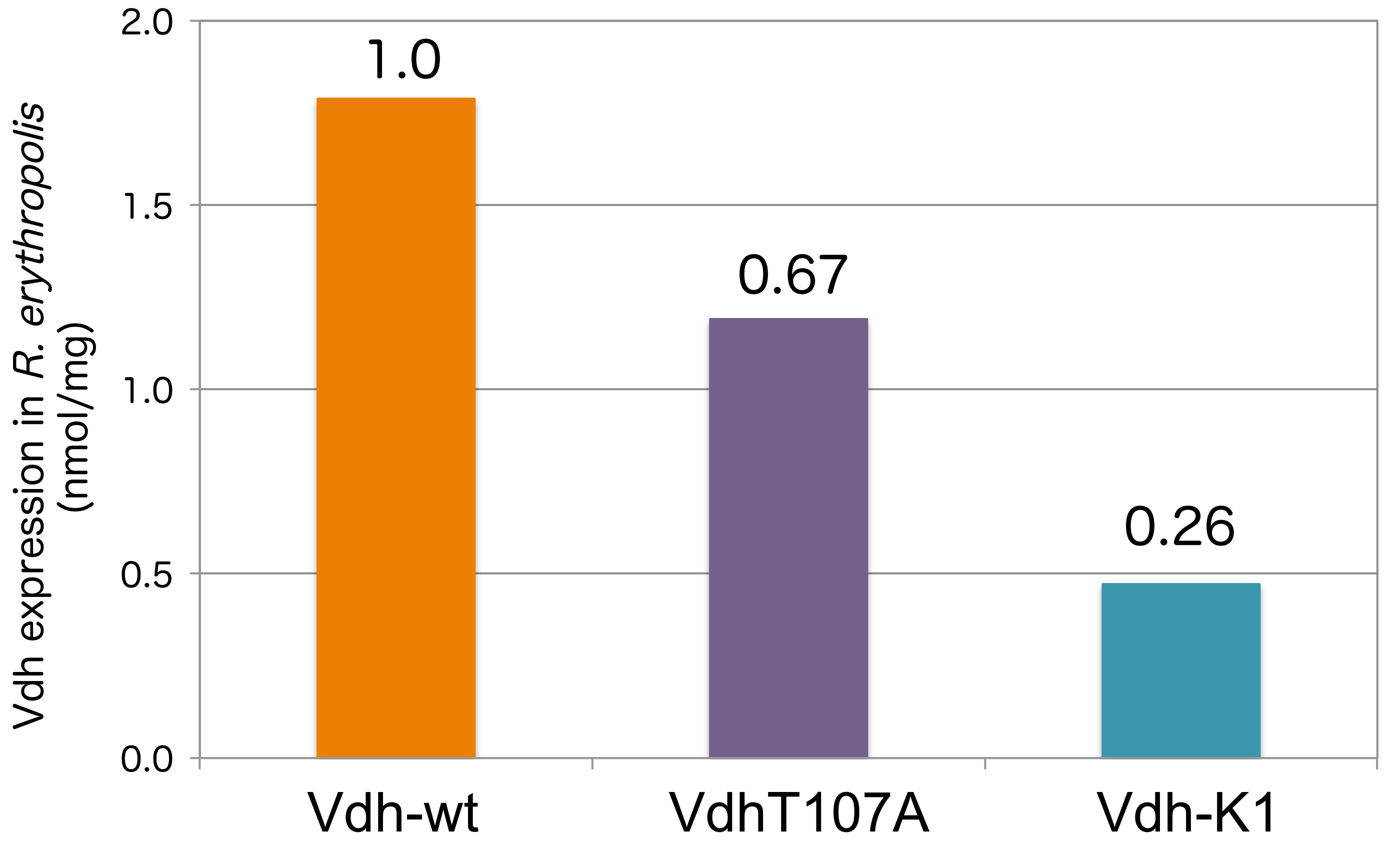


P450 Vdh

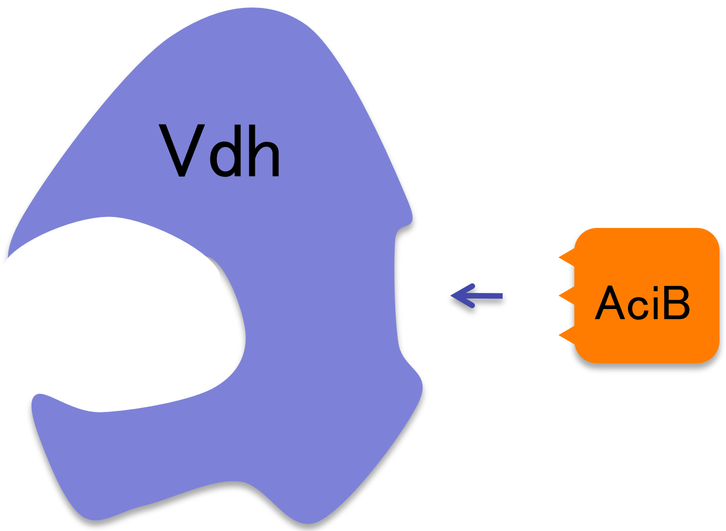
Mutants	relative activity
WT	1.0
T96D	0.5
F106V	ND
T107A	79.2
V108P	0.7
H342F	1.4
F346R	1.2
L348M	ND
Q351R	2.2

