Redesigning the Substrate Specificity of an Enzyme by Cumulative Effects of the Mutations of Non-active Site Residues*

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Directed evolution was used to change the substrate specificity of aspartate aminotransferase. A mutant enzyme with 17 amino acid substitutions was generated that shows a 2.1 \( \times 10^6 \)-fold increase in the catalytic efficiency \( (k_{cat}/K_m) \) for a non-native substrate, valine. The absorption spectrum of the bound coenzyme, pyridoxal 5'-phosphate, is also changed significantly by the mutations. Interestingly, only one of the 17 residues appears to be able to contact the substrate, and none of them interact with the coenzyme. The three-dimensional structure of the mutant enzyme complexed with a valine analog, isovalerate (determined to 2.4-Å resolution by x-ray crystallography), provides insights into how the mutations affect substrate binding. The active site is remodeled; the subunit interface is altered, and the enzyme domain that encloses the substrate is shifted by the mutations. The present results demonstrate clearly the importance of the cumulative effects of residues remote from the active site and represent a new line of approach to the redesign of enzyme activity.

Despite the impressive ability of natural enzymes to catalyze a broad array of reactions and utilize diverse substrates, attempts to make even minor modifications in enzyme activity or substrate specificity have proven to be difficult. Efforts to change the properties of existing enzymes (1–4) have highlighted a limitation to enzyme design; in most cases, we can consider only amino acid residues that constitute the active site. Enzymes, however, exert their functions not only through the chemical properties of the side chains of the amino acid residues that contact substrates and cofactors. Residues distant from an active site may be important in holding the catalytic residues in their required orientations. Charge distribution throughout a whole enzyme molecule may facilitate substrate binding by electrostatically guiding the substrate into the active site. These ideas are consistent with the fact that enzymes are macromolecules composed of hundreds of amino acid residues. It has been difficult to demonstrate by protein engineering the importance of residues that are remote from the active site because the number of these residues is large and the contribution of each residue may be modest. It is also beyond our present understanding to predict a priori the effects of the mutations of remote residues on enzymatic activity through changes in the complex architecture of tertiary and/or quaternary structure. Although random mutagenesis and directed evolution have recently proven to be useful in addressing such problems in the rational redesign of enzymes (5–10), creating enzymes with the desired activity remains challenging.

Aspartate aminotransferase (AspAT)\(^1\) is a homodimeric enzyme and catalyzes amino group transfer between acidic amino acids, aspartate and glutamate, and their corresponding 2-oxo acids (11). Each subunit has a pyridoxal 5'-phosphate (PLP) molecule at the active center. The structure (12, 13) and reaction mechanism (14) of AspAT have been studied extensively. AspAT from Escherichia coli shows moderate activity for aromatic amino acids. The activity for \( \beta \)-branched amino acids, valine and isoleucine, is barely detectable and even lower than that for basic amino acids (15). Several studies were previously done to increase the activity for basic or aromatic amino acids (16–18). These results imply that the substrate specificity of AspAT like many other enzymes cannot be easily manipulated by mutating one or a few active site residues.

To alter the substrate specificity of AspAT toward \( \beta \)-branched amino acids, we established an experimental system based on directed evolution where mutant AspATs with higher activity for branched-chain substrates, especially valine and 2-oxovaline, evolve during successive rounds of selection (10). Briefly, the selection system uses an auxotrophic E. coli strain, which is deficient in the gene for branched-chain amino acid aminotransferase. The higher the activity of a plasmid-encoded mutant AspAT for 2-oxovaline is, the faster the auxotrophic E. coli carrying the plasmid grows on a selection plate. After five rounds of the selection, the catalytic efficiency \( (k_{cat}/K_m) \) of mutant AspATs for \( \beta \)-branched substrates was increased about 10\(^5\)-fold. One of the mutant AspATs showing the highest activity (AV5A-7) was analyzed in detail (10). The mutant had 13 amino acid substitutions, but interestingly, only one of the mutated residues seemed to interact directly with the substrate based on the three-dimensional structure of the wild-type AspAT. To elucidate the effects of these mutations on substrate binding, we set out to determine the three-dimensional structure of the mutant AspAT complexed with a \( \beta \)-branched substrate analog. The affinity of AV5A-7 for valine was, however, still too low \( (K_m = 400 \text{ mM}) \) to yield a crystal of such an enzyme-inhibitor complex.

