Fast Kinetic Study of Yeast Phenylalanyl-tRNA Synthetase: Role of $tRNA^{Phe}$ in the Discrimination between Tyrosine and Phenylalanine†

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ABSTRACT: An extensive study of the discrimination between phenylalanine and tyrosine by yeast phenylalanyl-tRNA synthetase was carried out in the presence of native $tRNA^{Phe}$. Our results clearly show that, besides the previously reported dissociation of tyrosyl adenylate from the enzyme template [Lin, S. X., Baltzinger, M., & Remy, P. (1983) *Biochemistry 22*, 681–689], two more correction processes are involved in the rejection of tyrosine in the presence of $tRNA^{Phe}$. A minor part of the misactivated tyrosine is indeed transferred to $tRNA^{Phe}$, but the resulting misaminoacylated tRNA is very rapidly hydrolyzed ($k_h \simeq 60\ s^{-1}$), as it has already been shown for other systems. However, the major part of the misactivated tyrosine is rejected as the result of a pretransfer correction consisting of the fast hydrolysis ($k'_h \simeq 20\ s^{-1}$) of the enzyme-bound noncognate adenylate induced by the binding of native $tRNA^{Phe}$. The transfer step itself is found to be nonspecific, as the rate constant is almost the same for phenylalanine and tyrosine. This result is supported by the observation that tyrosine and phenylalanine are also transferred at the same rate to $tRNA^{Phe}_{ox\text{-}red}$. It is shown that the integrity of the 3′-terminal adenosine of the tRNA is critical for triggering the pretransfer hydrolysis of enzyme-bound noncognate aminoacyl adenylate. A detailed kinetic analysis is presented that shows that the observed rate constant of $tRNA^{Phe}$ tyrosylation and the rate of disappearance of enzyme–tyrosyl adenylate complex are in fact apparent rate constants. According to the proposed model, these apparent rate constants are equal to the sum of the rate constants for the transfer step and the pretransfer enzymatic hydrolysis of the noncognate aminoacyl adenylate.

For a long time, numerous studies have been devoted to the accuracy of the aminoacylation reaction, a crucial step for the fidelity of protein synthesis. In the literature, most reports have considered the enzymatic hydrolysis of the misaminoacylated tRNA as the dominant, if not unique, step ensuring the specificity.

Our previous results (Lin et al., 1983) showed that yeast phenylalanyl-tRNA synthetase is able to discriminate efficiently tyrosine from phenylalanine at the level of the adenylation reaction. However, tyrosine does give rise to tyrosyl adenylate–enzyme formation. It was therefore of interest to study the fate of the misactivated tyrosine in the presence of $tRNA^{Phe}$. This paper will report the results obtained in this study and propose a more complete mechanism ensuring the accuracy of tRNA aminoacylation.

Materials and Methods

L-Tyrosine and L-phenylalanine were bought from Merck (Darmstadt, FRG). As commercial tyrosine usually contains $\simeq$0.5% of phenylalanine, it was purified by nine successive recrystallizations, as previously described (Lin et al., 1983). Radioactive amino acids, L-[^{14}C]tyrosine (350–450 mCi/mmol) and L-[^{14}C]phenylalanine (400–500 mCi/mmol), were purchased from the Commissariat à l'Energie Atomique (Saclay, France) and were used without further purification. 6-*p*-Toluidinonaphthalene-2-sulfonate (TNS),[1] inorganic pyrophosphatase (EC 3.6.1.1), and ATP were from Sigma (St Louis, MO). Yeast $tRNA^{Phe}$ (1720 pmol/OD_{260}) was purified by countercurrent distribution (Dirheimer & Ebel, 1967) and BD-cellulose chromatography (Ehrlich et al., 1980). Yeast phenylalanyl-tRNA synthetase (EC 6.1.1.20) was purified according to Fasiolo & Ebel (1974). The specific activity usually ranges around 3600 nmol·min^{-1}·mg^{-1} under the conditions described by Fasiolo et al. (1977). All other chemicals used were of the best available purity.

Preparation of Modified tRNAs. (*A*) $tRNA^{Phe}_{ox}$. Native $tRNA^{Phe}$ (final concentration $\simeq$160 μM) was incubated with 10 mM $MgCl_2$ and 1 mM freshly prepared sodium periodate, in 0.1 M sodium acetate buffer, pH 4.5, for 30 min in the dark at 0 °C. The reaction was stopped by the addition of glycerol (10% v/v final concentration). The oxidized tRNA was then extensively dialyzed against distilled water.

(*B*) $tRNA^{Phe}_{ox\text{-}red}$. Oxidized $tRNA^{Phe}$ was subsequently reduced under the following conditions: $tRNA^{Phe}_{ox}$ ($\simeq$100 μM) was incubated for 50 min at 0 °C in the presence of 5 mM sodium borohydride freshly solubilized in 50 mM phosphate buffer, pH 8. To stop the reaction, the excess of borohydride was destroyed by acidification of the reaction mixture to pH 4–5, and the $tRNA^{Phe}_{ox\text{-}red}$ was recovered by ethanol precipitation at −20 °C.

(*C*) $tRNA^{Phe}_{2'deoxyA}$. This was synthesized according to the method of Sprinzl et al. (1973) as described by Remy & Ebel (1976).

Quenched-Flow Experiments. Fast kinetic measurements were carried out in the range of 5–500 ms to study both the aminoacyl-tRNA formation and the aminoacyl adenylate consumption after mixing the aminoacyl adenylate–enzyme complex with the tRNA. They were performed in a quenched-flow apparatus built in our laboratory (Gangloff et al., 1984) that allowed the measurement of a complete kinetic curve in a single stroke.

The experiments to detect the transfer of the misactivated tyrosine to $tRNA^{Phe}$ were performed as follows. The first syringe contained all compounds required to form the aminoacyl adenylate–enzyme complex: 5.9 μM phenylalanyl-tRNA synthetase, 64 μM [^{14}C]tyrosine (388 mCi/mmol) (this high

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[1] Abbreviations: TNS, 6-*p*-toluidinonaphthalene-2-sulfonate; Tris, tris(hydroxymethyl)aminomethane; $tRNA^{Phe}_{ox}$, $tRNA^{Phe}$ with the 3′-terminal adenosine oxidized by periodate; $tRNA^{Phe}_{ox\text{-}red}$, $tRNA^{Phe}$ oxidized by periodate and reduced by sodium borohydride; $tRNA^{Phe}_{2'deoxyA}$, $tRNA^{Phe}$ in which the 3′-terminal adenosine has been replaced by 2′-deoxyadenosine; BD-cellulose, benzoylated DEAE-cellulose.