Enzymatic activity of Vdh-WT
0.070 ± 0.011 mol/min/mol of P450

Expression level of Vdh-T107A mutant in *Rhodococcus erythropolis*



Kinetic parameters for AciB on VD_3 25-hydroxylation activity

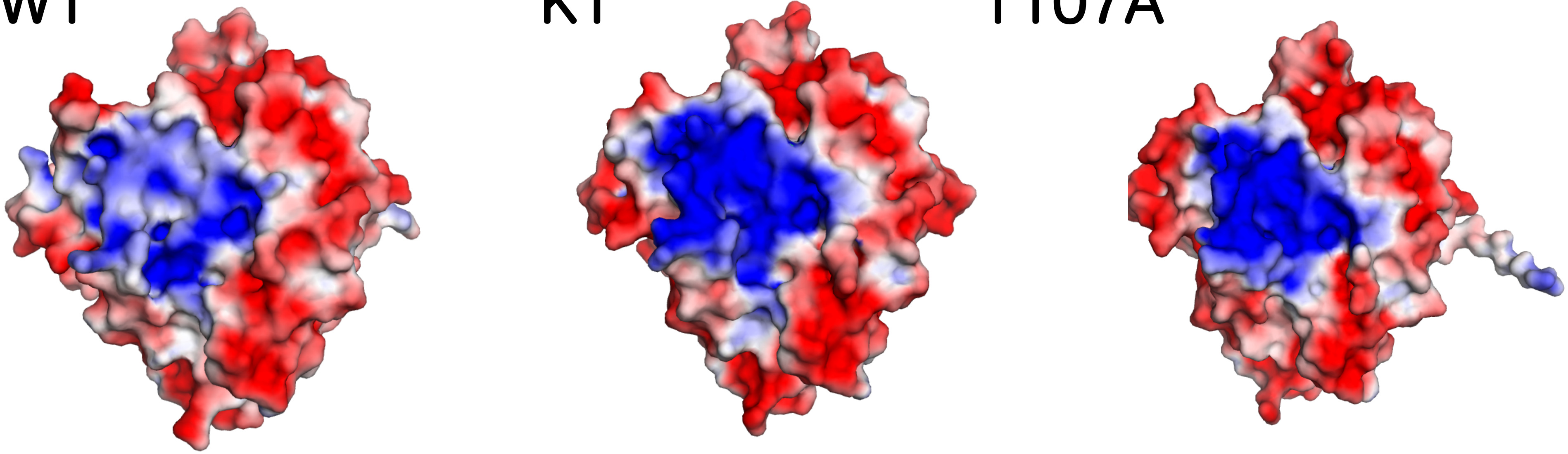


Vdh-K1 and Vdh-T107A enhance binding activity to AciB

	K_m (μM)	V_{max} (mol/min/mol of P450)
Vdh-WT	85.7 ± 8.4	0.81 ± 0.18
Vdh-K1	19.7 ± 4.1	20.8 ± 1.37
Vdh-T107A	24.5 ± 3.8	23.0 ± 1.40

