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Site-Directed Mutagenesis as a Probe of Enzyme Structure and Catalysis: Tyrosyl-tRNA Synthetase Cysteine-35 to Glycine-35 Mutation†

Anthony J. Wilkinson, Alan R. Fersht,* David M. Blow, and Greg Winter*

ABSTRACT: Oligodeoxynucleotide-directed mutagenesis has been used on the gene of tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* to produce mutant enzymes altered at the adenosine 5′-triphosphate (ATP) binding site. Deliberate attempts were made to alter rather than destroy enzymic activity so that kinetic measurements may be made to identify the subtle roles of the enzyme–substrate interactions in catalysis. Cys-35, the -SH group of which is involved in binding the 3′-OH of the ribose ring of ATP, has been mutated to a serine residue [Winter, G., Fersht, A. R., Wilkinson, A. J., Zoller, M., & Smith, M. (1982) *Nature (London) 299*, 756–758] or glycine residue. The mutant enzymes are less active than the wild type, and the reduction in activity can be attributed to a decrease in the value of k_{cat} and an increase in K_M. Thus, the interaction energy of the side chain of Cys-35 with the substrate is not fully realized in the enzyme–substrate complex but is used preferentially to stabilize the transition state. Relative to its absence in the Gly-35 mutant, the side chain of Cys-35 is calculated to stabilize the transition state for pyrophosphate exchange by 1.2 kcal/mol and the transition state for aminoacylation by 1.0 kcal/mol.

It is now possible to alter any amino acid residue of a protein at will by site-directed mutagenesis of its gene. The rapid advances in solid-phase synthesis of oligodeoxynucleotides and the introduction of the technique of oligodeoxynucleotide-directed mutagenesis (Hutchinson et al., 1978) have facilitated the construction of point mutations in DNA [see Itakura (1982) and Smith (1982) for brief reviews]. The application of this technique to the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* has previously been described (Winter et al., 1982). In outline (see Figure 1), the gene coding for the enzyme was cloned into the bacteriophage vector M13; a short

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oligodeoxynucleotide primer was synthesized that is complementary to the sequence of the DNA to be mutated apart from a single mismatch; the mismatch was designed to convert the codon for the target amino acid into that for the desired mutant amino acid residue; the oligodeoxynucleotide was annealed to the single-stranded DNA and used as a primer for replication catalyzed by a DNA polymerase; the new strand was ligated with DNA ligase to produce a heteroduplex of wild-type and mutant DNA; host cells were transfomed by the heteroduplex to give cells containing mutant or wild-type vector. Mutant plaques were identified using the ^{32}P-labeled mutagenic primer as a probe in a hybridization assay since it binds preferentially to the mutant DNA (Wallace et al., 1981). An alternative procedure to using M13 is to perform the mutagenesis in a double-stranded vector after exposing small regions of single strands by treatment with endonucleases and exonucleases prior to priming (Wallace et al., 1980).

Although oligonucleotide-directed mutagenesis has been used in the past few years to examine the effects of structural changes in polynucleotides, it is only recently that the technique has been successfully applied to studying the effects of mutation on enzyme structures and activity. Results may be derived at various levels. For example, in the absence of detailed three-dimensional structural information on a protein, site-directed mutagenesis may be used to probe which residues are essential for catalysis (Dalbadie-McFarland et al., 1982; Sigal et al., 1982) in a similar manner to the established procedures of chemical modification. Combined with a high-resolution crystal structure of a protein, however, site-directed mutagenesis provides a means of measuring the contribution of all residues at the active site to binding and catalysis. It may also allow an assessment of the roles of amino acid residues in the folding of the protein and the assembly of subunits where involved.

