The Michaelis constant ($K_m$) and $V_{\text{max}}$ ($E_0/k_{\text{cat}}$) values for two mutant sets of enzymes were studied from the viewpoint of their definition in a rapid equilibrium reaction model and in a steady state reaction model. The "AMP set enzyme" had a mutation at the AMP-binding site (Y95F, V67I, and V67I/L76V), and the "ATP set enzyme" had a mutation at a possible ATP-binding region (Y32F, Y34F, and Y32A/Y34A). Reaction rate constants obtained using steady state model analysis explained discrepancies found by the rapid equilibrium model analysis. (i) The unchanged number of bound AMP's for Y95F and the wild type despite the markedly increased $K_m$ values for AMP of the AMP set enzymes was explained by alteration of the rate constants of the AMP step ($k_{-\text{g}}$, $k_{-\text{g}}$) to retain the ratio $k_{-\text{g}}/k_{-\text{g}}$. (ii) A 100 times weakened selectivity of ATP for Y34F in contrast to no marked changes in $K_m$ values for both AMP and ATP for the ATP set of enzymes was explained by the alteration of the rate constants of the ATP steps. A similar alteration of the $K_m$ and $k_{\text{cat}}$ values of these enzymes resulted from distinctive alterations of their rate constants. The pattern of alteration was highly suggestive. The most interesting finding was that the rate constants that decided the $K_m$ and $k_{\text{cat}}$ values were replaced by the mutation, and the simple relationships between $K_m$, $k_{\text{cat}}$, and the rate constants of $K_m = k_{-\text{g}}/k_{-\text{g}}$ and $k_{\text{cat}} = k_{\text{f}}$ were not valid. The nature of the $K_m$ and $k_{\text{cat}}$ alterations was discussed.

The advantage of kinetic measurements is that the dynamic aspect of an enzyme molecule is given by kinetic parameters, and the events not included in its activity are automatically omitted. The importance of the linkage between dynamics and enzyme catalysis was indicated in a recent study of adenylate kinase (27). Kinetic parameters should be those that can describe the dynamic aspects of the enzyme and not the static binding of substrates. The results of the analysis of the two mutant sets by using rapid equilibrium and steady state methods are discussed, and their comparison shows advantages and disadvantages of each method.
**Materials**—The enzyme was ADK in porcine skeletal muscle obtained as a recombinant protein by expressing its cDNA. Enzymes for gene manipulation and deoxyribonucleotides were purchased from Toyobo Co. Ltd. and Takara Shuzo Co. Ltd. Substrate nucleotide species were purchased from Sigma and Roche Applied Science. All other chemicals were purchased from Wako Pure Chemicals Co. and Nacalai Tesque Inc.

**Preparation of Mutants**—Site-directed mutagenesis was done by using the methods described previously (33, 28) with mutagenic primer DNAs TACGCGTTAACCACC (16-mer) for the Y34F mutation, GAAGTGGTGCAGCA (16-mer) for the Y95F mutation, and GTCCAGACCTC (17-mer) for the Y32A/V344A mutations, and by using the method of Nakayama and Eckstein (30) with primers GACGGTTTCCCCGGG (16-mer), GCAGCTGATCCCACTGG (17-mer), and GGACATGTCGCCAGGC (17-mer) for Y95F, V67I, and L76V mutation, respectively. The primer DNAs were prepared by using high pressure liquid chromatography with a C18 reversed phase column on a 0.1–1 m KH2PO4 linear gradient system of 30% CH3CN solution and on 20% PAGE after 5-phosphorylation.

Alteration of the target nucleotide sequences was confirmed by DNA sequencing. Substitutions of amino acid residues were confirmed by amino acid composition analysis by using an automated amino acid analyzer (Beckman System 7300).

Mutated ADK proteins were expressed in Escherichia coli JM109 using the pMK2 expression vector (31) at 37 °C for 36 h. For Y95F, V67I, and V67L/V67F mutants, preliminary cultivation was done at 20, 25, 30, and 37 °C at various cultivation periods from 18 to 72 h. The optimal temperature and period of cultivation were 25 °C and 36 h, respectively, for Y95F mutant ADK and 30 °C and 24 h, respectively, for V67I and V67L/V67F mutant ADKs. The expressed ADK was extracted, purified, and refolded as described previously (32). The expressed wild type and six mutant proteins, as inclusion bodies, were obtained as a single band by protein by using SDS-PAGE (18%) analysis.

**CD and Protein Concentration Measurements**—The CD of the enzyme solution having extinction A220 = 1.0 was recorded using a CD spectrometer (Jasco J-100) with a 1-cm light path for 250–300 nm and a 0.2-mm light path for 200–250 nm wavelength region. The molar concentration of the enzyme solution was measured with the extinction coefficient of 1.16 × 104 dm2 mol−1 cm−1 for the wild type. Extinction coefficients for mutant enzymes were obtained as coefficients of the wild type value multiplied by the ratio of combined extinction coefficients of amino acid residues for mutant enzymes to that for the wild type.

**CD Spectra**—The CD spectra of the 220 nm region of Y95F and the other mutant enzymes were the same as for the wild type (data not shown). CD spectra in the 280 nm region of Y95F and the other mutant proteins lacking Tyr were less negative than for the wild type, presumably due to the substitution of Tyr by Phe. Thus, conformations of mutants had no effect due to the replacement of every target amino acid residue in the mutant enzymes.