Electrostatic potentials of Fdx-binding surface of Vdh-WT, Vdh-K1 and Vdh-T107A

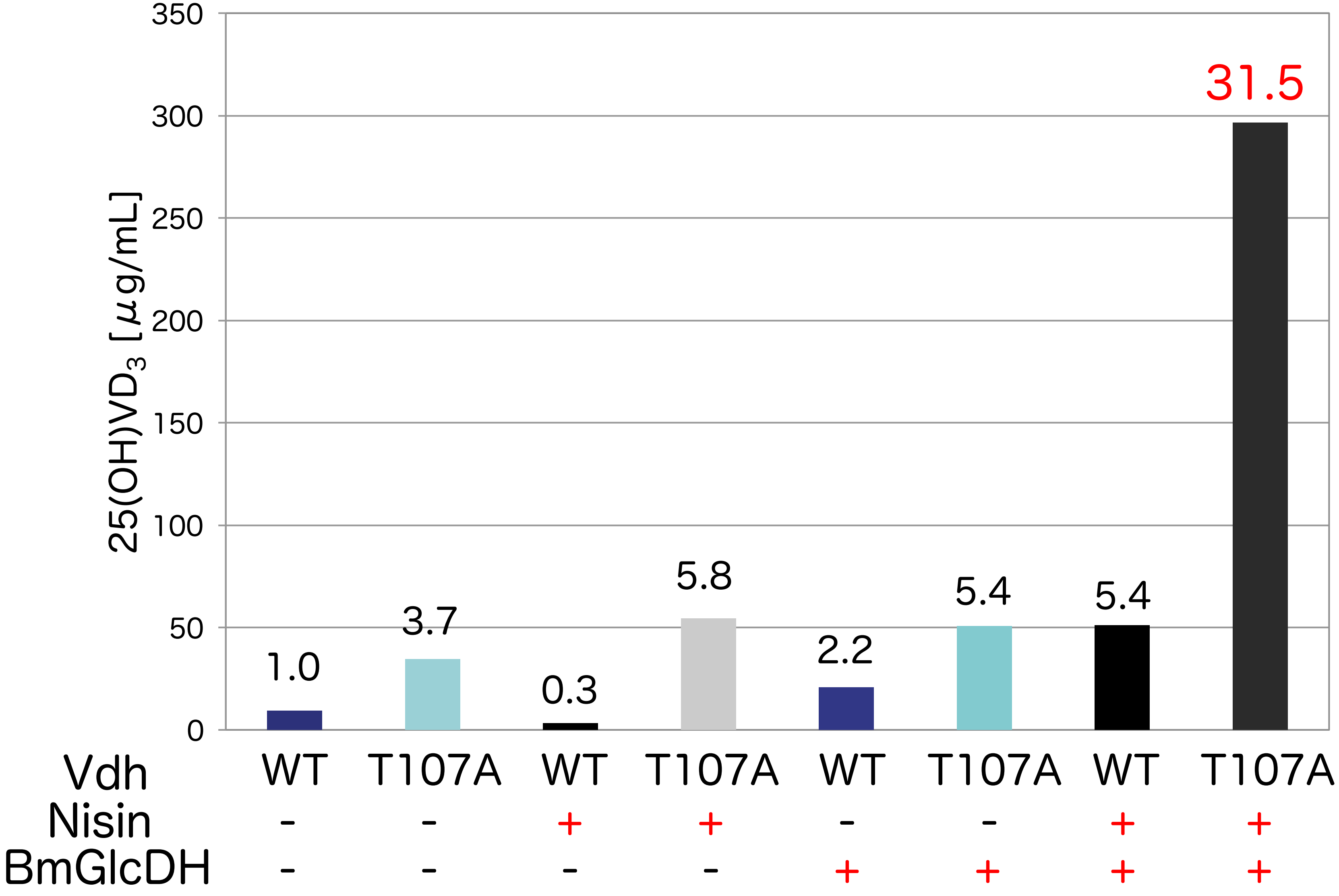
WT K1 T107A



It is known that the P450-binding surface of Fdx is negatively charged and the Fdx-binding surface of P450 is positively charged