The first such study has been initiated by using the tyrosyl-tRNA synthetase from *B. stearothermophilus* (Winter et al., 1982). This enzyme catalyzes the aminoacylation of $tRNA^{Tyr}$ in a two-step reaction (eq 1 and 2) (Fersht & Jakes,

$$E + Tyr + ATP \rightleftharpoons E\cdot Tyr\text{-}AMP + PP_i \quad (1)$$

$$E\cdot Tyr\text{-}AMP + tRNA^{Tyr} \rightleftharpoons Tyr\text{-}tRNA^{Tyr} + AMP + E \quad (2)$$

1975). It is a symmetrical dimer of M_r 2 × 47.5K (Koch, 1974; Irwin et al., 1976). The nucleotide sequence of the gene has been determined from a clone in the vector pBR322 (Winter et al., 1983). X-ray crystallographic studies on the enzyme at 0.3-nm resolution have been published (Bhat et al., 1982), and in subsequent work refinement has been extended to a nominal 0.21 nm (T. N. Bhat, P. Brick, and D. M. Blow, unpublished results). The enzyme is readily assayed by active site titration (Fersht et al., 1975a). Kinetic studies indicate that the two active sites interact: only 1 mol of tyrosine is bound (tightly) per dimer in solution (Fersht, 1975) as is only 1 mol of $tRNA^{Tyr}$ (Jakes & Fersht, 1975; Dessen et. al., 1982); 1 mol of tyrosyl adenylate is formed far more rapidly than the other at the two active sites (Mulvey & Fersht, 1977). Under the conditions of pyrophosphate exchange, only one active site is primarily responsible for the chemical catalysis (Fersht et al., 1975b).

The initial target for site-directed mutagenesis was Cys-35, a residue that is conserved in the tyrosyl- and methionyl-tRNA synthetases from *Escherichia coli* (Barker & Winter, 1982). Examination of the crystal structure of the complex between the tyrosyl-tRNA synthetase and tyrosyl adenylate revealed that the –SH group makes a contact with the 3′-OH of the ribose. The strategy of the mutagenesis was to alter, but not destroy, activity so that kinetic measurements could be made on the mutant enzyme. It was found that the wild-type enzyme could be cloned into M13 and expressed at exceptionally high levels (up to 50% of soluble protein). A mutant enzyme, containing Ser-35, was prepared and expressed at the same high levels. The activity of the mutant was lower and the reduction in activity attributable to a poorer binding of ATP (Winter et al., 1982). In the present study, we present our findings on the mutation of Cys-35 to Gly-35 in order to measure the contribution of the binding energy of the side chain of Cys-35 to catalysis.

Experimental Procedures

Materials

Reagents were obtained from Sigma. Enzymes were purchased from Bethesda Research Laboratories or Collaborative Research. Crude tRNA from *B. stearothermophilus* was obtained from the Imperial College Pilot Plant and purified by chromatography on BD-cellulose to an amino acid acceptance of 250 pmol/A_{260}. Bacteriophage M13mp93 and *E. coli* JM101 were generous gifts from Dr. J. Messing. The primer 5′-CAAACCCGCCGTAGAG was synthesized by the method of Gait et al. (1982) by using reagents purchased from Cruachem Ltd. It was purified by HPLC[1] on an ion-exchange resin (Partisil 10SAX). Preparation of mutant DNA and screening for mutant phage were performed precisely as described previously (Winter et al., 1982).

Preparation and Purification of Tyrosyl-tRNA Synthetase. An overnight culture of *E. coli* W71-18 in 2 × TY medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per L) was diluted 100-fold into fresh medium (to give $A_{550} \sim 0.05$) and infected with M13 at high multiplicity. Growth was continued at 37 °C with aeration for 5–6 h until A_{550} = 1–1.2. The cells were harvested by centrifugation at 5500 rpm for 15 min, resuspended in a buffer containing 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride. After three cycles of freezing and thawing from liquid nitrogen, lysis was completed by sonication. The lysate was clarified by centrifugation at 15 000 rpm for 30 min. The protein precipitating between 50 and 70% saturated ammonium sulfate was dialyzed against 50 mM potassium phosphate (pH 6.5) and 0.1 mM phenylmethanesulfonyl fluoride and applied to a column of DEAE-Sephacel equilibrated with the same buffer. After being washed with the buffer, the column was developed with a gradient of 50–300 mM potassium phosphate (pH 6.5). The enzyme eluted at about 200 mM buffer with 80% recovery of activity and was 95% pure as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Methods

Standard Conditions. All experiments were performed at 25 ± 0.1 °C in a standard buffer containing 144 mM Tris-HCl (100 mM Tris-HCl–44 mM Tris, pH 7.78), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride. (The mercaptoethanol is not strictly necessary for the maintenance of activity of the tyrosyl-tRNA synthetase from *B. stearothermophilus*.)

