AFFINITY LABELLING OF TRYPTOPHANYL-tRNA SYNTHETASE WITH MESITOYL-AMP

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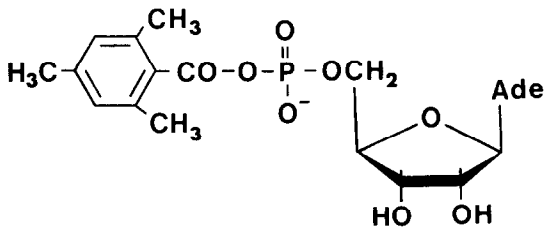
Received 3 November 1980

1. Introduction

The most effective inhibitors of aminoacyl-tRNA synthetases are derivatives of aminoacyl adenylates, intermediate compounds in the tRNA aminoacylation reaction [1–6]. Such 'multi-substrate' analogs simulate the activated state of substrates in their enzyme-catalysed transformation; because of their high affinity and, possibly, a higher selectivity, they are ideal as a basis for preparing affinity labels [7]. Attempts have been made to use analogs of aminoacyl adenylates for affinity modification of the synthetases. ATP γ-(*p*-azidobenzyl)-methylanilidate was shown to be a reversible competitive inhibitor of *E. coli* MRE-600 phenylalanyl-tRNA synthetase with respect to ATP and amino acid; UV-irradiation of the synthetase in the presence of this compound resulted in complete inactivation of the enzyme [8]. For methionyl-tRNA synthetase, despite the high affinity of methioninyl-8-azido-adenosine 5′-phosphate for the enzyme, only 5–15% of the photoreactive analog was bound covalently [6].

In this work, affinity labelling of beef pancreas tryptophanyl-tRNA synthetase (EC 6.1.1.2) (M_r 108 000–120 000, α_2 type (see [9])) was accomplished using a mixed anhydride of AMP with mesitylenecarboxylic acid [10]:



Mesitoyl-AMP

This compound, an acyl adenylate, contains a reactive adenylate phosphate group and an inert mesitoyl moiety possessing a considerable hydrophobicity [11].

2. Materials and methods

Electrophoretically homogeneous tryptophanyl-tRNA synthetase was isolated from beef pancreas as in [9]. Prior to use, the preparation of the enzyme was passed through a charcoal filter [12] (Ederol) or was precipitated at pH 5.0 and redissolved. The preparations of mesitoyl-AMP were synthesized as described [11].

The enzyme (1.6 μM) was incubated with mesitoyl-AMP (1 mM, unless specified) at 37°C in 0.05 M cacodylate buffer (pH 7.5). The activity of the enzyme was assayed in ATP–[^{32}P]pyrophosphate exchange and $tRNA^{Trp}$ aminoacylation reactions as in [9].

Unreacted mesitoyl-AMP was removed from the preparation of the inactivated enzyme by dialysis at 4°C against 0.05 M Tris–HCl buffer (pH 7.5) with or without 1 mM ATP. When the level of radioactivity in a dioxane scintillation cocktail for samples from the dialysis bag remained unchanged this was taken as a criterion for completeness of dialysis.

3. Results

Incubation of tryptophanyl-tRNA synthetase with mesitoyl-AMP at pH 7.5 and 37°C leads to a decrease in the ATP–[^{32}P]pyrophosphate exchange activity (fig.1A). The extent of inactivation depends on the concentration of mesitoyl-AMP and the time of incubation (pseudo-first order kinetics) and reaches 100% (fig.1A,2). The capabilities of the enzyme to catalyse

