Fine Structure–Activity Analysis of Mutations at Position 51 of Tyrosyl-tRNA Synthetase†

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ABSTRACT: Residue Thr-51 at the active site of tyrosyl-tRNA synthetase (*Bacillus stearothermophilus*) has been replaced with all the smaller amino acids by protein engineering to investigate direct and indirect effects of mutation on substrate binding and catalysis. The γ-hydroxyl group of Thr-51 was thought to be 0.5 Å too far from the ribose ring oxygen of ATP to form a hydrogen bond. Consistent with this, it is found that mutation of Thr-51 → Cys-51, which should place the γ-thiol group within its correct distance for hydrogen bonding, increases the affinity of the enzyme for ATP. Other mutations (Ser-51, Ala-51, and Gly-51) show the contributions to binding of the other atoms in the side chain of Thr-51. A family of enzymes has been produced, TyrTS(Thr-51) (wild type), TyrTS(Ala-51), TyrTS(Cys-51), and TyrTS(Pro-51), in which the value of k_{cat}/K_M for ATP in aminoacylation increases along the series. This is achieved by the value of K_M decreasing significantly (2.5, 1.25, 0.29, and 0.019 mM, respectively) while there are smaller decreases in k_{cat} (4.7, 4.0, 2.9, and 1.8 s^{-1}, respectively). These variations cause each one of the enzymes to be more active than the others at particular concentrations of ATP. For example, at concentrations of ATP greater than 5.9 mM, TyrTS(Thr-51) is the most active, while TyrTS(Ala-51), TyrTS(Cys-51), and TyrTS(Pro-51) are the most active at 5.9–2.2, 2.2–0.42, and less than 0.42 mM ATP, respectively. Interestingly, position 51 shows variation in tyrosyl-tRNA synthetases isolated from different organisms.

We are mapping the contribution of hydrogen bonding to catalysis and specificity in the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* by systematic application of site-directed mutagenesis (Winter et al., 1982; Fersht et al., 1984). The studies are based upon the crystal structures of the native enzyme and the enzyme-bound tyrosyl adenylate complex (Bhat et al., 1982; Blow & Brick, 1985; Monteilhet & Blow, 1978; Rubin & Blow, 1981). There are nine amino acid residues with polar side chains at the active site that could form hydrogen bonds with the tyrosyl adenylate. Eight of these residues are conserved in the tyrosyl-tRNA synthetase from *Escherichia coli*, whose primary structure is 56% homologous with the thermophilic enzyme (Winter et al., 1983). The exception is residue 51. There is a threonine at position 51 in the thermophilic enzyme (Figure 1) but a proline at this position in the enzyme from *E. coli*. Further, the tyrosyl-tRNA synthetase from the extreme thermophile *Bacillus caldotenax* has now been sequenced (M. D. Jones, D. M. Lowe, T. Borgford, & A. R. Fersht, unpublished results), and although it is found to be 99% identical with the enzyme from *B. stearothermophilus*, there is an alanine at position 51. The side chains of proline and alanine cannot form hydrogen bonds. A preliminary examination of the crystal structures suggested that a possible hydrogen bond between the hydroxyl group of Thr-51 and the ring oxygen of the ribose of the adenylate is too long to be energetically favorable: the distance between the two oxygen atoms is some 3.4 Å rather than the optimal 2.8 Å for the interatomic distance between two oxygen atoms that form a hydrogen bond. Strong evidence supporting an unfavorable interaction between Thr-51 and the substrate was adduced from an experiment in which Thr-51 was converted into an alanine residue. TyrTS(Ala-51) was found to exhibit an increased value of k_{cat} and a decreased value of K_M for ATP in the activation reaction (Wilkinson et al., 1983). An interatomic distance of 3.3–3.5 Å is optimal for an –SH···O hydrogen bond. It is therefore predicted that the mutation Thr-51 → Cys-51 could place the –SH group in a position to form a good hydrogen bond. In this study, we show that the mutation Thr-51 → Cys-51 does increase the affinity of the enzyme for ATP.