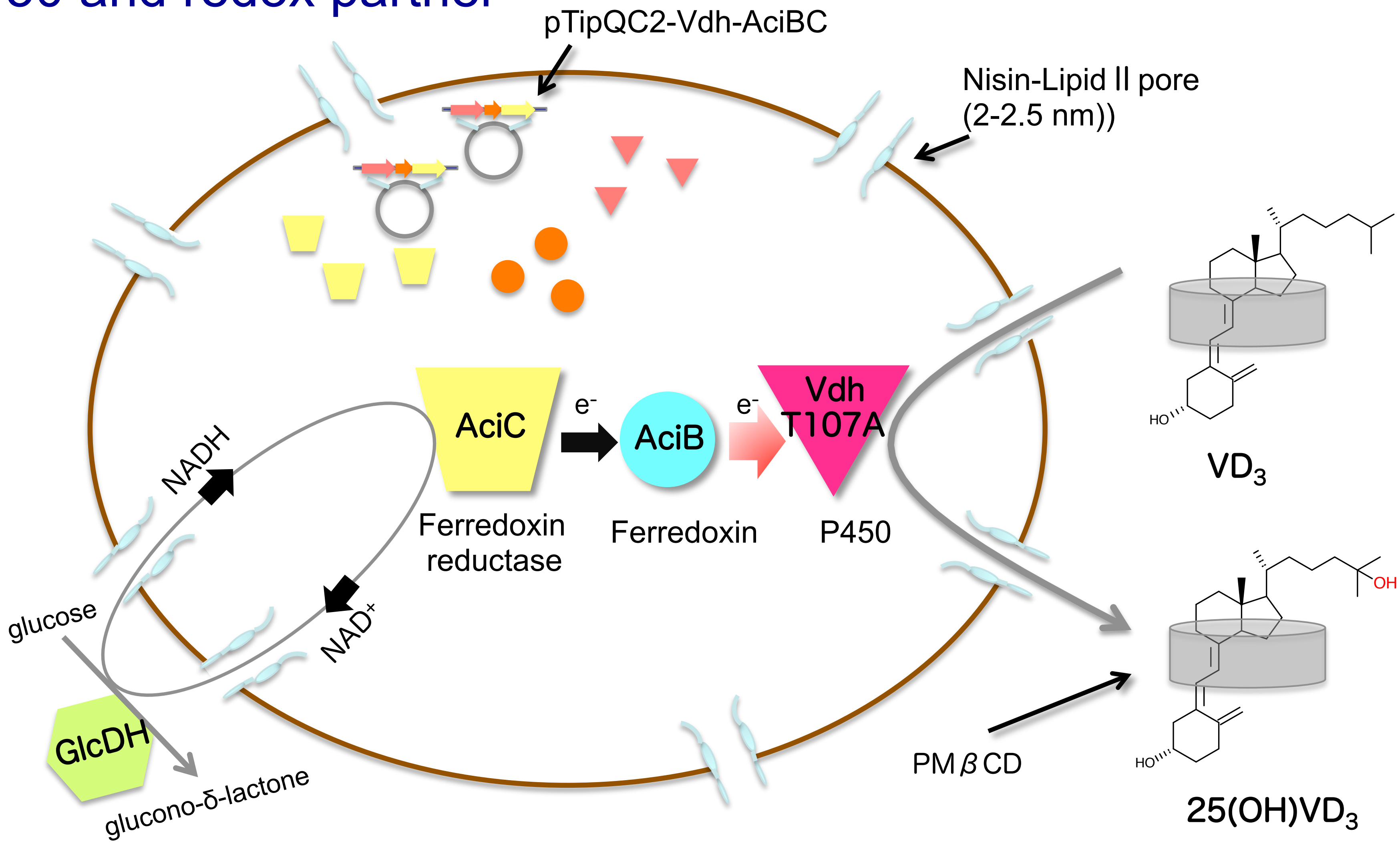
Blue：positive
Red：negative

Optimization of *Rhodococcus* cells



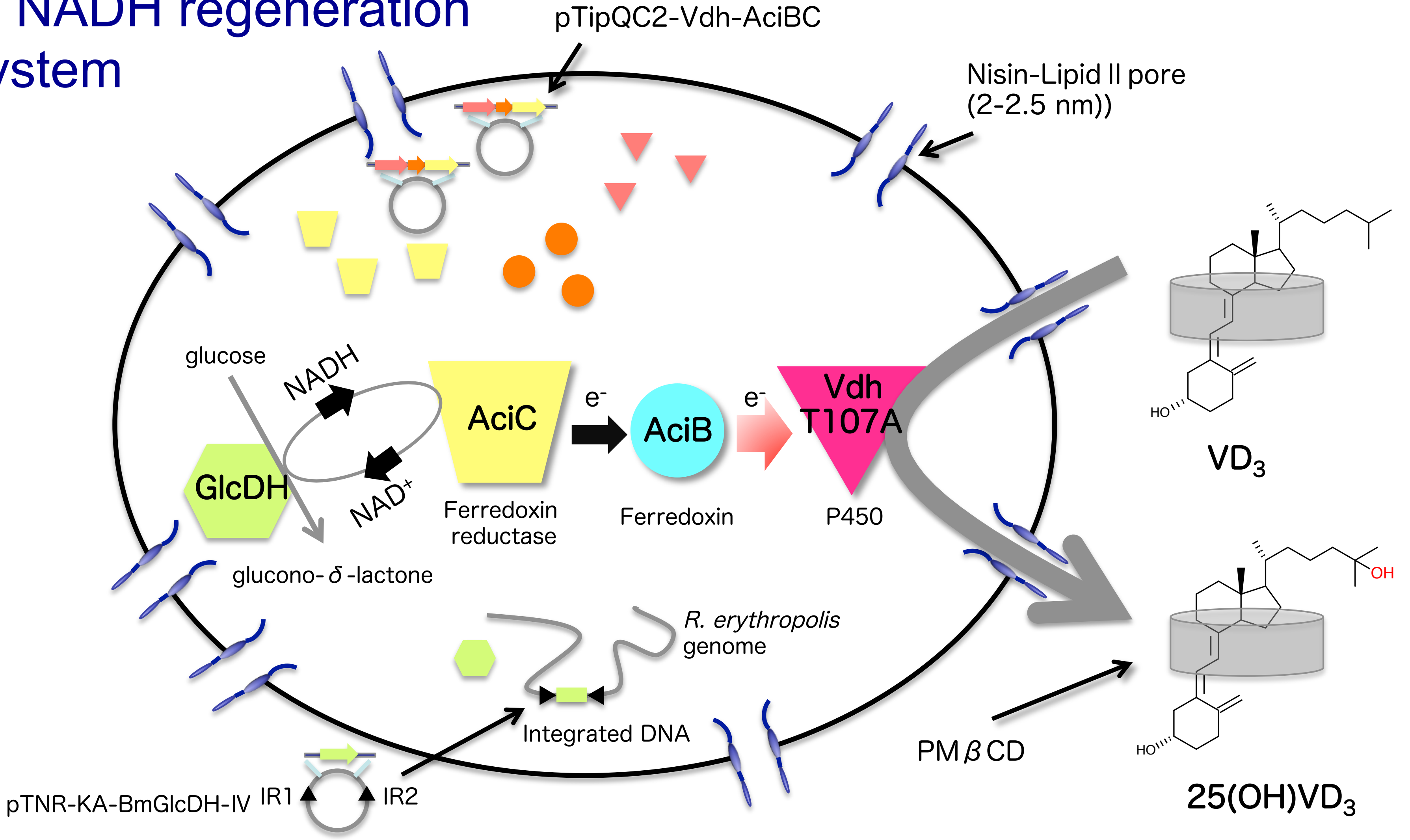
Development of a VD_3 hydroxylation system by using *Rhodococus erythropolis* as a host cell

