Hanke, T., Bartmann, P., Hennecke, H., Kosakowski, H. M., Jaenicke, R., Holler, E., & Böck, A. (1974) *Eur. J. Biochem. 43*, 601–607.
Hanke, T., Bartmann, P., & Holler, E. (1975) *Eur. J. Biochem. 56*, 605–615.
Hennecke, H., & Böck, A. (1974) *Eur. J. Biochem. 50*, 157–166.
Holler, E. (1976) *J. Biol. Chem. 251*, 7717–7719.
Holler, E. (1980) *Biochemistry 19*, 1397–1402.
Holler, E., & Calvin, M. (1972) *Biochemistry 11*, 3741–3752.
Holler, E., & Kosakowski, H. M. (1973) *Biochem. Biophys. Res. Commun. 54*, 1532–1539.
Holler, E., Schwartze, G., Scheibl, R., & Hammer-Raber, B. (1980) *Biochemistry 19*, 5403–5411.
Holler, E., Baltzinger, M., & Favre, A. (1981a) *Biochemistry 20*, 1139–1147.
Holler, E., Wang, C. C., & Ford, N. (1981b) *Biochemistry 20*, 861–867.
Kosakowski, H. M., & Böck, A. (1970) *Eur. J. Biochem. 12*, 67–73.
Kosakowski, H. M., & Holler, E. (1973) *Eur. J. Biochem. 38*, 274–282.
Krauss, G., Römer, R., Riesner, D., & Maass, G. (1973) *FEBS Lett. 30*, 6–10.
Krauss, G., Riesner, D., & Maass, G. (1976) *Eur. J. Biochem. 68*, 81–93.
Krauss, G., Von der Haar, F., & Maass, G. (1979) *Biochemistry 18*, 4755–4761.
Perrin, D. D. (1965) in *IUPAC Dissociation Constants of Organic Bases in Aqueous Solutions*, Butterworths, London.
Pimmer, J., & Holler, E. (1979) *Biochemistry 18*, 3714–3723.
Pingoud, A., Riesner, D., Boehme, D., & Maass, G. (1973) *FEBS Lett. 30*, 1–5.
Roe, B., Sirover, M., & Dudock, B. (1973) *Biochemistry 12*, 4146–4154.
Schimmel, P. R. (1973) *Acc. Chem. Res. 6*, 299–305.
Schoffield, P., & Zamecnick, P. C. (1968) *Biochim. Biophys. Acta 155*, 410–416.
Schuber, F., & Pinck, M. (1974) *Biochimie 56*, 383–390.
Sprinzl, M., & Cramer, F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3049–3053.
Sprinzl, M., & Faulhammer, H. G. (1978) *Nucleic Acids Res. 5*, 4837–4855.
Tanford, C., & Hauenstein, J. D. (1956) *J. Am. Chem. Soc. 78*, 5287–5291.
Yarus, M., & Berg, P. (1969) *J. Mol. Biol. 42*, 171–189.
Yarus, M., & Berg, P. (1970) *Anal. Biochem. 35*, 450–465.

Catalytic Mechanism of Phenylalanyl-tRNA Synthetase of *Escherichia coli* K10. Conformational Change and $tRNA^{Phe}$ Phenylalanylation Are Concerted†

Mireille Baltzinger‡ and Eggehard Holler*

ABSTRACT: Catalytic phenylalanylation of $tRNA^{Phe}$ and the reverse reaction, AMP-dependent deacylation of Phe-$tRNA^{Phe}$, have been measured by steady-state and pre-steady-state techniques, including rapid sampling and fluorescence stopped-flow methods. (1) Stoichiometry of adenylate synthesis under steady-state phenylalanylation of $tRNA^{Phe}$ indicates half-of-the-sites reactivity. (2) Identity of values of rate constants under pre-steady- and steady-state conditions demonstrates that the rate-limiting steps in catalysis are bond making for phenylalanylation and bond breaking for AMP-dependent deacylation, respectively. (3) Values of catalytic rate constants are the same as those for the conformational change of the Phe site directed enzyme·Phe-$tRNA^{Phe}$ complex [Baltzinger, M., & Holler, E. (1982) *Biochemistry* (preceding paper in this issue)]. (4) A model is developed that accounts for the observed concert of chemical and geometrical reactions as well as for experimental evidence that nascent Phe-$tRNA^{Phe}$ may not be the same as in solution. In this model, nascent Phe-$tRNA^{Phe}$ is thought to be the tetrahedral intermediate that is formed by nucleophilic attack of the adenylate by the tRNA. It awaits the conformational change in order to break down into Phe-$tRNA^{Phe}$ and AMP. The model can serve as a unifying basis for an interpretation of discrimination against noncognate amino acids and tRNAs and also gives an explanation why severe product inhibition is not observed [Güntner, C., & Holler, E. (1979) *Biochemistry 18*, 2028–2038].

The catalysis of tRNA aminoacylation by aminoacyl-tRNA synthetases involves, in a minimum mechanism, an activation of the amino acid by synthesis of an adenylate and a subsequent transfer of the activated amino acid to the tRNA. We have reported on the mechanism of aminoacyl adenylate synthesis (Holler & Calvin, 1972; Pimmer & Holler, 1979). The present paper deals with the mechanism of phenylalanylation of $tRNA^{Phe}$, catalyzed by *Escherichia coli* phenylalanyl-tRNA synthetase. The particular mechanism is considered to be representative for at least a group of aminoacyl-tRNA synthetases. The reaction under study is interesting with respect to the question of other (covalent) intermediates (Thiebe, 1975; Kovaleva et al., 1978; Remy & Ebel, 1976) and with regard to rejection of misactivated noncognate amino acids (Fersht, 1977). Problems of thermodynamics and kinetics of product release have also been connected with this

† From the Institut für Biophysik und Physikalische Biochemie, Universität Regensburg, D-8400 Regensburg, Federal Republic of Germany. *Received June 12, 1981.* The work was generously supported by the Deutsche Forschungsgemeinschaft and by an EMBO long-term fellowship to M.B. Part of this has been presented during the Proceedings of the International Conference on the Biological Implications of Protein–Nucleic Acid Interactions, Poznan, Poland, 1980.

‡ EMBO long-term fellow. Present address: Institut de Biologie Moleculaire et Cellulaire, Centre National de la Recherche Scientifique, 67000 Strasbourg, France.

0006-2960/82/0421-2467$01.25/0

part of the catalytic reaction (Güntner & Holler, 1979; Holler, 1976, 1980).

In the accompanying paper (Baltzinger & Holler, 1982), we have presented evidence for a conformational change of the nascent enzyme·aminoacyl-tRNA complex to be rate determining for the enzymatic phenylalanylation of $tRNA^{Phe}$. In the present paper, we demonstrate that this conformational change is also rate determining for the reverse reaction, AMP-dependent deacylation of Phe-$tRNA^{Phe}$. We demonstrate that esterification and deacylation are in concert with the conformational change in both directions. The concept, which we have derived from kinetics of partial reactions and under steady-state conditions, was verified by measurement of transients in the direction of synthesis and AMP-dependent deacylation of Phe-$tRNA^{Phe}$.

Materials and Methods

Chemicals. L-[^{14}C]Phenylalanine was a product of Amersham Buchler. 6-(*p*-Toluidinyl)naphthalene-2-sulfonate (TNS)[1] was obtained from Serva (Heidelberg) and L-phenylalaninol from Fluka (Buchs). ATP, AMP, and $tRNA^{Phe}$ (Phe acceptance 1200–1400 pmol/A_{260} unit) were purchased from Boehringer (Mannheim). All other chemicals were analytical grade and obtained from Merck (Darmstadt).

Enzymes. L-Phenylalanyl-tRNA synthetase (EC 6.1.1.20) from *E.coli* K10 was prepared as described by Hanke et al. (1974). The specific activity was 53 000 nmol mg^{-1} h^{-1} and 1.9 active sites per enzyme (M_r 270 000). Inorganic pyrophosphatase (EC 3.6.1.1) from yeast was purchased from Boehringer (Mannheim) in the form of a 1 mg/mL (200 units/mg) suspension in 3.2 M ammonium sulfate.

Aminoacyl-$tRNA^{Phe}$ was prepared as described by Bartmann et al. (1974).

Buffers. Routine buffer was 50 mM Tris-HCl buffer (pH 7.5; 25 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.2 mM dithioerythritol unless noted otherwise.

Steady-State Aminoacylation of tRNA. Unless stated otherwise, phenylalanylation of $tRNA^{Phe}$ was followed by the method of Kosakowski & Böck (1970) by measuring initial rates.

Steady-state AMP-dependent deacylation of [^{14}C]Phe-$tRNA^{Phe}$ was observed by the method described (Holler et al., 1981).

Quench-Flow Kinetic Experiments. ^{14}C phenylalanylation of $tRNA^{Phe}$ and AMP-dependent deacylation of [^{14}C]Phe-$tRNA^{Phe}$ (PP_i present) were measured at short time scales by employment of the quench-flow apparatus described previously (Kern & Gangloff, 1981). Phenylalanylation with in situ synthesized enzyme·[^{14}C]Phe~AMP was carried out as follows. Enzyme, ATP, [^{14}C]Phe, and inorganic pyrophosphatase (5 μg/mL) were incubated for 1–5 min in one syringe and then mixed with a solution of $tRNA^{Phe}$ in a 1:1 (v/v) ratio.