There are several mutations at position 51 that increase the affinity of the tyrosyl-tRNA synthetase for ATP and give higher values of k_{cat}/K_M in charging. Why have some of these changes occurred in evolution but not others? We show in this study that mutation at position 51 generates a family of enzymes that is fine tuned for optimal activities at different concentrations of ATP.

Many of the mutagenesis experiments are designed to examine the effects of mutating a specific portion of an aminoacyl side chain. But often other groups in the chain are concomitantly removed. To investigate the effects of such changes, we have analyzed a series of mutations at position 51 of the tyrosyl-tRNA synthetase in which the threonine side chain is replaced by all the smaller naturally occurring ones.

Experimental Procedures

Materials and methods are described in the preceding paper in this issue (Jones et al., 1985). There was an additional step in the purification of mutant enzymes. The crude cell lysates were heated at 56 °C for 30 min. This led to the precipitation of all of the tyrosyl-tRNA synthetase from *E. coli* and a considerable fraction of its other proteins while leaving the tyrosyl-tRNA synthetase from *B. stearothermophilus* unaffected. Mutations of Thr-51 → Cys-51, Thr-51 → Ser-51, and Thr-51 → Gly-51 were directed by the oligonucleotides 5′CAAAATGC*A*GGCCAAGT3′, 5′CAAAATGC*T-GGCCAAGT3′, and 5′CAAAATGC*C*GGCCAAG3′, respectively, where * indicates mismatches. All enzymes were

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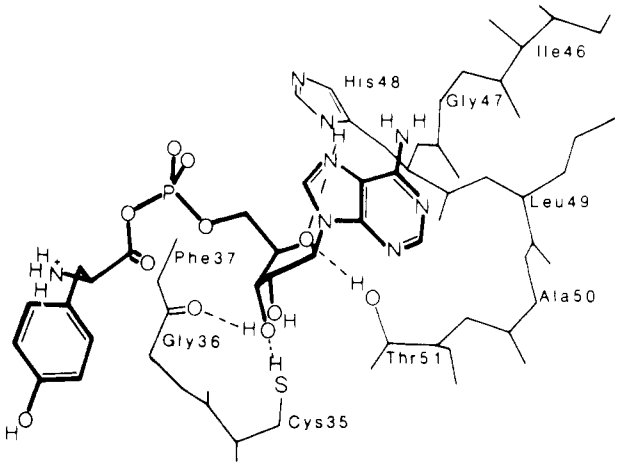

FIGURE 1: Structure of the tyrosyl-tRNA synthetase–tyrosyl adenylate complex, showing the relationship between Thr-51, Cys-35, and the substrate.

Table I: ATP Dependence of Activation of Tyrosine[a]

enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})
wild type	8.35	1.08	7730
Thr-51 → Cys-51	12.4	0.35	35400
Thr-51 → Ala-51	8.75	0.54	16200
Thr-51 → Ser-51	1.88	1.16	1620
Thr-51 → Gly-51	6.0	1.25	4800
Cys-35 → Gly-35	2.75	2.6	1130
Cys-35 → Gly-35; Thr-51 → Cys-51	9.4	0.97	9690

[a] At 25 °C, pH 7.78, 144 mM Tris-HCl, 10 mM $MgCl_2$, 0.05 mM tyrosine ($\sim 25K_M$), 2 mM pyrophosphate, 0.1 mM phenylmethanesulfonyl fluoride, and 10 mM 2-mercaptoethanol. Data extrapolated to infinite concentration of tyrosine.

purified to homogeneity by established criteria and were found to have 1.0 ± 0.05 active sites per mole of dimeric enzyme. Pyrophosphate exchange and aminoacylation kinetics were conducted in the standard buffer [144 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.78), 10 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, and 10 mM 2-mercaptoethanol]. Because of the possibilities of oxidation of Cys-51, controls were performed by replacing the mercaptoethanol with 1 mM dithiothreitol or oxidized dithiothreitol. There was no dependence of rate on the nature of the added thiol.

