Use of Indirect Site-directed Mutagenesis to Alter the Substrate Specificity of Methylamine Dehydrogenase*

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Methylamine dehydrogenase (MADH) is a tryptophan tryptophylquinone-dependent enzyme that catalyzes the oxidative deamination of primary amines. Native MADH exhibits a strong preference for methylamine over longer carbon chain amines. Residue αPhe55 controls this substrate specificity. When αPhe55 is replaced with Ala, this preference is reversed with αF55A MADH preferring long-chain amines with at least seven carbons (Zhu, Z., Sun, D., and Davidson, V. L. (2000) Biochemistry 39, 11184–11186). To further modulate the substrate specificity of MADH, the side-chain of αPhe55 was repositioned by site-directed mutagenesis of residue βIle107. This residue makes close contact with αPhe55 and restricts its movement. βI107V MADH exhibits a stronger preference for propylamine, and βI107N MADH exhibits a preference for 1-aminopentane. Thus, it has been possible to create forms of MADH that exhibit a preference for amines with carbon chain lengths of one, three, five, or seven carbons. The ability to discriminate between amines of different chain length was essentially abolished by an αF55I mutation. Molecular modeling studies with the known crystal structure are described that provide an explanation for these results. These results provide an example of a design-based approach to protein engineering in which site-directed mutagenesis on one residue can be used to reposition another residue to specifically alter enzyme specificity.

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‡ The abbreviations used are: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone.

The phenyl group of αPhe55 serves two functions in determining substrate specificity. It interacts with the methyl group of methylamine to help orient the amino group of the substrate for nucleophilic attack of TTQ, and it excludes long-chain amines from the active site (Fig. 1). Site-directed mutagenesis was previously used to create αF55A MADH, which exhibited a dramatically altered substrate specificity in which long-chain amines are preferred to short-chain amines (6).

In this paper we further examine the extent to which the nature and position of the side-chain of residue αP55 can determine the substrate specificity of MADH. In addition to directly mutating residue αPhe55, site-directed mutagenesis was used to alter the position of this residue. To reposition the phenyl side-chain of αPhe55, mutations were made of residue Ile107 of the β subunit. This residue is located adjacent to αPhe55 at the interface of the α and β subunits. From the crystal structure, it appears that the side-chain of βIle107 restricts the movement of the side-chain of αPhe55. We show that relatively conservative mutations of βIle107 significantly alter the substrate specificity of MADH. When βIle107 is converted to valine, the enzyme exhibits a strong preference for propylamine. When βIle107 is converted to asparagine, the preferred substrate is the five-carbon amylamine (1-aminopentane). Consistent with the importance of the position of the phenyl ring of αPhe55 in determining substrate specificity, conversion of αPhe55 to isoleucine yields an enzyme that exhibits no clear substrate specificity and shows relatively poor activity with amines of any carbon chain length. Thus, it has been possible to use site-directed mutagenesis to create forms of MADH that exhibit preference for amines with carbon chain lengths of one, three, five, or seven carbons; or which exhibit no preference at all. Molecular modeling studies with the known crystal structure are used to provide an explanation for these results.

EXPERIMENTAL PROCEDURES

Native MADH was purified from P. denitrificans as described previously (7). The βI107V, βI107N, and αF55I mutants of MADH were heterologously expressed in Rhodobacter sphaeroides (8) and purified as described previously for the αF55A MADH mutant (9). All reagents were purchased from Sigma or Aldrich and used without further purification.

Site-directed mutagenesis was performed on double-stranded pMEG976 (8) using the QuickChange Site-directed Mutagenesis Kit (Stratagene) and two mutagenic primers following a previously described procedure (8). In this construct, the gene that encodes the MADH β subunit possesses a polyhistidine tag at its C terminus to facilitate purification of the expressed protein. The primers used to create the site-directed mutations were: βI107V, 5’-CAAACGATGCTCGTCTGGCTTCGGCGCGGAGG-3’; βI107N, 5’-CAAACGATTACGCTCGTCTGGCTTCGGCGCGGAGG-3’; αF55I, 5’-GTAACGCGCCGCCATATGCCGGCGGTACCCACCAATTCG-3’. For each, the complementary sequence was used as the second primer for the mutagenesis. The underlined bases are those that were changed to create the desired mutation. The mutations were confirmed by sequencing 70 base pairs around the mutated site.