After incubation (10–500 ms), the reaction was stopped by mixing with an equal volume of 20% trichloroacetic acid containing 20 mM Phe. The precipitate was filtered over Whatman GF/C disks and counted for radioactivity. Reaction solutions, in particular those containing dithioerythritol or reduced glutathione, were freshly prepared.

In the case of AMP-dependent deacylation, a solution of [^{14}C]Phe-$tRNA^{Phe}$ (100 μCi/μmol) was mixed with a solution of enzyme. Solutions contained 50 mM Tris-HCl (pH 7.5; 25 °C), 10 mM $MgCl_2$, 10 mM AMP, 1 mM PP_i, 0.1 mM EDTA, and 0.2 mM dithioerythritol. Deacylation was stopped by mixing with a solution of 30 A_{260} units of unfractionated tRNA, 20 mM Phe, and 0.1 M sodium acetate buffer, pH 4. The mixture was added in portions of 140–200 μL of 0.15 mg/mL cetyltrimethylammonium bromide in water. After 5 min, the suspension of precipitated nucleic acids was transferred into a 1-mL plastic tube and cleared by centrifugation (4 min, Eppendorf centrifuge 3200). A 200-μL sample of the supernatant was counted in water-mixable scintillation cocktail (Scintillator 299, Packard). In the presence of PP_i, adenylate is rapidly turned into ATP and Phe by pyrophosphorolysis (50–100 s^{-1}; Pimmer & Holler, 1979) and will not accumulate in the deacylation experiments.

Stopped-Flow Measurements. Kinetics were observed via the fluorescence of added TNS ("TNS method") or the protein intrinsic fluorescence [see Baltzinger & Holler (1982)]. The Durrum-Gibson stopped-flow spectrophotometer equipped with a fluorescence attachment has been described (Bartmann et al., 1975a).

Enzyme·phenylalanyl adenylate was prepared in situ by incubation of enzyme, ATP, Phe, and inorganic pyrophosphatase (1–10 μg/mL) in one syringe of the stopped-flow apparatus. Enzyme·phenylalanyl adenylate complex free of reactants was prepared by passing the reaction mixture of Sephadex G50 coarse as described (Rainey et al., 1977). In one experiment, in situ prepared enzyme·adenylate was separated kinetically from reactive Phe by including L-phenylalaninol in the tRNA solution. This compound is a competitive inhibitor (K_i = 4 μM, at saturating ATP; Holler et al., 1975). The various methods gave identical results.

Data Processing for Single-Turnover Reactions. In an ideal situation, a single-turnover reaction follows the simple mechanism

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

where E denotes enzyme, S substrate, ES enzyme–substrate complex, and P product. Typically, the enzyme is regenerated during the reaction. In many cases, values of k_1 and k_{-1} are high compared with k_2, and the complex may be treated at preequilibrium with the subsequent reaction. If the concentration of active sites, $[E]_0$, is in very large excess over the concentration of substrate, $[E]_0 \gg [S]_0$, the time dependence of product formation is single exponential and follows a rate constant $k_{obsd} = k_2[E]_0/(k_{-1}/k_1 + [E]_0)$. For enzyme concentrations high compared with the dissociation constant, k_{-1}/k_1, of the enzyme–substrate complex, k_{obsd} equals k_2.

Our data processing was based on the above consideration. It deserves the following comments in the case of the deacylation of Phe-$tRNA^{Phe}$. (1) Phenylalanyl-tRNA synthetase contains two active sites per molecule of enzyme (Bartmann et al., 1975a,b); thus, concentrations of active sites were twice those of the enzyme. (2) For practical reasons (limited amounts of material, low background, nuclease or phosphatase activities in the enzyme preparation, etc.), the concentration of enzyme was similar to that of Phe-$tRNA^{Phe}$. Under this condition, one will find on derivating an expression for k_{obsd} that kinetics will no longer be single exponential because of the occurrence of a square term in the concentration of the enzyme–substrate complex. (3) The above difficulties can be overcome, in part, by evaluation of the late portion of the time dependence of product formation. According to the above mechanism, the enzyme regenerates while substrate is consumed. Thus, during the course of the reaction, the situation approaches the ideal case where enzyme is in large excess over substrate. This is indicated by an approach to a single-ex-

[1] Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; TNS, 6-(*p*-toluidinyl)naphthalene-2-sulfonate.

Table I: Kinetic Constants for AMP-Dependent Deacylation of [^{14}C]Phe-tRNA^{Phe} [a]

condition	k_{cat} (s^{-1})	K_m (μM)
steady state		
3.5 μM [^{14}C]Phe-tRNA^{Phe}, 2 mM PP_i, [AMP] varied (Figure 1A)	10 ± 0.5	(0.35 ± 0.03) × 10^3
10 mM AMP, 2 mM PP_i, [[^{14}C]Phe-tRNA^{Phe}] varied (Figure 1B)	10 ± 0.5	0.4 ± 0.1
10 mM AMP, 2 mM PP_i, 0.45 μM tRNA^{Phe}, [[^{14}C]Phe-tRNA^{Phe}] varied (Figure 1B)	7 ± 1	0.6 ± 0.1
10 mM AMP, 2 mM PP_i, 5.5 μM tRNA^{Phe}, [[^{14}C]Phe-tRNA^{Phe}] varied (Figure 1B)	4 ± 0.4	2 ± 0.5
single turnover		
2.5 μM enzyme, 2.2 μM [^{14}C]Phe-tRNA^{Phe}, 10 mM AMP, 1 mM PP_i (quench-flow experiment)	12 ± 1	nd [b]

[a] Tris-HCl (50 mM) (pH 7.5; 25 °C), 10 mM (free) $MgCl_2$, 0.1 mM EDTA, and 0.65 mM dithioerythritol. [b] nd = not determined.

ponential product formation. We found the method suitable for both the quench-flow measurements and the stopped-flow experiments in the presence of TNS. Single exponentials were observed after 50% and 60–70% product, respectively, had been liberated. The quality of the fits corresponded to standard deviations for the rate constant of 10% and 20% or better, respectively.

We emphasize that the method is an approach, especially with regard to the mechanism, which is more complicated than the one given above. It includes the formation of the enzyme·adenylate intermediate. Fortunately, because of rapid phosphorolysis, the intermediate does not substantially accumulate and thus occupies only a fraction of enzyme under the conditions used for computation of the rate constants.

Results

Conformational Change Is Rate Limiting for Synthesis and AMP-Dependent Deacylation of Phe-tRNA^{Phe}. We have previously reported that association between phenylalanyl-tRNA synthetase and Phe-tRNA^{Phe} involved a conformational change (Baltzinger & Holler, 1982). The reverse of this conformational change was noticed to be the rate-determining reaction during phenylalanylation of tRNA^{Phe}. We arrived at this conclusion from a comparison of kinetic constants of formation of the enzyme·Phe-tRNA^{Phe} complex and of tRNA phenylalanylation at steady state. In the following section, we measured the rate constant of enzymatic AMP-dependent deacylation and compared them with the rate constants previously obtained for the kinetics of complex formation. We verified by competition experiments with tRNA^{Phe} that the Michaelis–Menten complex is not consistent with tRNA site directed but with Phe site directed binding of Phe-tRNA^{Phe}.

The concentration dependence of steady-state rates of AMP-dependent deacylation of Phe-tRNA^{Phe}, with AMP or Phe-tRNA^{Phe} as the varied substrate, is shown in terms of Eadie (1942) plots in Figure 1. Kinetic constants are listed in Table I. The rate constant is 10 ± 0.5 s^{-1} and the Michaelis–Menten constant 0.4 ± 0.1 μM for Phe-tRNA^{Phe} as the varied substrate. This is to be compared with rate constant $k_f K_1$(obsd) = 13 ± 4 s^{-1} and K_1(obsd) = 0.4 μM for association of enzyme and Phe-tRNA^{Phe} (Baltzinger & Holler, 1982). k_f refers to the bimolecular rate constant of association in the Phe site directed mode and K_1(obsd) to an apparent dissociation the value of which resembles an averaged dissociation constant for binding of Phe-tRNA^{Phe} at the tRNA-specific site of the enzyme, and for binding in the collisional complex of the Phe site directed mode [for details, see Baltzinger & Holler (1982)]. These values of the compared reactions are the same within experimental accuracy and state that the conformational change of the enzyme·Phe-tRNA^{Phe} complex is also rate limiting for the AMP-dependent deacylation.