RESULTS

Pyrophosphate Exchange Kinetics. The activation of tyrosine was measured by the conventional pyrophosphate exchange procedure. The values of k_{cat} and K_M for ATP were determined at near-saturating concentrations of tyrosine [0.05 mM = $25K_{M(Tyr)}$]. The data are listed in Table I. The most active enzyme is TyrTS(Cys-51), where the –OH is mutated to an –SH. Its value for k_{cat} is 50% higher than that for wild-type enzyme while its K_M is 3 times lower. The specificity constant k_{cat}/K_M for the ATP dependence of TyrTS(Cys-51) is consequently some 5 times larger than the constant for wild-type enzyme. TyrTS(Cys-51) is also significantly more active than TyrTS(Ala-51), which has had the –OH deleted. Removal of the γ-methyl group of Thr-51 (Thr-51 → Ser-51) leads to a 5-fold decrease in k_{cat}/K_M. This decrease is paralleled in the change Ala-51 → Gly-51.

There are possible problems in the mutation Thr-51 → Cys-51 in that the γ-carbon of Thr-51 and the γ-sulfur atom of Cys-35 are only 3.9 Å apart in the native enzyme (Figure 1). They are thus in van der Waals contact. Further, on mutation of Thr-51 → Cys-51, the γ-sulfur of Cys-51 can rotate to aproach within 2.6 Å of the sulfur of Cys-35. Apart from the possible formation of strong van der Waals interactions between the two sulfur atoms, there is also the possibility of forming a covalent bond between the two. Accordingly, we performed additional experiments of mutating Thr-51 → Cys-51 on TyrTS(Gly-35) in which the offending side chain has been deleted. The double mutant TyrTS-(Gly-35; Cys-51) is a slightly better enzyme than wild type. TyrTS(Gly-35) is the appropriate comparison for the effects of mutation of Thr-51 → Cys-51, and the double mutant is considerably more active than this (Table I).

Table II: Effects of Structural Changes on Substrate Binding[a]

mutation	structural change in residue	ΔG (kcal/mol)
Thr-51 → Cys-51	$HOCH(CH_3)- \rightarrow HSCH_2-$	−0.90
Thr-51 → Cys-51 [TyrTS(Gly-35)]	$HOCH(CH_3)- \rightarrow HSCH_2-$	−1.27
Gly-51 → Cys-51	$H- \rightarrow HSCH_2-$	−1.18
Ala-51 → Cys-51	$CH_3- \rightarrow HSCH_2-$	−0.46
Thr-51 → Ser-51	$HOCH(CH_3)- \rightarrow HO(CH_2)-$	+0.92
Thr-51 → Ala-51	$HOCH(CH_3)- \rightarrow CH_3-$	−0.44
Ser-51 → Ala-51	$HOCH_2- \rightarrow CH_3-$	−1.36
Ser-51 → Gly-51	$HOCH_2- \rightarrow H-$	−0.64
Ala-51 → Gly-51	$CH_3- \rightarrow H-$	+0.72
Thr-51 → Gly-51	$HOCH(CH_3)- \rightarrow H-$	+0.28
Ser-51 → Cys-51	$HOCH_2- \rightarrow HSCH_2-$	−1.83
Gly-35 → Cys-35	$H- \rightarrow HSCH_2-$	−1.14
Ser-35 → Cys-35	$HOCH_2- \rightarrow HSCH_2-$	−1.18

[a] Binding of ATP in transition state calculated from k_{cat}/K_M for activation.

