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ASPECTS OF THE KINETIC PROPERTIES OF LYSYL-tRNA SYNTHETASE FROM *ESCHERICHIA COLI*, STRAIN B*

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SUMMARY

1. The amount of lysyl-tRNA formed is dependent on the quantity of lysyl-tRNA synthetase used. This is not compatible with the generally accepted mechanism whereby lysyl-tRNA is produced enzymatically.

2. The reactions for pyrophosphate exchange and for the overall formation of lysyl-tRNA have energies of activation of 16.6 and 29.0 kcal/mole, respectively over the temperature range 2–28°. The corresponding values for the entropies of activation are −1.4 and +38.4 entropy units, respectively. The second of these values is large and may indicate a marked conformational change in either tRNA or in the enzyme or in both when they interact.

3. Two values of the Michaelis constant, K_m, for lysine at 25° were found: 2.4 μM over the range of lysine concentrations 1.5–15 μM and 27 μM over the range 25–200 μM. Possible reasons for this are discussed.

4. At 25° the turnover numbers for the enzyme are 102 and 50 moles/mole of enzyme per min for the pyrophosphate exchange and formation of lysyl-tRNA, respectively, under our standard conditions.

5. It is postulated that the formation of lysyl-tRNA does not require lysyl-AMP–enzyme complex as an obligatory intermediate. A mechanism in which a dual pathway occurs is suggested for the production of the former.

INTRODUCTION

The usually accepted explanation of the mechanism whereby aminoacyl-tRNA is formed involves the production of an aminoacyl-adenylate–enzyme complex as an intermediate[1–3] (Eqn. 1),

$$\text{Amino acid} + \text{ATP} + \text{enzyme} \rightleftarrows \text{Aminoacyl-adenylate–enzyme complex} + \text{PP}_i \qquad (1)$$

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which subsequently undergoes reaction with tRNA[4] (Eqn. 2).

Aminoacyl-adenylate–enzyme complex + tRNA ⇋ Aminoacyl-tRNA + + AMP + enzyme (2)

The overall equilibrium reaction is usually represented as in Eqn. 3.

Amino acid + ATP + tRNA ⇋ Aminoacyl-tRNA + AMP + PP_i (3)

We studied some kinetic aspects of lysyl-tRNA synthetase and certain of our data make it seem likely that the formation of lysyl-tRNA, and by analogy of other aminoacyl-tRNA molecules, proceeds by a somewhat more complicated mechanism than that represented above (Eqns. 1–3). We found that our preparations of enzyme have two Michaelis K_m values for lysine and these are dependent on the substrate concentration range under investigation. We wish to compare our data with the two previously estimated values[5,6] for the Michaelis constant for lysine of this enzyme and which differed by a factor of 10. We wish to describe also the effects of changes in temperature on the catalytic properties of the enzyme, as well as the effects of tRNA on the pyrophosphate exchange reactions catalysed by lysyl and arginyl-tRNA synthetases.

EXPERIMENTAL

The preparation of lysyl- and arginyl-tRNA synthetases were described earlier[7].

The rate and extent of formation of L-*lysyl-tRNA* was generally carried out as described previously[7]. The concentrations in the assay of the various components of the reactions which were usually employed are shown in Column 3 of Table I.

The pyrophosphate exchange reaction was usually assayed with the use of a stock solution which consisted of (i) 0.5 ml 0.5 M Tris–HCl buffer (pH 7.8), which contained also 0.04 M $MgCl_2$, (ii) 0.125 ml tRNA (10 mg/ml) or 0.125 ml water depending on the assay, (iii) 0.05 ml 0.1 M L-lysine, (iv) 0.225 ml water, (v) 0.05 ml of a neutralised (NaOH) solution which was 0.1 M in both ATP and $MgCl_2$ and (vi) 0.05 ml 0.1 M

TABLE I

THE CONCENTRATIONS OF THE COMPONENTS USED IN THE PYROPHOSPHATE EXCHANGE REACTION AND IN THE FORMATION OF L-LYSYL-tRNA

Component	*Concn. in the assay mixture (mM)*	
	Pyrophosphate exchange	*Formation of* L-*lysyl-tRNA*
Tris buffer	100	100
Lysine	2	0.1
ATP	2	1
Mg^{2+}	10	9
tRNA*	50 μg/0.1 ml**	100 μg/0.1 ml***
Pyrophosphate	2	—

* In many of the pyrophosphate exchange experiments, no tRNA at all was added.

