Ligand Binding and Enzymic Catalysis Coupled through Subunits in Tyrosyl-tRNA Synthetase†

Alan R. Fersht,* Roderick S. Mulvey, and Gordon L. E. Koch

ABSTRACT: The interaction of the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* with its substrates in the aminoacyl adenylation reaction has been studied by stopped-flow fluorescence. The observed changes have been assigned to their chemical and physical processes by comparison with equilibrium dialysis, pyrophosphate exchange kinetics and rapid quenching and sampling techniques to give the rate constants for ligand binding, the formation of tyrosyl adenylate, and the reverse reaction. The stoichiometry of tyrosine and ATP binding in the catalytic process has been determined directly by equilibrium dialysis and equilibrium gel filtration under pyrophosphate exchange conditions, *i.e.*, where a steady state has been set up in which the equilibrium position favors starting materials. It is shown that the rate-determining step in the formation of tyrosyl adenylate involves 1 mole each of tyrosine and ATP. A second mole of tyrosine and ATP bind to the aminoacyl adenylate complex stabilizing the high-energy intermediate. The enzyme tyrosyl adenylate complex that is isolated by gel filtration is in a different conformational state from that in the presence of tyrosine and ATP.

In the two preceding papers (Fersht, 1975; Fersht *et al.*, 1975) it has been established that the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* rapidly forms one aminoacyl adenylate per dimer but the second site is only weakly active if at all. A general mechanism was postulated that could account for this type of behavior (Fersht, 1975). In this context we present a kinetic analysis of the formation of the aminoacyl adenylate using stopped-flow fluorescence, quenched flow, and conventional techniques.

Holler and Calvin (1972) used the fluorescence of an external probe to study the pre-steady-state kinetics of the isoleucyl activating enzyme from *Escherichia coli*. Blanquet *et al.* (1972) have shown that the tryptophanyl fluorescence of the methionyl-enzyme may be used to monitor ligand binding. In the present study the fluorescent changes that occur on mixing the tyrosyl-enzyme with ATP and tyrosine and the aminoacyl adenylate complex with pyrophosphate are directly correlated with ligand binding and chemical processes by comparison with more direct methods. It is also shown how the stoichiometry of ligand binding during the catalytic process may be determined by equilibrium dialysis or equilibrium gel filtration under the conditions of the pyrophosphate exchange reaction, *i.e.*, where there is no net turnover of substrate, but the system is at equilibrium (or steady state) and the equilibrium position favors starting materials. This is an analogous procedure to that used to study the binding of a substrate to chymotrypsin by X-ray diffraction (the "equilibrium" method, Fersht and Renard, 1974).

Experimental Section

Materials and apparatus have been described by Fersht *et al.* (1975) and Fersht (1975). The quenched-flow apparatus will be described elsewhere.

Methods. All experiments were performed under standard conditions of 25 ± 0.1°, Tris-Cl (144 mM, μ = 0.1) (pH 7.78) and 10 mM $MgCl_2$ unless otherwise stated.

Pyrophosphate Exchange. The K_M for tyrosine was found from the initial rate of incorporation of [^{32}P]pyrophosphate into ATP on incubating 0.05 μM enzyme, 2 mM ATP, and 2 mM [^{32}P]pyrophosphate (0.06–0.6 Ci/mol) and varying quantities of tyrosine under the standard conditions. Aliquots were periodically taken and quenched with 7% perchloric acid, and the [^{32}P]ATP was assayed by adsorption on charcoal and monitoring with a gas flow counter.

Stopped-Flow Fluorescence Studies. Protein tryptophan fluorescence was excited at 290 nm using a grating monochromator and emission at wavelengths greater than 325 nm monitored after passing through a cut off filter (Fersht *et al.*, 1975). The formation of aminoacyl adenylate was followed by noting the decrease in fluorescence yield on mixing enzyme (0.5–0.6 μM), incubated under the standard conditions with either tyrosine or ATP, with an equal volume of ATP or tyrosine, also incubated under the standard conditions. The binding of tyrosine to the enzyme was similarly monitored after mixing enzyme and tyrosine.

The reverse reaction was followed from the increase in fluorescence on mixing a 0.5 μM solution of enzyme bound aminoacyl adenylate (prepared by gel filtration as described by Fersht (1975) but omitting the inorganic pyrophosphatase) with pyrophosphate, both incubated under the standard conditions. For some experiments the adenylate complex was prepared in 20 mM bis-Tris-Cl (pH 6).