[1] Abbreviations: TyrTS, tyrosyl-tRNA synthetase; M13(TyrTS), M13 vectors containing the tyrosyl-tRNA synthetase gene; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate. Mutants are referred to as follows: TyrTS(Gly-35), enzyme with glycine at position 35.

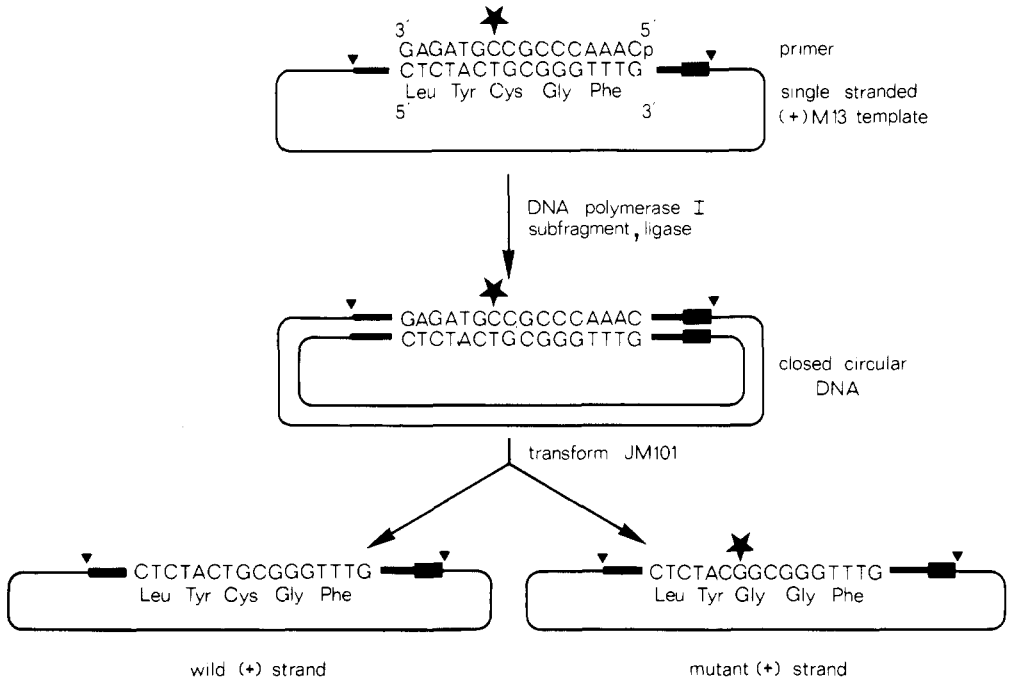

FIGURE 1: Scheme for oligodeoxynucleotide-directed mutagenesis using M13 single-stranded DNA template.

Active Site Titration. A solution of enzyme (50 μL of 0.5–5 μM) in the standard buffer was added to an equal volume of 4 mM ATP, 20 μM [^{14}C]Tyr (513 Ci/mol), and 1 unit of inorganic pyrophosphatase in the same buffer. After incubation for 1–2 min at 25 °C, aliquots (25 μL) were spotted onto presoaked nitrocellulose filters (Schleicher and Schüll BA85) and washed with 5.0 mL of cold 5 times diluted standard buffer. After they were dried, the amount of complex retained was monitored by scintillation counting in a toluene-based scintillant. The nitrocellulose disk assay was checked by the "burst" assay with [γ-^{32}]ATP exactly as described by Fersht et al. (1975a). Under these conditions, the enzyme is known to give reliably a stoichiometry of 1 mol of tyrosyl adenylate bound per dimer of M_r 95000 (Fersht et al., 1975a).