Here we describe the further improvement in both the catalytic efficiency and the \( K_m \) value of AV5A-7 for valine. The affinity of a new mutant AspAT for valine is high enough to

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The atomic coordinates and structure factors (code 1yyo) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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\(^1\) The abbreviations used are: AspAT, Aspartate aminotransferase; PLP, pyridoxal 5'-phosphate; 2-oxovaline, 2-ketoisovaleric acid; Pipes, 1,4-piperazinediethanesulfonic acid.
allow crystallization of a complex between the mutant enzyme and a valine analog. The crystal structure shows that the mutations in residues distant from the active site cause significant changes in the higher order structure of the enzyme, which influence substrate and cofactor binding.

**MATERIALS AND METHODS**

**Directed Evolution—**An auxotrophic E. coli strain, YJ103 (ΔilvE Δkat), the selection medium, 5’ and 3’ primers used for the polymerase chain reaction amplification of the coding region of *aspC*, and the construction of the expression plasmid were reported previously (10). Briefly, the first round of selection was done as follows. The coding region of the *aspC* gene containing the three mutations, Ser<sup>319</sup> → Gly, Asn<sup>142</sup> → Thr, and Asn<sup>297</sup> → Ser, was subjected to DNA shuffling (6, 9, 19), and the mutant genes were ligated downstream of the promoter of the tetracycline resistance gene of pBR322. After a 45-h incubation at 37 °C, 116 colonies from a library of 5.6 × 10<sup>6</sup> colonies were picked up, and a mixture of the plasmids was prepared. As for the second and third rounds of selection, the conditions were the same as those for the first round except the incubation time: the second round, a 40-h incubation, a library size of 7.9 × 10<sup>6</sup> colonies, and 136 colonies picked; the third round, a 28-h incubation, a library size of 2.8 × 10<sup>7</sup> colonies, and 48 colonies picked. Among the 48 clones, 9 clones exhibiting the highest activity for 2-oxovaline were chosen, and the coding regions of the *aspC* genes were sequenced.

**Expression, Purification, and Activity Measurement of Mutant AspATs—**The coding region of the mutant AspATs was subcloned into pUC18. The mutant enzymes were expressed in *E. coli* TY103 (20), which is deficient in the AspAT gene, and purified as described (21). The activity for each substrate was measured at 25 °C by spectrophotometrically monitoring the single turnover reaction using an Applied Photophysics stopped-flow apparatus (model SX.17MV) as described (15). The buffer system was 50 mM Hepes, pH 8.0, containing 0.1 mM KCl and 10 μM EDTA.

**Crystallography—**The crystals of ATB17 complexed with isovalerate were grown by the sitting drop vapor diffusion method. Three microliter drops containing 37 mg/ml protein were mixed with 1 μl of 0.2 mM sodium isovalerate and 3 μl of the reservoir solution containing 1.6 mM ammonium sulfate and 0.1 M Na-Hepes, pH 7.5. The drops were equilibrated against 0.5 ml of the reservoir solution at 20 °C. An x-ray data set was collected with a Rigaku R-AXIS IcE image plate detector mounted on a Rigaku RU-200 rotating anode generator operated at 40 kV and 100 mA with monochromated CuKα radiation at room temperature. The oscillation images were processed and reduced using a processing software, Rigaku PROCESS (22). Refinement of the structure began with the structure of the wild-type AspAT (12) as an initial model—i.e., the nature of these changes is difficult to predict.

Despite such drastic changes in the substrate specificity and the electronic distribution within the π-electron system of the bound PLP molecule is changed significantly by the mutations, although the nature of these changes is difficult to predict.