Comparison of Stopped-Flow Fluorescence with Quenched-Flow Studies on the Reverse Reaction. One syringe of the quenched-flow apparatus contained 0.546 μM enzyme–tyrosyl adenylate incubated in bis-Tris-Cl (pH 6, 20 mM) and 10 mM $MgCl_2$ at pH 6.0 and 25°. The other syringe contained 45 μM[^{32}P]pyrophosphate (46 Ci/mol) in 288 mM Tris (pH 7.78) and 10 mM $MgCl_2$ at 25°. Equal volumes of the two solutions were mixed and quenched automatically with 7% perchloric acid after a predetermined time (0.15–30 sec). The [^{32}P]ATP formed was monitored by adsorption on charcoal as described earlier.

Identical solutions (apart from the quenching) were mixed in the stopped-flow spectrofluorimeter as described

† From the MRC Laboratory of Molecular Biology, Cambridge CB2 2QH, England. *Received June 19, 1974.*

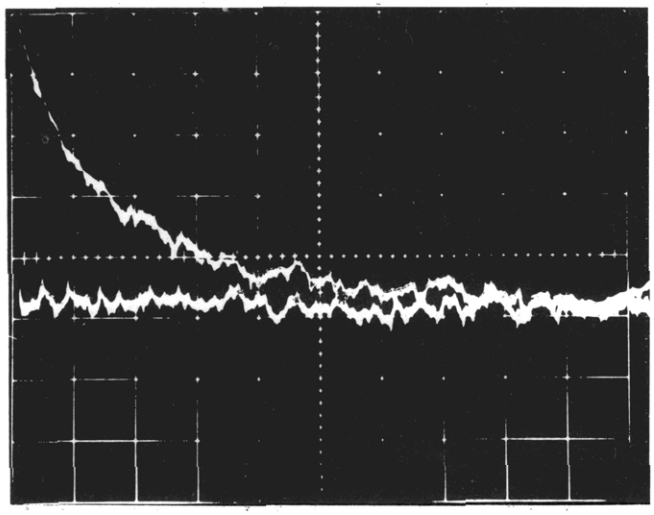


FIGURE 1: Decrease in fluorescence on mixing 25 mM ATP (pH 7.78, 25 mM $MgCl_2$) with an equal volume of 120 μM tyrosine and 0.5 μM tyrosyl-tRNA synthetase under the same conditions. Abscissa = 50 msec/(large) division; Ordinate = 20 mV/division; final total signal = 1.2 V, 10-nm slits, 2.2 msec time constant on electronic response.

above and the fluorescence changes compared with the ATP formation.

Equilibrium Dialysis. Equilibrium dialysis cells were set up as described in the preceding paper. The binding of tyrosine was measured by filling one chamber with 25.1 μM enzyme in the standard buffer and the other with 4–65 μM [^{14}C]tyrosine (30 Ci/mol) under the same conditions. The binding of tyrosine in the presence of ATP and pyrophosphate was determined using 13 μM enzyme in one chamber and initial concentrations of 15–250 μM and with either 2 mM ATP and 5 mM pyrophosphate or 10 mM ATP and 10 mM pyrophosphate in the other.

Binding of ATP. A lower limit to the binding of ATP was determined by the method of Hummel and Dreyer (1962). A 0.55 × 4.2 cm column of G-25 Sephadex (fine) was equilibrated with 21 μM [γ-^{32}P]ATP (23 Ci/mol) under the standard conditions. A freshly prepared solution (100 μl) of 24 μM enzyme in the same buffer and ATP was added to the column. Individual drops of eluant were collected in scintillation vials and the radioactivity was assayed. The individual drop sizes were measured using [^{3}H]H_2O as an internal probe as described in the previous paper. Similar experiments were performed with the column equilibrated with 21 μM [^{14}C]ATP (58 Ci/mol), 200 μM tyrosine, and 1 mM pyrophosphate under the standard conditions; 10.6 or 4.4 μM enzyme was added to the column as above.

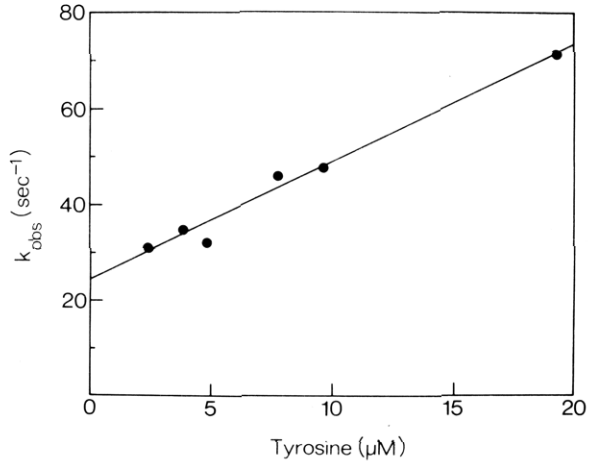


FIGURE 2: Rate constant for the binding of tyrosine to tyrosyl-tRNA synthetase (0.25 μM) both incubated at pH 7.78, 10 mM $MgCl_2$.