**Kinetic Measurements**—Kinetic measurements were done as described previously (33) at pH 7.0 and 25 °C. Sets of concentrations of ATP and AMP used as substrates were as follows: 100, 150, 200, 1000, 1500, and 2000 (μM) for the reactions of wild type enzyme; 50, 100, 150, 250, 500, 1000, and 4000 (μM) for the reactions of Y34F, Y35F, Y67F, and V67L/V67F; 50, 100, 150, 200, 300, 400, 500, 700, 1000, and 4000 (μM) for the reactions of Y32A/Y344A; 50, 100, 200, 300, 500, 700, 1000, 2000, and 5000 (μM) for the reactions of Y95F. Fig. 1 shows initial reaction rates but omit data that showed inhibitory effects by ATP or AMP. These reaction rates were further analyzed by using the random Bi Bi model with rapid equilibrium or steady state assumptions. For the rapid equilibrium model based on the rapid equilibrium model at a steady state is described by Equation 1.

\[
\frac{1}{v} = \frac{K_m^3 K_m^4}{S_r S_M} + \frac{K_m^3}{S_T} + \frac{K_m^2}{S_M} + 1 \left\{ \begin{array}{c}
\frac{1}{V_m} \\
\frac{1}{V_n}
\end{array} \right\}
\]

In the case of the steady state model based on the concept of steady state conditions (35), the enzyme species change to other species with rate constant k_F and k_n, respectively (Fig. 1). The condition of the steady state is written as shown in Equation 2.

\[
\partial [E] / \partial t = A [E] = 0
\]

where \(A\) is an enzyme species vector \([|E|, |ET|, |EM|, |ETM|]\), \(0\) is a zero vector, and \(A\) is the transformation matrix of the reaction described in Fig. 1.

\[
A = \begin{pmatrix}
-k_{14} & -k_{14} & -k_{33} & k_{13} & k_{14} \\
-k_{43} & -k_{43} & -k_{43} & k_{43} & k_{43} \\
-k_{14} & -k_{14} & -k_{33} & k_{13} & k_{14} \\
-k_{33} & -k_{33} & -k_{33} & k_{33} & k_{33}
\end{pmatrix}
\]

**Transformation Matrix A**

Parameter representation is the same as shown in Fig. 1. Because each element is a rate, the secondary rate constants, \(k_{n}\), contain an appropriate substrate concentration to give a rate when multiplied by the enzyme concentration (i.e., \(k_{n} = k_{E} \times S_{n}\), but \(S_{a}\) and \(S_{b}\) are not described explicitly for simplicity of expression in this section.

Each row of matrix \(A\) calculates a change in the amount of enzyme species during unit time, i.e., the top row calculates a change in free enzyme species as shown in Equation 3.

\[
\partial [E] / \partial t = -(k_{14} + k_{14}) [E] + k_{1} [ET] + k_{3} [EM] + k_{4} [ETM]
\]

By replacing an arbitrary row of matrix \(A\) by a unit vector by replacing a corresponding element of the zero vector by \(|E|\), which establishes conservation of the enzyme molecules |E| = |ET| + |EM| + |ETM|, the equation is transformed into \(A' = A + B\), where \(A'\) is the matrix replaced by a \(k\)th (arbitrary) row, and \(B\) is a modified zero vector of which the \(k\)th element is \(|E|\), and the other elements are zero. The enzyme species vector is calculated as \(A' = A' |E|\) and the concentration of ternary complex species is obtained by the \(k\)th element of \(A'\). So the reaction rate equation in the steady state model at a steady state (35, 36) is derived as shown in Equation 4.

\[
v = k_F \frac{\text{det}(A')}{\text{det}(A)}
\]
where \( \det A \) is the determinant of matrix \( A \), and \( A_{ij} \) is the cofactor matrix of \((i,j)\) element of transposed \( A \); \( k \) is the number of the row replaced by a unit vector, and \( l \) is the number of enzyme species with which the reaction rate is expressed as \( k[E_l] \) (\( l \) is 4 in the model of this study.) \( [E_l] \) is omitted from the equation by making its value unity.

The calculation of every kinetic constant was done as described previously (34) for a rapid equilibrium model, and by the least square minimization of the function \( F = \sum (v_i - v_i^F)^2 \) for a steady flow model, where \( v_i \) is an experimentally observed reaction rate; \( v_i^F \) is a rate calculated by Equation 4, and \( F \) is a summation of the square of the differences between all for every experimental points (36). For the minimization, the hybrid method of Powell (37, 38) was used. Correction vectors for the Gauss-Newton algorithm and for the steepest direction were calculated by using the Jacobian matrix derived from the analytically coded \( \det (A) / \det (b) \). The program was allowed to run until the correction vector decreased to a threshold value. A weighting function that decreases with increasing reaction rate was also used for the function \( F \) and the Jacobian matrix.

**Equilibrium Dialysis—**Experiments of equilibrium binding for the Y95F mutant and wild type enzymes were done by using a homemade equilibrium dialysis instrument with 200-\( \mu \)l chambers. Each chamber was divided into two 100-\( \mu \)l parts by a dialysis membrane (Spectrum Paste, cut-off molecular weight 10,000; Spectrum Medical Industries, Inc.) and was rotated at 10 rpm. Chambers on one side contained weak radiolabeled ATP or AMP (370 MBq/mmol) at one of the following concentrations: 50, 100, 200, 300, 400, and 500 (\( \mu \)M); the chambers on the other side contained the nucleotide and enzyme (100 nmol in each chamber). Substrate binding to the Y34F mutant was checked at 300 \( \mu \)M ATP and AMP. The amount of enzymes in the chambers was estimated as for the CD measurement. The equilibrium was established by overnight dialysis at 25 °C. The difference in radioactivity between the two sides was calculated by using a liquid scintillation counter (LKB model 2000), and the number of bound nucleotides was estimated without including the enzyme volume in the calculation.

