〈Original Article〉

Comparison of the substrate specificity of L-pipecolate oxidase and bacterial monomeric sarcosine oxidase, and structural interpretation of the enzymes

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Summary Mammalian L-pipecolate oxidase (PIPox) catalyzes the oxidative conversion of L-pipecolate to Δ^1-piperideine-6-carboxylate. Patients suffering from Zellweger syndrome are deficient in PIPox. We compared the substrate specificity of PIPox with that of bacterial monomeric sarcosine oxidase (mSox), which is used in commercial diagnostic tests for creatinine and creatine. The L-pipecolate to sarcosine oxidation activity ratio differed markedly between PIPox and mSox, at approximately 10 and 0.0036, respectively. The amino acid sequences of both enzymes exhibited approximately 30% identity and 54% similarity. Structural models of PIPox were constructed by homology modeling based on the tertiary structure of mSox. The catalytic centers of the open and substrate-binding forms of PIPox were more widely opened than those of mSox. Comparison of the local structure of the active sites of both enzymes led to the identification of three amino acid residues important for widening of the catalytic site. Conservation of these residues in PIPoxes from various eukaryotic species suggested a great impact on the substrate specificities.

Key words: L-pipecolate oxidase, Sarcosine oxidase, Substrate specificity, Homology modeling, Structural comparison

1. Introduction

L-Pipecolate oxidase (PIPox, EC 1.5.3.7; L-pipecolate:oxygen 1,6-oxidoreductase) is a flavoprotein enzyme that catalyzes the oxidative conversion of L-pipecolate (piperidine-2-carboxylate) to Δ^1-piperideine-6-carboxylate and hydrogen peroxide. The former product spontaneously opens to form 2-aminoadipate semialdehyde at physiologic pH. In mammals, L-pipecolate is an intermediate in the L-lysine degradation pathway[1]. Mammalian PIPoxes have a C-terminal tripeptide peroxisomal targeting signal 1 motif and are localized in the peroxisomes[1-3]. Patients suffering from Zellweger syndrome exhibit abnormally low PIPox activity[2, 3].

Another flavoprotein enzyme, monomeric sarcosine oxidase (mSox, EC 1.5.3.1; sarcosine: oxygen oxidoreductase), catalyzes the oxidative

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demethylation of sarcosine (N-methylglycine) to N-methylideneglycine and hydrogen peroxide. The former product spontaneously opens to form glycine and formaldehyde at physiologic pH. In bacteria, mSox is involved in the metabolism of creatinine along with two related enzymes, creatininase and creatinase. As it is used in combination with creatininase and creatinase in enzymatic assays for creatinine and creatine, mSox is a clinically important enzyme[4, 5]. Based on comparisons of amino acid sequences and enzyme activities, previous studies have found that PIPox is most closely related to mSox[3, 6, 7].

In a recent study, we investigated the substrate specificity and stereoselectivity of mSox isolated from bacteria of the genus *Bacillus* (SoxB)[8]. The results of that study were discussed in terms of enzyme-substrate complex models constructed using computer-aided docking analyses. In another study, we constructed a three-dimensional structural model of *Arthrobacter* mSox (SoxA) using homology modeling based on the X-ray structure of SoxB[9]. The binding properties of SoxA with respect to various substrate analogues were determined by computationally analyzing the fit of substrates in the active site[10]. Furthermore, we also characterized the structural changes that occur in SoxA as a result of various previously studied mutations[11, 12] that are known to alter the enzyme's substrate affinity[13].

In the present study, we conducted a detailed comparison of the substrate specificities and amino acid sequences of mSox and PIPox. A structural model of PIPox was constructed by homology modeling based on the tertiary structure of mSox. The quite different substrate specificities of these enzymes are interpreted from a structural perspective.

2. Materials and methods

Enzyme assay

Compounds used as substrates included sarcosine, L-proline, D-proline, L-pipecolate, and D-pipecolate (Fig. 1), which were purchased from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan). The enzyme assay was based on the measurement of hydrogen peroxide produced during substrate oxidation. The 4-aminoantipyrine peroxidase system was used for the enzyme assay, as described previously[11-13]. The final assay mixture contained 100 mmol/L substrate, 0.49 mmol/L 4-aminoantipyrine, 2.1 mmol/L phenol, 50 mmol/L Tris-HCl (pH 8.0), and 5 U/mL of horseradish peroxidase. Enzyme solution (35 μL) was incubated with the assay mixture (910 μL) at 37℃, and the amount of quinoneimine dye formed by the coupling of 4-aminoantipyrine, phenol, and horseradish peroxidase was measured spectrophotometrically at 500 nm against a sample blank. One unit of activity was defined as the formation of 1 μmol of hydrogen peroxide (0.5 μmol of quinoneimine dye) per minute at 37℃ and pH 8.0.