Detailed Energetic Changes on Mutating Thr-51. The changes in binding energy of the transition states of the reagents to the enzymes may be calculated from eq 1, where

$$\Delta G = -RT \ln [(k_{cat}/K_M)_{mutA}/(k_{cat}/K_M)_{mutB}] \quad (1)$$

mutA and mutB represent mutant enzymes, in a manner analogous to that described previously (Wilkinson et al., 1983). The effect of any individual change in the side chain may be calculated from the appropriate choice of mutants. The results are presented in Table II for the various permutations of mutants. For example, the γ-methyl group of Thr-51 is seen from the comparison of TyrTS and TyrTS(Ser-51) to contribute 0.92 kcal/mol to the binding energy, and the contribution of the β-methyl group of Ala-51 is seen from the comparison of TyrTS(Ala-51) and TyrTS(Gly-51) to be 0.72 kcal/mol.

Previously, the effect of removing the –OH of Thr-51 was estimated from the mutation Thr-51 → Ala-51 to improve binding energy by 0.44 kcal/mol (Wilkinson et al., 1983). This mutation, however, also includes the deletion of the γ-methyl group, which is seen to be worth 0.92 kcal/mol, and so the comparison underestimates the effect of the –OH group. The appropriate comparison for removal of the –OH group is TyrTS(Ser-51) to TyrTS(Ala-51). The hydroxyl group is seen in Table II to weaken binding by 1.36 kcal/mol. Similarly, comparing TyrTS(Ala-51) with TyrTS(Cys-51) shows that the –SH group contributes 0.46 kcal/mol to binding energy. The advantage of having an –SH rather than –OH in the γ position at residue 51 is 1.83 kcal/mol [TyrTS(Cys-51) vs. TyrTS(Ser-51)]. These data are summarized in Table III.

An interaction between Cys-35 and Thr-51 or Cys-51, which was expected from the crystal structure (see above), is indicated from application of the "double-mutation" procedure of Carter et al. (1984). If residues 51 and 35 do not interact directly or indirectly in the enzyme or enzyme–substrate complex, then the mutation Thr-51 → Cys-51 on TyrTS

Table III: Contributions of Individual Groups at Position 51 to Binding Energy[a]

group	contribution to binding energy (kcal/mol)
γ-methyl	−0.92
γ-SH	−0.46
γ-OH	+1.36
β-methyl	−0.72

[a] Contributions of the groups on Thr-51 to the binding energy of ATP (in the transition state) taken from Table II.

Table IV: ATP Dependence of Aminoacylation of tRNA[a]

enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})
TyrTS	4.7	2.5	1860
TyrTS(Ala-51)[b]	4.0	1.25	3200
TyrTS(Cys-51)	2.9	0.29	8920
TyrTS(Pro-51)[b]	1.8	0.019	95800
TyrTS(Gly-35;Cys-51)	3.0	1.1	2680
TyrTS(Gly-35)	1.9	6.1	320

[a] Conditions as in Table I but with 0.1 mM tyrosine and 0.002 units/mL inorganic pyrophosphatase replacing the pyrophosphate. [b] From Wilkinson et al. (1984).

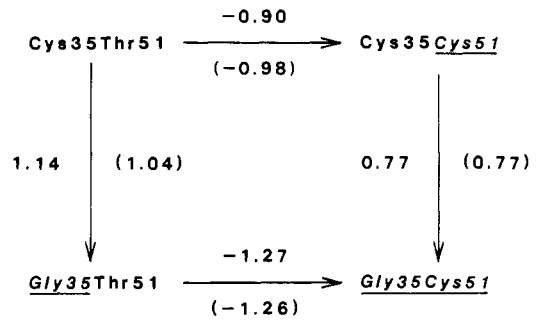

FIGURE 2: Differences in transition-state binding energy of ATP with wild-type (Cys-35;Thr-51) and mutant tyrosyl-tRNA synthetases calculated from values of k_{cat}/K_M [the double-mutant procedure of Carter et al. (1984)]. The values in parentheses are the binding energies in aminoacylation; the open values are those in pyrophosphate exchange.

should lead to the same change in binding energy as does Thr-51 → Cys-51 on TyrTS(Gly-35). But, as seen in Figure 2 and Table II, the changes differ by 0.37 kcal/mol.