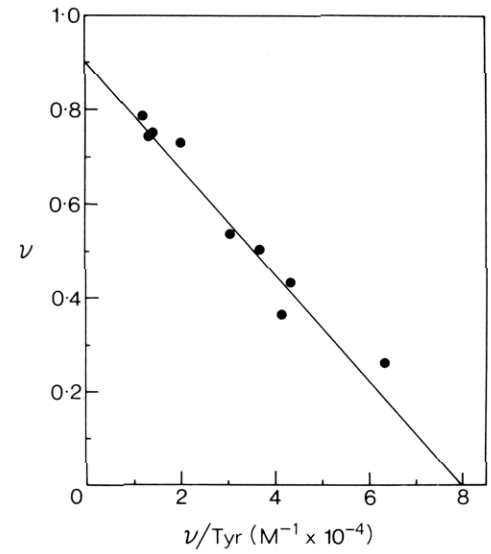


FIGURE 3: Plot of number of moles of tyrosine bound/mole of enzyme dimer (ν) against ν/[tyrosine] at pH 7.78, 10 mM $MgCl_2$.

Results

Stopped-Flow Fluorescence and Comparison with Other Data. On mixing enzyme, which had been incubated with tyrosine, with a solution of ATP a single relaxation corresponding to an 8% decrease in fluorescence is observed (Figure 1). The rate constant for this change follows saturation kinetics with respect to ATP concentration giving a K_M value of 3.9 ± 1.4 mM and a k_{cat} of 17.8 ± 1 sec^{-1}. (It is shown below that this corresponds to aminoacyl adenylate formation.) On mixing enzyme, which had been incubated with ATP, with a solution of tyrosine an initial fast decrease (~8%), in addition to the above slower change, is observed. This fast relaxation is also observed on mixing tyrosine with enzyme alone and represents binding of tyrosine to the enzyme. The rate law follows the equation: k_{obsd} = 24.4 ± 1.8 + (2.44 ± 0.18)[Tyr] (sec^{-1}) (see Figure 2). The process

$$E + Tyr \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\cdot}Tyr$$

requires a rate law: $k_{obsd} = k_{off} + k_{on}$[Tyr]. The ratio of the two derived rate constants gives a value of 10 ± 1 μM for the dissociation constant of the enzyme–tyrosine complex. This may be compared with the value of 11.6 ± 1 μM (0.92 binding site/mol) obtained from equilibrium dialysis (Figure 3).

The slower fluorescence decrease of the formation reaction is reversed in the reverse reaction. The mixing of tyrosyl-tRNA synthetase tyrosyl adenylate with pyrophosphate leads to an increase in fluorescence (see Figure 4), the rate constant of which follows saturation kinetics with a k_{cat} of 14 sec^{-1} and K_M of 0.74 mM. This fluorescence change is shown to be concurrent with the formation of ATP from the pyrophosphate and the adenylate by sampling the reaction by means of a quenched flow apparatus and directly monitoring the ATP produced. This is illustrated in Figure 5. Under conditions where the addition of pyrophosphate to the adenylate complex causes a fluorescence change with a

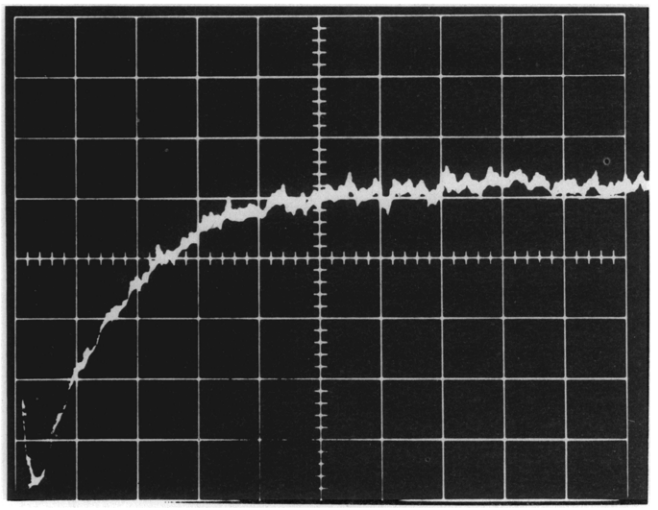


FIGURE 4: Increase in fluorescence on mixing 2.5 mM pyrophosphate (pH 7.78, 10 mM $MgCl_2$) with an equal volume of tyrosyl-tRNA synthetase–tyrosyl adenylate (0.5 μM) incubated with 250 μM tyrosine and 100 μM ATP. Instrument settings as in Figure 1 (50 msec, 20 mV/division).