The similarities of the values imply that the Phe site directed Michaelis–Menten complex competes with the tRNA site directed complex of Phe-tRNA^{Phe} [see also Güntner & Holler (1979)]. We have previously shown that tRNA^{Phe}, which

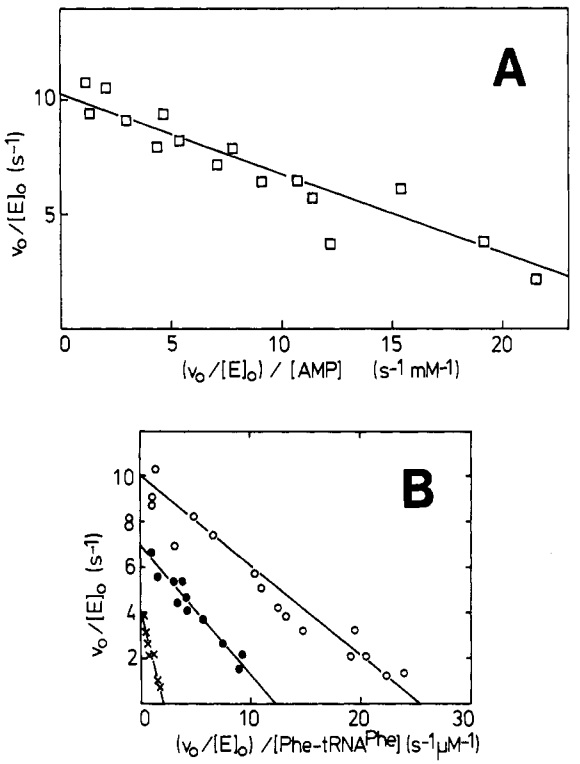
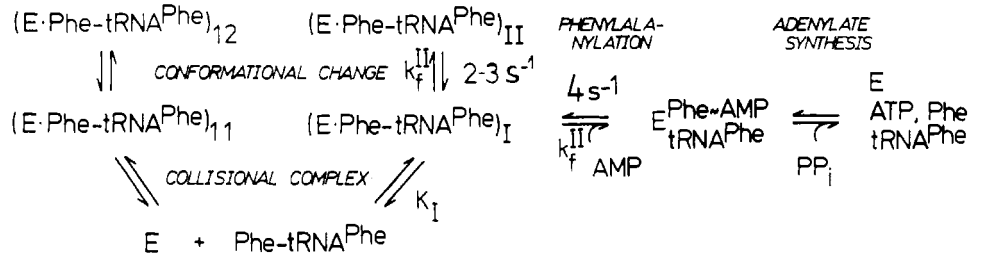


FIGURE 1: AMP-dependent deacylation of [^{14}C]Phe-tRNA^{Phe} under steady-state conditions. Inhibition by tRNA^{Phe}: (A) [AMP] varied, 3.5 μM Phe-tRNA^{Phe}; (B) [Phe-tRNA^{Phe}] varied, 10 mM AMP, tRNA^{Phe} absent (○) or present at 0.45 (●) or 5.5 μM (×). Conditions were 50 mM Tris-HCl (pH 7.5; 25 °C), 12 mM total $MgCl_2$, 2 mM PP_i, 0.1 mM EDTA, and 0.65 mM dithioerythritol. Lines represent best fits to experimental points and are used to determine the kinetic constants in Table I. Initial rates were measured by drawing 15–20-μL aliquots at various times. Deacylation was terminated by mixing these aliquots each with 120 μL of carrier tRNA (50 A_{260} units/mL) and 0.25 M sodium acetate (pH 5.0), and subsequently with 120 μL of cetyltrimethylammonium bromide (4 mg/mL). The mixture was cleared from precipitated nucleic acid by centrifugation, and 200 μL of the supernatant was counted for radioactivity in Scintillator 299 (Packard).

cannot bind in the Phe site directed mode, is noncompetitive with Phe-tRNA^{Phe} (Güntner & Holler, 1979; Holler, 1980). This mechanism should hold, and the values of the observable kinetic parameters should be predictable for deacylation in the presence of added tRNA^{Phe}. The results in Figure 1B and in Table I show the catalytic rate constant and the Michaelis–Menten constant being affected by added tRNA^{Phe}. A calculation is carried out in the Appendix that demonstrates the correctness of the proposed kinetic mechanism (Scheme I) by employing recently determined values and by comparing calculated with experimental values. The terminology in Scheme I is the same as in the preceding paper (Baltzinger & Holler, 1982).

tRNA Phenylalanylation and AMP-Dependent Deacylation of Phe-tRNA^{Phe} Are in Concert with the Conformational Change. The similarity of values for the rate constants of the

Scheme I



enzyme·Phe-tRNA^{Phe} complex and for the catalytic rate constants shows that a conformational change is rate determining. It does not, however, exclude the possibility that the conformational change and the chemical reaction are kinetically distinct. If they are not concerted, rapid sampling should reveal a burst of Phe-tRNA^{Phe} prior to steady-state phenylalanylation (Fersht, 1977) or a burst of tRNA^{Phe} prior to steady-state deacylation. With the technique of single-turnover measurements, observable rate constants should have higher values than those obtained at steady state.

For tRNA phenylalanylation, in situ synthesized enzyme·[^{14}C]Phe~AMP [1.15 μM enzyme, 16.5 mM MgATP, 80 μM [^{14}C]Phe (50 μCi/μmol), and 10–20 units/mL inorganic pyrophosphatase] was mixed in a quench-flow apparatus with a solution containing 9.9 μM tRNA^{Phe}. The mixing ratio was 1:1 (v/v). Both solutions contained 50 mM Tris-HCl (pH 7.5; 25 °C), 10 mM free $MgCl_2$, 0.1 mM EDTA, and 6.6 mM reduced glutathione. Prolonged incubation of adenylate with dithioerythritol gave erroneously high backgrounds of acid-precipitable radioactivity; this was not observed with freshly reduced glutathione. A burst was not observed. Production of [^{14}C]Phe-tRNA^{Phe} was linear with time. The rate constant, in terms of the slope of the line with the best fit to the experimental points, was 4.4 ± 0.2 s^{-1} and compares readily with k_{cat} = 3.8 ± 0.3 s^{-1} of steady-state phenylalanylation (Holler, 1980). In the case of AMP-dependent deacylation of Phe-tRNA^{Phe}, a quench-flow experiment was performed under conditions of excess active sites over [^{14}C]Phe-tRNA^{Phe} (see Materials and Methods and Table I). The rate constant of the single-exponential appearance of [^{14}C]Phe, 12 ± 1 s^{-1}, was in good agreement with k_{cat} = 10 ± 0.5 s^{-1} from steady-state deacylation (Table I). The results indicate that chemical synthesis and deacylation of Phe-tRNA^{Phe} were concerted with the conformational change.

Stoichiometry of Phenylalanine Transferring Sites. Part of an exact description of reaction kinetics is the knowledge of the number of functioning active sites per molecule of enzyme. In this section, we determined how many of the two active sites per molecule of enzyme (Hanke et al., 1974; Fayat et al., 1974; Bartmann et al., 1975b) could transfer phenylalanine during pre-steady-state and steady-state conditions. Consider, for instance, two active sites per molecule of enzyme, which are equivalent and independent. Catalytic rate constants at conditions of excess of enzyme over substrate (single-turnover experiment) would have smaller values that those measured at high excess of substrate over enzyme (steady-state experiment). Because the values were the same under both conditions (Table I), we would already conclude that phenylalanyl-tRNA synthetase had only a single transferring site. Stoichiometry measurements were performed under steady-state conditions of tRNA^{Phe} phenylalanylation (Table II). Enzyme·[^{14}C]Phe~AMP was synthesized in situ by the reaction of 69 μM [^{14}C]Phe (509 μCi/μmol), 2.6 mM ATP, and 1.7 μM enzyme in 100 μL of buffer (50 mM Tris-HCl, pH 7.5 at 22 °C, 10 mM free $MgCl_2$, 0.1 mM EDTA, 1.4 mM dithioerythritol, and 0.4 μg/mL inorganic pyrophosphatase) for 5 min. An aliquot of 10 μL was layered onto a parafilm plastic sheet and rapidly mixed with 3 μL of a solution of tRNA^{Phe} (139 μM in the same buffer). After 2–5 s, the reaction was terminated by addition of 7 μL of 0.5 M HCl containing 10 mM L-phenylalanine. Controls without tRNA were performed by mixing 10-μL aliquots of the enzyme·adenylate reaction mixture with 10 μL of 0.5 M HCl–10 mM Phe solution. The amount of [^{14}C]phenylalanyl adenylate was determined according to Jakubowski et al. (1977). Aliquots of 1 μL were applied to cellulose thin-layer sheets (Eastman 13254) and developed in butanol–acetic acid–water (4:1:1 by volume) at 4 °C. The results are summarized in Table II. In the absence of tRNA^{Phe}, 1.5 mol of adenylate was measured per mol of enzyme, which stands in close agreement with 2 mol of adenylate per mol of enzyme (Bartmann et al., 1975a,b). Under conditions of steady-state phenylalanylation, less than stoichiometric amounts of adenylate were observed. Under otherwise identical conditions, only half of the adenylate which had been measured in the absence of tRNA^{Phe} was found (compare the first and fourth lines in Table II). The results indicate that under steady-state conditions only a single active site is functioning. We have confirmed this by comparison with calculated stoichiometry values (Table II), and by the following chase experiment. Adenylate (1.5 mol/mol of enzyme) was mixed with a solution of tRNA^{Phe} (30 μM final concentration) containing unlabeled Phe (7 mM final concentration). The amount of acid-precipitable radioactivity corresponded to 0.83 mol of [^{14}C]Phe-tRNA^{Phe}, equivalent to approximately 50% transfer. The remaining fraction of the adenylate must have been hydrolyzed since other compounds besides [^{14}C]Phe could not be found by thin-layer chromatography (not shown). Phe-tRNA^{Phe} in place of tRNA^{Phe} did not diminish the amount of adenylate (Table II, last line) and