**RESULTS**

**Preparation of Enzymes—** The amino acid compositions of purified enzymes indicated a corresponding alteration to every amino acid residue in the mutant proteins.

**Enzymatic Reactions—** Profiles of the reaction rates for fixed AMP concentrations and increasing ATP concentrations were divided into two regions based on critical ATP concentrations at which concentration the reaction rate was largest for the fixed AMP concentration. In the ATP concentration range lower than the critical value, \( 1/v \) increased with increase in 1/ATP linearly (Fig. 2b), but in the concentration range higher than the critical value, \( 1/v \) increased with increase in [ATP], indicating inhibition effects. An apparent \( K_m \) was calculated for this inhibition as the ATP concentration that \( 1/v \) had markedly larger values of \( \alpha_{ATP} \) and \( \beta_{ATP} \) for increasing ATP were plotted against 1/[AMP] for these seven enzyme reactions (data not shown). Because all plots, including the wild type, lie on linear lines, the rapid equilibrium model was applicable in the concentration ranges used here despite modification of the respective sites, and \( K_m \) and \( V_{max} \) max values were calculated from the slopes and intercepts of \( \alpha_{ATP} \) and \( \beta_{ATP} \) plots by using Equations 6 and 7. \( K_m \) of these ATP site mutant enzymes were about one to two times larger than for the wild type. The \( K_m \) values for AMP (\( K_m^a \) and \( K_m^b \) ) of these ATP site mutant enzymes were about one to two times larger than for the wild type. Among them \( K_m^a \) of 800 \( \mu \)M for the Y32A/Y34A mutant was the largest. The catalytic rates were from 1.8 \( \times \) 10\(^{-3} \) s\(^{-1} \) for the wild type to 1.6 \( \times \) 10\(^{-3} \) s\(^{-1} \) for the Y32A/Y34A mutant enzyme, i.e. the rate constant of Y32A/Y34A was about 10\(^{-3} \) times that of the wild type enzyme. For Y95F, only a few parameters were derived because the intersections of the plots of \( \alpha_{ATP} \) against 1/[AMP] were negative or zero within experimental error, and therefore no reasonable values were obtained for \( K_m^b \). The intercept of \( \beta_{ATP} \) against 1/[AMP] was positive but very small for the Y95F mutant enzyme and was therefore assigned as zero. Accordingly, \( K_m^b \) for Y95F was estimated as 6.5 mM from the slope of \( \alpha_{ATP} \) and the intercept of \( \beta_{ATP} \) using the rate equation of the rapid equilibrium model. Y95F, V67I, and V67I/L76V, the mutant enzymes near the AMP site, had markedly larger values of \( \alpha_{AMP} \) and \( \beta_{AMP} \) for increasing AMP were plotted against 1/[ATP] for the wild type enzyme reaction for several kinds of substrate nucleotides, double-reciprocal plots of initial velocities against the inverse of a substrate concentration were made for the wild type, Y32F, Y34F and Y32A/Y34A (Fig. 3a–d). These plots were linear for many kinds of nucleotide species and for all applied enzyme reactions, except for CMP of the wild type (Fig. 3a). The inverse of the reaction rate for the wild type enzyme with a substrate set of ATP and CMP depended on the square of 1/[CMP], but for other mutant enzymes on the same substrate set it was linearly dependent on 1/[CMP]. Nucleotide species (UTP, GTP, and CTP for Y34F and CMP for Y32A/Y34A) showed an inhibitory effect (i.e. they were not linear but had a higher dependence) at a concentration range greater than the critical value that ATP showed for the wild type enzyme reaction.

Table I lists the calculated kinetic parameters. \( K_m \) values of Y32F, Y34F, and Y32A/Y34A mutant enzymes for ATP (\( K_m^a \) and \( K_m^b \) ) were about one, two, and two times, respectively, larger than for the wild type. The \( K_m \) values for AMP (\( K_m^a \) and \( K_m^b \) ) of these ATP site mutant enzymes were about one to two times larger than for the wild type. Among them \( K_m^b \) of 800 \( \mu \)M for the Y32A/Y34A mutant was the largest. By comparing Equation 5 with Equation 1, slope \( \alpha_{ATP} \) (apparent \( K_m/V_{max} \)) and intercept \( \beta_{ATP} \) (apparent \( 1/V_{max} \)) of the rate equation were written as shown in Equation 6 and Equation 7, respectively.

\[
\small
\frac{1}{v} = \frac{\alpha_{ATP}}{K_m} + \beta_{ATP} \quad \text{(Eq. 5)}
\]

\[
\alpha_{ATP} = (K_m^a K_m^b / S_M + K_m^b) / V_{max} \quad \text{(Eq. 6)}
\]

\[
\beta_{ATP} = (K_m^b / S_M + 1) / V_{max} \quad \text{(Eq. 7)}
\]