3. p450 and redox partner

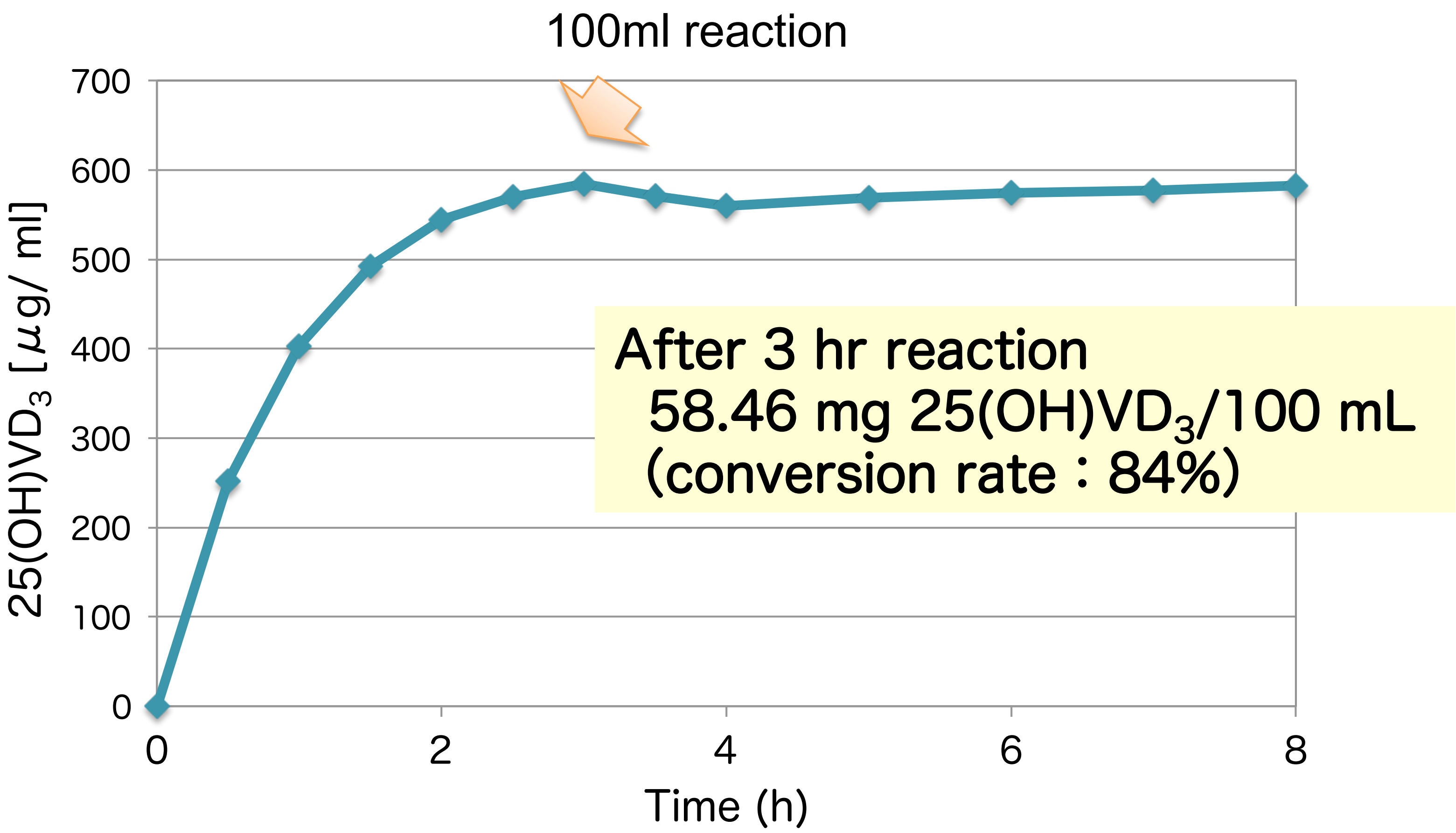


Development of a VD_3 hydroxylation system by using *Rhodococus erythropolis* as a host cell