** This is total stripped tRNA. A concentration of 50 μg/0.1 ml is equivalent to a concentration of $tRNA^{Lys}$ of 1 μM, on the assumption that tRNA has a molecular weight of 25 000 and that $tRNA^{Lys}$ constitutes 5% of the total.

*** Equivalent to a concentration of $tRNA^{Lys}$ of 2 μM.

[^{32}P]pyrophosphate (activity 0.05–0.1 μC/μmole). To 40-μl fractions of this cold (4°) solution were added 55-μl volumes of water followed by 5-μl volumes of enzyme in solution of bovine serum albumin (1 mg/ml). The concentrations of the various components present under assay conditions are summarised in Table I. After incubation of the tubes at the desired temperature for measured periods of time, including one at zero time, the reaction was stopped by the addition of 0.1 ml of 7% perchloric acid in 0.2 M sodium pyrophosphate solution. Acid-washed Norit-A was added (0.5 ml of a suspension in water of 30 mg/ml) and the mixture was filtered on a Millipore filter. The precipitate and the filter were thoroughly washed with cold water and transferred to an appropriate holder, where they were moistened with a drop or two of 0.06% polyvinylpyrrolidone solution. The holder was heated in an oven at 70° until the precipitate was dry (30 min). The radioactivities of the ATP in the samples were determined on a Nuclear Chicago gas flow counter, and compared with the specific activity of the pyrophosphate used at the same time.

RESULTS

The effect of enzyme concentration on the rate and extent of reaction

There is no rectilinear relationship between the amount of lysyl-tRNA formed and the time of reaction over any period at any of the enzyme concentrations which were used (Fig. 1). It should be emphasised that the true initial rate of formation of lysyl-tRNA could be obtained only by measurements of the extent of formation after various periods of time and with the use of these values to determine the tangent at the origin of curves of the form shown (see also ref. 8). The initial rates of formation of lysyl-tRNA are directly proportional to the amount of enzyme used (Fig. 2; see also ref. 9).

The total amount of lysyl-tRNA formed increases with the amount of enzyme used over a 10-fold range of concentrations of the latter. This is not attributable to

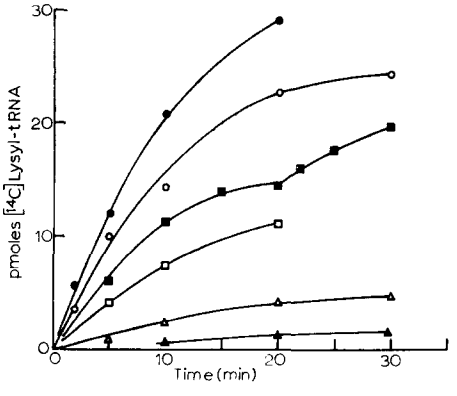


Fig. 1. The production at 27° of lysyl-tRNA as a function of time. Various dilutions, in bovine serum albumin, of lysyl-tRNA synthetase (originally 162 μg/ml) were employed: ●—●, 1:100 dilution; ○—○, 1:150; ■—■, 1:200; □—□, 1:300; △—△, 1:500; ▲—▲, 1:1000. In the experiment shown involving a 1:200 dilution of enzyme further amounts of tRNA (5 μl of a solution containing 5 mg/ml) were added after incubation for 20 min.