For the rapid equilibrium model, these two parameters were calculated by using the reaction rates of increasing ATP concentration from 50 \( \mu \)M to 1 mM ATP and fixed AMP concentrations for wild type, Y32F, Y34F, Y32A/Y34A, Y95F, V67I, and V67I/L76V mutant enzyme reactions. Then \( \alpha_{ATP} \) and \( \beta_{ATP} \) for increasing ATP were plotted against 1/[AMP] for these seven enzyme reactions (data not shown). Because all plots, including the wild type, lie on linear lines, the rapid equilibrium model was applicable in the concentration ranges used here despite modification of the respective sites, and \( K_m^a \) and \( V_{max} \) max values were calculated from the slopes and intercepts of \( \alpha_{ATP} \) and \( \beta_{ATP} \) plots by using Equations 6 and 7. \( K_m \) of these ATP site mutant enzymes were about one to two times larger than for the wild type. Among them \( K_m \) of 800 \( \mu \)M for the Y32A/Y34A mutant was the largest. The catalytic rates were from 1.8 \( \times \) 10\(^{-3} \) s\(^{-1} \) for the wild type to 1.6 \( \times \) 10\(^{-3} \) s\(^{-1} \) for the Y32A/Y34A mutant enzyme, i.e. the rate constant of Y32A/Y34A was about 10\(^{-3} \) times that of the wild type enzyme. For Y95F, only a few parameters were derived because the intersections of the plots of \( \alpha_{ATP} \) against 1/[AMP] were negative or zero within experimental error, and therefore no reasonable values were obtained for \( K_m^b \). The intercept of \( \beta_{ATP} \) against 1/[AMP] was positive but very small for the Y95F mutant enzyme and was therefore assigned as zero. Accordingly, \( K_m^b \) for Y95F was estimated as 6.5 mM from the slope of \( \alpha_{ATP} \) and the intercept of \( \beta_{ATP} \) using the rate equation of the rapid equilibrium model. Y95F, V67I, and V67I/L76V, the mutant enzymes near the AMP site, had markedly larger values of \( \alpha_{AMP} \) and \( \beta_{AMP} \) for increasing AMP were plotted against 1/[ATP] for the wild type enzyme reaction for several kinds of substrate nucleotides, double-reciprocal plots of initial velocities against the inverse of a substrate concentration were made for the wild type, Y32F, Y34F and Y32A/Y34A (Fig. 3a–d). These plots were linear for many kinds of nucleotide species and for all applied enzyme reactions, except for CMP of the wild type (Fig. 3a). The inverse of the reaction rate for the wild type enzyme with a substrate set of ATP and CMP depended on the square of 1/[CMP], but for other mutant enzymes on the same substrate set it was linearly dependent on 1/[CMP]. Nucleotide species (UTP, GTP, and CTP for Y34F and CMP for Y32A/Y34A) showed an inhibitory effect (i.e. they were not linear but had a higher dependence) at a concentration range greater than the critical value that ATP showed for the wild type enzyme reaction.

Table II summarizes the results of specificity measurements for the nucleotide substrates used: the catalytic ability indicated by \( V_{max}/(K_m V_{max})^{1/2} \) derived from the linear region in Fig. 3, and specificity \( R \), which is the ratio of the catalytic ability to that of ATP or AMP of the same enzyme.

In the case of ATP and other triphosphate substrates, the catalytic ability of ATP site mutant enzymes was smaller than...
FIG. 2. Linear part of catalytic reactions for wild type (a and b), Y32F(c), Y34F(d), Y32A/Y34A(e), Y95F(f), V67I(g), and V67I/L76V (h). AMP concentrations used were 100, 150, 200, 300, 500, 800, 1000, 2000, and 5000 μM from the bottom to the top, respectively, for the wild type-catalyzed reaction; 50, 100, 200, 300, 500, 1000, 2000, and 4000 μM for Y32F; 50, 100, 150, 250, 500, 1000, and 4000 μM for Y32F, Y95F, and
for wild type enzymes, but the specificities of the triphosphate species were classified into two distinct groups as follows: 1) wild type and Y32F enzymes, which have specificity $10^{-3}$ for triphosphate substrates, and 2) other enzymes that include Y95F and V67I/L76V, which do not have specificity for monophosphate substrates, and 2) other enzymes that include Y95F, V67I, and V67I/L76V (data not shown). The specificities of mutant enzymes were about 20–40% of the wild type activity, irrespective of nucleotide species, and was fairly constant compared with the changes in triphosphate substrate species. Mutated enzymes had no activity for monophosphate species examined, except for AMP and CMP.

**Calculation of Rate Constants of the Steady State Model**—To show that the minimization was done successfully, a minimum point view for the wild type parameter set was shown as a graph of $\Delta F - F_{\text{min}}/F_{\text{min}}$ with each rate constant independently increasing 10% away from its minimum point value (Fig. 4). Pareto-like graphs with the lowest points at the center indicated that the calculated rate parameters were for a minimum point. The $K_m$ and $k_{\text{cat}}$ values calculated from the simulated rate lines in Fig. 2 agreed well with those calculated by using the rapid equilibrium model (data not shown). But for Y32F, the rate parameter that gave the third lowest functions was chosen. The difference in value between the third and lowest functions was about 20–40% of the wild type activity, irrespective of nucleotide species, and was fairly constant compared with the changes in triphosphate substrate species. Mutated enzymes had no activity for monophosphate species examined, except for AMP and CMP.

| Enzyme          | $K_m$ | $K_m$ | $K_m$ | $k_{\text{cat}}^{-1}$ | ATP* | $K_i$ |
|-----------------|-------|-------|-------|-----------------------|------|-------|
|                 | $\mu M$ | $\mu M$ | $\mu M$ |                          |      | $\mu M$ |
| Wild type       | 100   | 250   | 400   | 150 1.83E + 03           | 500  | 800   |
| Y32F            | 70    | 400   | 700   | 120 1.17E + 02           | 500  | 1000  |
| Y34F            | 180   | 540   | 500   | 170 6.00E + 01           | 500  | 1000  |
| Y32A/Y34A       | 140   | 470   | 800   | 200 1.55E + 00           | 800  | 1500  |
| Y95F            | 290   | 500   | >6500 | 800 8.47E + 01           | 1000 | 2000  |
| V67I            | 250   | 1200  | 1600  | 140 6.18E + 00           | 1000 | 2200  |
| V67I/L76V       | 130   | 680   | 4800  | 900 3.11E + 01           | 1000 | 1800  |

$^a$ ATP is a critical ATP concentration above which the inhibition dominates in the ADK reaction. $K_i$ is the inhibition constant obtained as a value that gives the half of the observed maximal catalytic rate above the critical ATP concentration at 200 $\mu M$ AMP.