Table II: Stoichiometry of Enzyme·[^{14}C]Phenylalanyl Adenylate Complexes during Steady-State Phenylalanylation of tRNA^{Phe} [a]

[ATP] (mM)	[Phe] (μM)	% of total tRNA^{Phe} phenylalanylated	stoichiometry, [E·Phe~AMP]/[E]$_0$ exptl (mol/mol)	stoichiometry, [E·Phe~AMP]/[E]$_0$ calcd[c] (mol/mol)
2.0	53	0[b]	1.5	2.0[d]
0.15	5	5	0.36	0.34
		4.9	0.32	
		5.9	0.45	
0.20	20	10.4	0.49	0.63
		13.8	0.61	
		12.1	0.64	
2.0	53	8.5	0.82	0.85
		39	0.73	
2.0	53	93	1.8	2.0
		89	1.9	

[a] For conditions, see Materials and Methods. [b] tRNA^{Phe} not present. [c] Calculated on the basis of a single active site and eq 9. k_{ac} = 5 s^{-1}, k_{int} = 50 s^{-1}, S_{tRNA} = 1.0 (32 μM tRNA^{Phe}), K_{Phe} = 30 μM, K_{ATP} = 260 μM. [d] For saturation of two active sites.

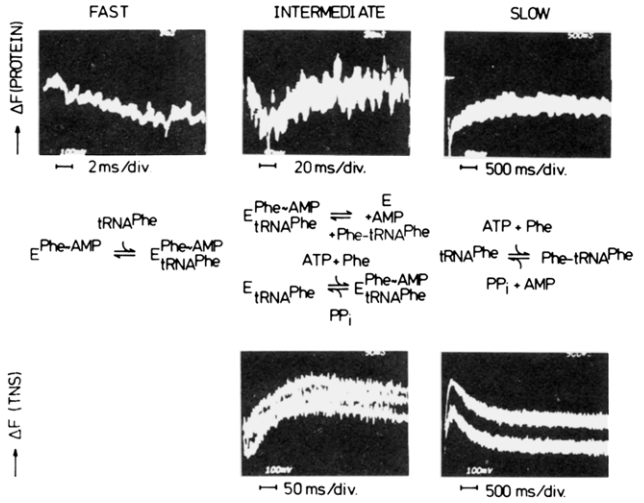


FIGURE 2: Reactions during catalysis observed by fluorescence. (Upper panels) Protein intrinsic fluorescence intensity; (lower panels) TNS fluorescence (TNS method). Solutions of enzyme and $tRNA^{Phe}$ were mixed in a stopped-flow apparatus. All solutions contained 50 mM Tris-HCl (pH 7.5; 25 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2–0.6 mM dithioerythritol, ATP, Phe, and respectively 5 μM TNS. (Upper panels) 0.12 μM enzyme, 0.52 μM $tRNA^{Phe}$, 0.2 mM ATP, and 5 mM; (lower panels) 0.094 μM enzyme, 0.054 μM $tRNA^{Phe}$, 0.2 mM ATP, and 19 μM Phe. The signal-to-noise ratio in the upper example is not representative. Accuracy was higher in most other cases. Note that the scale is 100 mV per division in the first upper photograph and 50 mV per division in the two other upper photographs. The assignment of the reactions is carried out in the text.

was not stimulatory for hydrolysis. We conclude from all of these results that only a single active site was functioning in the synthesis of adenylate and in transferring phenylalanine to tRNA under pre-steady-state and steady-state conditions of tRNA aminoacylation (half-of-the-sites reactivity).

Verification of Kinetics of Partial Reactions to Be Valid for the Overall Catalytic Reaction. Single-turnover and burst experiments as well as the measurement of the enzyme·Phe-$tRNA^{Phe}$ complex formation were considered as being partial reactions of the catalytic mechanism. We wanted to verify that the conclusions derived from these reactions were also valid under conditions where the complete sequence of reactions could be observed. Our approach was to observe various transients by the stopped-flow technique, measure their rate constants, and compare them with the results from the partial reactions.

(A) tRNA Phenylalanylation. Kinetics of association of $tRNA^{Phe}$ with enzyme·adenylate, of consumption of adenylate and its resynthesis, and of $tRNA^{Phe}$ phenylalanylation have been established by complementary fluorescence techniques as will be outlined in this section. One technique followed the fluorescence of enzyme-bound TNS [details in Kosakowski & Holler (1973), Bartmann et al. (1975a), Güntner & Holler (1979), Pimmer & Holler (1979), Holler (1980), and Holler et al. (1981)]. Note that by this technique only the reaction at one of the two active sites of the enzyme is observed (Pimmer & Holler, 1979; Holler, 1980). The other technique employed protein intrinsic fluorescence (Farelly et al., 1971; Bartmann et al., 1975b; Baltzinger & Holler, 1982). An example of the application of the two techniques is given in Figure 2. In situ synthesized enzyme·adenylate was rapidly mixed with a solution of $tRNA^{Phe}$. In the case of the protein intrinsic fluorescence (upper panels), we observed a fast intensity decrease (2–10 ms), an intermediate increase (20–200 ms), and a slow second increase (200 ms–3 s). In the case of the TNS method (lower panels), a fast reaction was not observed. An increase was followed by a decrease, the times of which corresponded to the intermediate and slow reactions, respectively. The reactions have been assigned to binding of $tRNA^{Phe}$, to consumption and simultaneous resynthesis of adenylate (while approaching steady state), and to overall phenylalanylation of $tRNA^{Phe}$, respectively.

The fast intensity decrease (upper panels) was identified with binding of $tRNA^{Phe}$ for the following reasons. (1) An intensity decrease as a result of enzyme·$tRNA^{Phe}$ complex formation (K_d = 0.12 μM) has been reported (Bartmann et al., 1975a). (2) The intensity decay followed a single-exponential time dependence (after a proper subtraction of the contribution from the intermediate reaction, where necessary). Observed rate constants were linear with respect to concentration of $tRNA^{Phe}$ according to $k_{obsd} = k_{off} + k_{on}[tRNA^{Phe}]$ with rate constants $k_{on} = 100 \pm 10\ \mu M^{-1}\ s^{-1}$ (association) and $k_{off} = 40 \pm 4\ s^{-1}$ (dissociation). Such kinetics resembled those of an apparent bimolecular recombination (Bernasconi, 1976) and have been reported in the case of other aminoacyl-tRNA synthetases (Krauss et al., 1973, 1979). The value of the calculated dissociation constant $K_d(calcd) = k_{off}/k_{on} = 0.4\ \mu M$ is not in good agreement with the above value of Bartmann et al. (1975b). (3) Neither noncognate tRNAs, like $tRNA^{Lys}$ or $tRNA^{Tyr}$, nor lysyl-tRNA synthetase or bovine serum albumin gave any results. The reaction was prevented by 0.5 M NaCl. In the absence of adenylate, the reaction was too fast to be measured.

The intermediate reaction was assigned to consumption and simultaneous resynthesis of adenylate because of the following reasons. (1) Reaction rates were of the same time scale as those measured previously for adenylate synthesis in the absence of tRNA (Pimmer & Holler, 1979). They varied with changing concentrations of Phe and ATP as was expected for adenylate synthesis (Figure 4). (2) They also depended on the concentration of $tRNA^{Phe}$, as was in accord with the consumption of adenylate during phenylalanylation of $tRNA^{Phe}$. It should be noted that this consumption had been not considered in previous work (Pimmer & Holler, 1979). (3) The intermediate reaction "fused" with the slow reaction at conditions of single turnover when adenylate could be consumed but not resynthesized (Figure 3B). (4) With the TNS method, the intensity in Figure 2 (lower panels) reached a steady-state level which was dependent of the concentrations of all substrates (Figure 5). In other reactions (not shown) and the TNS method, we started by mixing enzyme, Phe, ATP, and $tRNA^{Phe}$. The same steady-state level as above was obtained in this case by an intensity decrease.

Intensity changes were easily understood in the case of the TNS method from a competition between TNS and adenylate. In the case of protein intrinsic fluorescence, this mechanism was not known. The intensity decrease accompanying adenylate synthesis was absolutely dependent on the presence of $tRNA^{Phe}$. Phe-$tRNA^{Phe}$, which itself causes an intensity decrease (Baltzinger & Holler, 1982), could not replace $tRNA^{Phe}$. This explained the occurrence of the observed single exponential in Figure 3A in the presence of limiting $tRNA^{Phe}$.