**RESULTS AND DISCUSSION**

**Creation of a Mutant AspAT with Higher Affinity for Valine—**Sequence analysis of the mutant AspATs obtained from the previous selection showed that 5 of the 13 substitutions found in AV5A-7, Asp<sup>194</sup> → Asp, Ile<sup>134</sup> → Leu, Asn<sup>319</sup> → Gly, Asn<sup>142</sup> → Thr, and Asn<sup>297</sup> → Ser, were conserved among all of the mutants examined, and these substitutions were found to be functionally important (10). The fact that one substitution—Val<sup>357</sup> → Leu—was significantly increased in the catalytic efficiency for β-branched substrates was unique to AV5A-7 (10), however, implied that the sequence space had not yet been fully searched. Despite this finding, the initial selection system appeared to have reached its limit after five rounds of selection, because further increases in the catalytic efficiency for β-branched substrates did not benefit the growth of the host E. coli cells under the most stringent selection conditions.

In the present study, we used two different strategies to find additional beneficial mutations. In one approach, we added each of the 20 unique substitutions found in four selected mutant AspATs, AV5A-1, AV5B-1, AV5B-4, and AV5B-5 (10), to AV5A-7 one mutation at a time and assayed each new mutant for 2-oxovaline activity (data not shown). Two substitutions, Ser<sup>319</sup> → Phe and Ser<sup>363</sup> → Gly, were chosen in this manner. In a second strategy, directed evolution was once again employed. This time, however, the experiment was started from a mutant AspAT that had three substitutions, Ser<sup>319</sup> → Gly, Asn<sup>142</sup> → Thr, and Asn<sup>297</sup> → Ser, to facilitate the evolution. After three rounds of selection, mutant AspATs that showed similar activity for 2-oxovaline to that of AV5A-7 were obtained, and the coding regions of 9 mutant AspATs were sequenced. Each mutant AspAT had 4–9 additional substitutions, and, again, Asn<sup>34</sup> → Asp and Ile<sup>37</sup> → Met were conserved in all the mutants. After adding each potential substitution one at a time to AV5A-7/Ser<sup>319</sup> → Phe/Ser<sup>363</sup> → Gly, 3 substitutions, Ala<sup>11</sup> → Thr, Phe<sup>24</sup> → Leu, and Ile<sup>353</sup> → Thr, were chosen. One of the 13 substitutions of AV5A-7, Gly<sup>2</sup> → Val, was mutated back to the wild-type sequence because the substitution was found to decrease the expression level of AspAT while not affecting the activity for β-branched substrates. The resulting mutant, ATB17, thus has 17 substitutions (Fig. 1A), 11 of which are clearly functionally important. As for the other 6 substitutions, Lys<sup>41</sup> → Asn, Lys<sup>126</sup> → Arg, Ala<sup>269</sup> → Thr, Ala<sup>293</sup> → Val, Ser<sup>311</sup> → Gly, and Met<sup>397</sup> → Leu, of which the contribution to the total effect was 10–20% in AV5A-7, the importance of each substitution could not be determined (10).

**Characterization of the Mutant AspAT—**Compared with AV5A-7, the *k<sub>v</sub>/K<sub>v</sub>* values of ATB17 for branched-chain substrates are increased, whereas those for acidic 2-oxo acid substrates are decreased (Table I). In particular, the *K<sub>v</sub>* value for valine is decreased 76-fold to 5.5 mM. The *k<sub>v</sub>/K<sub>v</sub>* value of ATB17 for valine or 2-oxovaline is increased >2.1 × 10<sup>6</sup> or 6.7 × 10<sup>5</sup>-fold, respectively, and that for isoleucine or 2-oxoisoleucine is increased >6.0 × 10<sup>5</sup> or 5.4 × 10<sup>5</sup>-fold, respectively, compared with that of the wild-type AspAT. ATB17 retains significant activity for acidic substrates. Although this activity could have been easily eliminated by a single mutation at Arg<sup>292</sup> (see below and also Refs. 16 and 17), none of the mutants examined had such mutations. Probably Arg<sup>292</sup> was maintained simply because no selection pressure was applied to minimize the activity for acidic substrates or because acidic amino acids may serve as the amino group donors to 2-oxovaline in *E. coli* cells growing under the selection conditions.