$^b$ The value cannot be calculated exactly because the ordinate intersections of $\alpha(M)$ and $\beta(M)$ were zero or negative within experimental error. Thus, values of $\alpha$ and $\beta$ are estimated for $1/\text{AMP}$ first, and then slope and intersections of $\alpha$ and $\beta$ as a function of $1/\text{ATP}$ are determined.

**Table II**

| Substrate specificity of wild type and mutant enzymes
|---------------------------------|

$(V_{\text{max}}/K_m)^{\text{inv}}$ is an inverse of a slope of each reaction line in Fig. 3. $R$ is the ratio of $(V_{\text{max}}/K_m)^{\text{inv}}$ value of the enzyme to that of the ATP or AMP of the same enzyme. The unit of $(V_{\text{max}}/K_m)^{\text{inv}}$ is $s^{-1}$.

| Wild type | Y32F | Y34F | Y32A/Y34A |
|-----------|------|------|-----------|
| ATP       | 2.46E + 01 | 1.00E + 00 | 6.57E + 00 | 1.00E + 00 | 1.00E + 00 |
| GTP       | 1.48E - 02 | 6.00E - 04 | 2.53E - 03 | 3.90E - 04 | 2.30E - 03 |
| CTP       | 2.46E - 02 | 9.90E - 04 | 5.58E - 03 | 8.50E - 04 | 1.20E - 02 |
| TTP       | 1.12E - 02 | 4.60E - 04 | 3.94E - 03 | 6.00E - 04 | 1.90E - 03 |
| UTP       | 1.31E - 02 | 5.30E - 04 | 2.10E - 03 | 3.20E - 04 | 5.50E - 03 |
| AMP       | 7.55E + 00 | 1.00E + 00 | 2.00E + 00 | 1.00E + 00 | 1.00E + 00 |
| CMP       | 6.90E - 04 | 9.00E - 05 | 5.91E - 04 | 3.00E - 04 | 1.00E + 00 |
| GMP       | 6.24E - 03 | 8.10E + 00 | 6.24E - 03 | 8.10E + 00 | 6.24E - 03 |
| IMP       | 2.73E - 05 | 3.50E - 02 | 2.73E - 05 | 3.50E - 02 | 2.73E - 05 |
TABLE VI shows the value of each term in Equation 8 calculated by the obtained rate constants (Table IV) at the substrate concentration of 0.3 mM for ATP and AMP, where the random Bi Bi mechanism for all enzymes worked without substrate inhibition. At concentration 0.3 mM, $a_0$, $a_1$, $b_0$, and $b_1$ dominated in the denominator and $A_1$ dominated in the numerator. Thus, Equations 6 and 7 were written as $(a_0/A_1)_iS_{m,i} + a_1/A_1$ and $(b_0/A_1)_iS_{m,i} + b_1/A_1$, respectively, for the wild type, Y95F, Y32F, and V67I. Terms $a_1$, $b_1$, $b_2$, and $A_2$ dominated for Y34F, and $b_0$, $b_1$, $c_0$, $c_1$, and $B_1$ dominated for Y32A/Y34A. These results showed that in Equation 8 the terms contributing to the $K_m$ values were replaced by the mutation depending on their rate constants. As a result of the domination by these terms, the rate constants in the $K_m$ values were different for each enzyme. For example, $a_0$ is equal to $k_{1} - k_{-2} + k_{-7}k_{-2} + k_{-1}k_{-7}k_{-3} + k_{1}k_{-2}k_{-4}$ and was represented by the $k_{1}k_{-2}k_{-3}$ term for the wild type because the rate constants of the wild type made the middle term crucially larger than the other two terms. Also the $k_{cat}$ was altered in the same situations depending on the last term of Equation 7 as follows: $k_{cat}$ of the wild type group was $k_1$ multiplied by $A_1/b_2$ (i.e. 0.97, 0.31, 0.98, and 0.13 for the wild type, Y95F, Y32F, and V67I, respectively); the $k_{cat}$ of Y34F was multiplied by $A_2/b_2$ (1.00), and $k_{cat}$ of Y32A/Y34A was multiplied by $b_1c_1$ (1.00). In Y95F and V67I, the $k_{cat}$ of the rapid equilibrium model differed markedly from $k_f$ of the steady state model.

Equilibrium Binding of ATP and AMP—The number of bound substrate nucleotides for the wild type and Y95F was found by equilibrium binding experiments (Table VII). Although the $K_m$ value of Y95F for AMP markedly increased from that of the wild type (Table I), the number of enzyme-bound AMP seemed to increase by a small amount. AMP bound to the enzyme was 1 mol/mol of enzyme at concentration 500 μM. The number of ATP bound to the Y95F enzyme was 0.8 mol/mol of enzyme at 300 μM ATP, and the number of ATP was 0.7 and 1.6 at 300 and 400 μM, respectively, for the wild type enzyme. The number of bound ATP on the Y95F enzyme was equal to or a little larger than the wild type at 0.4 mM ATP concentration. The dissociation constants for these substrates were calculated as 300 and 980 μM for ATP and AMP of the wild type, respectively, and as 230 and 700 μM for ATP and AMP of Y95F enzyme, respectively. ATP and AMP binding onto Y34F enzyme were also checked at 300 μM concentration, but they showed no marked alteration from the wild type.