Active site titration of crude extracts requires modification to remove unlabeled tyrosyl adenylate that is bound to the enzyme in vivo. The crude extract (5 μL) was added to a solution (50 μL) of 20 μM [^{14}C]Tyr and 10 mM ATP in the standard buffer along with 1 mM pyrophosphate to scramble the labeled amino acid by the pyrophosphate exchange reaction. After incubation for 1 min at 25 °C, 1 unit of inorganic pyrophosphatase was added to remove the pyrophosphate and titration continued as above.

Kinetic Procedures. Pyrophosphate exchange, tRNA charging, and equilibrium dialysis experiments were performed as described previously (Calendar & Berg, 1966; Jakes & Fersht, 1975). Concentrations of reagents are given later in the tables.

Results

A total of 36 clones of M13 was isolated after mutagenesis and screened with ^{32}P-labeled probe as described previously (Winter et al., 1982). Three clones corresponded to TyrTS-(Gly-35) (i.e., tyrosyl-tRNA synthetase with Cys-35 mutated to Gly-35). These were plaque purified, and the DNA sequence in the region of the mutation was confirmed by the dideoxy procedure of Sanger et al. (1977) with a suitable restriction fragment primer. A 1-L culture of infected cells yielded up to 20 mg of tyrosyl-tRNA synthetase, the yield of protein in crude extracts being somewhat variable and dependent on the conditions of growth. *E. coli* W71-18 (Winter et al., 1981) was found to produce more enzyme than strain JM101.

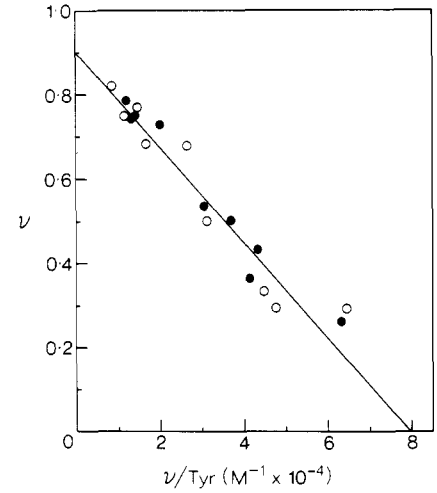

FIGURE 2: Determination of stoichiometry of binding (ν) of tyrosine to TyrTS(Gly-35) (○) and TyrTS(Cys-35) (●). The latter data are taken from Fersht (1975).

Effect of Mutation of Cys-35 on Tyrosine Binding. The mutation of Cys-35 to Gly-35 had no significant effect on the binding of tyrosine to the free enzyme or on the values of K_M for tyrosine in aminoacylation or pyrophosphate exchange reactions. Equilibrium dialysis measurements on the binding of tyrosine and TyrTS(Gly-35) under standard conditions (Figure 2) gave a dissociation constant of 11 μM and a stoichiometry of 0.9 mol of Tyr bound per mol of dimeric enzyme, values very close to those found previously for the native enzyme (Fersht et al., 1975b; Mulvey & Fersht, 1977). The kinetic data for pyrophosphate exchange and tRNA charging by the wild-type enzyme isolated from the M13 clone are also in excellent agreement with those obtained previously with authentic enzyme isolated from *B. stearothermophilus* (Fersht et al., 1975a,b; Jakes & Fersht, 1975). The mutant and wild-type enzymes have similar values of K_M for tyrosine in pyrophosphate exchange (Table I). The kinetics of