Homology searching

Homology searching and multiple alignment of the amino acid sequences of several PIPoxes[1, 3] and mSox[14] were performed using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2)[15]. The sequences (RefSeq or UniProt accession numbers are listed in Table 1) were aligned to examine sequence identity and identify conserved sequences.

Molecular modeling

Homology modeling was used to construct models of *Macaca mulatta* (Rhesus monkey) PIPox. Three-dimensional protein models were generated using MODELLER software[16], based on structures of SoxB[17, 18] (PDB ID: 2gb0 and 1el5). The program Pymol[13] was used for molecular visualization and simulation of substrate docking utilizing the pair-fitting function. A substrate-binding model of PIPox was obtained by superposing the coordinates of the active site of PIPox onto those of SoxB bound to a substrate analogue, dimethylglycine (DMG). The coordinates for sarcosine and L-pipecolate were then generated by superposing the positions of the C_α, methylamino, and carboxyl groups (total of 6 pairs) onto those of DMG, with root mean square deviations (RMSDs) of 0.154 and 0.160 Å, respectively. Models of the substrate binding sites of PIPox and

mSox (SoxB) were thus constructed.

3. Results and discussion

Comparison of substrate specificities

To further our understanding of the substrate affinity and stereoselectivity of PIPox, the known substrate specificity of *M. mulatta* PIPox1 was

Table 1 List of amino acid sequences of PIPoxes used in this study

Species	Accession number
Homo sapiens (human)	NP_057602
Macaca Mulatta (Rhesus monkey)	XP_001110686
Oryctolagus cuniculus (rabbit)	P79371
Mus musculus (mouse)	Q9D826
Gallus gallus (chicken)	XP_001235036
Danio rerio (zebrafish)	XP_686922
Arabidopsis thaliana (thale cress)	Q9SJA7
Glycine max (soybean)	XP_003522669
Schizosaccharomyces pombe (fission yeast)	NP_595239
[*Bacillus* mSox]	[P40859]

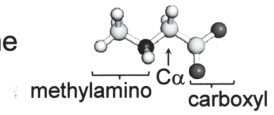
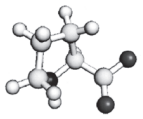
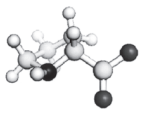
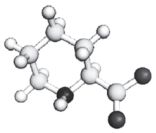
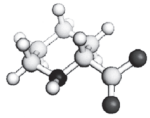
Substrate	Relative Activity	
	mSox	PIP ox
Sarcosine 	1.0	0.10
L-Proline 	0.0025	0.23
D-Proline 	0.0045	0.060
L-Pipecolate 	0.0036	1.0
D-Pipecolate 	n.d.	n.d.

Fig. 1 Comparison of PIPox and mSox substrate specificities. The substrate specificity of PIPox from *M. mulatta* is cited from reference 1. Substrate structures are shown as ball-and-stick drawings. Carbon, nitrogen, and oxygen atoms are represented as white, black, and dark-grey balls, respectively. Hydrogen atoms are represented as small balls.

compared with that of *Bacillus* mSox (SoxB). As shown in Figure 1, the enzymes exhibited different oxidation activity with respect to sarcosine, proline, and pipecolate. The L-pipecolate to sarcosine oxidation activity ratios for PIPox and mSox were approximately 10 and 0.0036, respectively. A structural comparison of sarcosine and L-pipecolate showed that the Cα, methylamino, and carboxyl groups have similar positions in the molecules (Fig. 1). Accordingly, we hypothesized that the extremely different L-pipecolate to sarcosine oxidation activity ratios of the enzymes are due to spatial variation around the active sites.