**DISCUSSION**

Enzymatic activities of the wild type and mutants Y32F, Y34F, Y32A/Y34A, Y95F, V67I, and V67I/L76V were measured at 25 °C. First, in the concentration ranges of nucleotide substrates chosen, ATP or AMP molecules had no inhibition effect, thus making the analyses by rapid equilibrium and steady state assumptions possible with the random Bi Bi model. Second, information on substrate specificity was obtained for a variety of nucleotide mono- and triphosphates. Third, a comparison of these kinetic parameters for the models together with substrate specificity and equilibrium binding results gave intuitive insight into the enzyme reaction.

**AMP Site Mutation—**The kinetic analysis of the rapid equilibrium model showed a marked increase in $K_m$ values for AMP of AMP site mutants Y95F, V67I, and V67I/L76V, but the $K_m$ values for AMP of the other three ATP site mutants did not increase markedly. This Tyr-95 is in a kinetically assignable AMP site and might be a possible AMP-binding site. The remaining AMP site mutant enzymes, V67I and V67I/L76V, had very similar kinetic parameters to those of Y95F (Table I); these sites were deduced to be $K_m$-modifying sites for AMP. Nevertheless, differences in these mutations existed outside of.

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**Note:** The text is a continuation of a scientific paper discussing enzyme kinetics and substrate specificity, focusing on the effects of mutations on reaction rates and substrate affinities. The paper explores how changes in enzyme structure affect catalytic efficiency and substrate binding, providing insights into the mechanisms of enzyme function.
Concentration of the paired substrate other than listed in the extreme left column is 200 μM of AMP or ATP. The amount of enzymes equal to 1 OD wild type enzyme is used.

|        | Wild type | Y32F     | Y34F     | Y32A/F34A |
|--------|-----------|----------|----------|-----------|
|        | \(K_m \)  | \(V_{max} \) | \(K_m \)  | \(V_{max} \) | \(K_m \)  | \(V_{max} \) | \(K_m \)  | \(V_{max} \) |
| ATP    | 25        | 9.85E + 02 | 40       | 2.89E + 02 | 85       | 1.51E + 02 | 95       | 1.41E + 01 |
| GTP    | 730       | 1.08E + 01 | 550      | 1.36E + 00 | 970      | 2.69E + 00 | 390      | 3.61E + 03 |
| CTP    | 350       | 8.54E + 00 | 210      | 1.21E + 00 | 150      | 2.15E + 00 | 480      | 3.25E + 02 |
| ITP    | 1800      | 2.04E + 01 | 380      | 1.44E + 00 | 2500     | 5.56E + 00 | 690      | 2.66E + 02 |
| UTP    | 810       | 1.05E + 01 | 430      | 9.19E - 01 | 830      | 4.27E + 00 | 1700     | 3.11E + 03 |
| AMP    | 280       | 2.13E + 03 | 280      | 5.91E + 02 | 330      | 2.76E + 02 | 290      | 2.27E + 01 |
| CMP    | 9400      | 2.56E + 02 | 1400     | 8.21E - 01 | 820      | 2.53E + 00 | 55       | 6.24E + 02 |
| GMP    |           |           |          |           | 25       | 1.64E + 01 |          |          |
| IMP    |           |           |          |           | 130      | 3.61E + 03 |          |          |
| UMP    |           |           |          |           |          |           |          |          |

FIG. 4. Profiles of target function around the obtained minimum point. Minimum point view given by (\(F_{max} - F_{min}\))/\(F_{min}\), with increasing rate parameters is shown for the wild type reaction. \(F_{min}\) is the target function value at the minimum point. When one rate constant is altered from its minimum, the value of target function is written as \(F_{min}\). A single rate constant is altered independently in the 10% range from the minimum point value, and the \(F_{min}\) given by the alteration of the rate constant \(k_4\) is plotted against the varying constant indexed by \(k_4\). Each curve is the \(F_{min}\) given by the alteration of the rate constant \(k_3\), \(k_{-3}\), \(k_{-1}\), \(k_{-1}\), \(k_{-2}\), \(k_{-2}\), \(k_{-2}\) from top to bottom, respectively, and the last three lines, \(k_{-3}\), \(k_{-3}\), \(k_{-2}\), \(k_{-2}\), \(k_{-2}\), look like one line because they overlap each other. The \(F_{min}\) curve of \(k_{-4}\) is in the inset graph.

the substrate concentration range in this study, where deviations from the linear relations were observed (data not shown).

Despite these results of rapid equilibrium analysis of AMP site mutants, the increase in \(K_m\), which is the decrease in binding affinity in the rapid equilibrium assumption, disagrees with the fact that the number of AMP first binding to the Y95F mutant did not decrease (Table VII). This analysis does not discriminate the mutants, because the alterations were all in \(K_m\) for AMP (and in \(k_{cat}\)). However, steady state analysis showed that alterations of Y95F were in the rate constants specifically corresponding to the AMP-binding step, and these alterations did not change their ratio. Steady state analysis discriminated V67I and V67I/L76V from Y95F by the alteration of all rate constants, in contrast to the alteration of rate constants specific for the AMP step of Y95F. These explanations seem more rational and acceptable for AMP site mutation.