The slow reaction was assigned to phenylalanylation of $tRNA^{Phe}$ on the basis of the following observations. (1) At conditions of excess $tRNA^{Phe}$ over enzyme using the TNS method, the intensity decrease followed a linear time dependence before it leveled off at chemical equilibrium (not shown). The duration of the linear portion was long in the presence of high concentrations of $tRNA^{Phe}$. In the case of the protein intrinsic fluorescence, this portion was never linear. This probably reflected binding of product Phe-$tRNA^{Phe}$ and the lack of a fluorescence change in the absence of $tRNA^{Phe}$. (2)

Table III: Kinetic Parameters of $tRNA^{Phe}$ Phenylalanylation at Various Conditions[a]

condition	obsd parameters[b]		
	dissociation constant (μM)	adenylate synthesis, k_{int} (s^{-1})	phenylalanylation, k_{ac} (s^{-1})
steady state (aminoacylation assay)[c]	nd[d]		3.8 ± 0.3
pre steady state (quench flow)	nd		4.4 ± 0.2
single turnover (as in Figure 3, stopped flow, fluorescence)	nd		4–6
pre steady state (intermediate reaction of Figure 2)			
[Phe] varied[e]	30	69 ± 10	6 ± 3
[ATP] varied,[f] 5 mM Phe	200	50 ± 10	5 ± 3
[ATP] varied, 20 μM Phe	200	25 ± 8	5 ± 3
$\Delta F_{interm}/\Delta F_{adenylate}$			
[ATP] varied[g]	500		
[Phe] varied[h]	40		

[a] Tris-HCl (50 mM), pH 7.5 (21–25 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.2 mM dithioerythritol. In most cases, inorganic pyrophosphatase had been added (see figures and text). [b] Determination of parameters from Figures 4 and 5 on the basis of eq 5, 7, and 8. [c] Holler (1980). [d] nd = not determined. [e] Figure 4A. [f] Figure 4B. [g] Figure 5B. [h] Figure 5C.

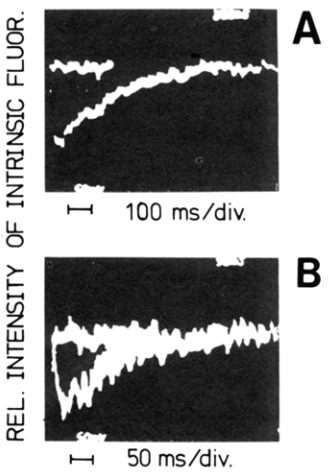
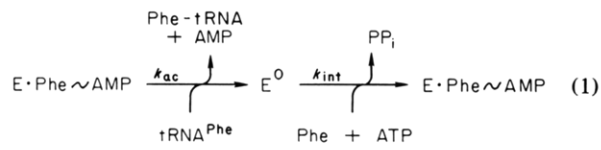


FIGURE 3: Single turnover of $tRNA^{Phe}$ phenylalanylation observed via protein intrinsic fluorescence. (A) Equal concentrations of $tRNA^{Phe}$ and enzyme active sites. A solution containing 0.5 μM enzyme was mixed in a 1:1 (v/v) ratio with a solution containing 1.0 μM $tRNA^{Phe}$. Both solutions were 5 mM in Phe and 1 mM in ATP. (B) Limiting amounts of adenylate. One solution contained 0.1 mM ATP, 0.13 mM Phe, and 0.2 μM enzyme and the other 10 mM L-phenylalaninol (inhibitor) and 1 μM $tRNA^{Phe}$. Buffer was 50 mM Tris-HCl (pH 7.5; 25 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.2 mM dithioerythritol. The fast intensity decrease reflects formation of the enzyme·$tRNA^{Phe}$ complex.

In the case of the protein intrinsic fluorescence, and either limiting adenylate or limiting $tRNA^{Phe}$, the slow reaction fused with the intermediate reaction to a single-exponential time dependence (Figure 3). Rate constants were 4–6 s^{-1}, provided that concentrations of enzyme and $tRNA^{Phe}$ exceeded the value of the enzyme·$tRNA^{Phe}$ dissociation constant (0.12 μM; Bartmann et al., 1975a); otherwise, reactions were slower. With the TNS method, a single-exponential intensity increase was seen for limiting adenylate (not shown). With limiting $tRNA^{Phe}$, the intensity first increased due to consumption and resynthesis of adenylate and then decreased exponentially due to phenylalanylation of all the $tRNA^{Phe}$ (Figure 2, lower panels). Rate constants of the single-turnover experiments are included in Table III.

Kinetics and amplitudes were analyzed in order to obtain a quantitative description. Kinetic equations have been derived on the basis of the following minimal reaction scheme:



(1)

with E·Phe∼AMP the enzyme·adenylate complex, E^0 the enzyme (complexes) with free binding sites for Phe (and TNS, respectively), k_{ac} the first-order rate constant of phenylalanylation of $tRNA^{Phe}$, and k_{int} the first-order rate constant of adenylate synthesis. Reverse reactions have been neglected because PP_i was hydrolyzed by added inorganic pyrophosphatase and AMP concentrations were small ($\ll K_m$-(AMP) = 0.35 mM, Table I). All reactions except synthesis and consumption of adenylate were assumed rapid and, where necessary, at preequilibrium. Substrates were thought to bind randomly (Santi et al., 1971), and coupling between sites was neglected (Bartmann et al., 1975a; Holler, 1980). To consider other assumptions, we refer to Pimmer & Holler (1979) and Holler (1980).

In the case of the TNS method, intensity (F) was proportional to [E·TNS], and for [TNS] $\gg E_0$, it was proportional to E^0. Similar considerations would be necessary in the case of the protein intrinsic fluorescence. The starting equation is

$$d[E_0]/dt = k_{ac}[E\cdot Phe\sim AMP]S_{tRNA} - k_{int}[E^0]S_{ATP}S_{Phe} \quad (2)$$

Concentration terms are

$$S_{tRNA} = [tRNA^{Phe}]/\{K_{tRNA} + [tRNA^{Phe}]\}$$
$$S_{ATP} = [ATP]/\{K_{ATP} + [ATP]\} \quad (3)$$
$$S_{Phe} = [Phe]/\{K_{Phe} + [Phe]\}$$

K_{tRNA}, K_{ATP}, and K_{Phe} are dissociation constants of the respective complexes (total enzyme $[E]_0 \ll$ [ATP], [Phe], and [$tRNA^{Phe}$]). The mass conservation equation is used in an approximation form.

$$[E]_0 = [E^0] + [E\cdot Phe\sim AMP] \quad (4)$$

The concentration of E·Phe has been neglected partly because at low Phe/K_{Phe} little complex is formed. Furthermore, if the intermediate reaction is evaluated, the small amounts of turnover hardly affect high concentrations of Phe and of enzyme·Phe complex. Errors due to high concentrations of the enzyme·Phe complex are avoided if reaction amplitudes are considered relative to reaction amplitudes observed in the case of adenylate synthesis upon rapid mixing of solutions containing enzyme, Phe, and ATP at the particular concentrations. Complexes of Phe-$tRNA^{Phe}$ are not considered for evaluation of the intermediate reaction because concentrations of Phe-$tRNA^{Phe}$ are below values of the particular dissociation constants at that time. The complexes cannot be neglected in the case of single-turnover experiments, where concentrations of reactants are high.

Table IV: Number of Reactions and Kinetic Constants for Rapid Mixing of Phe-tRNA^{Phe} with AMP and PP_i As Measured by the TNS Method[a]

conditions of mixing (final concn)	no. of reactions	rate constant (s^{-1})	reaction amplitude, $\Delta F/\Delta F_{Phe\to\infty}$[b]
enzyme (0.15 μM) vs. Phe-tRNA^{Phe} (0.78 μM)	1	13 ± 2	−0.6
enzyme (0.15 μM) vs. Phe-tRNA^{Phe} (0.78 μM), 1.2 mM PP_i	1	14 ± 2	−0.6
enzyme (0.15 μM) vs. Phe-tRNA^{Phe} (0.78 μM), 9 mM AMP	1	12 ± 2	−1.0
enzyme (0.75 μM) vs. Phe-tRNA^{Phe} (0.71 μM), 1 mM PP_i plus [AMP] (varied)	2	40 ± 5 6 ± 1[d] 7.6–9.2[e]	−0.4 +0.1[c]

[a] Tris-HCl buffer, pH 7.5 (25 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2–0.6 mM dithioerythritol, and 5 μM TNS. [b] Relative change in fluorescence intensity (in comparison to $\Delta F_{Phe\to\infty}$) by mixing solutions of enzyme and 5 mM Phe. The minus sign indicates a decrease in intensity, the plus sign an increase. [c] Not corrected for the effect of liberated Phe in the presence of AMP. According to synergism, Phe binds quite strongly to the enzyme in the presence of 9 mM AMP (Kosakowski & Holler, 1973). The effect was determined in a control experiment. After correction, the reaction amplitude amounts to $\Delta F/\Delta F_{Phe\to\infty} = +0.4$. [d] The maximum rate constant is given. The Michaelis-Menten constant is K_m(AMP) = 0.5 ± 0.15 mM. The rate constant of the fast reaction is independent of AMP concentration within experimental error. [e] After correction for incomplete saturation. See text.