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specific activity was used to increase the accuracy of the measurements), 10 mM ATP–$MgCl_2$, 20 units/mL inorganic pyrophosphatase, 0.5 mM dithioerythritol. The second syringe contained 20 μM $tRNA^{Phe}$. All solutions were buffered with 50 mM Tris-HCl, pH 7.5, containing 150 mM KCl and 10 mM $MgCl_2$. The reaction was carried out at 23 °C. The quencher used was solution A (20% w/v trichloroacetic acid containing 5 mM nonradioactive tyrosine). In order to avoid the retention of the precipitated aminoacyl-tRNA on the walls of the fraction-collecting rotor, each well of the latter was preloaded with 200 μL of quenching solution A. After the reaction, the different fractions were withdrawn from the collector with a smooth-tip Pasteur pipet, and each well was rinsed 3 times with 1 mL of solution B (10% trichloroacetic acid containing 5 mM nonradioactive tyrosine). The washings were added to the corresponding fractions, and the aminoacyl-tRNA was filtered on glass-fiber disks (Whatman GF/C). The filters were then dried, and the radioactivity was measured by scintillation counting in Omnifluor/toluene scintillator (4 g/L).

The same solutions were used in the measurement of the adenylate consumption after they were mixed with the tRNA, except that the quenching solution used was 0.4 M HCl. After the reaction, 4-μL aliquots were removed from the different wells of the fraction-collecting rotor and applied to thin-layer cellulose plates (Polygram CEL 300), which were developed at 4 °C in butanol–acetic acid–water (7:2:3 v/v/v) as described by Jakubowski et al. (1977).

Stopped-Flow Experiments. The consumption of the enzyme-bound tyrosyl adenylate after it was mixed with native $tRNA^{Phe}$ was also monitored by stopped-flow fluorescence spectroscopy, with TNS as the fluorescent reporter group in a Durrum-Gibson stopped-flow apparatus under the conditions previously described (Lin et al., 1983). Using the same technique, we also measured the kinetic association and dissociation rate constants of chemically synthesized tyrosyl adenylate [see Lin et al. (1983)] to phenylalanyl-tRNA synthetase in the presence of native $tRNA^{Phe}$ (0.5 μM).

Hydrolysis of Enzyme-Bound Tyrosyl and Phenylalanyl Adenylates in the Presence of Modified $tRNAs^{Phe}$. As the reactions were usually much slower with modified tRNAs, kinetics were followed in a Jobin Yvon JY3C fluorometer, under the conditions previously reported (Lin et al., 1983). The solution contained 50 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.1 mM dithioerythritol, 50 μM TNS, and 5 μM phenylalanyl-tRNA synthetase in the absence or in the presence of 11 μM $tRNA^{Phe}_{ox}$ or $tRNA^{Phe}_{2'deoxyA}$. The fluorescence intensity of this solution was considered as 100%, and the chemically synthesized adenylate was added at zero time, resulting in an important quenching of the fluorescence intensity. The recovery of luminescence with time was then recorded for several minutes, monitoring the hydrolysis of the added adenylate.

Measurement of the First Turnover of Phenylalanine and Tyrosine Transfer to $tRNA^{Phe}_{ox\text{-}red}$. As these reactions were too slow to be conveniently monitored with our quenched-flow apparatus and too fast for the conventional manual procedure, we used a special method, previously described by Baltzinger & Holler (1982), based on mixing of droplets of reagents and quenchers on a Parafilm sheet. The reaction mixtures and quenching solutions used were those described in section dealing with quenched-flow measurements. Several droplets of 20 μL of one of the reaction mixtures were placed on a Parafilm sheet. Close to them ($\simeq$5 mm) were placed droplets of 40 μL of quenching solution. With a Gilson micropipet,

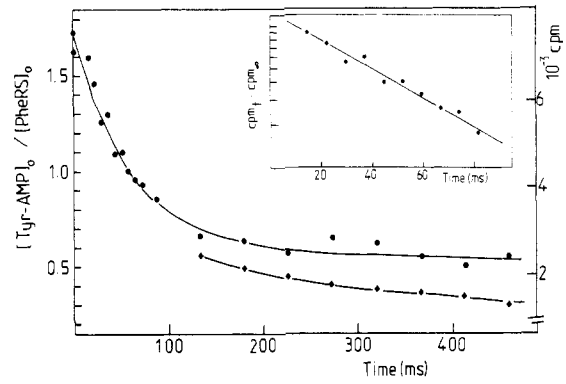


FIGURE 1: Disappearance of tyrosyl adenylate upon binding of native $tRNA^{Phe}$ to the enzyme template. A solution containing 5.9 (●) or 11.8 μM (♦) enzyme, 64 μM [^{14}C]tyrosine (388 mCi/mmol), 10 mM ATP–$MgCl_2$, 20 units/mL inorganic pyrophosphatase, and 0.5 mM dithioerythritol was mixed in a 1:1 (v/v) ratio in a quenched-flow apparatus with a solution containing 20 μM $tRNA^{Phe}$. Both solutions were buffered with 50 mM Tris-HCl, pH 7.5, 150 mM KCl, and 10 mM $MgCl_2$. Tyrosyl adenylate concentration was followed as described under Materials and Methods. The fast exponential decay is characterized by a rate constant of 27 s^{-1} as shown by the first-order plot in the insert.

20 μL of the second reaction mixture was rapidly introduced at zero time in the 20-μL droplet of the first one, mixed with the extremity of the tip, allowed to react for a short period of time, and finally mixed with the adjacent droplet of quenching solution. The droplets were then withdrawn from the Parafilm sheet with a micropipet, and the spot where each droplet stood was rinsed 3 times with 200 μL of quencher A. Reaction mixtures and washings were combined, and the aminoacyl-tRNA formed was filtered on glass-fiber disks. The radioactivity was then measured as described above.

Using this technique, it was possible to measure kinetics in the range of one second to a few minutes. Duplicate or triplicate measurements were done, especially for the shortest reaction periods (1–4 s).