During the purification of ATB17, we noticed that the color of the fractions containing the enzyme was orange, rather than yellow like the wild-type AspAT and AV5A-7. Thus, the absorption spectra of the purified AspATs were measured (Fig. 1B). AspAT has absorption bands in the region 300–500 nm, which derive from the bound PLP molecule and are influenced significantly by amino acid residues interacting with PLP (11, 26, 27). The wild-type AspAT and AV5A-7 exhibit two major bands around 360 and 430 nm, whereas in ATB17 the latter band is red-shifted to 450 nm and has a broad shoulder above 500 nm. The differences in the relative intensity of the two bands between the wild-type and mutant AspATs show that the pK<sub>a</sub> of the imine nitrogen of the Schiff base formed between PLP and Lys<sup>258</sup> (26, 27) is decreased in both mutant AspATs. These changes in the absorption spectra imply that the electronic distribution within the π-electron system of the bound PLP molecule is changed significantly by the mutations, although the nature of these changes is difficult to predict.

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absorption spectrum, only one of the mutated residues in ATB17 appears to be located at a position contacting the substrate, and none of them interact directly with PLP. The higher affinity of ATB17 for valine allowed us to crystallize ATB17 in the presence of an amino-free valine analog, isovalerate, to further investigate the effects of the mutations.

**Effects of the Mutations on the Tertiary and Quaternary Structure of AspAT**—The x-ray crystal structure of ATB17 complexed with isovalerate was solved at 2.4-Å resolution (Table II) and was compared with the wild-type AspAT complexed with an aspartate analog, maleate (Fig. 2). Three features stand out in the gross structure of ATB17. First, the spatial arrangement of the two subunits of the dimer is altered. Second, the domain motion may be enhanced in ATB17. Third, two absorption spectra of the wild-type AspAT (solid line), AV5A-7 (dotted line), and ATB17 (broken line). All the spectra were measured in a 50 mM Pipes buffer, pH 6.5, at a protein concentration of 19 μM.

### Table I

| Substrate       | WT | AV5A-7 | ATB17 |
|-----------------|----|--------|-------|
|                 | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ |
| L-Valine        | —  | —      | <1 × 10^{-3} | 46 | 420 | 110 | 12 (0.3) | 5.5 (0.4) | 2.1 × 10^{-3} |
| L-Isoleucine    | —  | —      | <1 × 10^{-3} | 60 | 140 | 30 | 110 (8) | 15 (2) | 7.3 × 10^{-3} |
| L-Leucine       | —  | —      | 2.4 | 160 | 120 | 1.3 × 10^{-3} | 8.4 (1.3) | 140 (30) | 60 |
| 2-Oxovaline$^d$ | 5.7 × 10^{-3} | 100 | 0.057 | 28 | 3.8 | 7.4 × 10^{-3} | 90 (2) | 2.4 (0.09) | 3.8 × 10^{-4} |
| 2-Oxoisoleucine$^d$ | 3.3 × 10^{-3} | 52 | 0.063 | 220 | 48 | 4.6 × 10^{-3} | 250 (6) | 7.3 (0.4) | 3.4 × 10^{-4} |
| 2-Oxocitrate$^d$ | 1.2 | 43 | 28 | 140 | 3.4 | 4.1 × 10^{-4} | 320 (10) | 2.2 (0.2) | 1.5 × 10^{-4} |
| L-Aspartate     | 550 | 4.5 | 1.2 × 10^{-3} | 220 | 68 | 3.2 × 10^{-3} | 9.2 | 2.8 | 3.3 × 10^{-3} |
| L-Glutamate     | 700 | 38 | 1.8 × 10^{-3} | —  | — | 380 | —  | —  | 570 (20) |
| Oxalacetate     | 800 | 0.035 | 2.3 × 10^{-3} | 650 | 0.52 | 1.3 × 10^{-6} | 120 (10) | 2.2 (0.5) | 5.5 × 10^{-4} |
| 2-Oxoglutarate  | 600 | 1.3 | 4.6 × 10^{-5} | 120 | 3.9 | 3.1 × 10^{-4} | —  | —  | 2200 (30) |

$^a$ Data from Ref. 15, except for those for branched-chain 2-oxo acids (10).

$^b$ Data from Ref. 10.

$^c$ Reactions did not show saturation kinetics in the substrate concentrations examined.

$^d$ Abbreviations used are: 2-oxovaline, 2-ketoisovaleric acid; 2-oxoisoleucine, 2-keto-3-methyl-n-valeric acid; 2-oxocitrate, 2-ketoisocaproic acid.