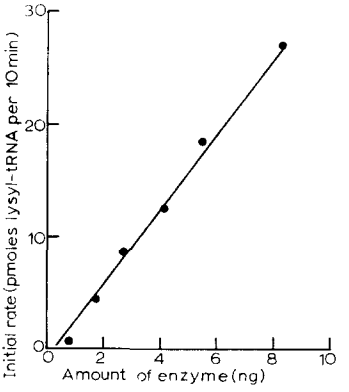
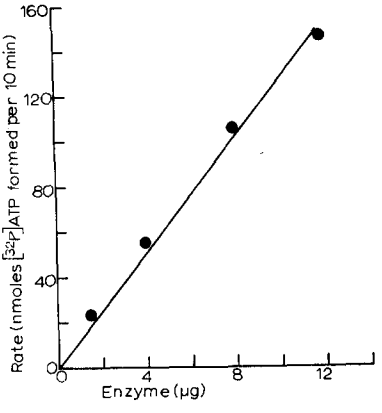


Fig. 2. The relationship between the initial rate of formation of lysyl-tRNA and the amount of lysyl-tRNA synthetase used. Temp. 27°.



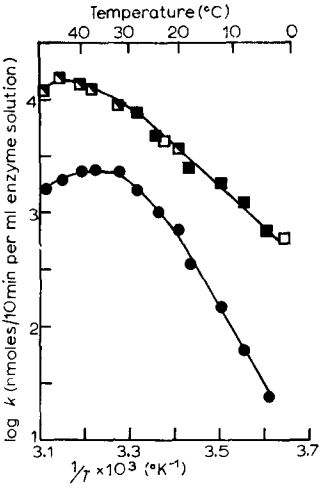


Fig. 3. The relationship between the initial rate of incorporation of [^{32}P]pyrophosphate into ATP and the amount of lysyl-tRNA synthetase used. Temp. 25°.

Fig. 4. The initial rates at various temperatures of (a) Lysyl-tRNA formation catalysed by lysyl-tRNA synthetase (●—●) and (b) the incorporation of [^{32}P]pyrophosphate into ATP in absence of tRNA (□—□) and in presence of tRNA (■—■). Coincident points for the pyrophosphate exchange reaction (b) are indicated thus ◥◣.

instability of the enzyme because the addition of further tRNA to the system at equilibrium causes the reaction to proceed further (Fig. 1). The finding that the amount of lysyl-tRNA formed depends on the quantity of enzyme employed is thermodynamically at variance with the proposition that the equilibrium reaction is represented by Eqn. 3.

The initial rate of incorporation of [^{32}P]pyrophosphate into ATP is rectilinearly dependent on the amount of enzyme used (Fig. 3).

Increasing the temperature was found to increase the rates of formation of lysyl-tRNA and also that of incorporation of pyrophosphate into ATP. From 2° to about 28° the changes in rate observed obey the Arrhenius equation of the form $k = Ae^{-E/RT}$ (Fig. 4). The values of the energies of activation were calculated from these data for temperatures up to 28° (Column 2, Table II).

Above 28° there is a progressive falling off in the rate of both reactions until at about 40° there is a rapid loss in enzymatic activity.

TABLE II

KINETIC CONSTANTS FOR LYSYL-tRNA SYNTHETASE

Reaction	*E (cal)**	*Frequency factor (moles/mole enzyme per min)*	*ΔS* (25°) (e.u.)*
Pyrophosphate exchange	16 600	$1.5 \cdot 10^{14}$	−1.4
Lysyl-tRNA formation	29 000	$9.1 \cdot 10^{22}$	+38.4

* In an abstract[10] we have erroneously assigned the lower value for the activation energies shown here to the reaction involving formation of lysyl-tRNA.

Changes in the concentration of lysine affect the initial rate of formation of lysyl-tRNA. The curve obtained by plotting the rate, v, at 25° against lysine concentrations, at a constant concentration of all the other components (Table I), is not of a simple form. The characteristics of the curve are more apparent by plotting v against $v/[S]$ (Fig. 5) (refs. 11–13). This line has two distinct regions and the values of K_m at 25° calculated for these are $2.4 \cdot 10^{-6}$ M over lysine concentrations from 1.5 to 15 μM, and $27 \cdot 10^{-6}$ M over the range 25–200 μM. The biphasic form of the curve may explain in part the apparent discrepancy between the results of STERN AND MEHLER[5] who calculated a K_m value for lysine at a higher temperature (37°) of $1.6 \cdot 10^{-6}$ M over part of the lower range of substrate concentrations which we used (0.5–4 μM) and those of WALDENSTRØM[6] who reported a K_m value, also at 37°, of $18 \cdot 10^{-6}$ M. WALDENSTRØM[6] did not state the range of substrate concentrations at which his measurements were made.