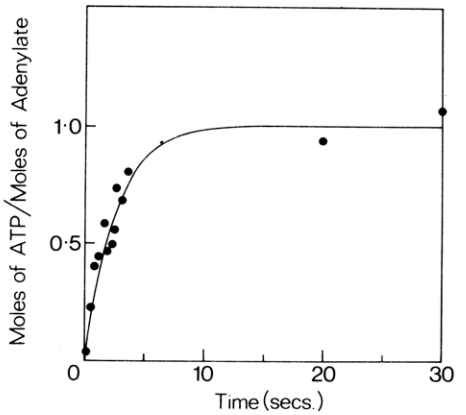


FIGURE 5: Comparison of quenched flow and fluorescence stopped flow. Conditions as in text. (●) Number of moles of [^{32}P]ATP/mol of enzyme formed from mixing 45 μM[^{32}P]pyrophosphate with [E · Tyr ~ AMP]; solid curve is that observed for the fluorescence change under the same conditions.

rate constant of 0.39 ± 0.003 sec^{-1} there is the simultaneous formation of 1 mol of ATP/mol of complex with rate constant 0.37 ± 0.04 sec^{-1}. This suggests that the decrease in fluorescence on mixing ATP with enzyme and tyrosine and also the second relaxation on mixing tyrosine with enzyme and ATP correspond to the formation of tyrosyl adenylate. This is confirmed from the pyrophosphate exchange assay experiment. In the presence of saturating tyrosine and 2 mM ATP and pyrophosphate the turnover number for the initial rate of incorporation of [^{32}P]pyrophosphate into the ATP, which is generally the rate constant for the formation of aminoacyl adenylate (Loftfield, 1972), is 5 ± 1 sec^{-1}. The corresponding rate constant for reaction of similar tyrosine concentrations with 2 mM ATP and the enzyme monitored by stopped-flow spectrofluorimetry is 6 sec^{-1}.

The K_M for tyrosine from the pyrophosphate exchange assay is 1.8 ± 0.5 μM. Stopped-flow fluorescence experiments substituting phenylalanine (which had been freed from all tyrosine by recrystallization) for tyrosine show the fluorescence change associated with the formation of aminoacyl adenylate but with a k_M of 50 mM and k_{max} of 5 sec^{-1} in the presence of 2 mM ATP (120 mM phenylalanine gave a pyrophosphate exchange rate of about 40% that of tyrosine).

Effect of ATP and Tyrosine on the Reaction of Pyrophosphate with Tyrosyl-tRNA Synthetase Tyrosyl Adenylate. The data are summarized in Table I. It has been shown (Fersht, 1975) that tyrosine binds to the adenylate with a K_S of 144 μM as does ATP with a K_S of ~3 μM. When both ligands are present the K_S values decrease to 4 μM and ~0.1 μM. It is seen in the table that the binding of these ligands causes small changes of reactivity. It will be shown later that the absence of large decreases in reactivity is important. It is of interest that the fluorescence change on formation of ATP from the reaction of the complex is greater when the complex is initially incubated with tyrosine and ATP than in the absence of ligands. The latter corresponds to just the reverse of aminoacylation, while the former involves the expulsion of the ligands from their binding sites in addition to the chemical process.

Binding of ATP to Free Enzyme. Owing to the ATPase activity of the preparation (Fersht, 1975) the binding of ATP could not be measured by equilibrium dialysis. Application of the equilibrium gel filtration procedure of Hummel and Dreyer (1962) revealed no binding of ATP under conditions of 24 μM enzyme and 21 μM ATP. As the method is sufficiently reproducible to detect at least 5% binding, this requires that the dissociation constant of the enzyme–ATP complex is greater than 400 μM. This is 200 times greater than the K_M value for the ATPase activity of the enzyme preparation (Fersht, 1975). The activity is presumably due to an impurity, present in 1 part in 10^6 or less (the observed turnover number based on total enzyme concentration being only 2×10^{-4} sec^{-1}, some six orders of magnitude below usually encountered values).

Binding of ATP during the Catalytic Process. (a) KINETIC. The variation of the rate constant, k, for the formation of tyrosyl adenylate as a function of ATP concentration is plotted in Figure 6 in the form of a Hill (1910) plot. The slope in the concentration range 0.25–25 mM is 0.95 ± 0.05. A single experiment using 1 μM [^{14}C]ATP and 0.2 μM enzyme under the standard conditions and determining the time course of [E · Tyr ~[^{14}C]AMP] formation by nitrocellulose filtration suggests this relationship holds down to 1 μM ATP.