Results and Discussion

Consumption of Enzyme-Bound Tyrosyl Adenylate upon Binding of $tRNA^{Phe}$. The fate of enzyme-bound tyrosyl adenylate after being mixed with native $tRNA^{Phe}$ was studied as described under Materials and Methods. As can be seen in Figure 1, the disappearance of the radioactive tyrosyl adenylate is clearly biphasic. The first part of the curve can be fitted with an exponential decay of rate constant 27 ± 2 s^{-1}. The asymptote of this first process would correspond to a 0.7 stoichiometry (moles of adenylate per mole of input enzyme). Indeed, the concentration of bound adenylate could be calculated after measurement of the total tyrosyl adenylate concentration in a parallel experiment, where the tRNA solution was replaced by buffer. The total adenylate concentration was found to be 1.7-fold the enzyme concentration. Since the equilibrium dissociation constant is close to 2 μM (Lin et al., 1983), it can easily be calculated that, for the enzyme concentration used in the experiment (2.94 μM), the free tyrosyl adenylate concentration is equal to 2 μM, which corresponds to a 0.69 stoichiometry with respect to the input enzyme. This result supports the interpretation that only the enzyme-bound tyrosyl adenylate hydrolyzes rapidly after being mixed with native $tRNA^{Phe}$. The second slow process that is observed corresponds to the disappearance of the free tyrosyl adenylate, its binding to the enzyme being now rate limiting. This hypothesis is confirmed by the observation that this second process is faster when the enzyme concentration is higher (see legend to Figure 1).

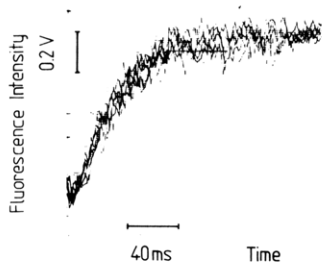


FIGURE 2: Disappearance of tyrosyl adenylate–enzyme complex upon binding of $tRNA^{Phe}$. Fluorescence intensity of TNS is measured in a stopped-flow apparatus after a solution containing 5.9 μM enzyme, 64 μM tyrosine, 10 mM ATP–$MgCl_2$, and 10 units/mL inorganic pyrophosphatase is mixed with solution containing 24 μM $tRNA^{Phe}$. Standard buffer (see Figure 1) contained 20 μM TNS. The intensity increase followed a k_{obsd} of 28 s^{-1}.

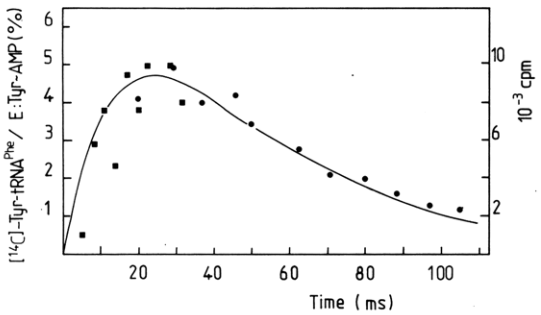


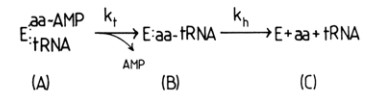
FIGURE 3: Transient aminoacylation of $tRNA^{Phe}$ with tyrosine catalyzed by phenylalanyl-tRNA synthetase. Conditions were described under Materials and Methods. The solid line is the theoretical curve drawn for k_t = 5,5 s^{-1}, k_h = 62 s^{-1}, and k'_h = 22 s^{-1}. (■ and ●) Two sets of experiments.

The above result is confirmed by the study of the disappearance of the tyrosyl adenylate–enzyme complex, using TNS as a fluorescent probe. A fast exponential decay with a rate constant of 28 s^{-1} is observed (Figure 2). The decay appears to be monophasic since the fluorescent reporter group only monitors the variation in the concentration of adenylate–enzyme complex. After the initial important decrease in the preexisting equilibrium concentration of this complex, its residual variations (corresponding to a steady state between slow binding of the free tyrosyl adenylate and its fast hydrolysis on the enzyme template) are too faint to be detected.

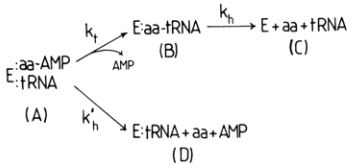
At this stage, the above results could be considered as supporting the existence of a transfer of the misactivated tyrosine to $tRNA^{Phe}$ much faster (k_t = 28 s^{-1}) than that of phenylalanine [k_t = 6 s^{-1}; according to Fasiolo & Fersht (1978)] as it has already been described for threonine misactivated by valyl-tRNA synthetase (Fersht & Kaethner, 1976): threonine was apparently transferred at a rate of 36 s^{-1} as compared to 12 s^{-1} for the cognate valine. The following section on the measurement of the formed tyrosyl-$tRNA^{Phe}$ will disprove this conclusion in our system and lead us to propose a more sophisticated correction mechanism.

Transient Transfer of Misactivated Tyrosine to Native $tRNA^{Phe}$. The quenched-flow experiments at room temperature brought evidence for a clear transient formation of tyrosyl-$tRNA^{Phe}$ (Figure 3), although the maximum efficiency of transfer is surprisingly low, since the maximum extent of tyrosyl-$tRNA^{Phe}$ formed after 24 ms of reaction only amounts to 4–5% of the input aminoacyl adenylate–enzyme complex. The transient aminoacyl-tRNA formed is indeed tyrosyl-$tRNA^{Phe}$ and cannot result from some contamination of phenylalanine in radioactive tyrosine, since the enzyme-catalyzed deacylation of phenylalanyl-$tRNA^{Phe}$ is much slower (1 min^{-1}) than the observed reaction. Obviously, a transfer-correction mechanism takes place, as already observed in several other cases (Fersht & Kaethner, 1976; Fersht, 1977a; Igloi et al., 1978; Jakubowski & Fersht, 1981; Gabius et al., 1983).