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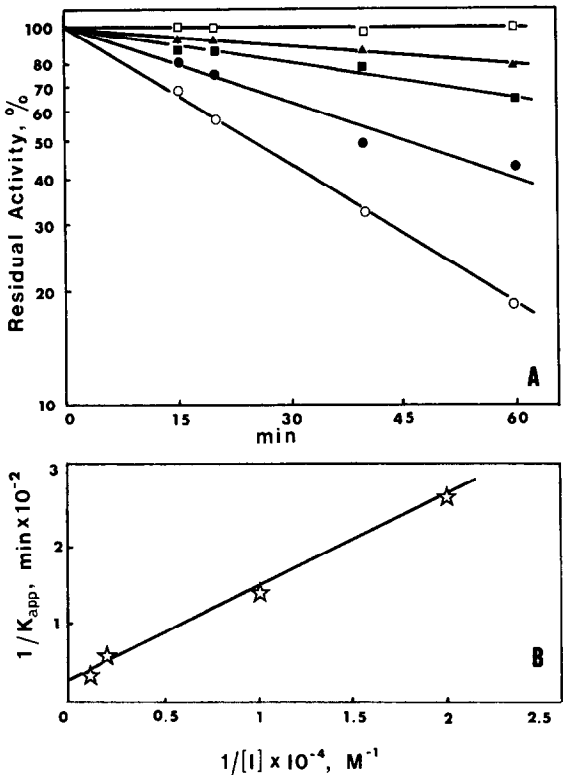

Fig.1. Inactivation of tryptophanyl-tRNA synthetase with mesitoyl-AMP. (A) Semilogarithmic plot of residual activity in the reaction of ATP–[^{32}P]pyrophosphate exchange against time. Concentrations of mesitoyl-AMP/incubation (mM): (○) zero; (●) 0.05; (■) 0.1; (▲) 0.5; (□) 1. (B) The rate constant, k_{app}, for inactivation of enzyme as a function of mesitoyl-AMP concentrations (using the data from (A) above).

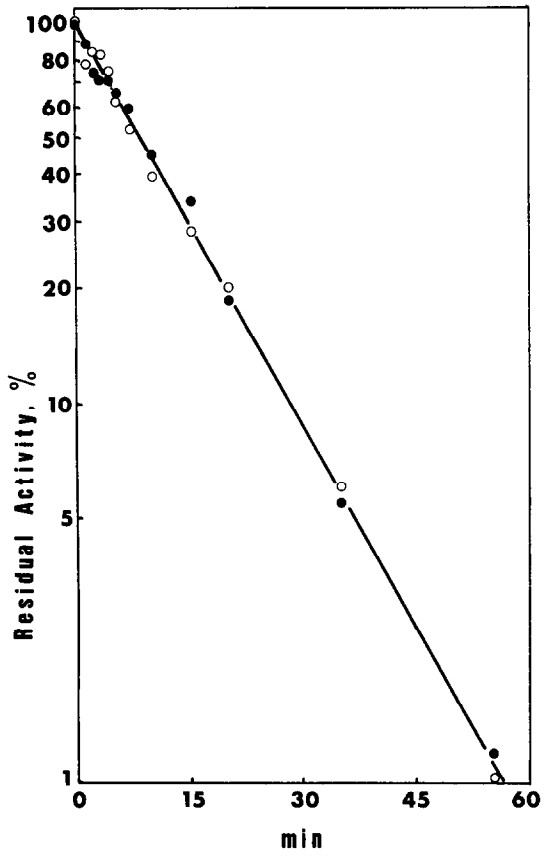

Fig.2. Inhibition of the activity of tryptophanyl-tRNA synthetase with mesitoyl-AMP in the exchange (●) and $tRNA^{Trp}$ aminoacylation (○) reactions.

the ATP–[^{32}P]pyrophosphate exchange and tRNA aminoacylation reactions fall in parallel (fig.2). The comparison of a protective action of the substrates all of which are taken at saturating concentrations shows that tryptophan and ATP protect the enzyme against inactivation more effectively than $tRNA^{Trp}$ (fig.3A).

If the enzyme preparation completely inactivated with mesitoyl-AMP was dialysed for 18 h at 4°C with 3 changes of buffer against 0.05 M Tris–HCl buffer (pH 7.5) or against 1 mM ATP in the same buffer no restoration of the enzyme activity occurred. These data as well as the time course of inactivation (fig.1A,2) indicate that the inhibition is irreversible.