(b) EQUILIBRIUM GEL FILTRATION. Application of the Hummel and Dreyer (1962) procedure to the binding of

TABLE I: Effect of ATP and Tyrosine on the Reaction of Pyrophosphate with Tyrosyl-tRNA Synthetase–Tyrosyl Adenylate.[a]

ATP (μM)	Tyrosine (μM)	k_{cat}[b] (sec^{-1})	K_M[b] (mM)	k_{cat}/K_M ($\equiv k_{-1}$) (sec^{-1} M^{-1} × 10^{-3})
0	0	14 ± 1.6	0.74 ± 0.15	19 ± 2
50	0	10 ± 1	0.30 ± 0.08	34 ± 5
0	125	31 ± 2	1.1 ± 0.1	28 ± 2
50	125	38 ± 5	1.6 ± 0.3	23 ± 2

[a] 0.25 μM adenylate (pH 7.78) and 10 mM $MgCl_2$, 25°. [b] Maximum rate constant and Michaelis constant for the reaction of pyrophosphate with the aminoacyl adenylate complex.

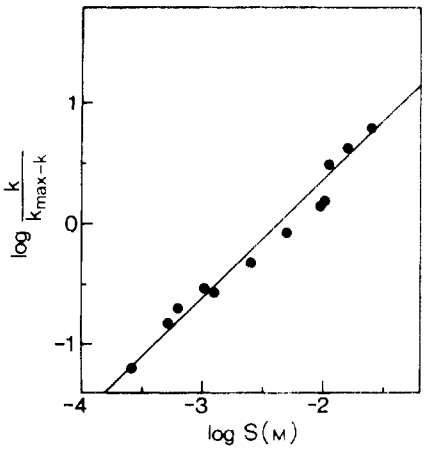


FIGURE 6: Hill plot for the rate constant (k) for aminoacyl adenylate formation against log [ATP].

ATP under catalytic conditions (1 mM pyrophosphate, 200 μM tyrosine) failed to detect any binding at 21 μM ATP and either 4.4 or 10.6 μM enzyme.

Binding of Tyrosine under Catalytic Conditions. In the presence of 1 mM ATP and 2.5 mM pyrophosphate the equilibrium dialysis studies show 1.02 mol of tyrosine bind to the enzyme with a dissociation constant of about 7.4 μM (Figure 7). At 5 mM ATP and 5 mM pyrophosphate the stoichiometry was 1.05 and the dissociation constant about 5 μM.

Discussion

The protein fluorescence changes observed on mixing the enzyme with its substrates must be assigned to the chemical and physical processes involved by correlating with additional physical measurements. This has been done by comparison of the kinetic data with those derived from equilibrium dialysis, pyrophosphate exchange kinetics, and direct sampling. On mixing tyrosine with the enzyme incubated with ATP and $MgCl_2$ there is an initial rapid decrease in fluorescence corresponding to binding followed by a slower decrease which corresponds to aminoacyl adenylate formation.

The binding of tyrosine to the tyrosyl-tRNA synthetase is slow, $k_{on} = 2.4 \times 10^6$ sec^{-1}, some two to three orders of magnitude below that of a diffusion controlled reaction (Eigen and Hammes, 1963) and also 20 times slower than the association of a small aromatic substrate with chymotrypsin (Renard and Fersht, 1973). The binding of isoleucine to the isoleucyl ligase from *E. coli* is similarly slow and a substrate induced conformational change has been suggested (Holler and Calvin, 1972). The binding of tyrosine probably similarly involves a conformational change of the enzyme since (a) the reaction is slow, (b) fluorescence changes are involved, and (c) binding of tyrosine at one site prevents binding at the second site of the tyrosyl-enzyme (Bosshard *et al.*, 1974) (and the same is proposed to occur for the isoleucyl-enzyme (Fersht, 1975)).

The formation of aminoacyl adenylate may also involve a conformational change as this is accompanied by fluorescence changes. If this is so it could be a general phenomenon as the valyl- and methionyl-enzymes from *E. coli* and *B. stearothermophilus* also undergo fluorescence changes on aminoacyl adenylate formation (Hélène *et al.*, 1971; Blanquet *et al.*, 1972; unpublished data, this laboratory).