The observed first-order rate constant of the intermediate reaction is then obtained from a combination of eq 2 and 4:

$$k_{obsd} = k_{ac}S_{tRNA} + k_{int}S_{Phe}S_{ATP} \quad (5)$$

Expressions for the reaction amplitude have been derived by employing the steady-state condition

$$d[E^0]/dt = 0 \quad (6)$$

and $[E{\cdot}Phe{\sim}AMP] = [E]_0 - [E^0]$ from eq 4:

$$\frac{\Delta F_{interm}}{\Delta F_{Phe\sim AMP}} = \frac{[E^0]}{[E]_0} = \frac{k_{ac}S_{tRNA}}{k_{ac}S_{tRNA} + k_{int}S_{ATP}S_{Phe}} \quad (7)$$

$$\frac{\Delta F_{interm}}{\Delta F_{Phe\sim AMP}} = 1 - \frac{k_{int}S_{ATP}S_{Phe}}{k_{obsd}} = \frac{k_{ac}S_{tRNA}}{k_{obsd}} \quad (8)$$

$$\frac{[E{\cdot}Phe{\sim}AMP]}{[E]_0} = 1 - \frac{k_{ac}S_{tRNA}}{k_{ac}S_{tRNA} + k_{int}S_{ATP}S_{Phe}} \quad (9)$$

Equation 5 has been employed in the case of Figure 4, eq 7 in the case of Figure 5B,C, eq 8 in the case of Figure 5D, and eq 9 for calculation of the values in Table II. Parameters were determined by curve fitting or in the usual way by employing linearization methods [e.g., see Bartmann et al. (1975a)]. The values of k_{ac} were calculated for K_{tRNA} = 0.12 μM (Bartmann et al., 1975a). Dissociation constants obtained in Table III agree with those previously determined (Bartmann et al., 1975a; Pimmer & Holler, 1979). Values of rate constants of adenylate synthesis, k_{int}, in Table III are lower than those measured in the absence of tRNA^{Phe}. A similar result has been forwarded for the yeast enzyme (Fasiolo et al., 1981). In the case of varying [tRNA^{Phe}], a stoichiometry of 1 mol of tRNA^{Phe} per mol of enzyme·adenylate was obtained by the tangent method (Figure 5A). This finding supports the data in Table II and supports the idea of half-of-the-sites reactivity.

(B) AMP-Dependent Deacylation of Phe-tRNA^{Phe}. Kinetics of AMP-dependent deacylation were measured by the TNS method. We observed a complete extinction of the fluorescence intensity. Kinetics were identical with those for enzyme·Phe-tRNA^{Phe} formation from free enzyme and Phe-tRNA^{Phe} in the absence of AMP (Table IV). The extinction of the fluorescence in the presence of AMP and the kind of kinetics were in agreement with formation of adenylate and tRNA^{Phe} by a mechanism in which the conformational change was rate limiting. In a further experiment, we mixed a solution of enzyme with a solution of Phe-tRNA^{Phe}, containing AMP and PP_i (Table IV). We saw a rapid intensity decrease and a subsequent recovery of intensity (Figure 6). Such a sequence was consistent with the intermediate synthesis of adenylate from Phe-tRNA^{Phe} and AMP, followed by pyrophosphorolysis.

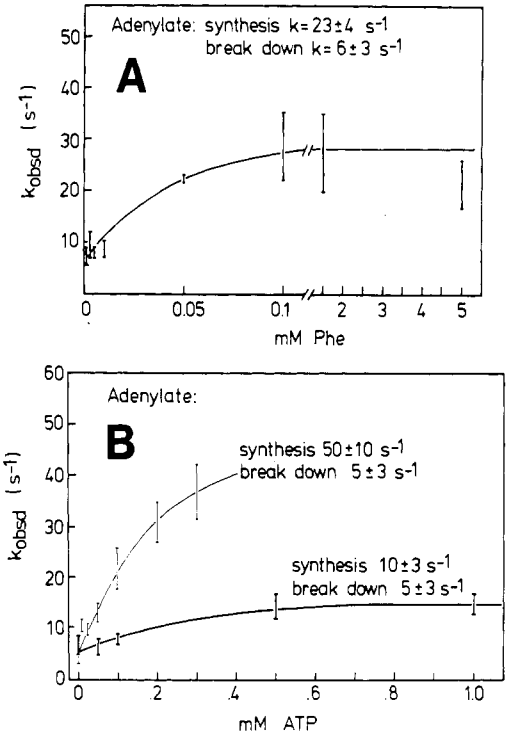


FIGURE 4: First-order rate constants of the intermediate reaction as a function of L-phenylalanine (A) and ATP concentrations (B). (A) Concentrations after mixing were 0.10 μM enzyme, 1.0 μM tRNA^{Phe}, 0.1 mM ATP, and variable [Phe]. (B) Concentrations after mixing were 0.02–0.5 μM enzyme, 1.4 μM tRNA^{Phe}, and 20 μM Phe (lower curve, TNS fluorescence) and 0.1–0.2 μM enzyme, 1.0 μM tRNA^{Phe}, and 5 mM Phe (upper curve, intrinsic protein fluorescence). Solutions contained 50 mM Tris-HCl (pH 7.5; 25 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM dithioerythritol, and 5 μM TNS (except for the upper curve). Curves are computed on the basis of eq 5, setting $k_{ac}S_{tRNA}$ = 6 s^{-1}, K_{Phe} = 30 μM, and $k_{int}S_{ATP}$ = 23 s^{-1} (A), $k_{ac}S_{tRNA}$ = 5 s^{-1}, K_{ATP} = 200 μM, and $k_{int}S_{Phe}$ = 10 s^{-1} (B, lower curve), and $k_{ac}S_{tRNA}$ = 5 s^{-1}, K_{ATP} = 200 μM, and $k_{int}S_{Phe}$ = 50 s^{-1} (B, upper curve).

The level of fluorescence intensity for the intermediate was dependent on the concentrations of reactants (not shown).

The rapid intensity decrease followed a single-exponential time dependence over more than 80% of its amplitude. The rate constant, 40 s^{-1}, was almost insensitive against a variation of [AMP]. We assigned the reaction to pyrophosphorolysis. A value of 80 s^{-1} has been reported for the rate constant under somewhat different conditions (Pimmer & Holler, 1979). The finding of pyrophosphorolysis as being the fast reaction was consistent with general kinetics derived for a consecutive slow synthesis and rapid breakdown of an intermediate (Fersht,

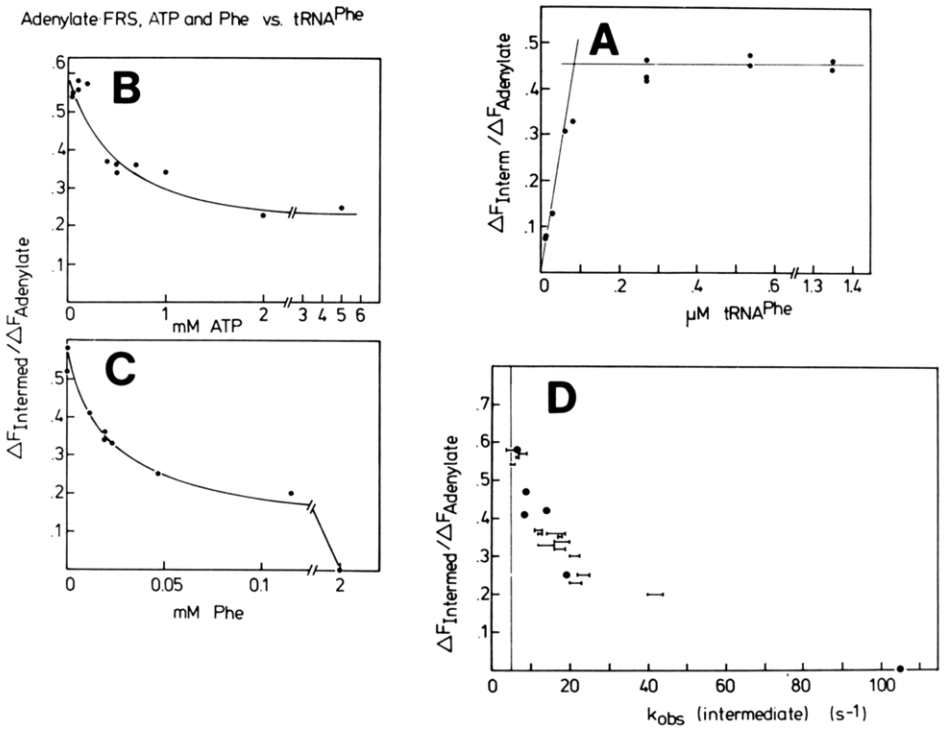


FIGURE 5: Amplitude of the intermediate reaction. A solution of in situ synthesized enzyme·adenylate was mixed in a 1:1 (v/v) ratio with a solution of $tRNA^{Phe}$. The TNS method was used. The amplitude, ΔF_{interm}, was measured as the increase in fluorescence intensity from its initial to its maximum value (Figure 2). Reference $\Delta F_{Phe\sim AMP}$ was measured by mixing a solution of enzyme and Phe (actual concentration) with a solution of ATP and Phe in the absence of $tRNA^{Phe}$. Curves in panels B and C are drawn for dissociation constants of ATP and Phe, respectively, indicated in Table III. Conditions were 50 mM Tris-HCl (pH 7.5; 25 °C), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM dithioerythritol, 5 μM TNS, 1–10 μg of inorganic pyrophosphatase, and 94 nM enzyme (final). (A) [$tRNA^{Phe}$] varied, 10 μM Phe, and 0.5 mM ATP. (B) [ATP] varied, 19 μM Phe, and 0.54 μM $tRNA^{Phe}$. (C) [Phe] varied, 0.5 mM ATP, and 0.54 μM $tRNA^{Phe}$. (D) Relative amplitude as a function of the first-order rate constant, k_{obsd} (0.54 μM $tRNA^{Phe}$). Note that intercept 1.0 cannot be obtained because of the intensity decrease caused by formation of enzyme·Phe-$tRNA^{Phe}$ at the end of the reaction (Baltzinger & Holler, 1982).