Steady-state kinetic assays (7) were performed in 10 mM potassium phosphate, pH 7.5, at 30 °C. The assay mixture contained 16 mM MADH,
MADH Substrate Specificity

Native MADH exhibits a strong preference for methylene, and \( K_m \) increases with increasing length of the carbon chain (10). For \( \alpha F55A \) MADH the preference is reversed with the enzyme preferring long-chain amines, and \( K_m \) increases with decreasing length of the carbon chain (6). Steady-state kinetic analysis with a variety of amines as substrates showed that the substrate specificities of \( \beta 107V, \beta 107N, \) and \( \alpha F55I \) MADH were each different from that of either the native MADH or \( \alpha F55A \) MADH (Table I). Monoamines longer than amylamine (1-aminopentane) are not soluble enough in aqueous solution to achieve concentrations necessary for the kinetic studies. Primary 1-N-diamines, which are more soluble than their corresponding monoamines, were tested as substrates to examine the effect of increasing the carbon chain length beyond five. The \( \beta 107V \) MADH exhibits a relatively strong preference for propylamine with \( K_m \) increasing as the carbon chain length is either increased or decreased. The \( K_m \) value for propylamine is approximately the same as the \( K_m \) value that native MADH exhibits for methylene. The \( \beta 107N \) MADH exhibits a preference for 1-aminopentane with \( K_m \) increasing as the carbon chain length is either increased or decreased. The \( K_m \) value for 1-aminopentane is also approximately the same as the \( K_m \) value that native MADH exhibits for methylene. The same patterns of substrate preference for these enzymes are indicated when \( k_{cat}/K_m \) values are compared (Fig. 2) as when examining the \( K_m \) values.

Previous studies have suggested that the position of \( \alpha Phe^{55} \) is important for stabilizing interactions with the methyl group of methylene and that steric hindrance restricted entry of amines with carbon chain lengths of three or more (6). This can be seen in Fig. 1 in which a five-carbon amine has been modeled into the active site of MADH. The crystal structure of native MADH further shows that the \( \delta 1 \)-methyl group of \( \beta le^{107} \) points toward the center of phenyl ring of \( \alpha Phe^{55} \) (Fig. 3). The distance between the \( \delta 1 C \) of \( \beta le^{107} \) and the \( \delta 1 C \) of \( \alpha Phe^{55} \) is \( \approx 4.0 \) Å. Thus, \( \beta le^{107} \) restricts the rotation of the phenyl group of \( \alpha Phe^{55} \). This methyl group is removed by the \( \beta 107V \) mutation. Thus, substitution of valine for isoleucine will allow limited rotation of the phenyl ring of \( \alpha Phe^{55} \). One can see in Fig. 4 that a small rotation of the phenyl ring about its bond with its \( \beta \)-carbon could reposition this residue such that the stabilizing interaction for the C-1 carbon of the substrate amine is lost, and stabilizing van der Waals' interactions with the C-3 carbon of the substrate may occur. The specificity would decrease as the carbon chain length increases beyond three because there