The values of the pseudo-first order rate constants of inactivation, k_{app}, for each mesitoyl-AMP concentration were obtained from the data of fig.1A and $1/k_{app}$ was plotted *vs* $1/[I]$ (fig.1B). The curve corresponds to the saturation of the enzyme with the inhibitor and indicates (see [13] and references therein) that intermediate reversible enzyme · inhibitor complex is formed prior to the chemical reaction according to the scheme:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot I \xrightarrow{k_{+2}} E - I$$

Table 1 presents the K_i-values characterizing the affinity of mesitoyl-AMP for tryptophanyl-tRNA synthetase and the rate constants for modification of the enzyme, k_{+2}, determined in various experiments. The rate of reaction is moderate, which fits in with the moderate reactivity of mixed anhydrides of mesitoylenecarboxylic acid and adenine nucleotides in solution [11]. It provides the possibility of achieving spe-

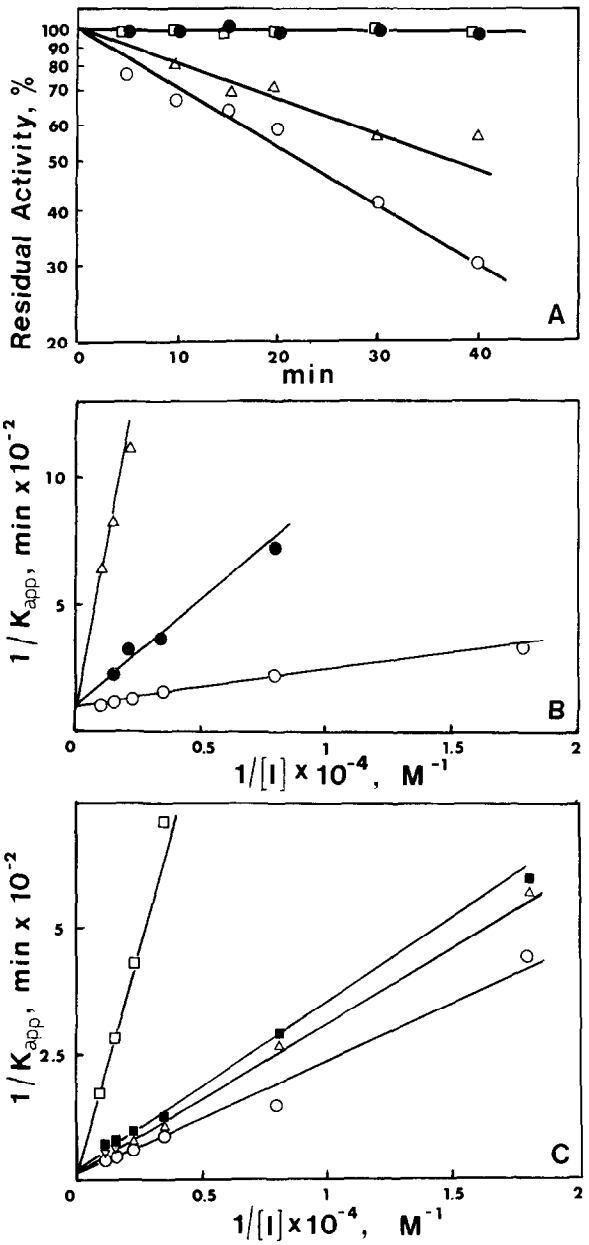

Fig.3. Protection by substrates of tryptophanyl-tRNA synthetase inactivation with mesitoyl-AMP (in the presence of 13 mM $MgCl_2$). (○) represents the incubations preformed in the presence of modifying agent but in the absence of substrates. (A) Kinetics of inactivation of the enzyme in the presence of 0.1 mM L-tryptophan (●); 10 mM ATP (□); 40 mM $tRNA^{Trp}$ (△). In the case of simultaneous addition of ATP and tryptophan the curve coincides with (●) and (□). (B) The rate constant of inactivation, k_{app}, as a function of mesitoyl-AMP concentration in the presence of (●) 1 μM and (△) 10 μM L-tryptophan. (C) The rate constant of inactivation, k_{app}, as a function of mesitoyl-AMP concentration in the presence of: (△) 0.5 mM; (■) 1 mM; (□) 10 mM ATP.

Table 1
Kinetic constants characterizing irreversible inhibition of tryptophanyl-tRNA synthetase with mesitoyl-AMP

Expt. no.	$K_i = \frac{k_{-1} + k_{+2}}{k_{+1}}$	k_{+2} (min^{-1})
1	0.33 mM	0.03
2	0.23 mM	0.01
3	0.60 mM	0.03
4	0.22 mM	0.02
Av.	0.34 ± 0.12	0.02 ± 0.01

cific labelling of the enzyme as shown by stoichiometry of the analog-to-enzyme binding [14] and by tryptic peptide mapping of the modified synthetase where only one neutral peptide was predominantly labelled with mesitoyl-[^{14}C]AMP (not shown).