Scheme I



Scheme II



Let us consider the minimum scheme for such a model (Scheme I), already described by Fersht & Kaethner (1976), where E = enzyme protomer, aa = amino acid, aa~AMP = aminoacyl adenylate, and aa-tRNA = aminoacyl-tRNA. Then

$$[A] = [A]_0 e^{-k_t t} \tag{1}$$

$$[B] = \frac{k_t}{k_h - k_t}[A]_0(e^{-k_t t} - e^{-k_h t}) \tag{2}$$

It should be kept in mind that in such a model, when k_h is much larger than k_t (which should be the case to account for the low maximum level of B), the intermediate appears to be formed with its decomposition rate constant and to be decomposed with its formation rate constant (Fersht, 1977b). Indeed, the tyrosyl-$tRNA^{Phe}$ formed seems to disappear with a rate constant of 27 s^{-1}, very similar to the rate constant observed for the decrease in the aminoacyl adenylate–enzyme complex. Once more, this result would favor the existence of a fast transfer reaction for the misactivated tyrosine. However, some difficulties are encountered when one tries to fit the experimental data with theoretical curves. In Scheme I, the maximum concentration of intermediate B is given by

$$\frac{[B]_{max}}{[A]_0} = \frac{k_t}{k_h} e^{-k_t t_{max}} \tag{3}$$

where t_{max} is the time needed to reach the maximum concentration of B. If $k_t = 27 \pm 2$ s^{-1} and t_{max} = 0.024 s as determined above, k_h has to be as large as 300 s^{-1} to account for a maximum transfer efficiency of 4.7%. Besides the fact that the deacylation reaction would be surprisingly fast, such a constant is not compatible with the experimental t_{max}. Indeed, the value of t_{max} can be calculated according to (see Appendix)

$$t_{max} = \frac{\ln k_h - \ln k_t}{k_h - k_t} \tag{4}$$

If k_h = 300 s^{-1} and k_t = 27 s^{-1}, the maximum concentration of aminoacyl-tRNA should be reached after 9 ms, far below the experimental value of 24 ms. The transfer efficiency of the misactivated amino acid therefore appears to be much lower than expected from the apparent rate constants measured. This seems to be a rather general phenomenon, since in previous studies Fersht & Kaethner (1976) had to introduce a "transfer efficiency" to account for the abnormally low value of the transient aminoacyl-tRNA. But, the origin of this transfer efficiency remained unclear. However, Fersht (1977) proposed the possible existence of a pretransfer correction mechanism. This could be as shown in Scheme II.

It only differs from the preceding one by a supplementary step consisting in the hydrolysis of the enzyme-bound noncognate aminoacyl adenylate upon binding of tRNA. This extra step has to be a hydrolysis of the noncognate adenylate and not merely a triggered release of the latter in solution (which could look like an "active" Hopfield's mechanism; Hopfield, 1974). Indeed, (i) the kinetic association and dissociation rate constants of tyrosyl adenylate for the tRNA–enzyme complex were found similar to those measured with naked enzyme (data not shown), and (ii) the thin-layer chromatographic analysis of the fate of enzyme-bound tyrosyl adenylate after being mixed with $tRNA^{Phe}$ revealed that is was actually hydrolyzed with formation of tyrosine.

This last model leads to

$$\frac{d[A]}{dt} = -(k_t + k'_h)[A] \quad (5)$$

$$\frac{d[B]}{dt} = k_t[A] - k_h[B] \quad (6)$$

The resolution of this set of differential equations leads to

$$[A] = [A]_0 e^{-(k_t+k'_h)t} \quad (7)$$

$$[B] = \frac{k_t}{k_h - (k_t + k'_h)}[A]_0[e^{-(k_t+k'_h)t} - e^{-k_h t}] \quad (8)$$

where $[A]_0$ is the input concentration of aminoacyl adenylate–enzyme complex.

The maximum transient level of the misaminoacylated tRNA is given by (see Appendix)

$$[B]_{max} = \frac{k_t}{k_h}[A]_0 e^{-(k_t+k'_h)t_{max}} \quad (9)$$

These equations clearly show that the apparent rate constant controlling the decrease of the enzyme-bound aminoacyl adenylate is in fact the sum of the transfer rate constant (k_t) and the pretransfer hydrolysis rate constant (k'_h). Parallelly, the evolution of the concentration of the intermediate misaminoacylated tRNA, described by eq 8, can be deduced from the previous, more simple model (eq 2) by replacement of the transfer rate constant k_t by an apparent rate constant equal to $k_t + k'_h$. Assuming this latter scheme, a good fitting of the experimental data of Figure 2 is obtained for the following values of the rate constants: $k_t = 5.5\ s^{-1}$; $k'_h = 22\ s^{-1}$; $k_h = 62\ s^{-1}$.

It is interesting to compare our data to the ones published by Gabius et al. (1983), which show at low temperature (0 °C) an important accumulation of the intermediate tyrosyl-$tRNA^{Phe}$ (40–45% with respect to the input aminoacyl adenylate–enzyme complex). Such an accumulation would only be possible if the transfer and hydrolysis rate constants were almost identical (the limit of $[B]_{max}$ in Scheme I when k_h and k_t approach the same value is, expressed in percent, $1/e \simeq 37\%$, close to the experimental value measured by these authors). The similarity between k_h and k_t is supported by the observation that the curve observed for the transient accumulation of tyrosyl-$tRNA^{Phe}$ is much more symmetrical than our one. But, it must be emphasized that the experimental data of Gabius et al. (1983) at 0 °C cannot be reconciled with our ones at 23 °C, assuming a reasonable temperature dependence for the kinetic constants. Indeed, the t_{max} observed in our experiments (24 ms at 23 °C as shown in Figure 3) would correspond to a t_{max} of roughly 120 ms at 0 °C, assuming a slowing down of the reaction by a factor $q_{10} = 2$ for a decrease of the temperature corresponding to 10 °C. Increasing the t_{max} to 40 s as shown by Gabius et al. when passing from 23 to 0 °C would require a q_{10} factor equal to 25, which is clearly prohibitive. Furthermore, using our preparation of yeast phenylalanyl-tRNA synthetase, we were unable to reproduce the experiments of Gabius et al. (1983). No satisfactory explanation can be proposed for these discrepancies.

The correction mechanism that we propose in Scheme II reconciles the low efficiency of transfer of the misactivated amino acid with rather large apparent rate constants for the transfer reaction. It also accounts for a transfer of the misactivated amino acid apparently faster than for the cognate one, since the apparent rate constant in the former case is the sum of the true transfer rate constant and the pretransfer hydrolysis rate constant.

The most interesting feature of this model is not only to bring a basis for a pretransfer correction mechanism but also to show that such a hydrolysis step affects the whole transient transfer reaction: not only does it lower the transient transfer level but it also changes the derived rate constants. Therefore, the experimental results cannot be interpreted by the simple juxtaposition of Scheme I and another separate step that leads to a reduced percentage of transfer, as proposed by Fersht & Kaethner (1976) and Fersht (1977a). It is, for instance, striking to observe that Scheme II allows a much better fitting of Fersht & Kaethner's (1976) experimental data in the threonine–valyl-tRNA synthetase system, without requiring the introduction of an arbitrary transfer yield of 62%. In this case, values of k'_h, k_t, and k_h, respectively, equal to 34, 18, and 32 s^{-1} give a maximum transfer ratio of 20% after 30 ms of reaction. Such a mechanism would probably also account for the very poor transfer efficiency observed in the valine–isoleucyl-tRNA synthetase system (Fersht, 1977a), where the maximum extent of transfer was 0.8% instead of 10% expected from the measured rate constants. The author himself suggested the possible involvement of an induced pretransfer hydrolysis. Unfortunately, at that time, no measurement of the overall consumption of valyl adenylate was carried out, so that it is impossible to try to fit these data with our present model.