Binding Stoichiometry and Reaction Mechanism. The

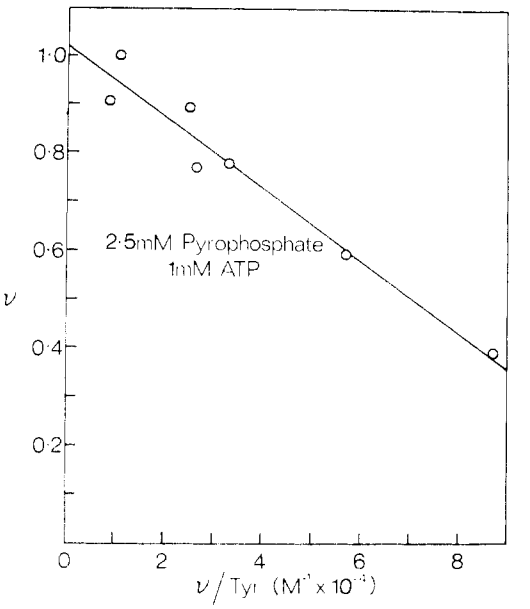
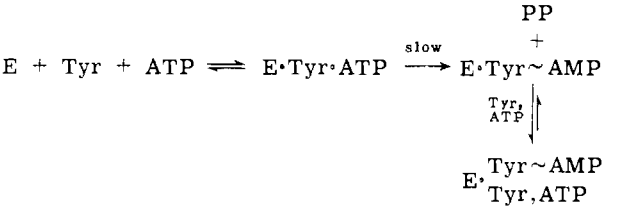


FIGURE 7: Stoichiometry of tyrosine binding (ν) under pyrophosphate exchange conditions determined by equilibrium dialysis.

formation of tyrosyl adenylate involves a single K_M for tyrosine (1.8 μM, pyrophosphate exchange) and also for ATP (3.9 mM, stopped-flow fluorescence). A Hill (1910) plot for the ATP binding gives a slope of close to one from 25 to 0.25 mM and probably down to 1 μM ATP. This suggests only 1 mol of ATP is concerned in the rate-determining step. Direct measurement of the stoichiometry of tyrosine binding under the pyrophosphate exchange conditions by equilibrium dialysis shows that only 1 mol of tyrosine is bound. Equilibrium gel filtration analysis of the binding of ATP under similar conditions shows that there is not a tightly bound ATP as well as the one binding with a K_M of 3.9 mM (the data are summarized in Table II). This means that the second ATP and the second tyrosine that bind to the tyrosyl adenylate complex bind after the rate-determining step. The simplest scheme (Scheme I) to account for the observed behavior is

SCHEME I



Only one K_M for each of ATP and tyrosine would be detected by the standard assay methods as there is always a sufficient excess of ATP or tyrosine to displace the equilibrium.

Does This Mechanism Contribute to Catalysis? In the presence of pyrophosphate the binding of additional ATP and tyrosine serves to displace the equilibrium toward products. In the presence of inorganic pyrophosphatase this would appear to be at first sight of no importance. However, the additional binding may still be of catalytic importance if the release of pyrophosphate from the products is slow and rate determining.

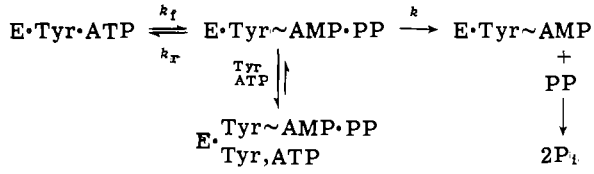
In Scheme II, if $k_f \ll k_r$, the rate constant for the formation of [E · Tyr ~ AMP] is $k_f k/k_r$. But [E ·

TABLE II: Stoichiometry of Ligand Binding.[a]

Ligand (μM)	Other Ligands	Stoichiometry and Dissociation Constant	Method
Tyrosine (4–10^3)		0.92 (10 μM)	Eq. dialysis
Tyrosine (1–10^3)	2 mM ATP, 2 mM PP	(1.8 μM)	Kinetic
Tyrosine (7–120)	1 mM ATP, 2.5 mM PP	1.02 (7.4 μM)	Eq. dialysis
Tyrosine (7–120)	5 mM ATP, 5 mM PP	1.05 (5 μM)	Eq. dialysis
Tyrosine (8–125)	6 mM ATP[b]	1.6	Eq. dialysis
ATP (20 μM)		(>400 μM)	Gel filtration
ATP (20 μM)	200 μM Tyr, 1 mM PP	(>200 μM)	Gel filtration
ATP (0.25–25 μM)	50 μM Tyr	1 (3.9 mM)	Kinetic
ATP (20 μM)	120 μM Tyr[b]	1.9	Gel filtration

[a] Standard buffer (Tris–$MgCl_2$). [b] Inorganic pyrophosphatase added.

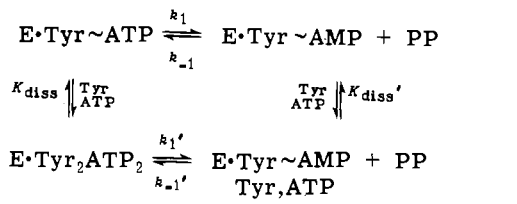
SCHEME II



Tyr ~ AMP, Tyr,ATP · PP] is formed rapidly with rate constant k_f. This could be an example of Scheme III of the proposed mechanism (Fersht, 1975).

