Alteration of Substrate Specificity and Optimum pH of Sarcosine Oxidase by Random and Site-Directed Mutagenesis

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The sarcosine oxidase gene was randomly mutated, and F103V, which showed altered specificity, was screened with the plate assay. Five different mutants at position 103 were also constructed. Each mutant exhibited a rather striking change in substrate specificity, except for F103W. The optimum pH was also shifted, depending on the substrate.

Sarcosine oxidase (EC 1.5.3.1; sarcosine:oxygen oxidoreductase [flavoprotein]) is one of many industrially important enzymes. It catalyzes the oxidative demethylation of sarcosine (N-methylglycine) and forms equimolar amounts of formaldehyde, glycine, and hydrogen peroxide (3, 5). It is useful for the enzymatic determination of creatinine, creatine, or sarcosine in clinics by coupling with related enzymes (2).

We have cloned and sequenced the sarcosine oxidase gene from Arthrobacter sp. strain TE1826 (soxA) (4), and SoxA was purified from the recombinant strain to homogeneity. The enzyme is a monomeric form, and its molecular weight was estimated to be 43,249 on the basis of the sequence data.

Suzuki (7) reported that sarcosine oxidase from Corynebacterium sp. acts on N-ethylglycine or N-methylalanine but hardly acts on other N-methyl amino acids. If the substrate specificity of sarcosine oxidase can be changed as directed, it will be useful for the enzymatic determinations of other substances. We tried to change the substrate specificity of SoxA, although the tertiary structure and active site of SoxA are still unknown. One feasible approach to obtain mutant enzymes with the desirably changed properties is the use of phenotypic selection from recombinant cells carrying a randomly mutated gene.

Mutant soxA by random mutagenesis. Random mutagenesis of the soxA gene was carried out with PCR techniques (1), as shown in Fig. 1. The PstI-EcoRI fragment of pSAOEP3 encoding SoxA (4) was chosen as the target DNA. PCR was done under standard conditions (8) with Tth DNA polymerase (Toyobo Co., Ltd., Osaka, Japan), except that dTTP was one-fifth the volume of other deoxyribonucleotide phosphates. The amplified fragments and pSAOEP3 were digested with NcoI and EcoRI, respectively. The NcoI-EcoRI fragment encodes the major part of SoxA (from amino acid residue 17 to the C terminus), except for the flavin adenine dinucleotide-binding site of the N terminus (4). The insert fragments and vector were separated by agarose gel electrophoresis, purified, and ligated. These were used to transform Escherichia coli JM109, and the transformants were plated on aldehyde indicator plates containing ampicillin (4). The plates were prepared by adding 50 mg of pararosaniline, 250 mg of sodium hydrogen sulfite, and 1 g of N-methyl-DL-valine to 1 liter of Luria agar. If N-methylvaline can be converted to formaldehyde and valine by an enzyme reacting with formaldehyde to form a Schiff base that is intensely red. E. coli JM109(pSAOEP3) producing the wild-type SoxA was negative by the plate assay, and one positive clone was found among about 3,000 colonies.

The recombinant plasmid was extracted from this clone, and the DNA sequence of the insert fragment was determined by the dideoxy method (6). As a result, only one T → G change was found at position 307 of the soxA gene, resulting in the replacement of the phenylalanine residue at position 103 by valine. The mutant SoxA and the recombinant plasmid were designated F103V and pSAOEP3-F103V, respectively.

The sarcosine oxidase and N-methylvaline oxidase activities of F103V were compared with those of the wild-type enzyme by using the plate assay (Fig. 2). E. coli JM109(pSAOEP3) was positive on the indicator plate containing 0.1% sarcosine and negative on the plate containing 0.1% N-methyl-DL-valine. In contrast, E. coli JM109(pSAOEP3-F103V) was negative on the former and positive on the latter. Accordingly, the substrate specificity of SoxA was changed by a point mutation at position 103.

Construction of other mutant enzymes by site-directed mutagenesis. The amino acid substitution of F103V leads to a change into a small side chain. In order to investigate the role of phenylalanine at position 103, other mutants were constructed by replacing it with amino acids of different sizes,

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ranging from glycine to tryptophan. Site-directed mutagenesis was performed by using the Transformer kit (Clontech Laboratories, Inc., Palo Alto, Calif.) according to the manufacturer's instructions. The oligonucleotides used were synthesized with DNA synthesizer 381A (Applied Biosystems, Inc., Foster City, Calif.). pSAOEP3 was used as the mutagenesis template, and E. coli JM109 was used as a host strain. Successful mutations were verified by DNA sequencing. The mutant enzymes in which glycine, alanine, threonine, leucine, and tryptophan were substituted for phenylalanine at position 103 were designated F103G, F103A, F103T, F103L, and F103W, respectively.