Another remarkable result is that, in the case of phenylalanyl-tRNA synthetase, the transfer step itself appears to be nonspecific, since tyrosine and phenylalanine are transferred with the same rate constant ($6\ s^{-1}$). This could also be a more or less general feature, since the reinterpretation of Fersht and Kaethner's data with the present model leads to a rate constant of $18\ s^{-1}$ for the transfer of threonine by valyl-tRNA synthetase (much closer to that measured for the cognate amino acid, 12 s^{-1}). This lack of specificity in the transfer step itself is further supported by the study of the transfer of phenylalanine and tyrosine to $tRNA^{Phe}_{ox\text{-}red}$.

Transfer of Phenylalanine and Tyrosine from Their Adenylate–Enzyme Complexes to $tRNA^{Phe}_{ox\text{-}red}$. The transfer of phenylalanine and tyrosine to $tRNA^{Phe}_{ox\text{-}red}$ followed very similar time courses (Figure 4). Both reactions proceeded rather slowly to a plateau (with a half-reaction time close to 1.3 s). The aminoacyl-tRNA formed only corresponded to roughly 15% of the input adenylate–enzyme complex. This result demonstrates that, even in the case of the aminoacylation of $tRNA^{Phe}_{ox\text{-}red}$, a pretransfer hydrolysis takes place, which consumes most of the enzyme-bound aminoacyl adenylate. However, it should be emphasized that this pretransfer hydrolysis triggered by $tRNA^{Phe}_{ox\text{-}red}$ does not seem specific, since the transfer efficiency is not higher for phenylalanine than for tyrosine. This point will be further discussed in the last section.

The experimental data of Figure 4 could be treated with a simplified form of Scheme II, omitting the posttransfer

Scheme III

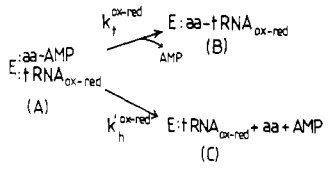


Table I: Kinetic Parameters for the Transfer of Phenylalanine and Tyrosine to $tRNA^{Phe}_{ox\text{-}red}$

amino acid	k_{app} (s^{-1})	$[B]_{t\to\infty}/[A]_0$ (%)	$k_t^{ox\text{-}red}$ (s^{-1})	$k'^{ox\text{-}red}_h$ (s^{-1})
Phe	0.48	15.5	0.074	0.40
Tyr	0.54	14[a]	0.073	0.47

[a] This number is a mean value resulting from the two experiments of Figure 4; it was calculated with respect to the total tyrosyl adenylate concentration because, in contrast to what was observed with native $tRNA^{Phe}$, the equilibrium

$$E + Tyr\sim AMP \underset{2\ s^{-1}}{\overset{10^6\ M^{-1}s^{-1}}{\rightleftharpoons}} E\text{–}Tyr\sim AMP$$

can be considered, in the concentration range used (several micromolar), as being in fast preequilibrium with respect to the decomposition of the enzyme–adenylate complex ($k_{app} \simeq 0.5\ s^{-1}$). The efficiency of transfer determined in this way is probably slightly underestimated, but the calculated rate constants are close to the actual values.

hydrolysis step. Indeed, the plateau concentration of aminoacyl-tRNA was found to be stable, except in the case of tyrosine, where a very slow hydrolysis of tyrosyl-$tRNA^{Phe}_{ox\text{-}red}$ occurs ($0.012\ s^{-1}$), too sluggish to interfere with the transfer reaction. The modified scheme can therefore be written as shown in Scheme III.

The kinetic analysis leads to

$$[A] = [A]_0 e^{-(k_t^{ox\text{-}red} + k'^{ox\text{-}red}_h)t} \quad (10)$$

$$[B] = \frac{k_t^{ox\text{-}red}}{k_t^{ox\text{-}red} + k'^{ox\text{-}red}_h}[A]_0[1 - e^{-(k_t^{ox\text{-}red} + k'^{ox\text{-}red}_h)t}] \quad (11)$$

where $[A]_0$ is the input adenylate–enzyme concentration. The apparent time constant for the formation of the aminoacylated-$tRNA_{ox\text{-}red}$ is

$$k_{app} = k_t^{ox\text{-}red} + k'^{ox\text{-}red}_h \quad (12)$$

and the plateau concentration of aminoacyl-$tRNA^{Phe}_{ox\text{-}red}$ is given by

$$[B]_{t\to\infty} = \frac{k_t^{ox\text{-}red}}{k_t^{ox\text{-}red} + k'^{ox\text{-}red}_h}[A]_0 \quad (13)$$

The transfer and hydrolysis rate constants can therefore be deduced from the combination of eq 12 and 13. The results are shown in Table I. Obviously, the rate of transfer is once more the same for phenylalanine and tyrosine.

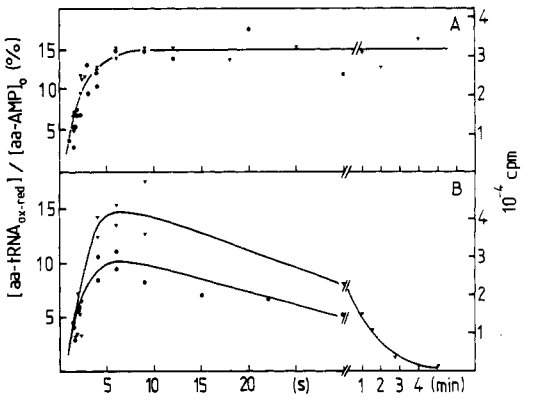


FIGURE 4: Single turnover experiment of the transfer of phenylalanine (panel A) or tyrosine (panel B) from enzyme-bound aminoacyl adenylates to $tRNA^{Phe}_{ox\text{-}red}$. A solution containing 5.9 μM enzyme, 64 μM ^{14}C-labeled amino acid, 10 mM ATP–$MgCl_2$, and 20 units/mL inorganic pyrophosphatase was mixed as described under Materials and Methods with a solution containing 12 μM $tRNA^{Phe}_{ox\text{-}red}$ and 0.9 mM nonradioactive amino acid in the standard buffer. (● and ▼) Two sets of experiments in each case.