This hypothesis may be tested by determining the rate constant for the attack of pyrophosphate on [E · Tyr ~ AMP], *i.e.*, k_r. We have done this but have come to the conclusion that the complex [E · Tyr ~ AMP] which is isolated by gel filtration exists in a different conformational state from that immediately formed on the reaction pathway. This stems from the argument based on the following thermodynamic cycle.

SCHEME III



k_1, k_1' and k_{-1}, k_{-1}' are the overall rate constants for the interconversion of substrates and products. k_{-1} (or k_{-1}') may be shown to be identical with k_{cat}/K_M for the attack of pyrophosphate (PP) on the aminoacyl adenylate complex (see Table I).

The ratio of the rate of the forward reaction by the lower route (V') to that by the upper route (V) in the cycle is given by

$$V'/V = k_1'[Tyr_2ATP_2]/k_1[Tyr,ATP] \quad (1)$$

The thermodynamic constraints on the cycle show

$$k_1'/k_1 = k_{-1}'K_{diss}/k_{-1}K_{diss}' \quad (2)$$

Substituting eq 2 and the relationship

$$[EAAATP][Tyr][ATP]/[EAA_2ATP_2] = K_{diss}$$

into eq 1 gives

$$V'/V = k_{-1}'[Tyr][ATP]/k_{-1}K_{diss}' \quad (3)$$

K_{diss} is equal to the product of the dissociation constants of ATP from the [E · AA ~ AMP,ATP] complex and the dissociation constant of tyrosine from the [E · AA ~ AMP,ATP,Tyr] complex, which we have shown to be 3 and 4 μM, respectively. It is seen in Table I that k_{-1} and k_{-1}' are approximately equal, $\therefore V'/V = [Tyr][ATP]/12 \times 10^{-12}$; *i.e.*, it is predicted that when the product of the concentrations of ATP and tyrosine is greater than 12×10^{-12} M the reaction occurs predominantly *via* the complex [E · Tyr_2ATP_2]. The reverse is found! One (or both) of the aminoacyl adenylate complexes which were isolated cannot be on the immediate reaction pathway. (Even if additional intermediates occur between [E · Tyr;ATP] and [E · Tyr ~ AMP] and [E · Tyr_2ATP_2] and [E · Tyr ~ AMP, Tyr,ATP] the same holds.) The true value of k_{-1}/k_{-1}' must be large so either the measured k_{-1}' is too high or k_{-1} too low in order for eq 3 to satisfy the observation that $V' < V$. As the equilibrium dialysis experiments show that only one tyrosine is bound in the presence of millimolar concentrations of ATP and pyrophosphate the tyrosyl adenylate cannot be accumulating under reaction conditions. Since the k_{cat} for formation of aminoacyl adenylate in the pyrophosphate exhange reaction is ~5 sec^{-1}, k_{-1}' must be much greater than 5 sec^{-1}. Clearly the value of k_{-1} is wrong and so the complex [E · Tyr ~ AMP] which is isolated is in the "wrong" conformation. The value of k_{-1} for the real intermediate [E · Tyr ~ AMP] must be very high, probably close to diffusion controlled, in order to satisfy eq 3 and the observed stoichiometry of reaction.

A high value for k_{-1} is consistent with k_r being fast in Scheme II so that the mechanism in Scheme III of Fersht (1975) could be effective here.

The demonstration of two sets of binding sites and the various binding and conformational equilibria involved effect several conclusions previously drawn by other workers and may explain some earlier observations. For example, Lotfield (1972) has pointed out that the rate of transfer of amino acid from isolated aminoacyl-tRNA synthetase aminoacyl adenylate complexes is often too slow to account for the complex being on the reaction pathway. Binding of amino acid and ATP at the second site could affect the rate and preliminary investigation suggests this is so (A. R. Fersht, unpublished data). The observation that additional amino acid and ATP may speed up the rate of dissociation of charged tRNA from the synthetase (Yarus and Berg, 1969; Hélène *et al.*, 1971) may also be accounted for by binding at the second site. Previous analyses of rate data

from pyrophosphate exchange and pre-steady-state kinetics have to be reviewed. In particular, the calculations of the free energy of formation of aminoacyl adenylates are presumably incorrect as additional binding constants are involved.