Aminoacylation Kinetics. The value of k_{cat}/K_M for the variation of ATP in the aminoacylation of $tRNA^{Tyr}$ catalyzed by TyrTS(Cys-51) is again some 5 times higher than that for wild-type enzyme (Table IV). The energy changes on mutation of residues 35 and 51, which are calculated from the values of k_{cat}/K_M, are illustrated by application of the double-mutant procedure in Figure 2. It is seen that the changes in k_{cat}/K_M for aminoacylation follow very closely those for pyrophosphate exchange. But, whereas the value of k_{cat} for TyrTS(Cys-51) in exchange is higher than that for wild-type enzyme, the k_{cat} for aminoacylation is lower in the mutant. Instead, the value of K_M for ATP is radically lowered for TyrTS(Cys-51). This is similar to the changes found previously on mutation of Thr-51 → Pro-51 (Wilkinson et al., 1984; Carter et al., 1984).

Comparison of the kinetics of TyrTS, TyrTS(Ala-51), TyrTS(Cys-51), and TyrTS(Pro-51) reveals that k_{cat}/K_M increases monotonically along the series while both k_{cat} and K_M decrease monotonically.

Discussion

Interaction of the γ-Hydroxyl of Thr-51 with ATP. All evidence from the mutational studies is consistent with the γ-hydroxyl of Thr-51 being too far from the substrate to form an energetically favorable −OH···O hydrogen bond and suggests that the distance is more appropriate for the longer −SH···O bond: as found previously (Wilkinson et al., 1984; Carter et al., 1984), removal of the −OH group, as in Thr-51 → Ala-51, improves the binding of ATP by 0.44 kcal/mol, and as found here, substitution of the −OH by a thiol group, as in Thr-51 → Cys-51, improves binding by 0.9 kcal/mol. The individual contributions of the hydrogen bonds to binding energies are estimated better from comparison of mutants in which the only structural changes are the removal of the −OH or −SH groups and not the methylene groups (Tables II and III). For example, the contribution of the −SH group to binding energy is found from the comparison of the binding of ATP to TyrTS(Cys-51) and TyrTS(Ala-51) to be 0.46 kcal/mol. Comparison of TyrTS(Ser-51) with TyrTS(Ala-51) shows that the −OH group weakens binding by 1.36 kcal/mol. The reason why the presence of the −OH group weakens binding is seen readily from looking at the hydrogen bond inventory on substrate binding to wild-type (eq 2) and mutant

$$[E\text{–}H\cdots OH_2] + [H_2O\cdots S] = [E\text{–}H \quad S] + 2H_2O \quad (2)$$

$$[E \quad H_2O] + [H_2O\cdots S] = [E \quad S] + 2H_2O \quad (3)$$

(eq 3) enzymes (Fersht et al., 1985). In eq 2, the wild-type enzyme with its hydrogen bond donor −H has to break its hydrogen bond with water to form the E·S complex, which lacks a hydrogen bond between E and S because the interatomic distance is too large. In eq 3, the enzyme does not break a hydrogen bond on desolvation but just loses some dispersion energy with water. In both eq 1 and eq 2, there is no hydrogen bond in the enzyme–substrate complex, and so binding is favored in eq 3.

The comparison of cysteine and serine at position 51 is similar to that at position 35, and the relevant data are listed at the bottom of Table II. Cys-35 is the residue found in wild-type tyrosyl-tRNA synthetase, and so mutation to Ser-35 places the γ-hydroxyl too far away from the substrate (Wilkinson et al., 1983). By analogy, Cys-51 rather than Thr-51 would appear to be the "natural" residue at position 51. The reason why cysteine is not found here is seen below.