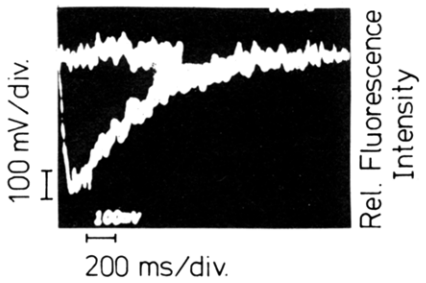


FIGURE 6: AMP-dependent deacylation of Phe-$tRNA^{Phe}$ under pre-steady-state conditions. Fluorescence intensity of TNS is measured after mixing of a solution containing 1.5 μM enzyme and a solution containing 1.42 μM Phe-$tRNA^{Phe}$, 2.06 mM pyrophosphate, and 0.55 mM AMP in the stopped-flow apparatus. The fast intensity decrease followed a $k_{obsd} = 40\ s^{-1}$ and the slow increase a $k_{obsd} = 2.3\ s^{-1}$. The fast reaction is almost insensitive to variation of the AMP concentration.

1977). In such kinetics, the intermediate appears with a rate constant similar to that of its breakdown and disappears with kinetics similar to those of its formation. This mechanism was qualitatively true in the observed case, since the slow reaction in Figure 6 was dependent on the concentration of AMP. In accordance with this, the slow reaction became longer as the concentration of Phe-$tRNA^{Phe}$ increased in excess over enzyme. Qualitatively, the slow reaction may be seen as the deacylation reaction of Phe-$tRNA^{Phe}$ yielding free enzyme, which can rebind TNS under concomitant increase in fluorescence intensity. Rate constants of this portion were evaluated as has been described under Materials and Methods (data processing for single-turnover reactions). without further correction, they were evaluated for the concentration dependence on AMP. A plot according to Eadie (1942) was linear with K_m(AMP) = 0.5 ± 0.15 mM and k_{obsd}(max) = 6 ± 1 s^{-1}. As indicated under Materials and Methods, this value had to be corrected for incomplete saturation of Phe-$tRNA^{Phe}$ by enzyme, that is, $[E]_0 \simeq K_s$, if we identify K_s with K_m(Phe-$tRNA^{Phe}$) = 0.4 μM from the steady-state deacylation experiments (Table I). Setting $[E]_0$ equal to the concentration of active sites, 2 × 0.75 μM = 1.5 μM, we obtain a corrected k_{obsd}(max) = 7.6 s^{-1}. This must be a lower limit since the actual concentration of available enzyme is somewhat reduced by the occurrence of the enzyme·adenylate intermediate. Another estimate takes into account anticooperativity and half-of-the-sites reactivity accounting for an available active-site concentration of, at most, 0.75 μM and thus a corrected rate constant k_{obsd}(max) = 9.2 s^{-1}. At any rate, the estimated value is in the range of that of k_{cat} for the steady-state deacylation (Table I).

The above results for the transient kinetics (Figures 2–6; Tables III and IV) are quantitatively consistent with the results from radioactive pre-steady-state and steady-state experiments (Figure 1; Tables I and II) in the case of tRNA aminoacylation, and qualitatively consistent in the case of AMP-dependent deacylation of Phe-$tRNA^{Phe}$. We consider our mechanism and the assignment of the rate-determining reaction validated by these findings. The results indicate also that other mechanisms which involve intermediates like aminoacyl enzymes (Thiebe, 1975) are not likely, at least in the case of *E. coli* phenylalanyl-tRNA synthetase.

Discussion

Rate-Determining Reaction. We have compared the kinetics at pre-steady-state and at steady-state conditions in both directions of the catalytic reaction. Agreement of rate constants, the absence of burst phenomena, and the principle of micro-

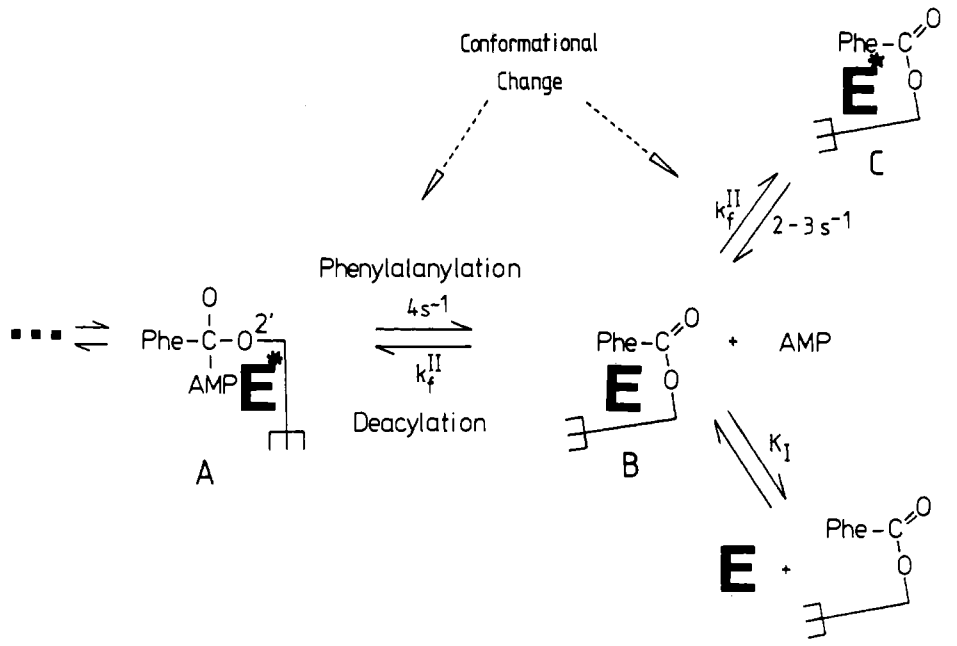


FIGURE 7: Proposed model of the catalytic pathway including the tetrahedral adenylate–tRNA intermediate and Phe site directed binding. (A) Tetrahedral complex. (B) Collisional enzyme·Phe-tRNA^{Phe} complex. (C) Dead-end complex. Phe site directed binding (complexes B and C) has been described in the accompanying paper (Baltzinger & Holler, 1982). The tetrahedral complex holds tRNA^{Phe} in the tRNA specific binding site of the enzyme. In the tetrahedral intermediate, tRNA^{Phe} binds with the 2′ carbon of the terminal adenosine (Sprinzl & Cramer, 1975). In the dead-end complex, the ester bond aligns with the 3′ carbon [see Baltzinger & Holler (1982)]. Enzyme and tRNA are symbolized by heavy lines and by forks, respectively.

scopic reversibility lead to the unambiguous conclusion that the formation of the Phe-tRNA^{Phe} ester bond and its breaking, respectively, are rate determining. The overall reaction, as measured by transient kinetics in both directions, is in full agreement with this conclusion. The results of our kinetic analysis are in full support of the classical mechanism with the aminoacyl adenylate on the catalytic pathway. We have no evidence for an aminoacyl enzyme intermediate. Kinetics of enzyme·tRNA^{Phe} complex formation, although slower in the presence of adenylate than in the case of free enzyme, are not rate limiting. Dissociation of the enzyme·Phe-tRNA^{Phe} complex, as it may appear from kinetics of complex formation (Holler, 1976), is also not rate determining.

Synthesis and AMP-Dependent Deacylation of Phe-tRNA^{Phe} Are in Concert with a Conformational Change. The kinetics of enzyme·Phe-tRNA^{Phe} complex formation have been shown to involve a slow conformational change (Baltzinger & Holler, 1982). The rate constant of the rearrangement of the complex and the rate constants of the catalytic reactions have identical values (Table V). Nevertheless, the results of the pre-steady-state kinetic experiments demonstrate that the enzyme·Phe-tRNA^{Phe} complex does not accumulate and, thus, cannot be on the catalytic pathway. This apparent paradox is resolved if we assume that nascent Phe-tRNA^{Phe} in the complex is similar to but not identical with Phe-tRNA^{Phe} of the association experiments. This idea has been put forward in the accompanying paper (Baltzinger & Holler, 1982). The fact that synthesis of Phe-tRNA^{Phe} does not show a burst in the quench-flow experiments indicates that the nascent bond between Phe and tRNA^{Phe} is labile under experimental conditions. We believe that the observed hydrolysis of phenylalanyl adenylate and the apparent half-of-the-sites reactivity (Table II) are indicative of just this lability. Clearly, further work along this line is required.