| Substrate | Native MADH | \( K_m \) | \( k_{cat} \) | \( K_m \) | \( k_{cat} \) | \( K_m \) | \( k_{cat} \) | \( K_m \) | \( k_{cat} \) |
|-----------|------------|---------|---------|---------|---------|---------|---------|---------|---------|
| Methylamine | 9 ± 1      | 30 ± 1  | 69 ± 2  | 20 ± 1  | 250 ± 22 | 34 ± 1  | 14900 ± 1100 | 77 ± 2  | 60 ± 8  | 2.0 ± 0.1 |
| Ethylene   | 19 ± 1     | 24 ± 1  | 340 ± 59 | 62 ± 4 | 840 ± 80 | 45 ± 0.2 | 9200 ± 1300 | 23 ± 1 | 360 ± 40 | 15 ± 1   |
| Propylene  | 36 ± 2     | 27 ± 1  | 64 ± 1 | 3.1 ± 0.1 | 8 ± 1  | 41 ± 0.2 | 1300 ± 1500 | 24 ± 1 | 200 ± 12 | 5.2 ± 0.1 |
| Butylene   | 870 ± 59   | 22 ± 1  | 88 ± 9 | 14 ± 1 | 7 ± 1 | 42 ± 0.1 | 240 ± 28 | 34 ± 1 | 370 ± 40 | 5.4 ± 0.1 |
| 1-Aminopentane | 2500 ± 290 | 17 ± 1 | 130 ± 25 | 3.4 ± 0.2 | 4 ± 1 | 42 ± 0.2 | 47 ± 5 | 20 ± 1 | 250 ± 21 | 5.4 ± 0.8 |
| 1,6-Diaminoheptane | 720 ± 83 | 17 ± 3 | 170 ± 10 | 26 ± 1 | 31 ± 3 | 16 ± 31 | 21 ± 73 | 43 ± 6 | 99 ± 6 | 3.4 ± 0.1 |
| 1,7-Diaminoheptane | 380 ± 46 | 27 ± 1 | 290 ± 40 | 19 ± 2 | 68 ± 7 | 15 ± 1 | 7 ± 1 | 32 ± 1 | 57 ± 4 | 3.4 ± 0.1 |

**FIG. 1.** Molecular modeling of 1-aminopentane into the active site of native MADH. The TTQ prostatic group, \( \alpha Phe^{55}, \beta Tyr^{119}, \) and the amine are drawn as space-filling models to highlight potentially favorable and unfavorable van der Waals' interactions. Heteroatoms are indicated by darker shading. The C-6 carbon of TTQ is indicated.**

**FIG. 2.** Effects of site-directed mutagenesis on \( k_{cat}/K_m \) values for different carbon chain length amines. Data are taken from Table I. Values are normalized to the \( k_{cat}/K_m \) value that each MADH exhibits for its preferred substrate. The actual maximum values are: MADH ( ), \( 3.3 \times 10^6 \) \( M^{-1} \) \( s^{-1} \) for methylene; \( \beta 107V \) MADH ( ), \( 5.2 \times 10^6 \) \( M^{-1} \) \( s^{-1} \) for propylamine; \( \beta 107N \) MADH ( ), \( 1.1 \times 10^6 \) \( M^{-1} \) \( s^{-1} \) for 1-aminopentane; \( \alpha F55A \) MADH ( ), \( 4.6 \times 10^6 \) \( M^{-1} \) \( s^{-1} \) for 1,7-diaminoheptane.
will be an overlap of van der Waals' radii between the longer chain substrates and $\beta$Tyr$^{119}$. This accounts for the strong preference of $\beta$I107V MADH for propylamine over longer and shorter amines.

The $\delta$1-methyl group of $\beta$Ile$^{107}$ is also removed by the $\beta$I107N mutation; however, $\beta$I107N MADH prefers amines with carbon chain lengths of five rather than three. Inspection of the crystal structure reveals that the $\gamma$2-methyl group of $\beta$Ile$^{107}$ points toward the $\beta$-methyl group of $\beta$Tyr$^{119}$ (Fig. 3) and may help to orient the position of the phenol ring of $\beta$Tyr$^{119}$. The distance between the $\gamma$2C of $\beta$Ile$^{107}$ and the $\beta$C of $\beta$Tyr$^{119}$ is ~4.2 Å. In $\beta$I107N MADH, this interaction is also eliminated. Therefore, the position of the phenol ring could be more flexible with less restricted rotation around the $\gamma$C-$\beta$C and $\beta$C-$\alpha$C bonds of $\beta$Tyr$^{119}$. At the same time, the amide group of asparagine has a smaller van der Waals' radius than the corresponding $\gamma$1- and $\delta$1-methyl groups of Ile, which also gives $\alpha$Phe$^{55}$ more freedom of rotation. These two factors together make a more flexible gate to the active site in $\beta$I107N MADH than in native MADH. Thus, the substrate preference for $\beta$I107N MADH is also not as strict as was that of $\beta$I107V MADH. Although the five-carbon chain is preferred, propylamine and butylamine are not that much poorer substrates.