Kinetic parameters of wild-type enzyme and its mutants. Each recombinant strain producing a mutant of SoxA was cultured, and all six mutant enzymes were purified to homogeneity as reported previously (4). The specific activities (with 95 mM sarcosine as the substrate at 37°C and pH 8.0) of F103W, F103L, F103T, F103V, F103A, and F103G were found to be 25.3, 8.1, 5.9, 5.2, 3.0, and 4.4 U/mg, respectively, although that of the wild type was 19.8 U/mg. The $K_m$ and $k_{cat}$ (saturation constant [number of substrate molecules transformed per second per molecule of enzyme]) values of the wild type and its mutants for four kinds of N-methyl amino acids were estimated from Lineweaver-Burk plots, and the results are shown in Table 1.

The $K_m$ values of the wild type for sarcosine and N-methylalanine are much smaller than those for N-methylvaline and N-methylleucine. It appears that the binding affinity is higher for the smaller substrate than for the larger one. F103W also shows the same tendency as the wild type. In contrast, other mutants show quite different tendencies. The $K_m$ values of these mutants for N-methylvaline and N-methylleucine are much smaller than those of the wild type. However, the $K_m$ values for sarcosine and N-methylalanine increased remarkably. As a result, the substrate specificities of these mutants were considerably changed from that of the wild type. For example, the catalytic efficiency ($k_{cat}/K_m$) of F103V for N-methylvaline is about 15 times higher than that of the wild type because of a corresponding decrease in the $K_m$. The $k_{cat}/K_m$ of F103A for N-methylvaline is about 40 times higher than that of the wild type, although the $k_{cat}/K_m$ for sarcosine is about 1/100th that of wild-type enzyme. The amino acid residue at position 103 influences the SoxA-substrate interaction.

Optimum pH of wild-type enzyme and its mutants. Since the amino acid at position 103 was related to the substrate specificity, the effect of pH on the activities of the wild type and its mutants was investigated with two kinds of substrates (Fig. 3). The wild type and F103W showed maximum activities for sarcosine at around pH 7.5. On the other hand, the maximum activities of the other mutants were observed at around pH 8.5. This shows that the pH profile of SoxA is shifted by changing the amino acid residue at position 103, although the charge is not altered.

For N-methylvaline, the pH profiles of each SoxA were different, as shown in Fig. 3B. The optimum pH values of N-methylvaline oxidase activities of almost all enzymes were neutral (between 7.0 and 8.0) and did not exhibit marked differences by amino acid replacement. These results indicate that the effect of pH on activity was also influenced by the substrate. In the case of SoxA, the substrate specificity can be significantly altered by substituting a variety of amino acids, and these changes also affect the pH optimum response to several substrates.
TABLE 1. Kinetic parameters of wild-type enzyme and its mutants*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Sarcosine</th>
<th>N-Methyl-αalanine</th>
<th>N-Methyl-αvaline</th>
<th>N-Methyl-αleucine</th>
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<tr>
<td></td>
<td>KM (mM)</td>
<td>kcat (1/s)</td>
<td>kcat/KM (%)</td>
<td>KM (mM)</td>
<td>kcat (1/s)</td>
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<td>Wild type</td>
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<td>100</td>
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<tr>
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<td>7.9</td>
<td>1.1</td>
<td>54</td>
<td>5.9</td>
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</tbody>
</table>

*Enzyme activity was assayed as follows. The production of hydrogen peroxide was measured with the 4-aminoantipyrine peroxidase system (4). An enzyme solution (0.05 ml) was incubated with a mixture (1.0 ml) of 95 mM substrate, 0.47 mM 4-aminoantipyrine, 2.0 mM phenol, 50 mM Tris-HCl (pH 8.0), and 5 U of horseradish peroxidase per ml at 37°C for 10 min. The reaction was stopped by adding 2.0 ml of 0.25% sodium dodecyl sulfate solution, and the A405 was measured against the blank. One unit was defined as the amount of the enzyme that catalyzed the oxidation of 1 μmol of substrate per min under the conditions described above. Reaction mixtures composed of 0.2 to 500 mM substrate solution were used for calculating the KM and kcat values.

The kcat/KM values are presented as percentages of those of the wild-type enzyme with sarcosine as the substrate.

REFERENCES