**ATP Site Mutation**—The analysis of the rapid equilibrium model showed that the ATP site mutation gave rise to a marked decrease in \(k_{cat}\) but little alteration in \(K_m\). These sites seem not to participate in substrate binding. Because two kinds of mutants were prepared, one lacking the hydroxyl group of a tyrosine residue (Y32F and Y34F) and the other replacing the phenol group by hydrogen (Y32A/Y34A), the role of those groups should be deduced by these parameters. When hydroxyl group and phenol groups were removed, \(k_{cat}\) decreased by 1 order at least (Table I), suggesting that the groups at the mutated sites participate in the chemical step, perhaps in formation of a transition intermediate. The effect of the hydroxyl group on Tyr-32 and Tyr-34 was identified clearly in the substrate specificity by comparing \((V_{max}/K_m)_{\text{wild}}\) and \((V_{max}/K_m)_{\text{mutant}}\), for Y32F, and ratio \(R\) or \(V_{max}\) for Y34F with those of the wild type. Because \(R\) values for Y34F were around 10–100 times greater than for the wild type, in contrast to the same order for Y32F, the hydroxyl group on Tyr-34 would have a role in recognizing substrate nucleotides through hydrogen bonding. Removal of the phenol groups at Tyr-32 and Tyr-34 produced more marked effects on \((V_{max}/K_m)_{\text{wild}}\), which decreased \((V_{max}/K_m)_{\text{mutant}}\) by a hundred-fold, but were somewhat greater for \(R\) (Table II). The \(R\) of Y32F and the wild type seem to be identical as are the \(R\)s of Y34F and Y32A/Y34A, thus dividing these enzymes into two groups, one with a hydroxyl that recognizes ATP and the other without a hydroxyl and is less effective in ATP recognition. The value of \(V_{max}\) for Y32A/Y34A was a thousandth as small as for the wild type, and the effect of the mutation was very large. More of consequence was that the substrate specificity for the monophosphate substrate was affected by the removal of phenyl rings. Although some thought is required to interpret the kinetic parameters for CMP, the substrate specificity alterations listed in Tables II and III were high for the \(V_{max}\) term and were small for the \(K_m\) term, which was the same as for \(K_m\) and \(k_{cat}\) alteration with ATP. The analysis by using the rapid equilibrium assumption indicated that recognition of substrates was modified by mutation through a catalytic or chemical process, and the binding process might not be markedly affected.

However, the result of the analysis by using the steady state model explains these changes in enzyme reactions as follows: 1) \(k_{cat}\) alone decreasing with Y32F; 2) rate parameters related specifically to the ATP-binding step and \(k_{cat}\) decreased with the Y34F mutant; and 3) all rate constants altered with Y32A/Y34A. These results indicate that the hydroxyl group of Tyr-34 specifically participates in ATP binding and that the enzyme region, including Tyr-34 and Tyr-32, participates in the chemical step. This participation is also in the Y32A/Y34A reaction. The \(k_{cat}\) value markedly decreased in this mutant in addition to alteration of all other rate constants. The alteration related to the ATP-binding step was also observed for this mutant lacking two hydroxyl groups and phenyl rings as \(k_{cat}\) decreased. These results indicate that the origin of specificity, which seemed to be in the chemical step by using rapid equilibrium analysis, was in the binding step, although the hydroxyl group of Tyr-34 and Tyr-32 participated in the chemical step at the same time, as shown by using rapid equilibrium analysis. Steady state analysis discriminated these mutants, which looked the same in the change in \(K_m\) and \(k_{cat}\), but they
had characteristic differences in specificity by specific alteration of the rate constants (Y32F and Y34F) and a change in all constants (Y32A/Y34A).

**How Rate Constants Are Affected**—The rapid equilibrium analysis indicated that the hydroxyl group and phenyl ring of Tyr-32 and Tyr-34 participated in the chemical step, which was also deduced from the steady state analysis. But these groups are distantly apart from the site where the chemical reaction takes place, which is assumed to be near the γ-phosphate of ATP, so the modifying effects of V_{max} and K_{f} should be assessed through an alteration of enzyme conformation. This is not extraordinary, because a large conformational change by this enzyme has been proposed during enzymatic reactions (11, 39–44). Although a conformational change exists, the interactions between enzyme and substrate and in the enzyme itself or the enzyme species before and after ATP binding or AMP binding or both must be the same for the mutant enzyme and the wild type, because K_{m} and K_{f} (e.g., K_{f} \text{ (Y34A)}) of these enzymes were the same. When the K_{m} and the K_{f} values were the same, the two interactions were the same, and is the case of first priority. So the ternary complexes (ETM in Fig. 1) of mutant enzymes were the same with that of the wild type and conformational change in which the hydroxyl group and phenyl ring participate has to differ after nucleotide binding, which causes a transitional state or pathway different from the wild type. This seems a reasonable explanation for V_{max} and K_{f} alteration, and also explains why the hydroxyl group of Tyr-34 participated in ATP recognition but that of Tyr-32 did not as explained as follows. This enzyme has a large conformational change in the first binding of ATP that accompanies movement of a fragment containing Tyr-32 and Tyr-34. During the movement a specific interaction of the hydroxyl group of Tyr-34 with ATP acted to facilitate the movement, and after the ETM formation, the difference in the region that included Tyr-32 and Tyr-34 caused by the loss of hydroxyl affects the transitional state or pathway. This effect is specific for the binding of ATP because the interaction is direct but is nonspecific (equal effects by the two mutations) for the transitional state (or pathway) modification because of the long range. These views from the steady state concept explain clearly and reasonably the specific change in the ATP-binding step of the Y34F reaction, and the alteration at the substrate site found by using the rapid equilibrium analysis that affects the chemical reaction sites distantly apart does not affect the substrate steps, even though a clear specificity difference exists. The change of Y94F parameters can also be explained in the same way as above with the conformational change accompanying substrate binding.