* Parameters could not be determined experimentally because spectral changes were too small to obtain reliable parameters. Therefore, parameters of ATB17 for aspartate were estimated as follows (15): $k_1 = k_2 \cdot K_k (k_3 + k_3)$, $K_1 = K_k \cdot k_3 (k_3 + k_3)$, where $k_1$ and $K_1$ are the $k_{\text{cat}}$ and $K_m$, respectively, values for the overall reaction, respectively; $k_2$ and $K_2$ are the $k_{\text{cat}}$ values for the single turnover reactions with aspartate and 2-oxovaline, respectively, and $K_3$ is the $K_m$ value for the single turnover reaction with aspartate. The parameters for the overall transamination reaction with aspartate and 2-oxoglutarate were determined as described (35) in the 50 mM Hepes buffer, pH 8.0, containing 0.1 mM KCl and 10 μM EDTA except 2-oxovaline was used instead of 2-oxoglutarate.
The other cluster (Fig. 2, red) is in the core of the small domain, except Leu-397, and may affect the domain motion given that these residues are located at the domain interface. Thus, these two clusters may influence in concert the domain motion.

Detailed analysis may help us to understand how the two clusters of mutated residues cause the observed conformational changes. Fig. 3 shows that the enzyme structure is changed especially around the two clusters. The Leu-20–Ile-33 loop (the lid of the active site which closes against the bound substrate). The other cluster (Fig. 2, red) is in the core of the small domain, except Leu-397, and may affect the domain motion given that these residues are located at the domain interface. Thus, these two clusters may influence in concert the domain motion.

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TABLE II

| Crystallographic parameters | Value |
|-----------------------------|-------|
| **Parameter**               | **Value** |
| Diffraction data            |       |
| Space group                 | C222_1 |
| Unit cell dimensions        |       |
| a (Å)                       | 157.00 |
| b (Å)                       | 84.84 |
| c (Å)                       | 78.62 |
| Total reflections           | 61,169 |
| Unique reflections          | 17,348 |
| Completeness (%)            | 98.6 (98.3)% |
| Resolution range (Å)        | 75.0–2.4 |
| Rmerge (%)                  | 5.6   |
| Refinement statistics       |       |
| Resolution range (Å)        | 10.0–2.4 |
| Number of reflections       | 16,005 |
| Number of protein atoms     | 3,091  |
| Number of water molecules   | 134    |
| Rcryst (%)                  | 18.7   |
| Rwork (%)                   | 23.5   |
| Deviations from ideal geometry (rms) |       |
| Bond lengths (Å)            | 0.007  |
| Bond angles (°)             | 1.4    |
| Improper angles (°)         | 1.27   |
| Dihedrals (°)               | 23.6   |

* Highest resolution bin given in parentheses.

**Rmerge** = \( \frac{\sum I - \langle I \rangle}{\langle I \rangle} \) where I is the intensity of an individual reflection and \( \langle I \rangle \) is the mean intensity of that reflection.

**Rcryst** = \( \sqrt{\frac{\sum (F_{\text{obs}} - F_{\text{calc}})^2}{\sum F_{\text{obs}}^2}} \)

where \( F_{\text{obs}} \) and \( F_{\text{calc}} \) are the observed and calculated structure factors, respectively.