In contrast to the other substrates, the methylamino group of L-proline holds a different position in the molecule (Fig. 1). RMSD values of 0.154, 0.630, 0.111, 0.160, and 0.145 Å were obtained for sarcosine, L-proline, D-proline, L-pipecolate, and D-pipecolate, respectively, when the target atoms (3 carbon, 2 oxygen, and 1 nitrogen atom) were superposed onto those of DMG fitted in the active site of SoxB. The markedly higher RMSD value for L-proline suggests that the lower activity of the enzyme against this substrate is due to weak interaction with the isoalloxazine ring of the coenzyme, flavin adenine dinucleotide (FAD). Through examination of enzyme-substrate complex models, in a previous study we found that L-proline interacts with the isoalloxazine ring with lower affinity than other substrates[8].

Low activity was also observed for D-isomers. Neither PIPox nor mSox exhibited activity against D-pipecolate, which contains the larger-size ring. This result suggests that the tertiary structure of the heterocyclic nitrogen ring of D-proline and D-pipecolate causes steric interference with the active site residues and the isoalloxazine ring of FAD, resulting in an extremely low frequency of enzyme-substrate complex formation (Fig. 1).

Amino acid sequence alignment

As the first step in constructing PIPox structure models, the amino acid sequences of *M. mulatta* and human PIPox were aligned with the sequence of SoxB (Fig. 2). The *M. mulatta* PIPox sequence was 95%

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Homo sapiens (human)                                                    MAAQK  5
Macaca mulatta (rhesus monkey)  MIREKAVPVSLDSSHQLVLMVQGLKLKEARGVLHKGLKVLAFQQPLSVAGGLVGIMAAQK 60
Bacillus mSox (SoxB)                                                      S  1
                                                                          .

Homo sapiens (human)            DLWDAIVIGAGIQGCFTAYHLAKHRKRILLLEQFFLPHSRGSSHGQSRIIRKAYLED-FY 64
Macaca mulatta (rhesus monkey)  DLWDAIVIGAGIQGCFTAYHLAKHRKRVLLLEQFFLPHSRGSSHGQSRIIRKAYLED-FY 119
Bacillus mSox (SoxB)            THFDVIVVGAGSMGMAAGYQLAKQGVKTLLVDAFDPPHTNGSHHGDTRIIRHAYGEGREY 61
                                  :*.**:***  *  :.*:***:  : **:: *  **:.** **::****:** *.  *

Homo sapiens (human)            TRMMHECYQIWAQLEHEAGTQLHRQTGLLLLGMK-ENQELKTIQANLSRQRVEHQCLSSE 123
Macaca mulatta (rhesus monkey)  TQMMHECYQIWAQLEHEAGTQLHRQTGLLLLGMK-ENQELKTIQASLSRQRVEHQCLSSE 178
Bacillus mSox (SoxB)            VPLALRSQELWYELEKETHHKIFTKTGVLVFGPKGESAFVAETMEAAKEHSLTVDLLEGD 121
                                . :  .. ::* :**:*:  ::. :**:*::* * *.  :       ..: :  : *..:

Homo sapiens (human)            ELKQRFPNIRLPRGEVGLLDNSGGVIYAYKALRALQDAIRQLGGIVRDGEKVVEINPG-L 182
Macaca mulatta (rhesus monkey)  ELKQRFPNIRLTRGEVGLLDNSGGVLYAYKALRALQDAVRQLGGIVHDGEKVVEINPG-L 237
Bacillus mSox (SoxB)            EINKRWPGITVPENYNAIFEPNSGVLFSENCIRAYRELAEARGAKVLTHTRVEDFDISPD 181
                                *:::*:*.* :...  .::: ..**::: :.:** ::  .  *. *    :* ::: .

Homo sapiens (human)            LVTVKTTSRSYQAKSLVITAGPWTNQLLRPLGIEMPLQTLRINVCYWREMVPGSYGVSQA 242
Macaca mulatta (rhesus monkey)  LVKVKTTTRSYQAKSLVITAGPWTNQLLRPLGIELPLQTLRINVCYWRERVPGSYGVSQA 297
Bacillus mSox (SoxB)            SVKIETANGSYTADKLIVSMGAWNSKLLSKLNLDIPLQPYRQVVGFFESDES-KYSNDID 240
                                 *.::*:. ** *..*::: *.*..:** *.:::***. *  * ::..  . .*. .