3. NADH regeneration system



Large scale bioconversion of vitamin D_3 to $25(OH)VD_3$



Vitamin D_3 hydroxylation in different bacterial platform

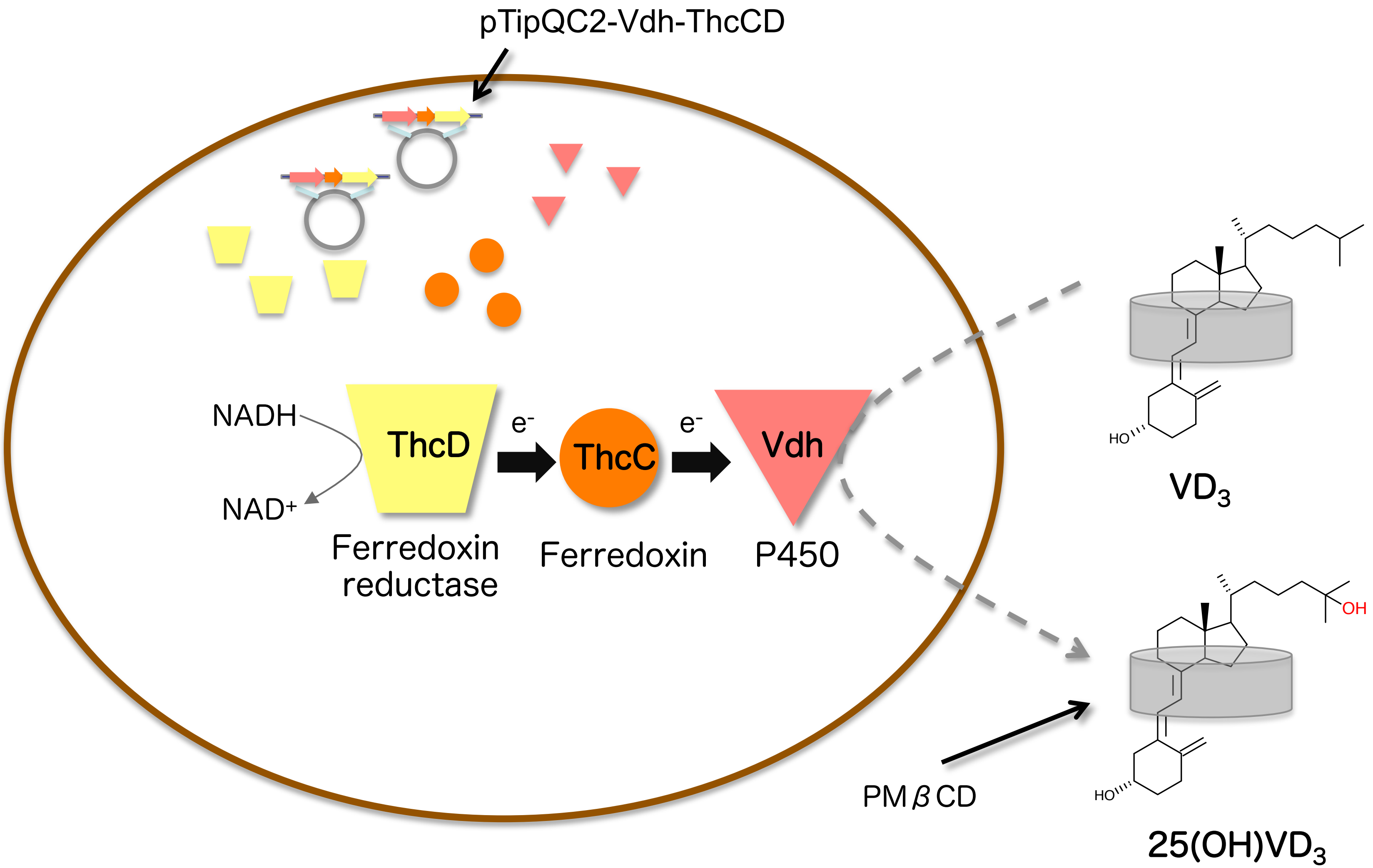
host	enzyme	25(OH)VD_3 production rate	25(OH)VD_3 production/h (fold)	Ref.
P. autotrophica	Vdh-WT	137μg/ml/48h	2.85 (1)	(1)
E. coli *	Vdh-WT	216μg/ml/24h	9.00 (3.2)	(2)
S. lividans	SU-1-R73V/R84V	7.8μg/ml/24h	0.33 (0.1)	(3)
R. erythropolis	Vdh-WT	53μg/ml/24h	2.21 (0.8)	(5)
R. erythropolis ‖	Vdh-WT	342μg/ml/16h	21.38 (7.5)	(4)
R. erythropolis ‡	Vdh-T107A	573μg/ml/2h	286.50 (100.4)	(5)

＊ tolC acrAB mutant
‖ nisin treated、BmGlcDH was added in the reaction
‡ nisin treated、BmGlcDH was expressed

(1) Takeda et al., *JFB* (1994)
(2) Fujii et al., *BBB* (2009)
(3) Hayashi et al., *FEBS* J (2010)
(4) Imoto et al., *BBRC* (2011)
(5) Yasutake et al., *ChemBioChem* (2013)