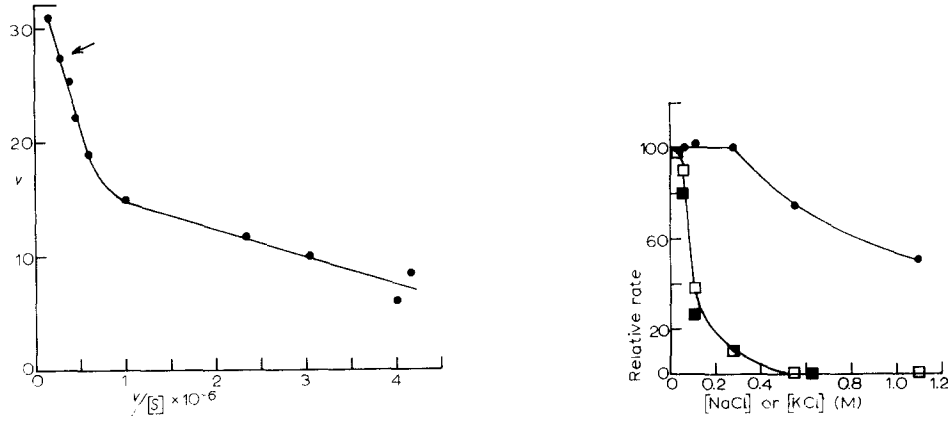


Fig. 5. Relationship between the initial rate of formation of lysyl-tRNA at 25° (v) and the lysine concentration [S]. The data are plotted as v against $v/[S]$ (refs. 11–13). The point marked with an arrow indicates that for which the substrate concentration is 0.1 mM, which was that used in the standard conditions (Table I).

Fig. 6. The effect of Cl^- on the activity of lysyl-tRNA synthetase: ●—●, relative rates of incorporation of [^{32}P]pyrophosphate into ATP in presence of added NaCl. The effects of NaCl (□—□) and of KCl (■—■) on the rate of formation of lysyl-tRNA are also shown: Coincident points for the latter reactions are represented thus ◥.

There are clearly two calculated values of v_{max} which are 6300 and 3100 nmoles lysyl-tRNA formed per 10 min per mg enzyme for the high and low ranges of substrate concentrations, respectively. These values correspond to catalytic constants at 25° of 50 and 25 moles product per min per mole enzyme on the assignment of a molecular weight of 80 000 to the enzyme[7]. The higher of these values was used to calculate the values of A and of the entropy of activation shown in Table II.

NaCl and KCl markedly reduce the rate of production of lysyl-tRNA, but they have very much less effect on the rate of the pyrophosphate exchange (Fig. 6). Thus 0.3 M NaCl has little effect on the rate of the latter reaction but it reduces the rate of formation of lysyl-tRNA by over 90%. This is similar to the effects of NaCl on valyl-tRNA synthetase[14].

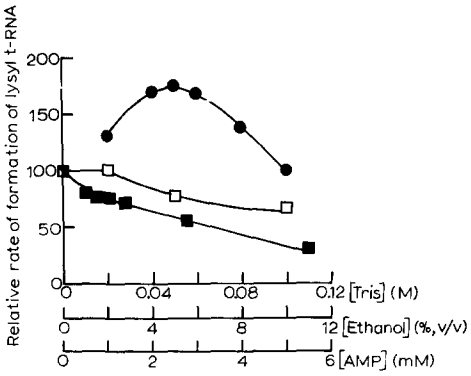


Fig. 7. The variation of the initial rate of formation of lysyl-tRNA (a) with changes in the concentration of Tris buffer used (●—●) expressed as a percentage of the rate under standard conditions (0.1 M Tris buffer); (b) in presence of ethanol at various concentrations (□—□) and (c) in presence of AMP (■—■). The temperature was 25°.