References

Blanquet, S., Fayat, G., Waller, J.-P., and Iwatsubo, M. (1972), *Eur. J. Biochem. 24,* 461.
Bosshard, H. R., Koch, G. L. E., and Hartley, B. S. (1974), submitted for publication.
Eigen, M., and Hammes, G. G. (1963), *Advan. Enzymol. Relat. Areas Mol. Biol. 24,* 13.
Englund, P. T., Huberman, J. A., Jovin, T. M., and Kornberg, A. (1969), *J. Biol. Chem. 244,* 3038.
Fersht, A. R. (1975), *Biochemistry 14,* 5.
Fersht, A. R., Ashford, J. S., Bruton, C. J., Jakes, R., Koch, G. L. E., and Hartley, B. S. (1975), *Biochemistry 14,* 1.
Fersht, A. R., and Renard, M. (1974), *Biochemistry 13,* 1416.
Hélène, C., Brun, F., and Yaniv, M. (1971), *J. Mol. Biol.* 58, 349.
Hill, A. V. (1910), *J. Physiol. (London) 40,* iv.
Holler, E., and Calvin, M. (1972), *Biochemistry 11,* 3741.
Hummel, J. P., and Dreyer, W. J. (1962), *Biochim. Biophys. Acta 63,* 530.
Loftfield, R. B. (1972), *Progr. Nucl. Acid Res. Mol. Biol. 12,* 87.
Renard, M., and Fersht, A. R. (1973), *Biochemistry 12,* 4713.
Yarus, M., and Berg, P. (1969), *J. Mol. Biol. 42,* 171.

Rabbit Skeletal Muscle Protein Kinase. Conversion from cAMP Dependent to Independent Form by Chemical Perturbations†

Laura C. Huang and Ching-hsien Huang*

ABSTRACT: Protein kinase isolated from rabbit skeletal muscle can be reversibly converted from the cAMP dependent form to the independent form by chaotropic salts and urea. A similar but irreversible conversion can also be induced by trypsin digestion of the holoenzyme. The dissociation of cAMP dependent protein kinase by low concentrations of thiocyanate raises the possibility of isolating both *native* regulatory and catalytic subunits. From various changes in enzymatic activity caused by urea and trypsin perturbation, it is proposed that the conversion of protein kinase from the cAMP dependent to the independent form is due primarily to preferential modification of the regulatory subunit of the holoenzyme.

Cyclic adenosine 3′,5′-monophosphate dependent protein kinases have been a subject of great interest in recent years (see Langan (1973); Walsh and Ashby (1973) for reviews). Several independent groups (Brostrom *et al.,* 1970; Gill and Garren, 1970; Kumon *et al.,* 1970; Tao *et al.,* 1970) have proposed the "dissociation model" to explain the mode of action of cyclic adenosine 3′,5′-monophosphate (cAMP)[1] on the protein kinase. In this model, cAMP promotes the dissociation of inactive holoenzyme to yield a regulatory subunit–cAMP complex and a free, active catalytic subunit. This dissociated catalytic subunit behaves as a cAMP independent protein kinase. Based on the simple "dissociation model," one would expect that agents which facilitate the dissociation would activate the cAMP dependent protein kinase, thus converting the protein kinase from a cAMP dependent form to a cAMP independent form.

Recently, it has been reported that chaotropic salts can induce dissociation of proteins such as β-lactoglobulin A, concanavalin A, and hemoglobin at relatively low concentrations without causing major shifts in protein conformation (Sawyer and Puckridge, 1973). In addition, it has been observed that potassium thiocyanate promotes dissociation of glutamine-dependent carbamyl phosphate synthetase into catalytically dissimilar subunits (Trotta *et al.,* 1974). These studies led us to investigate the proposal that the catalytic subunit of the protein kinase may be released from the inhibitory binding subunit upon the addition of chaotropic salts and thus make the catalytically active subunit available for phosphorylation in the absence of cAMP. In addition, other chemical agents such as urea and trypsin are well-known for their effects on the structural properties of water-soluble enzymes. They may also shift the equilibrium of the holoenzyme toward the dissociation and, accordingly, convert the protein kinase from a cAMP dependent form to a cAMP independent form.

The objectives of the present study were twofold: (1) to isolate purified cAMP dependent protein kinase from rabbit skeletal muscle and (2) to convert the enzyme from its cAMP dependent form to its independent form by chemical perturbation. From these studies, it is suggested that modification of the regulatory subunit is of primary importance in activating cAMP dependent protein kinase.

† From the Departments of Pharmacology (L.C.H.) and Biochemistry (C.H.), University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received August 22, 1974.* This research was supported in part by National Science Foundation Grant GB-38432 and U. S. Public Health Service Grant GM-17452.

[1] Abbreviations used are: cAMP, cyclic adenosine 3′,5′-monophosphate; DTT, dithiothreitol (Cleland's reagent).