Hydrolysis of Enzyme-Bound Phenylalanyl and Tyrosyl Adenylates Triggered by Modified $tRNAs^{Phe}$. Several modified $tRNA^{Phe}$ ($tRNA^{Phe}_{2'deoxyA}$, $tRNA^{Phe}_{ox}$, $tRNA^{Phe}_{ox\text{-}red}$) were tested for their ability to trigger an efficient pretransfer hydrolysis of enzyme-bound aminoacyl adenylates. Results are presented in Table II. As can be seen, all modifications of the 3′-terminal adenosine tested result in the almost complete suppression of the ability to induce a pretransfer correction mechanism. Indeed, the hydrolysis rate constants measured in the presence of the modified tRNAs do not significantly differ from the spontaneous rate of hydrolysis, at least when compared to the 22-s^{-1} rate constant measured in the presence of native tRNA. Only $tRNA^{Phe}_{ox\text{-}red}$ (which can be aminoacylated) induces a significant increase in the pretransfer hydrolysis rate constant, but it should be noted that this increase does not contribute to an improvement of the discrimination between phenylalanine and tyrosine, since the stimulation is identical for both amino acids. Only native $tRNA^{Phe}$ leads to a preferential increase of the pretransfer hydrolysis of tyrosyl adenylate. Although the corresponding hydrolysis rate constant (k'_h) has not been determined experimentally for phenylalanyl adenylate, an upper limit can be fixed for this constant: $k'_h \leq 33\ s^{-1}$ (see legend to Table II). Therefore, the above results strongly support a critical importance of the 3′-terminal adenosine of tRNA in triggering the hydrolysis of the enzyme-bound noncognate aminoacyl adenylate; the integrity of the *cis*-diol of adenosine is particularly required, since $tRNA^{Phe}_{2'deoxyA}$ is unable to trigger an efficient pretransfer hydrolysis of the enzyme-bound tyrosyl adenylate. This strict requirement for the 2′- and 3′-hydroxyl groups has already been proposed as an evidence that the correction mechanism

Table II: Values of the Pretransfer Hydrolysis Rate Constants and Transfer Rate Constants for Phenylalanine and Tyrosine in the Presence of Native and Modified $tRNA^{Phe}$

	tRNA				
const	in the absence of tRNA	native $tRNA^{Phe}$	$tRNA^{Phe}_{ox\text{-}red}$	$tRNA^{Phe}_{ox}$	$tRNA^{Phe}_{2'deoxyA}$
k'_h(Phe∼AMP) (s^{-1})	1.5×10^{-3}	nd (2.2)[a]	0.40	1.5×10^{-3}	2.3×10^{-3}
k'_h(Tyr∼AMP) (s^{-1})	2.3×10^{-3}	22	0.47	1.6×10^{-2}	7.4×10^{-3}
k_t(Phe) (s^{-1})		6.6[b]	0.074		
k_t(Tyr) (s^{-1})		5.5	0.073		

[a] nd, not determined. This value was not directly measured, but a maximum value can be estimated from the known efficiency of transfer and transfer rate constant (Fasiolo & Fersht, 1978) according to efficiency of transfer $\leq k_t/(k'_h + k_t)$. [b] Single-turnover rate constant according to Fasiolo & Fersht (1978).

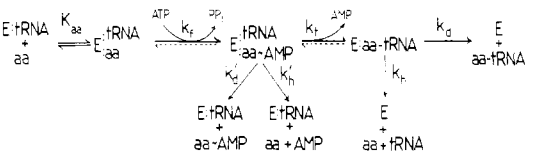
does involve a transient transfer of the misactivated amino acid to the tRNA (Igloi et al., 1978). This appears to be a convincing argument, should the transient yield of aminoacyl-tRNA formation correspond to the transfer and posttransfer hydrolysis rate constants measured. If, in the threonine–valyl-tRNA synthetase system, the discrepancy between expected and measured transient yields of aminoacylation is minor [since Fersht & Kaethner (1976) only need to introduce a 62% "transfer efficiency"], this is no longer the case in the tyrosine–phenylalanyl-tRNA synthetase and valine–isoleucyl-tRNA synthetase systems, where the actual maximum efficiency of transient aminoacylation is lower than expected by a factor of 8–12. On the contrary, such reduced yields are easily explained in the case of a correction mechanism combining both a pretransfer hydrolysis of the noncognate aminoacyl adenylate and a posttransfer hydrolysis of the misaminoacylated tRNA.

This pretransfer hydrolysis could be reconciled with the apparent requirement for the tRNA to undergo the aminoacylation reaction. The basis for this explanation could be the model proposed by Baltzinger & Holler (1982) involving an unstable, strained tetrahedral intermediate. In the cognate system, this intermediate will of course give rise preferentially to phenylalanyl-tRNA^{Phe}. On the contrary, in the noncognate system, the strained intermediate will decompose with formation of tyrosine and AMP. The formation of such a strained intermediate would of course be very much dependent on the exact geometry of the terminal ribose of the tRNA molecule. Therefore, the difference observed between native tRNA^{Phe} and tRNA$^{Phe}_{ox\text{-}red}$ is not surprising.

Conclusions

The above results demonstrate the existence of a double-rejection mechanism of tyrosine by phenylalanyl-tRNA synthetase in the presence of native tRNA^{Phe}. Besides the well-documented transfer–hydrolysis scheme, an efficient pretransfer hydrolysis of the wrong aminoacyl adenylate is triggered by native tRNA^{Phe}. This pretransfer hydrolysis is mainly responsible for tyrosine rejection, since around 75% of the misactivated amino acid is destroyed in this step. Reexamination of earlier results concerning the threonine–valyl-tRNA synthetase and valine–isoleucyl-tRNA synthetase systems (Fersht & Kaethner, 1976; Fersht, 1977; Igloi et al., 1978; Jakubowski & Fersht, 1981) suggests that the pretransfer hydrolysis mechanism could be involved in almost all the systems studied so far. Indeed, in these systems, the maximum amount of transient misaminoacylated tRNA is always substantially lower than expected from the measured rate constants. One of the main features of the proposed model is that the observed rate constants for the transfer of noncognate amino acids to tRNAs appear as the sum of the true transfer rate constant and the pretransfer hydrolysis rate constant. This could explain the somewhat surprising observation that noncognate amino acids appear to be transferred faster than the cognate ones. It should be mentioned that, in the valine–isoleucyl-tRNA synthetase system, the transfer rate constant, which has been deduced from the overall ATP consumption and found equal to that of the cognate isoleucine (Fersht, 1977), could be largely underestimated, should the adenylation step itself be rate limiting. Consequently, the ATP hydrolysis rate constant appears as a poor estimation of the adenylate consumption for noncognate amino acids. Therefore, the discrimination between tyrosine and phenylalanine by yeast phenylalanyl-tRNA synthetase appears to rely on a rather complicated multistep process, at the level of both the adenylation (Lin et al., 1983) and transfer reactions. The complete sequence of events can be described as shown in Scheme IV. In Scheme IV, K_{aa} = the equilibrium dissociation constant for the amino acid and k_f, k_d, k_h, k_t, k'_h, and k'_d are the rate constants for the corresponding steps.