Table I: Effect of Mutation on K_M for Tyrosine in Charging and Pyrophosphate Exchange[a]

enzyme	[ATP] (mM)	[Tyr] (μM)	lower[c] k_{cat} (s^{-1})	lower[c] K_M (μM)	upper[c] k_{cat} (s^{-1})	upper[c] K_M (μM)
charging[b]						
TyrTS(Cys-35)	10	0.5–350	1.2	2.0	7.1	100
TyrTS(Ser-35)	10	0.5–350	0.7	3.6	2.0	100
TyrTS(Gly-35)	10	0.5–350	0.7	2.0	1.9	110
pyrophosphate exchange[d]						
TyrTS(Cys-35)	2	0.5–50	6.0	2.4		
TyrTS(Ser-35)	2	0.5–50	1.0	2.6		
TyrTS(Gly-35)	2	0.5–50	1.0	2.7		

[a] 25 °C in standard pH 7.78 buffer (see text). Rate constants are quoted per mol of dimeric enzyme as measured by active site titration. [b] 20 μM $tRNA^{Tyr}$ and 20–50 nM tyrosyl-tRNA synthetase. 0.1-mL reaction volume per kinetic run. [c] Biphasic for charging reaction. [d] 200–700 nM enzyme and 2 mM ^{32}P-labeled pyrophosphate.

Table II: Effect of Mutation on K_M and k_{cat}/K_M for ATP in Charging and Pyrophosphate Exchange[a]

enzyme	[Tyr] (μM)	[ATP] (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})
charging[b]					
TyrTS(Cys-35)	20	0.5–10	3.0	2.2	1.38×10^3
TyrTS(Ser-35)	20	0.5–10	0.8	5.1	0.16×10^3
TyrTS(Cys-35)	100	0.12–16	4.7	2.5	1.86×10^3
TyrTS(Ser-35)	100	0.12–16	1.3	6.4	0.20×10^3
TyrTS(Gly-35)	100	0.12–16	1.9	6.1	0.32×10^3
pyrophosphate exchange					
TyrTS(Cys-35)	50	0.5–10	7.6	0.9	8.40×10^3
TyrTS(Ser-35)	50	0.5–10	2.4	2.4	1.00×10^3
TyrTS(Gly-35)	50	0.5–10	2.8	2.6	1.12×10^3

[a] Conditions as in Table I. [b] $[MgCl_2] = [ATP] + 10$ mM.

charging are more complicated to analyze because they are biphasic with respect to tyrosine concentration (Jakes & Fersht, 1975). Nevertheless, Table I shows that the two sets of values of K_M for tyrosine for the three enzymes are very similar.

Effect of Mutation of Cys-35 on ATP Binding and Catalytic Constants. It is seen in Table II that the value of K_M for ATP in the aminoacylation reaction increases 2–3-fold on mutation of Cys-35 to either Gly-35 or Ser-35. This is accompanied by a 3–4-fold decrease in the value of k_{cat} for TyrTS(Ser-35) and a 2–3-fold decrease for TyrTS(Gly-35). The values of k_{cat}/K_M are some 9 times and 6 times lower, respectively, for the two mutants. There are corresponding increases in K_M and decreases in k_{cat} for pyrophosphate exchange on mutation of Cys-35 (Table II).

Differences in values of K_M are usually reliable because measurements of these do not depend on knowing the absolute activity of the enzyme. Small differences in values of k_{cat} are often less reliable because their calculation depends on the absolute concentrations of active sites. However, because of the reproducibility of active site titration and kinetic experiments with this enzyme, we consider the differences in k_{cat} measured for the different mutants to be real and significant.

Discussion

Catalytic Efficiency of Wild-Type Tyrosyl-tRNA Synthetase (Cys-35) Compared with Gly-35 and Ser-35 Mutants. It was seen in Table II that the wild-type enzyme is more efficient than its mutants by reason of a higher value of k_{cat} and a lower value of K_M for ATP. Just how much more active the wild-type enzyme will be depends on the concentration of ATP. At saturating ATP, the value of K_M is unimportant, and so, activity is determined by k_{cat}; wild-type is thus 3.6 times more active than TyrTS(Ser-35) and 2.5 times more active than TyrTS(Gly-35). At concentrations of ATP below K_M, the rate is proportional to k_{cat}/K_M [see Fersht (1977), p 85, for example]. At low concentrations of ATP, wild-type enzyme is 9 times more active than TyrTS(Ser-35) and 6 times more active than TyrTS(Gly-35). It is often found in practice in vivo that metabolites are present at concentrations below K_M (Fersht, 1977, pp 257–259). For example, the total concentration of ATP in *E. coli* is 2–3 mM (Mathews, 1972), a value similar to that found for other organisms, and free ATP must be lower than this. The concentration is below the K_M for the mutant enzymes (6 mM, Table II) and similar to the value for the wild-type enzyme (2.5 mM). Thus, the presence of Cys at position 35 rather than Gly or Ser could provide a rate advantage of up to a factor of 6 or 9 in the cell. Cells containing TyrTS(Cys-35) presumably have a selective advantage in needing to synthesize less enzyme than those containing the mutants.