Proposal of a Catalytic Mechanism That Requires a Conformational Change. A mechanism (Figure 7) which fulfills the demands of all the results presented in this and the accompanying paper is presented as follows. (1) Adenylate and tRNA^{Phe} form a reactive tetrahedral intermediate. The reaction is fast and reversible. (2) Bond angles and lengths at the carbonyl C are geometrically fixed by the interactions of the phenylalanyl, AMP, and tRNA^{Phe} moieties with their respective binding sites of the enzyme. (3) The geometrical constraint is relieved, sp^2 hybridization of the carbon orbitals, and thus formation of Phe-tRNA^{Phe} becomes feasible by the release of the tRNA^{Phe} moiety from the tRNA-binding site of the enzyme concomitantly with the observed conformational change. The tetrahedral intermediate is identical with "nascent Phe-tRNA^{Phe}". It would be acid labile and could not be found under our experimental conditions. The tetrahedral intermediate would not be detectable by the TNS method because there would be no change in occupancy of the Phe-binding site during its synthesis from adenylate. The formation of the tetrahedral bond might be the mechanism causing the observed intensity changes in the case of the protein intrinsic fluorescence.

Table V: Comparison of Rate Constants of Catalytic Phenylalanylation of tRNA^{Phe} and of AMP-Dependent Deacylation with Rate Constants of Enzyme·Phe-tRNA^{Phe} Complex Formation[a]

reaction	rate constant (s^{-1})
Phe site directed enzyme·Phe-tRNA^{Phe} [b]	
association	13 ± 2[d]
dissociation	2–3
catalysis[c]	
phenylalanylation	3.8 ± 0.3[e]
	4.4 ± 0.2[f]
AMP-dependent deacylation	10 ± 0.5[e]
	12 ± 1[f]

[a] Tris-HCl (50–100 mM), pH 7.5, 25 °C, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM dithioerythritol, or 6.6 mM reduced glutathione (phenylalanylation by quench flow). [b] Baltzinger & Holler (1982). [c] Radioactive work, this publication. [d] First-order rate constants in terms of k_f/K_i (obsd) (association) and k_b (dissociation) as defined (Baltzinger & Holler, 1982). [e] Steady state. [f] Measured by quench flow.

The following considerations are in further support of the proposed model. (1) We have evidence that Phe-tRNA^{Phe} can interchange within the enzyme complex from the tRNA site directed into the Phe site directed binding mode under particular experimental conditions (unpublished experiments). The rate of the interchange is fast (k_{obsd} = 40 s^{-1}). (2) We have shown that Phe-tRNA^{Phe} is hydrolyzed independently of AMP if it binds to the tRNA^{Phe}-specific site of the enzyme (Güntner & Holler, 1979). We can explain recognition of a noncognate amino acid at the onset of hydrolytic proofreading (Fersht, 1977) by assuming that, contrary to the cause of a cognate amino acid, the noncognate aminoacyl moiety leaves the aminoacyl binding site at the time of the conformational change (conceivably because of less favorable interactions). The tRNA^{Phe} moiety remains bound (because of favorable interactions), and hydrolysis occurs. A similar mechanism might contribute to recognition of noncognate tRNA and subsequent hydrolysis because, as we have shown in the accompanying paper (Baltzinger & Holler, 1982), noncognate tRNA moieties are not admitted in the Phe site directed binding mode. (3) On the basis of free-energy calculations, we understand that the product aminoacyl-tRNA, if it could bind with its respective moieties simultaneously to the corresponding substrate binding sites, would cause very severe product inhibition (Güntner & Holler, 1979). Our present model describes a possibility of how to bypass this binding mode.

The task that remains is to furnish experimental support for the tetrhedral intermediate and to demonstrate that the proposed mechanism is also valid for other aminoacyl-tRNA synthetases. We have published several techniques in the present and previous publications that can well serve this purpose.

Appendix

Rate constants k_{cat}^{i} and Michaelis–Menten constants K_m^{i}(Phe-tRNA) of AMP-dependent deacylation of [^{14}C]-Phe-tRNA^{Phe} (Figure 1B, Table I) in the presence of inhibitor tRNA^{Phe} were calculated as follows: (1) $k_{cat}^{i} = k_f^{i}$(calcd)$K_i^{Phe\text{-}tRNA}$(calcd) = 4.4 s^{-1}. $K_i^{Phe\text{-}tRNA}$ represents K_1(obsd) in eq 4 of Baltzinger & Holler (1982). (2) k_f^{i}(calcd) = 2.15 μM^{-1} s^{-1} from eq 16 of Holler (1980), where k_f (in the nomenclature of this reference) = k_{cat}/K_m(Phe-tRNA) in the absence of tRNA^{Phe} (values from Table I, present publication) = 27 μM^{-1} s^{-1}, and $\beta = k_f^{i}/k_f$ = 0.0125 and K_i = 0.4 μM according to the values of Holler (1980, Table I and same nomenclature as used there). (3) $K_i^{Phe\text{-}tRNA}$(calcd) = aK_i = 2 μM with K_i = 0.4 μM (Holler, 1980). (4) $a = K_m^{i}$(Phe-tRNA)/K_m(Phe-tRNA) (Table I) = 5.07 in analogy to eq 7 of Kosakowski & Holler (1973). (5) K_m(Phe-tRNA) = 0.4 μM from Table I (present publication). (6) K_m^{i}(Phe-tRNA) = 2 μM as calculated from eq 6 of Kosakowski & Holler (1973) with K_2 (in their nomenclature) identical with K_m-(Phe-tRNA) = 0.4 μM (Table I, present publication), K_{21} (Kosakowski & Holler, 1973; their nomenclature) identical with K_i^{L} = 2.88 μM (Holler, 1980; calculated from Table I in this nomenclature), and K_1 (Kosakowski & Holler, 1973) = K_i = 0.4 (Holler, 1980) and $[L_1]_0$ = [tRNA^{Phe}] = 5.5 μM (Table I, present publication). In the case of 5.5 μM tRNA^{Phe}, calculated values are k_{cat}^{i} = 4.4 s^{-1} and K_m^{i}(Phe-tRNA) = 2 μM; in the case of 0.45 μM tRNA^{Phe}, these constants are 9.5 s^{-1} and 0.7 μM, respectively. The calculated values should be compared with the experimental values of Table I.

References

Baltzinger, M., & Holler, E. (1982) *Biochemistry* (preceding paper in this issue).

Bartmann, P., Hanke, T., Hammer-Raber, B., & Holler, E. (1974) *Biochemistry 13*, 4171–4175.

Bartmann, P., Hanke, T., & Holler, E. (1975a) *Biochemistry 14*, 4777–4786.

Bartmann, P., Hanke, T., & Holler, E. (1975b) *J. Biol. Chem. 250*, 7668–7674.

Bernasconi, C. F. (1976) in *Relaxation Kinetics*, Academic Press, London.

Eadie, G. S. (1942) *J. Biol. Chem. 146*, 85–93.

Farelly, J. G., Longworth, J. W., & Stulberg, M. P. (1971) *J. Biol. Chem. 246*, 1266–1270.

Fasiolo, F., Remy, P., & Holler, E. (1981) *Biochemistry 20*, 3851–3856.

Fayat, G., Blanquet, S., Dessen, P., Batelier, G., & Waller, J. P. (1974) *Biochimie 56*, 35–41.

Fersht, A. (1977) in *Enzyme Structure and Mechanism*, W. H. Freeman, San Francisco, CA.

Güntner, C., & Holler, E. (1979) *Biochemistry 18*, 2028–2038.

Hanke, T., Bartmann, P., Hennecke, H., Kosakowski, H. P., Jaenicke, R., Holler, E., & Böck, A. (1974) *Eur. J. Biochem. 43*, 601–607.

Holler, E. (1976) *J. Biol. Chem. 251*, 7717–7719.

Holler, E. (1980) *Biochemistry 19*, 1397–1402.

Holler, E., & Calvin, M. (1972) *Biochemistry 11*, 3741–3752.

Holler, E., Hammer-Raber, B., Hanke, T., & Bartmann, P. (1975) *Biochemistry 14*, 2496–2503.

Holler, E., Baltzinger, M., & Favre, A. (1981) *Biochemistry 20*, 1139–1147.

Jakubowski, H. Z., Pastuzyn, A., & Loftfield, R. B. (1977) *Anal. Biochem. 82*, 29–37.

Kern, D., & Gangloff, J. (1981) *Biochemistry 20*, 2065–2075.

Kosakowski, H. M., & Böck, A. (1970) *Eur. J. Biochem. 12*, 67–73.

Kosakowski, H. M., & Holler, E. (1973) *Eur. J. Biochem. 38*, 274–282.

Kovaleva, G. K., Moroz, S. G., Favorova, O. O., & Kisselev, L. L. (1978) *FEBS Lett. 95*, 81–84.

Krauss, G., Römer, R., Riesner, D., & Maass, G. (1973) *FEBS Lett. 30*, 6–10.

Krauss, G., von der Haar, F., & Maass, G. (1979) *Biochemistry 18*, 4755–4761.

Pimmer, J., & Holler, E. (1979) *Biochemistry 18*, 3714–3723.

Rainey, P., Hammer-Raber, B., Kula, M. R., & Holler, E. (1977) *Eur. J. Biochem. 78*, 239–249.

Remy, P., & Ebel, J. P. (1976) *FEBS Lett. 61*, 28–31.

Santi, D. V., Danenberg, P. V., & Satterly, P. (1971) *Biochemistry 10*, 4804–4812.

Sprinzl, M., & Cramer, F. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3049–3053.

Thiebe, R. (1975) *FEBS Lett. 60*, 342–345.