Mutation of Residue 51 Generates a Family of Enzymes That Is Fine Tuned for Different Concentrations of ATP. TyrTS(Cys-51) is more active than wild-type enzyme in pyrophosphate exchange at all concentrations of ATP since the mutant has a higher value of k_{cat} and a lower value of K_M. Although TyrTS(Cys-51) has a higher value of k_{cat}/K_M than wild type for ATP in aminoacylation, it has a lower value of k_{cat}. Thus, at saturating concentrations of ATP, wild-type enzyme is more active than TyrTS(Cys-51); but TyrTS(Cys-51) is more active than wild type at low concentrations of ATP where the rate is determined by k_{cat}/K_M. The relative activities of TyrTS(Cys-51) and TyrTS thus vary according to the concentration of ATP.

The optimization of rate with available substrate concentration has been analyzed in detail (Fersht, 1974, 1985). The rate at different concentrations of ATP is given by $v = [E]_0[ATP]k_{cat}/(K_{M(ATP)} + [ATP])$ (at a constant concentration of tyrosine). The evolutionary pressure to increase rate is on the term $k_{cat}[ATP]/(K_{M(ATP)} + [ATP])$. This can be achieved by changes in the structure of the enzyme so that the values of k_{cat} and K_M are increased individually while their ratio (k_{cat}/K_M) is maintained. The selective pressure is for the K_M to increase to above the normal steady-state concentration of the substrates in vivo. The concentration of ATP in vivo tends to be in the region 1–10 mM for many organisms.

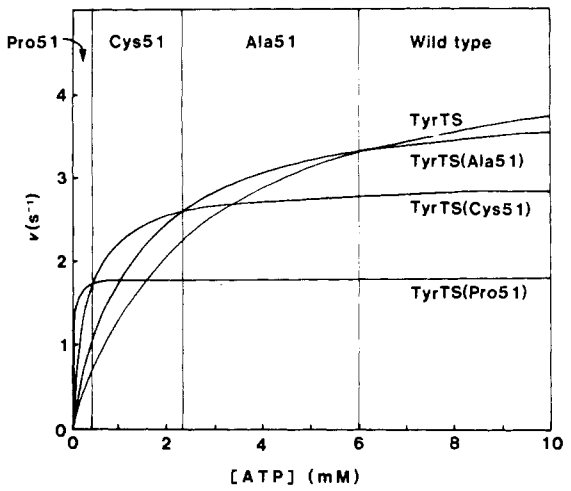

FIGURE 3: Rate of aminoacylation of tRNA at varying concentrations of ATP calculated from the data and the conditions of Table IV. The zones of ATP concentration are indicated in which each of the variants at position 51 is the most active of the four.

The activities of the mutant enzymes in this concentration range have been calculated from the Michaelis–Menten equation with the data from Table IV and are plotted in Figure 3. Above 2 mM ATP, there is very little to choose between TyrTS, TyrTS(Ala-51), and TyrTS(Cys-51). But nevertheless, each enzyme is tailored to be the best at particular concentrations. At greater than 5.9 mM ATP, wild-type TyrTS, the enzyme with the highest K_M, is the most active. At 2.2–5.9 mM ATP, TyrTS(Ala-51), the enzyme with the next highest K_M, is the most active. At 0.42–2.2 mM ATP, TyrTS(Cys-51), with a lower K_M, is the most active. At 0.42 mM ATP and below, TyrTS(Pro-51) with the lowest K_M is the most active. Thus, although the mutants at position 51 have higher affinities for ATP than does wild type, the higher affinities are at the expense of lower values of k_{cat} and lower rates at high substrate concentrations: the mutants utilize too much of the binding energy in binding the substrate. Although the rate constants we have measured in vitro will not be the same as those measured in vivo, it is likely that similar trends will occur and that TyrTS(Thr-51) or TyrTS(Ala-51) could be slightly more suitable for one organism than another.