Homo sapiens (human)            FPCFLWLGLCPHHIYGLPTGEYPGLMKVSYHHGNHADPEERDCPTARTDIGDVQILSSFV 302
Macaca mulatta (rhesus monkey)  FPCFLWLGLYPHHLYGLPAGEYPGLMKVSYHHGNHADPEERDCPTARADIRDVQIVSSFV 357
Bacillus mSox (SoxB)            FPGFMVEVPN-GIYYGFPSFGGCGLKLGYHTFGQKIDPDTINREFG-VYPEDESNLRAFL 298
                                ** *:        **:*:   **    : .*:: **:  :  . .  * . : :*:

Homo sapiens (human)            RDHLPDLKPEPAVIESCMYTNTPDEQFILDRHPKYDNIVIGAGFSGHGFKLAPVVGKILY 362
Macaca mulatta (rhesus monkey)  RDHFPDLKPEPAVMESCMYTNTPDEHFILDRHPKYDNIVIGAGFSGHGFKLAPVVGKILY 417
Bacillus mSox (SoxB)            EEYMPGANGELKRGAVCMYTKTLDEHFIIDLHPEHSNVVIAAGFSGHGFKFSSGVGEVLS 358
                                .:::*. : *      ****:* **:**:* **::.*:**.*********::. **::*

Homo sapiens (human)            ELSMKLTPSYDLAPFRISR---FPSLGKAHL 390
Macaca mulatta (rhesus monkey)  ELSMKLTPSYDLAPFRISR---FPSLGKAHL 445
Bacillus mSox (SoxB)            QLALTGKTEHDISIFSINRPALKESLQKTTI 389
                                :*::. ...:*:: * *.*     ** *: :
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Fig. 2 Comparison of PIPox (upper and middle) and mSox (lower) amino acid sequences. Identical and similar residues are indicated by asterisks and dots, respectively. Residues of *H. sapiens* and *M. mulatta* PIPox that differ are underlined.

identical to that of the human enzyme. Only 20 residues differed between *M. mulatta* and human PIPox (Fig. 2). The enzymatic properties of both enzymes were thus presumed to be similar.

The amino acid sequences of *M. mulatta* PIPox and SoxB exhibited approximately 30% identity and 54% similarity. In particular, the two regions involved in FAD binding (Phe4-Gly58 and Cys315-Thr363 in SoxB) exhibited higher levels of homology (49 and 57% identity, respectively), without any gaps.

The structure around the active site

Three-dimensional open and closed structure models of *M. mulatta* PIPox were constructed by homology modeling based on the SoxB sequence and its X-ray structures (the open form [PDB ID: 2gb0] and the DMG-binding form [PDB ID: 1el5]), as described in the Materials and methods section.

The overall structures of the open and closed forms of PIPox and SoxB superimposed well upon one another, with RMSD values of 0.17 and 0.15 Å for the atomic Cα position of PIPox and SoxB, respectively. To further elucidate the enzyme-substrate interaction, the tertiary structure of sarcosine or L-pipecolate was fitted in the active sites of the closed forms of PIPox and SoxB, based on structural data generated for the SoxB-DMG complex. We found that the catalytic centers of the open and substrate-binding forms of PIPox were more widely opened than those of SoxB (Fig. 3). A significant conformational change should not be required to enclose the active sites of SoxB and PIPox for catalytic reaction. A narrowing of the active site entrance and catalytic cleft for mSox to more effectively accommodate sarcosine would be reasonable. However, in this circumstance it would be more difficult for L-pipecolate, which is bulkier than sarcosine, to reach the active site. Furthermore, even if L-pipecolate could be bound in the active site, its heterocyclic ring would be too close to the side chains of several residues, especially that of Met245 (a distance of 2 Å or less).

In contrast, because PIPox has a wider entrance and catalytic cleft, it was thought that L-pipecolate would be a more preferable substrate than sarcosine. A comparison of the local structure around the active site of both enzymes led to the identification of three amino acid residues that are important in widening the space: Phe100, Met245, and His269 in SoxB and Glu157, Leu302, and Tyr327 in *M. mulatta* PIPox (Fig. 3). His269 and Tyr327 appear to form part of the wall of the catalytic cleft and would thus be expected to affect substrate binding. A previous report indicated that His269 is important for the enzymatic activity of SoxB, even though it is not the active-site base[19]. Perhaps the residue at this position plays a role in appropriately fitting the substrate for interaction with the isoalloxazine ring of FAD. Met245 and Leu302 appear to be directly involved in not only controlling the size of the catalytic cleft but also the size of the active site entrance. On the other hand, Phe100 and Glu157 were indirectly implicated in being involved in widening of the catalytic cleft because they are located in positions in which they can influence the flexibility of the Met245 and Leu302 side chains. These findings are consistent with those of our previous molecular docking study of mSox, which indicated that these residues play a role in widening the catalytic cleft[13].