Site-Directed Mutagenesis as a Probe of Theories of Enzyme Catalysis. An analysis of the role of enzyme–substrate bindng energy in catalysis suggests that (Fersht, 1974; Fersht, 1977, chapter 10) (i) the intrinsic binding energy of groups on the enzyme and substrate is used to increase k_{cat}/K_M and there is an evolutionary pressure to maximize this kinetic quantity, (ii) the intrinsic binding energy may be utilized to increase k_{cat}, as well as decrease K_M, and (iii) maximal rate is obtained when binding energy is used to increase the rate constant k_{cat} rather than reduce K_M to a value below the substrate concentration normally encountered in vivo. In the present example, the value of K_M for ATP with the TyrTS-(Cys-35) is, as mentioned above, comparable with the concentration of ATP found in vivo at about 2.5 mM (Table II). The full binding energy of ATP is thus not used to minimize its K_M value [the K_M for ATP and myosin, for example, is 10^{-13} M (Mannherz et al., 1974)]. On adding the $-CH_2SH$ side chain to TyrTS(Gly-35) to produce TyrTS(Cys-35), the additional binding energy is used to increase k_{cat}/K_m, and the increase is distributed between an increase in k_{cat} and a decrease in K_M.

The utilization of the binding energy of a group to increase k_{cat} rather than reduce K_M implies that the full binding energy of the group is not realized in the enzyme–substrate complex but is used to stabilize the enzyme–transition-state complex. The geometry of the enzyme is such that the small changes in bond lengths and angles during the reaction enable a better fit between the enzyme and the transition state of the substrate than between the enzyme and the substrate itself.

Stabilization of the Transition State by the Binding Energy of the Side Chain of Cys-35 with ATP. The binding energy in the transition state may be measured from the term k_{cat}/K_M in the Michaelis–Menten equation (Fersht, 1974, 1977, chapters 9 and 10). k_{cat}/K_M is related to the total binding energy of an enzyme and a substrate ($=\Delta G_S$) by the equation

$$RT \ln (k_{cat}/K_M) = RT \ln (kT/h) - \Delta G^{\ddagger} - \Delta G_S \quad (3)$$

(where $\Delta G^{\ddagger}$ is the chemical activation energy for the bond making and breaking steps, R is the gas constant, T is the absolute temperature, k is Boltzmann's constant, and h is Planck's constant). Equation 3 may readily be applied to calculating the binding energies of groups on a series of substrates reacting with the same enzyme (Fersht, 1974, 1977, chapters 9 and 11) as follows. Suppose there is a substrate (ns) which is smaller than the specific substrate (ss) by lacking a group R (where R is involved in binding interactions only

and not directly in the chemical step). Then providing $\Delta G^{\ddagger}$ is unaffected, the binding energy of the group R during the transition state (ΔG_R) is given by

$$\Delta G_R = RT \ln [(k_{cat}/K_M)_{ns}/(k_{cat}/K_M)_{ss}] \quad (4)$$

Equation 4 may equally well be applied to the binding of the *same* substrate to wild-type and mutant enzymes where the mutant enzyme has a larger active site cavity because it lacks a group R at the active site (provided again that R is again involved only in binding and not directly in chemical catalysis). The contribution of R to binding in the wild-type enzyme relative to its absence in mutant is given by

$$\Delta G_R = RT \ln [(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}] \quad (5)$$