Substitution of isoleucine for $\alpha$Phe$^{55}$ essentially eliminates the substrate specificity of MADH, converting it to an enzyme that is a relatively poor catalyst for the oxidative deamination of primary amines without regard for their carbon chain length. The most likely explanation for this is that the Ile side-chain is free to rotate about several carbon–carbon bonds and cannot be placed into a rigid conformation like that of $\alpha$Phe$^{55}$ in the native enzyme. As such, there is less restriction of long-chain amines but also less specific stabilization of short-chain amines.

It is difficult to analyze these data in terms of a quantitative comparative binding analysis because these are steady-state $K_m$ values and not true association constants. Some qualitative
MADH Substrate Specificity

comments, however, are in order. It is noteworthy that the $K_m$ values for the preferred substrate for the native, $\beta$107V, $\beta$107N, and $\alpha$F55A MADHs are approximately the same. For the reaction of native MADH with methyamine, the $K_m$ value of $13 \mu M$ that was obtained from transient kinetic studies (11) is similar to the steady-state $K_m$ value (Table I). If we regard the relative values of $\Delta G^*$ as an indication of the relative values of $\Delta G^*$, this therefore suggests that the sum of the binding energies that stabilize the enzyme-substrate complex are approximately the same for each mutant with its preferred substrate. This means that these mutations need not have changed the mechanism or nature of the interactions that stabilize substrate binding. They have simply changed the position at which the stabilizing interactions occur on substrates of different carbon chain lengths. This is consistent with our earlier discussion.

There are two main factors affecting substrate preference of MADH. One is the ability of $\alpha$Phe$^{55}$ and $\beta$Tyr$^{119}$ to stabilize the binding of the amine substrate and orient its amino group for nucleophilic attack of TTQ. The other function of $\alpha$Phe$^{55}$ and $\beta$Tyr$^{119}$ is to exclude long-chain amines from the active site due to steric hindrance. These facts have been demonstrated by site-directed mutagenesis and exploited to specifically alter the substrate specificity of MADH. Single site-directed mutations of residues $\alpha$Phe$^{55}$ and $\beta$Ile$^{107}$ of MADH significantly change the substrate specificity of this enzyme. The results show that MADH can be changed from an enzyme with a strong preference for methyamine to enzymes that prefer amines with carbon chain lengths of three ($\beta$107V), five ($\beta$107N), or seven or more ($\alpha$F55A). A single mutation was also used to render MADH unable to discriminate between amines with varying length carbon chains ($\alpha$F551).

From a protein engineering perspective, it is desirable to develop simple approaches for altering substrate specificity of enzymes without causing major structural changes that could otherwise affect the function or stability of the enzyme, or both. There are relatively few examples of the use of site-directed mutagenesis to rationally redesign substrate specificity. In most cases, multiple changes are required. For example, the substrate preference of aspartate aminotransferase was altered using homology modeling to prefer tyrosine (12) and using directed evolution to prefer branched-chain amino acids (13). However, in each case it was necessary to change at least six amino acid residues to alter the substrate specificity. Trypsin has been converted to chymotrypsin by replacing two surface loops containing 13 amino acids (14). It has been possible to alter substrate specificity by single amino acid substitutions. Examples of this include isocitrate dehydrogenase (15) and glutathione reductase (16). These results showed that interactions between neighboring residues in the active site may strongly influence each other. In the latter case, it was shown that an A34E mutation caused a significant movement of Arg$^{347}$ due to steric hindrance, which in turn altered substrate specificity. We report here how it was possible to use a rational approach to redesign substrate specificity by using site-directed mutagenesis of a single amino acid residue to reposition another critical residue in the active site. The demonstration with MADH that the specificity of the enzyme may be modulated over a wide range of substrates, in a predictable manner, by single mutations suggests that such an approach may be applicable to other enzymes.

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