Conservation of the three residues involved in widening the catalytic space

The three amino acid residues described in the previous section were presumed to be of importance in determining substrate affinity. We therefore examined the conservation of these residues in PIPoxes from various eukaryotic species (Fig. 4). At position 157 of *M. mulatta* PIPox, the residues in PIPoxes from nine different species were small (Gly, Ala, or Ser) or charged (Glu, Lys, or Arg), whereas the corresponding residue in SoxB or SoxA was Phe. In contrast, the residues in PIPoxes at position 302 of *M. mulatta* PIPox were nonpolar and aliphatic, like the corresponding residue in SoxB or SoxA. The side chains of the residues at position 302 in PIPox can be more easily moved and undergo conformational changes because of the flexible side chain of the residue at position 157. It is most likely that the flexibility of the side chain of the residue at position 302, which is ensured by the effect of the side chain of the residue at position 157, plays an important role in the oxidation

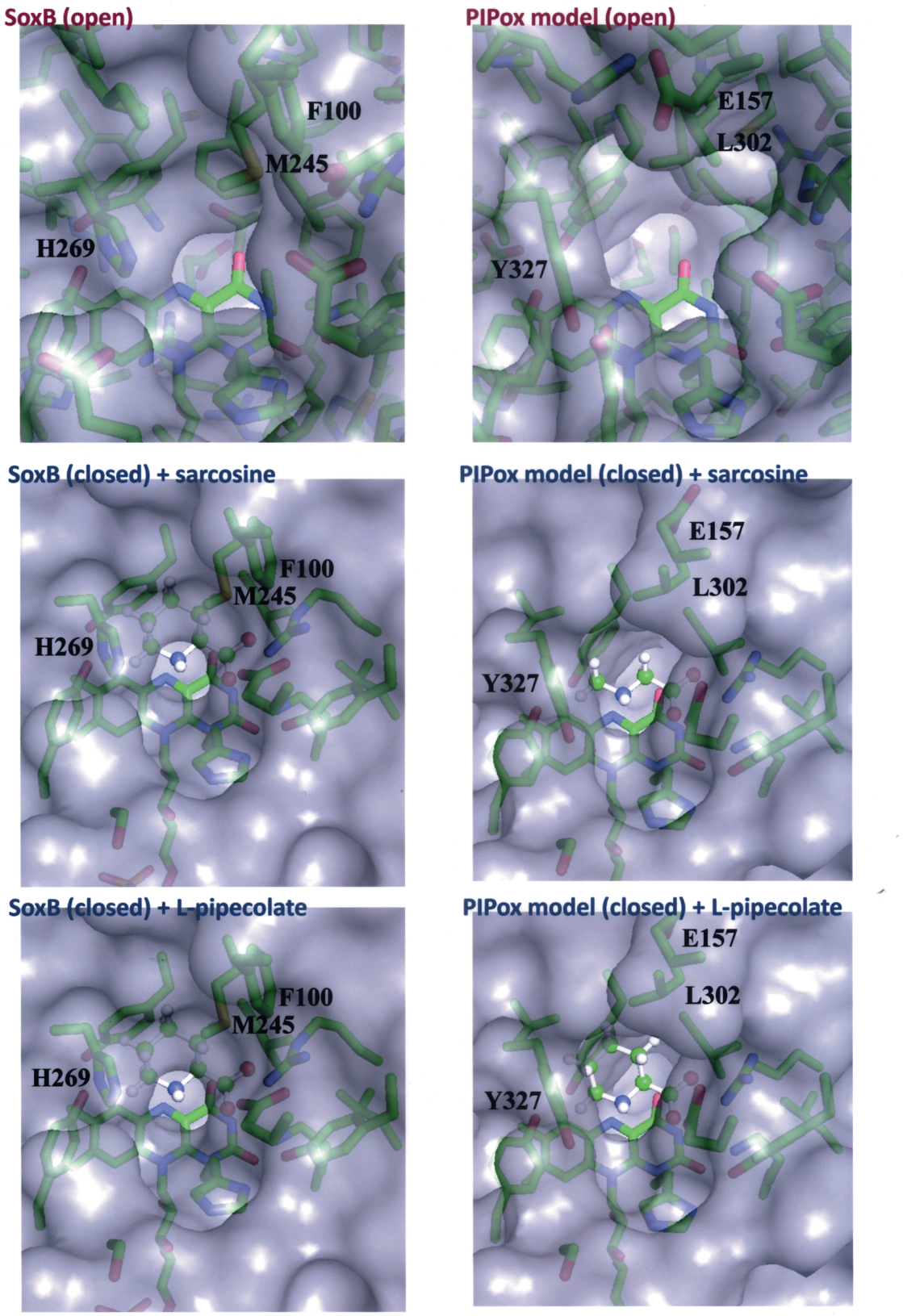

Fig. 3 Close-up views of the active site regions of PIPox and mSox (upper: open forms, middle: closed forms containing sarcosine, lower: closed forms containing L-pipecolate). Each structure was constructed using molecular modeling and substrate docking simulations, as described in the Materials and methods section. Amino acid residues and FAD are shown as stick drawings. The substrates, sarcosine and L-pipecolate, are shown as ball-and-stick drawings. Carbon, nitrogen, and oxygen atoms are shown in green, blue, and red, respectively. The molecular surface of each structure was calculated using Pymol and is shown transparently.