Scheme IV



In the case of the tyrosine–phenylalanine discrimination, the error rate in tRNA^{Phe} aminoacylation can be described by

$$\frac{[Tyr]}{[Phe]}\frac{K_{Phe}}{K_{Tyr}}\frac{k_f^{Tyr}}{k_f^{Phe}}\left(\frac{k_t}{k_t + k'_d + k'_h}\right)^{Tyr}\left(\frac{k_d}{k_h + k_d}\right)^{Tyr}$$

adenylation step | transfer step | post-transfer deacylation

which calls for several comments.

(i) The terms corresponding to the transfer step and post-transfer deacylation should be divided by the homologous ratios referring to phenylalanine. But as a first approximation, the latter can be considered as equal to 1, because k'_d and k'_h are negligible with respect to k_t in the transfer of phenylalanine and k_h is negligible with respect to k_d, for phenylalanyl-tRNA^{Phe}.

(ii) The difference in the affinities for the cognate and noncognate aminoacyl adenylates is roughly the same as that for cognate and noncognate amino acids (Lin et al., 1983). However, the improvement in the accuracy does not exactly correspond to the square of this affinity factor, since both the transfer rate constant and the pretransfer hydrolysis rate constant are not negligible with respect to the adenylate dissociation rate constant.

(iii) In vivo, the post-transfer hydrolysis cannot be thought of as an absolute process, because of the presence of the Tu factor. Indeed, once complexed by Tu factor, the aminoacyl-tRNA is efficiently protected against deacylation. Therefore, the gain in accuracy at that step depends on both the posttransfer hydrolysis and the dissociation rate constants of aminoacyl-tRNA. In the case of tyrosyl-tRNA^{Phe}, the dissociation rate constant is probably lower than or equal to the hydrolysis rate constant. Assuming that tyrosine and phenylalanine are present in vivo at equivalent concentrations (Thalhammer et al., 1982; Delhotal et al., 1983), the overall error rate in tRNA^{Phe} aminoacylation by tyrosine will be equal to or possibly lower than

$$1 \times \frac{40 \times 10^{-6}}{9 \times 10^{-3}} \times \frac{10}{100} \times \frac{6}{6 + 2 + 22} \times \frac{1}{2} = 5 \times 10^{-5}$$

adenylation step (Lin et al., 1982) | transfer step | post-transfer deacylation

This estimated upper limit is substantially lower than the measured error rate in protein synthesis, $(1–4) \times 10^{-4}$ (Lehninger, 1970; Loftfield & Vanderjagdt, 1972; Ellis & Gallant, 1982; Yarus, 1979), which is necessary to account for the other possible sources of errors like (i) aminoacylation of a noncognate tRNA, although this type of error is probably very rare, due to the reduced affinity for noncognate tRNAs (usually $\leq 1/100$ of that for cognate tRNAs) and the reduced rate of aminoacylation (usually $\leq 1/1000$ of that for cognate tRNAs) and (ii) misreading of codons, which we feel is probably the major contribution to the error rate in protein biosynthesis.

The main advantage of a multistep discrimination pathway like that presented here is a high flexibility allowing an efficient response to various conditions. Indeed, it should be kept in mind that the efficiency of the posttransfer hydrolysis of the misaminoacylated tRNA will only have a limited efficiency, due to the protection against deacylation brought by the Tu factor, as already underlined (Hopfield et al., 1976; Mulvey & Fersht, 1977). The residual error rate after this posttransfer correction will be limited by the value of the ratio $k_d/(k_h + k_d)$, which is probably comprised between 0.5 and 0.1. Ensuring a 10-fold increase in the accuracy at that step would require that the hydrolysis rate constant is roughly 10-fold higher than the dissociation rate constant, which might be difficult to achieve in most cases. The best illustration, although completely artificial, of the flexibility of our model is given by $tRNA^{Phe}_{ox\text{-}red}$. Since the latter is only very slowly deacylated when esterified with tyrosine and since no selective pretransfer hydrolysis is induced by this modified tRNA, one could think that the discrimination between tyrosine and phenylalanine will be strongly reduced. This is not completely true, because of the poor affinity of tyrosyl adenylate for phenylalanyl-tRNA synthetase. Indeed, the dissociation rate constant for tyrosyl adenylate (2 s^{-1}) is much higher than the transfer rate constant (0.074 s^{-1}) and even the pretransfer hydrolysis rate constant (0.4 s^{-1}). The high yield of transfer observed in vitro is only due to the fact that the dissociation of tyrosyl adenylate from phenylalanyl-tRNA synthetase is artificially reversible, because of the absence of tyrosyl-tRNA synthetase. In the presence of the latter, the maximum transient yield for the transfer of tyrosine would fall down, to $0.074/(2 + 0.4) \simeq 0.03$, which is comparable to the efficiency observed with native tRNA.