There is a complication when analyzing the effects of mutations on multisubstrate enzymes in that a mutation could affect the binding of more than one of the substrates. However, if it is known from X-ray diffraction and kinetic studies that the binding site of only one substrate is involved, then eq 5 may be applied to the substrate affected by the mutation providing the other substrates are held at saturating concentrations (for example, here, when [ATP] is varied and tyrosine and tRNA are saturating).[2]

Applying eq 5 to TyrTS(Cys-35) and TyrTS(Gly-35) for the binding of ATP during the pyrophosphate exchange reaction gives a value of 1.19 kcal/mol for the stabilization of the transition state by the binding energy of the $HSCH_2$– side chain of cysteine relative to the H– of glycine. A slightly lower value of 1.04 kcal/mol is calculated for the bindng of ATP during the charging reaction. Since charging and pyrophosphate exchange have different transition states, it is not surprising that the interactions between the 3′-OH of the ATP ribose and Cys-35 are slightly different in the two reactions.

It is tempting to equate the value of ~1 kcal/mol with the binding energy of the –SH group of Cys-35 and the 3′-OH of ATP. Although this is likely to be true, there could be a complication, however, if the absence of the group R in the mutant enzyme causes a conformational change. Then, a conformational change may be required in the mutant to accommodate the binding of the substrate. The energy required to induce this change will also appear in eq 5 and so lead to an overestimate of the contribution of R to the enzyme–substrate binding energy. Thus, strictly speaking, the net contribution to the binding energy of the interaction between the –SH of the enzyme and the –OH of the substrate is ≤1 kcal/mol.

Comparison of Binding of TyrTS(Cys-35) and TyrTS(Ser-35) with ATP. As the hydroxyl group of serine forms far stronger hydrogen bonds than does the thiol group of cysteine (Crampton, 1974), it would appear at first sight that TyrTS(Ser-35) should bind ATP more tightly than does TyrTS(Cys-35) and far more tightly than TyrTS(Gly-35). However, a serine hydroxyl is hydrogen bonded to water molecules in the absence of substrate and so on binding a substrate it just exchanges a hydrogen bond with water for a bond with the –OH of a substrate. [Hydrogen bonding of an enzyme with a substrate is considered to be entropy driven (Jencks, 1975).] This minimizes the difference in binding energy between a substrate that hydrogen bonds to an enzyme hydroxyl group and one that makes optimal contact with an enzyme thiol group. However, the optimum O–H···O hydrogen bond distance is at least 0.04 nm shorter than the corresponding S–H···O distance, a distance that will be enhanced by the different geometries of serine and cysteine (Figure 3) [see Frey et al. (1973) and Kerr et al. (1975)]. Thus, since the –SH of Cys-35 is in position to make contact with the ribose oxygen, a hydrogen bond of Ser-35 is at least 0.05 nm longer than the optimum and would contribute little to the binding energy. Accordingly, TyrTS(Ser-35) would have to lose a good hydrogen bond with a bound water molecule in the free enzyme to form a poor hydrogen bond with ATP in the enzyme-substrate or enzyme–transition-state complexes. This would cause poorer binding and catalysis.

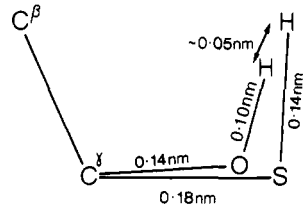

FIGURE 3: Geometry of C–O–H bonds in serine and C–S–H bonds in cysteine. The β and γ carbon atoms of both amino acids are superimposed. The two H atoms are drawn at their closest approach with data from Frey et al. (1973), Kistenmacher et al. (1974), and Kerr et al. (1975).