**Rwork** is calculated in the same manner as **Rcryst** with 10% of random reflections excluded from refinement.

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**FIG. 2.** Superimposition of the ATB17-isovalerate complex (pink, large domain; gray, small domain) and the wild-type AspAT-maleate complex (purple). The backbone of one subunit of ATB17, of which the large domain (residues 49–325) was superimposed on that of the wild-type AspAT (31), is indicated by a thick line. The NH_2- and COOH terminals of the subunit are indicated (N and C). The side chains of the residues that were mutated in ATB17 are shown as follows: the two clusters of the residues (see text) are in red and green, and the other residues are in light blue. The coenzyme, pyridoxal 5'-phosphate (yellow), and the bound valine analog, isovalerate (dark blue), are also shown. Maleate of the wild-type AspAT-maleate complex is omitted. Figs. 2 and 4 were produced with MOLSCRIPT and RAS-TER 3D (32–34).
methyl group at Cβ of the side chain. Thus, the PLP molecule may be required to be pushed back by Trp140 for AspAT to bind β-branched substrates. The wild-type AspAT has two arginine residues, Arg292 and Arg386, that are essential for substrate binding. Arg292 interacts with the side chain carboxylate group of acidic substrates. The side chain of Arg292 of ATB17 protrudes into solvent, whereas that of the wild-type AspAT flips toward the active site interacting with the carboxylate group of maleate (Fig. 4A). The same shift in the position of Arg292 was observed previously in the crystal structure of a mutant AspAT complexed with aromatic substrate analogs (28). It is therefore possible that the difference in the position of Arg292 between the two enzymes is caused by the difference in the bound substrate analogs, dicarboxylic maleate and monocarboxylic isovalerate, rather than by the mutations of ATB17. Arg386 is also an important residue of which the side chain interacts with the α-carboxylate group of all amino acid substrates. The orientation of the Arg386 side chain of ATB17 remains essentially unaltered despite the large conformational changes adjacent to Arg292 (Fig. 4, A and C).

One of the side walls of the active site consists of the residues belonging to the other subunit (Fig. 4A, asterisks). Thus, the changes in the subunit interface in ATB17 have caused deviations of these residues. This portion of the active site also contains two substitutions, Ala293→Val and Asn297→Ser. Although the functional importance of the Ala293→Val substitution is not clear, the Asn297→Ser substitution increased the activity of AV5A-7 for 2-oxovaline (10). It was reported previously for the mutant AspAT with increased activity for aromatic substrates that the Asn297→Ser substitution elimi-
nates a structural water molecule which is placed in hold by the side chains of Ser<sup>296</sup> and Asn<sup>297</sup> in the wild-type AspAT, concomitantly introducing new water molecules (28). Malashkevich et al. (28) proposed that this substitution makes space for the binding of bulky aromatic substrates. In our case, however, the side chain of isovalerate is much smaller than those of aromatic substrates, and, indeed, a water molecule (Fig. 4A, \( \text{WATI} \)) is re-introduced at almost the same position, constituting the side wall of the isovalerate-binding pocket together with the other newly introduced water molecules. We thus cannot explain how the Asn<sup>297</sup> \( \rightarrow \) Ser substitution increases the activity for valine.

Met<sup>37</sup> constitutes the upper wall of the isovalerate binding pocket and is the only mutated residue contacting isovalerate. The Ile<sup>37</sup> \( \rightarrow \) Met substitution would apparently benefit the binding of \( \beta \)-branched substrates by removing the steric hindrance between the \( \gamma \)-methyl groups of the Ile<sup>37</sup> side chain and the bound substrate. To determine how important this substitution is, we mutated Met<sup>37</sup> of ATB17 back to isoleucine. The total effect of all the mutations of ATB17 (calculated as follows: 
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(1 - \frac{<k_{cat}>_{ATB17/Met37}<k_{cat}>_{WT}}{<k_{cat}>_{ATB17/Met37}<k_{cat}>_{WT}})} \times 100 (\%)
\]

Conclusions—The isolation of a mutant AspAT with a million-fold increase in the catalytic efficiency for a non-native substrate shows that directed evolution is a powerful technique for altering enzyme activity. Our present selection system could also be used to produce an enzyme with transaminase activity from that with an unrelated activity. The three-dimensional structure of a complex between the mutant AspAT and a substrate analog suggests that conformational changes in the enzyme are responsible for the alteration in the substrate specificity. Significantly, only one of the 17 mutated residues contacts the substrate directly. Several of the mutations that particularly enhance the activity for \( \beta \)-branched substrates are located \( >10 \) Å distance from the active site.

Most attempts to redesign enzyme activity by mutating only active site residues have met with limited success. It may turn out to be common rather than exceptional that changing enzyme activity requires remodeling the active site through changes in the backbone flexibility, domain motion, or subunit arrangement. Results supporting this idea were recently reported for a catalytic antibody (29, 30). These findings emphasize the benefits of directed evolution over rational design and would justify present and future intense efforts to develop new strategies for directed evolution.
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