The concentration of Tris buffer used in the reaction also affects the rate of lysyl-tRNA formation (Fig. 7), and the amount which was usually employed was not that at which maximum rates could be obtained.

Small concentrations of ethanol have little effect on the rate of aminoacylation, and larger concentrations (> 0.3 M) markedly inhibit the reaction (Fig. 7), in contrast to the results obtained with valyl-tRNA synthetase[14].

Adenosine 5'-monophosphate inhibits the rate of aminocylation (Fig. 7).

The concentration of ATP was varied from 0.1 to 1.5 mM with all other concentrations remaining constant (Table I), and this resulted in no changes in the rate of formation of lysyl-tRNA at 37°. The ATP was thus always present in saturating concentrations.

The effect of tRNA on the rate of pyrophosphate exchange. It was found that lysine and lysyl-tRNA synthetase would effect an incorporation of [^{32}P]pyrophosphate into ATP as well in absence as in presence of tRNA (Fig. 4). On the other hand arginyl-tRNA synthetase catalyses the pyrophosphate exchange reaction only in presence of added tRNA. In these experiments, the results of which are described in Table III, the conditions used were similar to those described in Table I, but the values of pH were different: for the pyrophosphate exchange reaction the pH was 7.2 and for arginyl-tRNA formation it was 7.4. The concentrations of arginine were the same as those of lysine which was used in other experiments. The rate of the pyrophosphate exchange reaction in the presence of unfractionated tRNA was found to be about 60 times that of the formation of arginyl-tRNA (Table III). No incorporation of [^{32}P]pyrophosphate into ATP occurred at all when no tRNA was added. When partially fractionated tRNA, which was about 2.5 times richer in $tRNA^{Arg}$, was used there was a corresponding increase in the rate of the pyrophosphate exchange reaction. Similarly when tRNA which contained proportionately less $tRNA^{Arg}$ was used the rate of the pyrophosphate exchange reaction was decreased. These data, concerned with arginyl-tRNA synthetase, were obtained by Dr. Claude Janeway and they confirm the more extensive studies of MEHLER AND MITRA[15,16].

The ratio of absorbances at 280 and 260 mμ for lysyl-tRNA synthetase at pH 7.2

TABLE III

THE EFFECT OF ADDED tRNA ON THE RATE OF INCORPORATION OF ^{32}P-PYROPHOSPHATE INTO ATP

tRNA used	*Rate of reaction (nmoles/10 min per mg tRNA*	
	Pyrophosphate incorporation	*Arginyl-tRNA formation*
Unfractionated	78	1.3
*Enriched in $tRNA^{Arg}$	169	3.2
*Impoverished in $tRNA^{Arg}$	5	0.08

* These fractions were given to us by Dr. P. Schofield.

is 1.32. This might correspond to a nucleic acid content of 1.16% if we accept the proposition of WARBURG AND CHRISTIAN[17], which is equivalent to not more than 0.03 mole of tRNA per mole of enzyme of molecular weight 80 000 (ref. 7).

The stability of the enzyme to heat. Measurements of the stability of the enzyme to the effects of increases of temperature appeared to indicate that at 40° and above the enzyme rapidly lost its enzymatic activity (Fig. 8). These findings are in keeping with an earlier statement[5].

DISCUSSION

The findings that the amount of tRNA which becomes aminoacylated is dependent on the amount of enzyme used is not in accord with the simple interpretation of an equilibrium reaction of the form depicted in Eqn. 3. Such studies confirm the earlier work of ZILLIG *et al.*[18] as well as other studies carried out with heterologous systems[19,20], that is ones in which activating enzymes and their cognate tRNA molecules were from different species.