At the same time, mesitoyl-AMP is characterized with a relatively high affinity, for an ATP derivative, for the enzyme: K_i is 0.34 mM (table 1) as compared with a K_m of 2 mM for ATP and with a K_i of 3 mM for adenosine (see [9]). The value of K_i for mesitoyl-AMP as well as the protective action of both ATP and tryptophan against inactivation of tryptophanyl-tRNA synthetase (see fig.3A) allow one to suggest that mesitoyl-AMP being bound to the active centre of the enzyme behaves as an analog of tryptophanyl adenylate. Fig.3B,C show the effect of different concentrations of tryptophan and ATP, respectively, on the rate of modification of the enzyme. One can see the competition between mesitoyl-AMP and both the substrates, which indicates that not only ATP but also the tryptophan binding sites of the enzyme are involved in the formation of the reversible E · I complex.

The data in fig.3B,C make it possible to determine also the values of K_s for tryptophan and ATP. This is significant in the case of ATP since a relatively low affinity of this substrate for tryptophanyl-tRNA synthetase does not allow one to determine the K_s by the direct method of equilibrium dialysis. Fig.4 presents $1/k_{app}$ as a function of [ATP] using the data of fig.3C. The value of K_s from this curve is 1 mM which is in good agreement with the K_m for this substrate (see above). The value of K_s for tryptophan determined from fig.3B is 2 μM; according to the data of equilibrium dialysis and gel filtration, the K_s for the strongly bound tryptophan molecule is 1–2 μM [15].

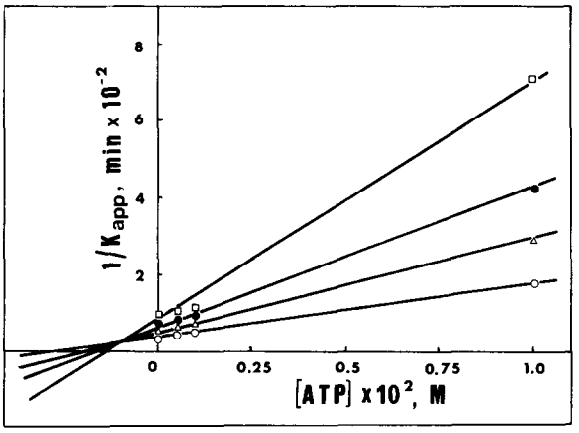

Fig.4. Determination of K_s for ATP from the dependence of k_{app}, the rate constant for modification of tryptophanyl-tRNA synthetase with mesitoyl-AMP, on the concentration of ATP (the secondary plot of the data of fig.3C): (□) 0.29; (●) 0.44; (△) 0.66; (○) 1 mM inhibitor.

Mesitoyl-AMP possesses a reactive phosphoanhydride group in the same position with respect to the 5′-oxygen atom in the ribose ring of the molecule as in aminoacyl adenylate; therefore mesitoyl-AMP being bound to synthetase could react with pyrophosphate. However, incubation of 0.5–1 mM mesitoyl-AMP with 1.6–2.0 μM tryptophanyl-tRNA synthetase and 1 mM sodium [^{32}P]pyrophosphate at 30°C for 40 min did not result in accumulation of [^{32}P]ATP (not shown). This can be attributed to the fact that the enzyme did not catalyse pyrophosphorolysis of the phosphoanhydride bond of this compound as observed with mixed anhydrides of AMP and aminophosphonic acids, potent reversible inhibitors of aminoacyl-tRNA synthetases [4]. Alternatively, it might be due to a much lower rate of mesitoyl-AMP pyrophosphorolysis as compared to the rate of modification of the functional group in the enzyme molecule.

4. Discussion

Mesitoyl-AMP is a true affinity label for tryptophanyl-tRNA synthetase as follows from:

(i) The existence of a reversible E · I complex prior to irreversible modification;

(ii) The competition with substrates for the same site on the enzyme;

(iii) The 1:1 correspondence between loss of activity and AMP residues incorporated from mesitoyl-AMP into the protein [14].