Influence of the Hydrocarbon Groups on the Side Chain of Thr-51 on Kinetics. The γ-methyl group and the β-CH group of Thr-51 make significant contributions to the enzyme–substrate binding energy (Table III). There are direct interactions of these groups with the substrate that could cause these effects. The methyl group probably makes van der Waals contact with the 3′-hydroxyl of the ribose ring of the substrate, and the β-CH group is close to doing the same. The β-CH group is also sufficiently close to carbons 1–4 of the ribose ring that there are favorable dispersion energies. These could well increase in TyrTS(Ala-51) on just a slight movement of enzyme or substrate so that the β-methyl group of Ala-51 has a significant binding energy with the ribose ring. Both the γ-methyl and β-CH groups of Thr-51 in wild-type enzyme make van der Waals contacts with the γ-SH of Cys-35, which is known to form a hydrogen bond with the 3′-hydroxyl of the substrate. An interaction, either direct or indirect, between Cys-35 and residue 51 is indicated by the observation (Table III) that whereas mutation of Thr-51 → Cys-51 on native TyrTS leads to an increase of 0.9 kcal/mol in binding energy, the same mutation on TyrTS(Gly-35) leads to a significantly higher change of 1.27 kcal/mol. The interaction may be formally expressed in terms of the double-mutant thermodynamic cycle of Carter et al. (1984) in Figure 2. It has been found that a $-CH_2$ group on a substrate can contribute up to 3.5 kcal/mol to binding energy when the group is presumably close-packed with enzyme side chains (Fersht, 1985), and so the contributions of hydrocarbon groups on Thr-51 to binding energy are not unreasonable.

It must be borne in mind that mutational changes could cause changes in enzyme–substrate affinity from a variety of other effects. At one extreme, they could leave the structure of the enzyme totally unaffected but alter the structure of the solvent shell around the enzyme. This would perturb the energetics of solvation and desolvation of the enzyme and hence the equilibrium constant for substrate binding. Alternatively, the creation of a small cavity could affect just the conformation of the side chain of an immediate neighbor. This might simply allow more flexibility of the side chains at the active site so that the entropy of the free enzyme is raised. If the side chains are more constrained in the enzyme–substrate complex, then the loss of entropy would decrease the enzyme–substrate affinity. It is clearly important in conducting a fine structure analysis of the effects of mutation that as many mutational changes must be made as is reasonably possible so that the effects of mutation can be assigned to specific interactions.

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REFERENCES

Bhat, T. N., Blow, D. M., Brick, P., & Nyborg, J. (1982) *J. Mol. Biol. 122*, 407–417.

Blow, D. M., & Brick, P. (1985) in *Biological Macromolecules and Assemblies: Nucleic Acids and Interactive Proteins* (Jurnak, F., & McPherson, A., Eds.) Vol. 2, pp 442–469, Wiley, New York.

Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell (Cambridge, Mass.) 38*, 835–840.

Fersht, A. R. (1974) *Proc. R. Soc. London, B 187*, 397–407.

Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, 2nd ed., Freeman, New York.

Fersht, A. R., Shi, J. P., Wilkinson, A. J., Blow, D. M., Carter, P., Waye, M. M. Y., & Winter, G. (1984) *Angew. Chem., Int. Ed. Engl. 23*, 467–473.

Fersht, A. R., Shi, J. P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature (London) 314*, 235–238.

Jones, D. H., McMillan, A. J., Fersht, A. R., & Winter, G. (1985) *Biochemistry* (preceding paper in this issue).

Montheilet, C., & Blow, D. M. (1978) *J. Mol. Biol. 122*, 407–417.

Rubin, J., & Blow, D. M. (1981) *J. Mol. Biol. 145*, 489–500.

Wilkinson, A. J., Fersht, A. R., Blow, D. M., & Winter, G. (1983) *Biochemistry 22*, 3581–3586.

Wilkinson, A. J., Fersht, A. R., Blow, D. M., Carter, P., & Winter, G. (1984) *Nature (London) 307*, 187–188.

Winter, G., Fersht, A. R., Wilkinson, A. J., Zoller, M., & Smith, M. (1982) *Nature (London) 299*, 756–758.

Winter, G., Koch, G. L. E., Hartley, B. S., & Barker, D. G. (1983) *Eur. J. Biochem. 132*, 383–387.