Apparent equilibrium constants, K_3, for the reaction represented in Eqn. 3 were reported to be 0.32 for valyl-tRNA synthetase of *Escherichia coli*[21], and 0.37 and 0.7 for threonyl-tRNA synthetases of calf[22] and guinea pig[23] liver, respectively. In all cases the amounts of tRNA and enzyme did not vary markedly in a given series of experiments. Lysyl-tRNA synthetase, as well as a number of other analogous enzymes, catalyses the incorporation of [^{32}P]pyrophosphate into ATP by a reaction path which

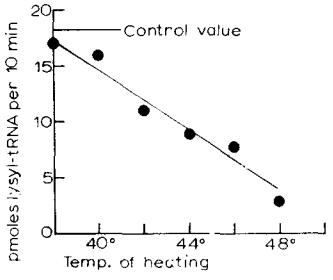


Fig. 8. The stability of lysyl-tRNA synthetase at different temperatures. Enzyme solutions were heated for 10 min at various temperatures. After dilution they were assayed at 25°.

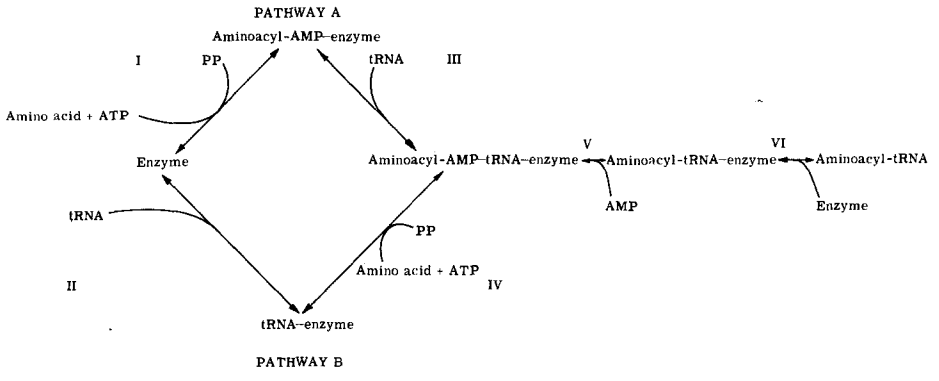


Fig. 9. A simplified representation of two possible pathways for the formation of lysyl-tRNA catalysed by lysyl-tRNA synthetase.

does not involve the participation of tRNA (Fig. 4). It is reasonable to believe that the enzyme interacts directly with tRNA, in the manner which has been demonstrated directly for some of these types of enzymes[24,25]. We should like to propose, therefore, that these enzymes in general interact with the various substrates in a scheme such as is represented in a simplified form in Fig. 9.

Step I represents the pyrophosphate incorporation reaction which occurs in the absence of tRNA. It is possible that this is the dominant reaction concerned with the pyrophosphate exchange reaction catalysed by lysyl-tRNA synthetase in view of the fact that tRNA has no effect on the rate of this reaction under the conditions employed (Fig. 4). Steps III, V and VI are those concerned with the interconversion of aminoacyl-adenylate–enzyme and aminoacyl-tRNA. Whether lysyl-tRNA formation proceeds wholly or in part by this pathway is unknown, but it is certainly possible since direct observations have been made with lysyl-adenylate–enzyme complex[26] as well as with other aminoacyladenylate–enzyme complexes[27–30].

As already mentioned the reaction represented by Step II is known to occur with certain of these enzymes[24,25] and a conformational change in tyrosyl-tRNA synthetase when it interacts with tRNA was deduced to have occurred from optical rotatory dispersion measurements[31]. The analogous reaction, Step VI, was demonstrated for several of the enzymes[24]. It would seem probable that the incorporation of [^{32}P]pyrophosphate into ATP, which is catalysed by arginine and arginyl-tRNA synthetase only in presence of $tRNA^{Arg}$, proceeds through the equilibria represented by Steps II and IV (Table III and refs. 15 and 16).