Suitability of Tyrosyl-tRNA Synthetase for Study by Site-Directed Mutagenesis. Sufficient enzyme may be isolated from a small-scale fermentation of *E. coli* infected with M13mp93(TyrTS) (10–20 mg from 1 L in a shaker flask) for several thousand kinetic assays. There is also adequate material for direct measurements of binding of tyrosine by equilibrium dialysis since only 0.3 mg of enzyme is required for constructing a binding isotherm of ten data points. The simple active site titration procedure enables the ready quantification of enzyme and has facilitated the acquisition of reproducible kinetic data. A means of active site titration is essential for a systematic study of the roles of the individual residues in catalysis. The stoichiometry of binding of tyrosine determined from equilibrium dialysis also affords a measure of concentration of enzyme. This will enable mutant tyrosyl-tRNA synthetases to be assayed in those cases where mutation of the ATP-binding site destroys the binding completely or prevents catalysis. The experimental ease of producing and assaying mutants of the tyrosyl-tRNA synthetase in combination with its interesting properties of being an oligomer with subunit interactions makes the enzyme a most attractive target for an extensive study by site-directed mutagenesis.

It is seen so far in this study that oligodeoxynucleotide-directed mutagenesis is a powerful probe for studying enzyme–substrate interactions. The initial results show that even a small interaction energy is used in an efficient manner to stabilize the transition state more than the enzyme–substrate complex. We are currently preparing mutant enzymes with more radical changes in the ATP binding site to discover how larger changes in binding energies can affect catalysis. In addition, we are attempting to cause mutations in the tyrosine binding site, in residues apparently involved in catalysis, and are exploring the subunit interfaces.

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[2] A rigorous mathematical treatment shows that eq 5 should be modified for multisubstrate systems by replacing K_M with the product of the K_Ms for all substrates, each measured under conditions where the other substrates are well below their K_M value. However, if the values of K_M for the substrates not directly affected by the mutation are the same in wild-type and mutant enzyme, then eq 5 holds.

Registry No. TyrTS, 9023-45-4; ATP, 56-65-5; Tyr, 60-18-4; Cys, 52-90-4; Gly, 56-40-6.

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Possible Association of NADPH–Cytochrome P-450 Reductase and Cytochrome P-450 in Reconstituted Phospholipid Vesicles†

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ABSTRACT: A fluorescent probe, *N*-(1-anilinonaphth-4-yl)-maleimide (ANM), was specifically labeled to SH group(s) in the hydrophilic moiety of NADPH–cytochrome P-450 reductase at a ratio of 1 ± 0.1 ANM/mol of protein. The ANM-labeled reductase and P-450 were reconstituted in phosphatidylcholine–phosphatidylethanolamine–phosphatidylserine vesicles in which all of the enzymes were functionally active. The reconstitution of the mixed-function oxidase system was found to be strongly dependent on both the lipid to protein molar ratio and phospholipid composition. The interactions of ANM-labeled reductase with P-450 in proteoliposomes were investigated by perturbation of the fluorescence of ANM. Upon incorporation of P-450 into the phospholipids vesicles (ANM-reductase/P-450/lipids ≡ 1:1.4:800), a significant decrease of total fluorescence intensity and slight increase of emission anisotropy of ANM were observed. In the average fluorescence lifetime of ANM bound with reductase, an appreciable change was shown between the absence and presence of P-450 in the vesicles. These data provide clear evidence that significant molecular interactions occur between the two proteins in a membranous reconstituted system.

Both NADPH–cytochrome P-450 reductase and P-450[1] are essential components of the hepatic microsomal mixed-function oxidase system catalyzing the oxidative metabolism of endogenous substrates such as fatty acids, prostaglandins, and steroids as well as many xenobiotics such as drugs, petroleum

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[1] Abbreviations: DLPC, dilauroylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PE, bovine brain phosphatidylethanolamine; PS, bovine brain phosphatidylserine; ANM, *N*-(1-anilinonaphth-4-yl)maleimide; DPH, 1,6-diphenyl-1,3,5-hexatriene; pCMB, *p*-(chloromercuri)-benzoate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ANM-reductase, ANM-labeled NADPH–cytochrome P-450 reductase; P-450, the cytochrome P-450LM_2 induced in rabbit liver by phenobarbital.

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