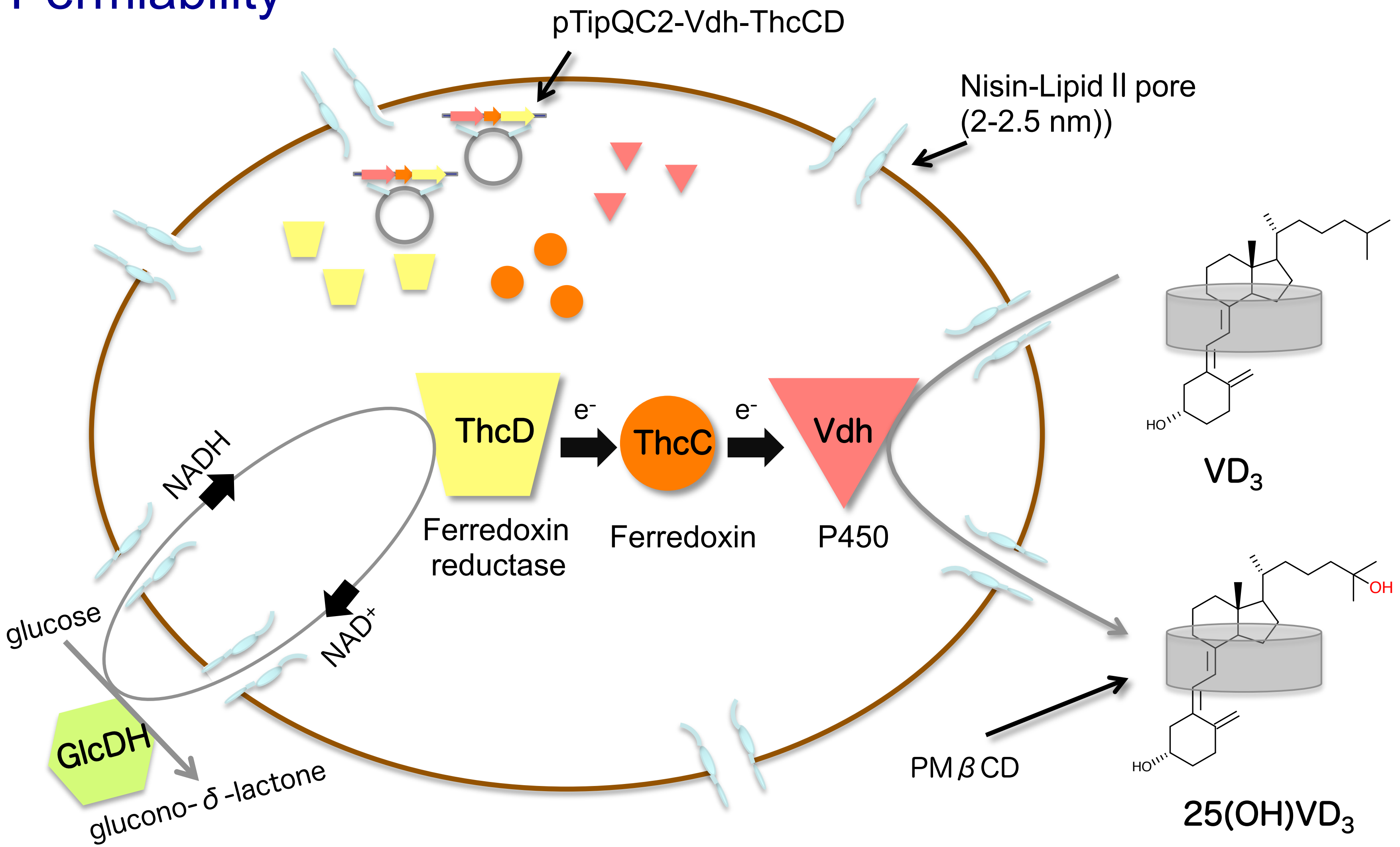
Development of a VD_3 hydroxylation system by using *Rhodococus erythropolis* as a host cell

1. Solubilizer



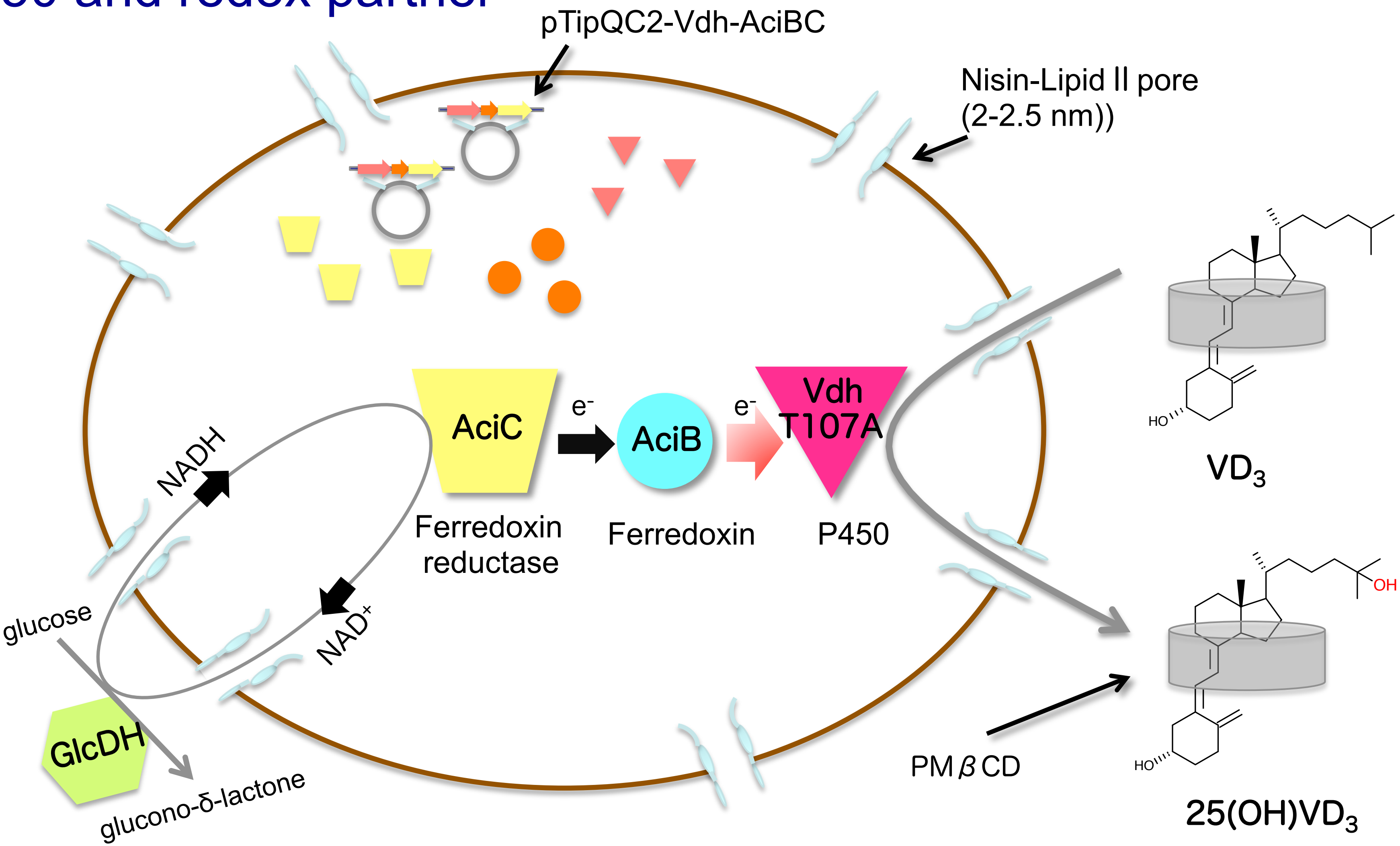
Development of a VD_3 hydroxylation system by using *Rhodococus erythropolis* as a host cell