It is reasonable to believe that the various rate constants for the reactions indicated in Fig. 9 may all vary from one enzyme and its cognate tRNA to another, and hence the extents to which Pathways A and B are followed. This implies that the absolute specificities of an aminoacyl-tRNA synthetase and the corresponding enzyme–tRNA complex may be different, as has been stressed by LOFTFIELD AND EIGNER[8,32,33]. It may be seen in a qualitative way from the scheme represented in Fig. 9 how tRNA may affect the rate of pyrophosphate incorporation into ATP markedly differently from one enzyme to another[34], for the effect observed will depend upon the relative values of the rate constants and on the concentrations of amino acid and of ATP.

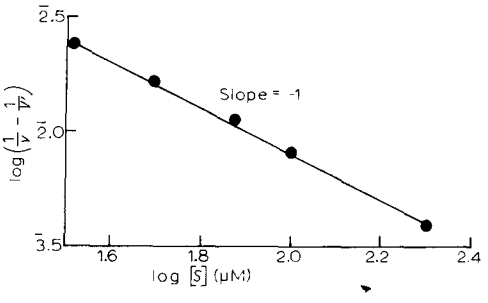


Fig. 10. The relationship between log lysine concentration [S] and log ((1/v)-(1/V')) (ref. 38), where v is the rate at substrate concentration [S], and V' is the maximum rate derived from the higher levels of substrate concentrations described in Fig. 3.

There are several possible explanations for the finding of a biphasic form of the curve of v against $v/[S]$ (Fig. 5). In the first place it is possible that the enzyme consists of one or more closely related molecular forms[7] each giving rise to a different value of K_m for lysine. It might result from the presence of two binding sites for lysine on the enzyme, since a plot of log $((1/v)-(1/V'))$ against log $[S]$ for the data shown in Fig. 5 where V' is the maximum rate of reaction at the higher substrate concentrations, gives rise to a straight line whose slope is unity (Fig. 10). This finding could indicate a two to one substrate–enzyme interaction at the higher substrate levels if one accepts the theoretical considerations of KWON AND BROWN[35].

Finally one must take into account the likelihood that kinetic data will be complicated for a scheme such as that envisaged in a simplified way in Fig. 9. Even more equilibria which involve the enzyme with ATP on the one hand and with lysine on the other may well be involved.

The activation energies for the two reactions catalysed by the enzyme differ markedly (Table II). The ratio of the rate of pyrophosphate exchange compared with that of formation of lysyl-tRNA thus varies from about 22 at 5° to a value of about 5 at 25°, under the particular conditions that we have employed. Above about 28° there is a marked falling off in the rates of both reactions. At least in the case of the transfer reaction this is not due wholly to reversible inactivation of the enzyme until temperatures of about 40° are reached (Fig. 8). It is reasonable to suggest that at temperatures between 28 and 40° the enzyme takes up, to an increasing extent, conformation(s) which are not fully enzymatically active at those temperatures, but which may revert to an active form.

The large positive value of the entropy of activation (Table II) in the formation of lysyl-tRNA is of interest. A definitive interpretation of this finding cannot be made at present in view of the large number of intermediates likely to be involved in the overall reaction. It may be an indication that formation of the kinetically important enzyme–substrate complexes involves an interaction of two oppositely charged species. These species are probably lysyl-tRNA synthetase on the one hand and $tRNA^{Lys}$ on the other. Since both of these are negatively charged, overall, at the pH at which the transfer reaction is carried out it is suggested that the metal ions, which are necessary for reaction to occur, function by becoming absorbed to either enzyme or tRNA in such a way as to completely change its charge characteristic.

Recently experiments were described the results of which cast doubt on the occurrence of valyl-adenylate–enzyme complex as an intermediate in the formation of valyl-hydroxamate catalysed by valyl-tRNA synthetase[36].

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The interpretation of the data in Fig. 1 must be considered in the light of the use of unfractionated tRNA, so that there may be competition for the recognition site on the enzyme among the various amino acid specific tRNA molecules present. We do not know what effect this may have on the total yield of lysyl-tRNA and it is clear that such experiments must be carried out with pure $tRNA^{Lys}$.

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