Conformational effects are more obviously seen in the Y32A/Y34A mutant reaction, because removal of the phenyl ring seems to hinder every conformational change during the catalytic cycle by choking the conformational pathway and deforming it by its bulky alteration and changing all of the rate parameters. The change in monophosphate specificity (V_{max}/K_{m})_{app} for Y32A/Y34A showed a great alteration, which seems to be the same for V67I and V67I/L76V mutants when the bulky alteration affected all rate constants, but in these cases the specificity was not changed. Nonlinearity of the conformational effects is shown in the V67I/L76V rate constants as the effect of the V67I mutation, which was markedly favorable for both ATP steps of the V67I reaction, became enormously unfavorable for the first ATP step but was favorable for the second ATP step when L76V mutation was added. Here the fact is stressed that for a small change in amino acids such as Tyr to Phe, the changing parameter is specific, and for large changes, such as for Val to Ile and Tyr to Ala, many or all parameters became variable through conformational effects. Also noted here is that K_{f} is affected by two factors as follows: 1) a rate with which ETM (Fig. 1) changes to an activated state, *ETM, 2) an efficiency with which *ETM species transfer the phosphate group. These two factors were not distinguished in this study, and what part of the process, i.e. the pathway to *ETM or the structure of *ETM, changed was not found. However, the difference in target interactions that act between ETM and *ETM through conformational change affects one or both of the factors. For some steps with markedly changed K_{f} values (for the first ATP of V67I/L76V, the second AMP of V67I, and the

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**Table IV**

| Coefficient | Components |
|-------------|------------|
| a_{0}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| a_{2}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| b_{0}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| b_{1}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| c_{0}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| c_{1}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| A_{1}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| A_{2}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| B_{1}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |

**Table V**

| Coefficient | Components |
|-------------|------------|
| a_{0}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| a_{2}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| b_{0}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| b_{1}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| c_{0}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| c_{1}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| A_{1}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| A_{2}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
| B_{1}       | k_{-2} \cdot k_{-1} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} + k_{-1} \cdot k_{-2} |
were mainly calculated by the largest component (coefficients in the same magnitude of rate constants; also the effective rate constants for the substrate concentration. Many rate constants are smaller than 1, the dissociation constant of the wild type and of several mutant enzymes, including Y95F, were mainly calculated by $k_{-2}$, but $K_{d}$ was mainly calculated by $k_{+1}$. $K_{d}$ of Y34F and Y32A/Y34A was calculated by $k_{+2}$. $K_{d}$ of the wild type and Y32A/Y34A, in which expressions $K_{d}$ of the wild type and Y32A/Y34A contained the dissociation constant, $k_{+2}$ of the second AMP-binding step. However, they were modified by other rate constants and were not equal to the dissociation constant itself. The rate constants of Y34F were totally unrelated to the dissociation or association of the step for $K_{d}$. Domination by the rate parameter was dependent on the magnitude of each rate constant and on the kinetic model. The discrepancies in the kinetic parameters in the rapid equilibrium model are explained by $K_{d}$ and $k_{cat}$ being determined with the same reaction scheme and with the different major rate constants contributing to $K_{d}$ and $k_{cat}$ which was occasionally replaced by other rate constants through mutation. Therefore, the correspondence between rate constants and $K_{m}$ and $k_{cat}$ was not fixed. Thus, a direct correspondence between $K_{m}$ and $k_{cat}$ was not certain, which caused alteration of $K_{m}$ and $k_{cat}$ to be different from the alteration of the rate constant at the corresponding step. The correspondence of $K_{m}$ and $k_{cat}$ to the corresponding step was not found.

Closing Remarks—The discrepancies between $K_{m}$ and $k_{cat}$ alterations and the mutations are well explained in this study. The rate constants obtained explain well the properties of the enzyme and the change in properties by mutation. The steady state model explains the alteration of $K_{d}$ and the null change of $K_{d}$ and discriminates between specific alterations and all alterations of rate constants introduced by the mutation. These explanations discussed above, together with the presumed structural alteration during enzymatic reactions, seem more rational and plausible for the ADK reaction. Therefore, analysis by using a steady state model is concluded to be more appropriate than analysis by using a rapid equilibrium model, especially for reactions of site-specifically mutated enzymes. These events are generally applicable for enzyme reactions, including those with a single substrate, if the reaction process bifurcates. Additional and more precise analyses by using a steady state model coupled with structural analysis are needed to have more precise and consistent views of enzyme reactions.

One more ATP molecule participates in the ADK reaction to bind to an AMP site at a higher concentration of ATP than used in this study, which prevents AMP binding and causes a decrease in reaction rate (inhibition). This effect was omitted from this study, but the binding of inhibitory ATP was present at a lower concentration and has to be incorporated in the mechanism in further analysis. Properties of other kinds of nucleotides, especially of CMP, such as the square dependence on $1/[\text{CMP}]$ must be considered. In this study, the rate constant at which enzyme species change to other species is preferred to explain the enzyme properties than the dissociation or association constant for the substrate and seems to give consistent views. Together with presumed conformational
change, dynamic aspects may be included in the rate constant. The substrate-binding step may have been handled unsatisfac-
torily in the kinetic analysis of this study based both on rapid
equilibrium and on steady state methods. These unsatisfactory
points also need to be improved in future studies.

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