Acknowledgments

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Appendix

Let us consider Scheme I under Results and Discussion. Then

$$\frac{d[A]}{dt} = -k_t[A] \quad (A1)$$

which leads to

$$[A] = [A]_0 e^{-k_t t} \quad (A2)$$

where $[A]_0$ = concentration of A at zero time.

$$\frac{d[B]}{dt} = k_t[A] - k_h[B] = k_t[A]_0 e^{-k_t t} - k_h[B] \quad (A3)$$

which can be integrated under the form

$$[B] = \frac{k_t}{k_h - k_t}[A]_0(e^{-k_t t} - e^{-k_h t}) \quad (A4)$$

The maximum concentration of the transient intermediate B will be reached when

$$\frac{d[B]}{dt} = \frac{k_t}{k_h - k_t}[A]_0(-k_t e^{-k_t t_{max}} + k_h e^{-k_h t_{max}}) = 0 \quad (A5)$$

which leads to

$$t_{max} = \frac{\ln k_h - \ln k_t}{k_h - k_t} \quad (A6)$$

Therefore

$$[B]_{max} = [B(t_{max})] = \frac{k_t}{k_h}[A]_0 e^{-k_t t_{max}} = \left(\frac{k_h}{k_t}\right)^{k_h/(k_h - k_t)} [A]_0 \quad (A7)$$

When one replaces Scheme I by Scheme II (see Results and Discussion), the set of differential equations becomes

$$\frac{d[A]}{dt} = -(k_t + k'_h)[A] \quad \text{or} \quad [A] = [A]_0 e^{-(k_t + k'_h)t} \quad (A8)$$

$$\frac{d[B]}{dt} = k_t[A] - k_h[B] = k_t[A]_0 e^{-(k_t + k'_h)t} - k_h[B] \quad (A9)$$

which leads to

$$[B] = \frac{k_t}{k_h - (k_t + k'_h)}[A]_0[e^{-(k_t + k'_h)t} - e^{-k_h t}] \quad (A10)$$

The same calculation as above will lead to

$$[B]_{max} = \frac{k_t}{k_h}[A]_0\left(\frac{k_h}{k_t + k'_h}\right)^{-(k_t + k'_h)/[k_h - (k_t + k'_h)]} \quad (A11)$$

with

$$t_{max} = \frac{\ln k_h - \ln (k_t + k'_h)}{k_h - (k_t + k'_h)} \quad (A12)$$

Registry No. Phe, 63-91-2; Tyr, 60-18-4; Tyr-AMP, 50466-77-8; Phe-AMP, 35874-27-2; phenylalanyl-tRNA synthetase, 9055-66-7.

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Kinetic Mechanism of the Interaction of D-Cycloserine with Serine Hydroxymethyltransferase†

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ABSTRACT: The kinetic mechanism for the interaction of D-cycloserine with serine hydroxymethyltransferase (EC 2.1.2.1) from sheep liver was established by measuring changes in the activity, absorbance, and circular dichoism (CD) of the enzyme. The irreversible inhibition of the enzyme was characterized by three detectable steps: an initial rapid step followed by two successive steps with rate constants of 5.4×10^{-3} s^{-1} and 1.4×10^{-4} s^{-1}. The first step was distinguished by a rapid disappearance of the enzyme absorbance peak at 425 nm, a decrease in the enzyme activity to 25% of the uninhibited velocity, and a lowering of the CD intensity at 432 nm to about 65% of the original value. The second step of the interaction was accompanied by a complete loss of enzyme activity and a marginal increase in the CD intensity at 432 nm. The final step resulted in the complete loss of the enzyme absorbance at 425 nm and of the CD band at 432 nm. The products of the reaction were identified as (a) apoenzyme by absorbance measurements, CD spectra, and reconstitution with pyridoxal 5′-phosphate and (b) a pyridoxal 5′-phosphate–D-cycloserine Schiff's base complex identified by its fluorescence and absorbance spectra. The Schiff base complex was expelled from the enzyme active site in the final step of the reaction. The proposed mechanism, which is different from those operative in other pyridoxal phosphate dependent enzymes, probably accounts for the selective inhibition of serine hydroxymethyltransferase by the drug in vivo.

The study of specific irreversible inhibition of pyridoxal phosphate (PLP)[1] dependent enzymes has attracted considerable attention because of the potential therapeutic value of these inhibitors and also in view of their usefulness as probes to study the physiological role of these enzymes (Bey, 1981). The antibiotic D-cycloserine (oxamycin, 4-amino-3-isoxazolidinone) used in the therapy of tuberculosis interferes with cell wall synthesis in the pathogenic bacteria by competitively inhibiting the enzyme D-alanylalanine synthetase (EC 6.3.2.4) (Strominger et al., 1960; Nehaus & Lynch, 1964). D-Cycloserine and its substituents in carbon 5 are rigid cyclic analogues of D-alanine and its higher homologues, respectively (Figure 1). These compounds slowly and irreversibly inhibit several PLP-dependent enzymes following an initial selective and reversible binding as quasi-substrates (Khomutov et al., 1968). Bukin & Sergeev (1968) reported that D-cycloserine selectively inhibited serine hydroxymethyltransferase (EC 2.1.2.1) in mouse liver extracts and in the liver of mice receiving a diet deficient in PLP. The pronounced antineoplastic activity of D-cycloserine and its dimer (Bukin et al., 1970; Sergeev et al., 1971; Draudin-Krylenko, 1976; Bukin & Draudin-Krylenko, 1980), especially in combination with 4-vinylpyridoxal (Bukin et al., 1979), suggested that serine hydroxymethyl transferase might play a critical role in maintaining neoplasia (Bukin & Draudin-Krylenko, 1980).

Although extensive investigations were carried out on the interaction of D-cycloserine with several PLP-dependent enzymes (Braunstein et al., 1961; Dann & Carter, 1964; Karpeiskii et al., 1964; Brown et al., 1969; Roze & Strominger, 1966; Wang & Walsh, 1978), very little information is available on the mechanism of interaction of this drug with serine hydroxymethyltransferase. This enzyme was isolated from several sources and was a homotetramer with a M_r of around 200000 ± 20000 and contained 1 mol of PLP/monomer (Schirch, 1982). We had earlier described the purification of the sheep liver enzyme to homogeneity and described some of its physicochemical, kinetic, and regulatory properties (Manohar et al., 1982). In this paper, we report the kinetic mechanism of the interaction of D-cycloserine with sheep liver serine hydroxymethyltransferase.

Experimental Procedures

Materials

The following biochemicals were obtained from Sigma Chemical Co., St. Louis, MO: 2-mercaptoethanol (2-ME), PLP, ethylenediaminetetraacetic acid (EDTA), D-cycloserine, pyridoxamine 5′-phosphate hydrochloride (PMP), and L-cysteine. L-[3-^{14}C]Serine (58.5 mCi/mmol) was purchased from New England Nuclear, Boston, MA; tetrahydrofolate (H_4-folate) was prepared by the method of Hatefi et al. (1959);

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; EDTA, ethylenediaminetetraacetic acid disodium salt; PMP, pyridoxamine 5′-phosphate hydrochloride; H_4-folate, tetrahydrofolate; CM, carboxymethyl; M_r, molecular weight; 2-ME, 2-mercaptoethanol; UV, ultraviolet; CD, circular dichroism; DTT, dithiothreitol.

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