The competitive manner of the enzyme protection against modification by both tryptophan and ATP, as well as a higher affinity of mesitoyl-AMP as compared to ATP, indicate that the affinity label behaves as an analog of tryptophanyl adenylate.

As was shown for various aminoalkyl adenylates, their capability to form complexes with aminoacyl-tRNA synthetases is due to varying contributions of the amino acid moiety and a constant contribution of the AMP moiety [3,5]. From the value of K_i for mesitoyl-AMP (3.4×10^{-4} M), one may assume that the affinity of this compound increased by about one order of magnitude, as compared to AMP, due to the formation of a phosphoanhydride bond with mesitylenecarboxylic acid. Such a small contribution to binding can be made by the mesitoyl group if its aromatic residue is involved in hydrophobic interactions with the indole binding site of the active centre. On the other hand, comparison of the affinity for synthetases of different analogs of aminoacyl adenylates (aminoalkyl adenylates and compounds containing the $-CO-CH_2-P(O)(O^-)$-group instead of a phosphoanhydride group) suggests that the anhydride linkage of adenylates participates in binding to the enzyme [16]. The role of the anhydride oxygen atom in binding to tryptophanyl-tRNA synthetase was postulated from the comparison of the K_i values for several adenosine derivatives which serve as irreversible inhibitors of this enzyme [17]. We have shown the competitive character of inhibition by mesitylenecarboxylic acid, with respect to tryptophan, with the K_i of 2.1×10^{-2} M (unpublished). However, orientation of the mesitoyl moiety of mesitoyl-AMP with respect to the AMP group is unknown, and, therefore, the data obtained do not allow one to discriminate between the alternative contributions of anhydride oxygen and mesitoyl moiety in binding with the enzyme.

References

[1] Cassio, D., Lemoine, F., Waller, J. P., Sandrin, E. and Biossonnas, R. A. (1967) Biochemistry 6, 827–835.

[2] Holler, E., Raney, P., Orme, A., Bennett, E. L. and Calvin, M. (1973) Biochemistry 12, 1150–1159.

[3] Flossdorf, J., Marutzky, R., Messer, K. and Kula, M.-R. (1977) Nucleic Acids. Res. 4, 673–683.

[4] Biryukov, A. I., Ishmuratov, B. Kh. and Khomutov, R. M. (1978) FEBS Lett. 91, 249–252.

[5] Lavrik, O. I., Moor, N. A. and Nevinsky, G. A. (1978) Bioorg. Khim. 4, 1480–1487.
[6] Wetzel, R. and Söll, D. (1977) Nucleic Acids Res. 4, 1681–1694.
[7] Wolfenden, R. (1977) Methods Enzymol. 46, 15–28.
[8] Lavrik, O. I., Nevinsky, G. A., Riazankin, I. A. (1979) Molekul. Biol. 5, 1001–1010.
[9] Kisselev, L. L., Favorova, O. O. and Kovaleva, G. K. (1978) Methods Enzymol. 59, 234–257.
[10] Nosova, V. V., Sokolova, N. I. and Shabarova, Z. A. (1975) Bioorg. Khim. 1, 1130–1133.
[11] Shumyantzeva, V. V., Sokolova, N. I. and Shabarova, Z. A. (1976) Nucleic Acids Res. 3, 903–916.
[12] Favorova, O. O., Madoyan, I. A. and Kisselev, L. L. (1978) Eur. J. Biochem. 86, 193–202.
[13] Brooklehurst, K. (1979) Biochem. J. 181, 775–778.
[14] Favorova, O. O., Madoyan, I. A. and Drutsa, V. L. (1981) FEBS Lett. 123, 161–164.
[15] Graves, P.-V., Mazat, J.-P., Yuguelin, H., Labouesse, J. and Labouesse, B. (1979) Eur. J. Biochem. 96, 509–518.
[16] Southgate, C. C. B. and Dixon, H. B. F. (1978) Biochem. J. 175, 461–465.
[17] Kovaleva, G. K., Ivanov, L. L., Madoyan, I. A., Favorova, O. O., Severin, E. S., Gulyaev, N. N., Baranova, L. A., Shabarova, Z. A., Sokolova, N. I. and Kisselev, L. L. (1978) Biokhimiya 43, 525–533.