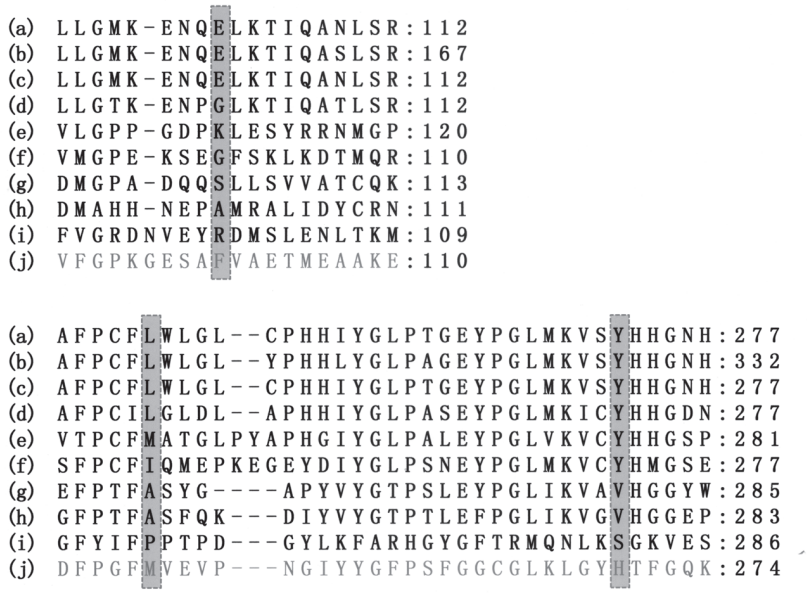

Fig. 4 Partial amino acid sequence alignments of several eukaryotic PIPoxes. Sequences (a) through (i) are for *H. sapiens, M. mulatta, Oryctolagus cuniculus* (rabbit), *Mus musculus* (mouse), *Gallus gallus* (chicken), *Danio rerio* (zebrafish), *Arabidopsis thaliana* (thale cress), *Glycine max* (soybean), and *Schizosaccharomyces pombe* (fission yeast) PIPox, respectively. The *M. musculus*, *G. gallus*, *D. rerio*, and *G. max* enzymes are encoded by the corresponding orthologous gene. The SoxB sequence (j) is also aligned for comparison. The positions of the three important residues discussed in the text are highlighted by grey boxes.

activity against L-pipecolate. In SoxB, Met245 is held rigid due to the bulky side chain of Phe100, thus explaining the extremely low L-pipecolate oxidation activity of this enzyme.

The residue at position 327 of *M. mulatta* PIPox was conserved among different PIPoxes. However, Val, Tyr, and Ser were in the corresponding position in *Arabidopsis thaliana* (thale cress), *Glycine max* (soybean), and *Schizosaccharomyces pombe* (fission yeast) (Fig. 4). Although these residues are slightly too small to form the wall of the catalytic cleft, we could not speculate on their function in the active site of the enzyme.

Our results suggest that the three conserved residues have a greater impact on the substrate specificities of PIPox and mSox than the overall homology between these enzymes. Prediction of substrate specificity may be possible to a certain degree based upon the extent of conservation of these residues, which could be utilized as targets for altering the enzymatic function of PIPox and mSox.

Acknowledgment

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