2. Permiability



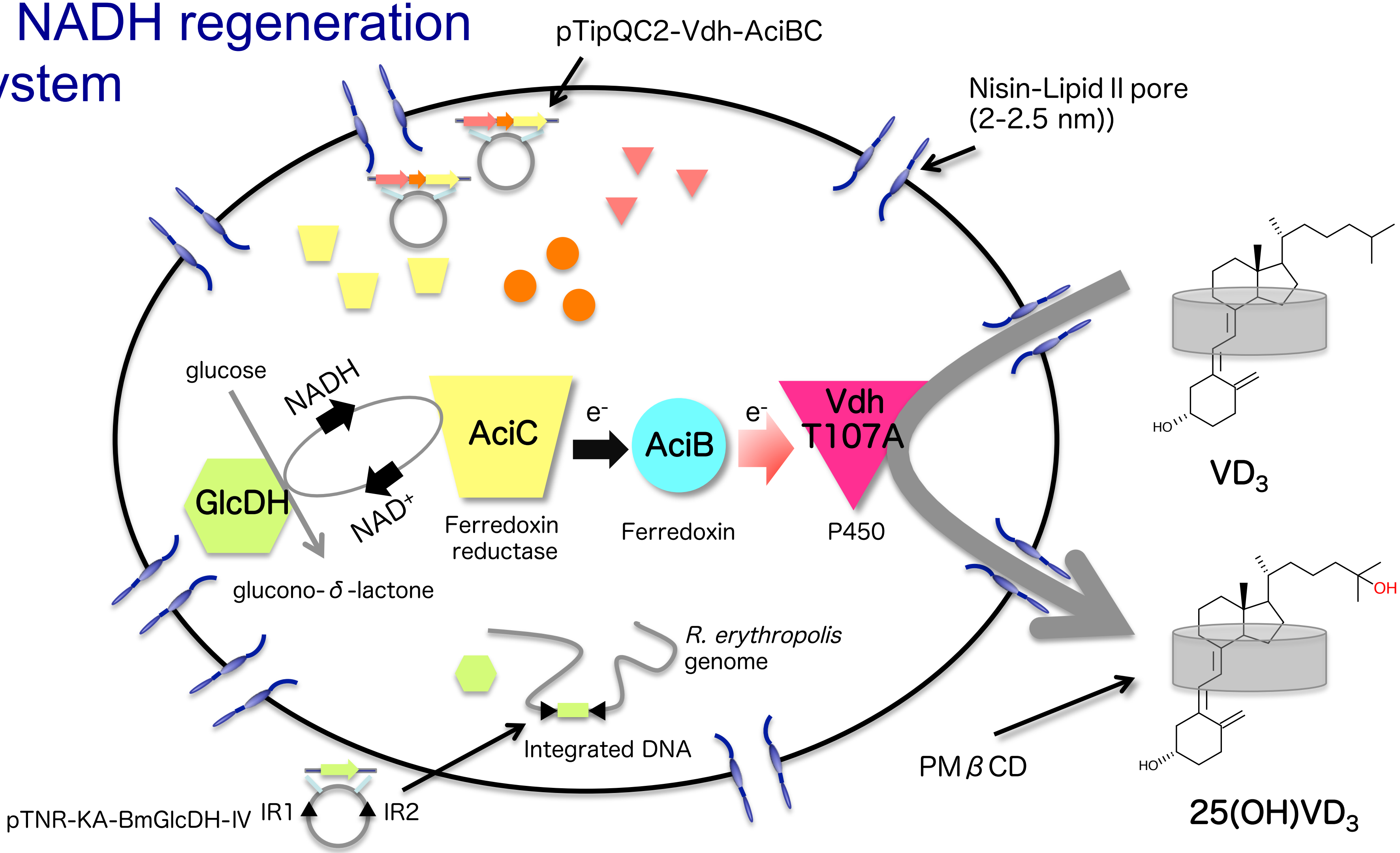
Development of a VD_3 hydroxylation system by using *Rhodococus erythropolis* as a host cell

3. p450 and redox partner



Development of a VD_3 hydroxylation system by using *Rhodococus erythropolis* as a host cell

3. NADH regeneration system



Thank you !!!