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A STEREOCHEMICAL AND POSITIONAL ISOTOPE EXCHANGE STUDY OF THE MECHANISM OF ACTIVATION OF TYROSINE BY TYROSYL-tRNA SYNTHETASE FROM *BACILLUS STEAROTHERMOPHILUS*

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Abstract—Tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* catalyses the activation of [$^{18}O_2$]-tyrosine by adenosine 5′[(*R*)α-^{17}O]triphosphate with inversion of configuration at P_α. It also catalyses positional isotope exchange in adenosine 5′[(β-$^{18}O_2$]triphosphate in the presence of tyrosine, but not in its absence or in the presence of the competitive inhibitor tyrosinol. Together these results imply that the enzyme catalyses an associative "in line" displacement of pyrophosphate from P_α of ATP by tyrosine.

The aminoacyl-tRNA synthetases are a family of enzymes which couple amino acids to their cognate tRNAs with remarkable fidelity.[1] This is achieved by the two step process involving first the activation of the amino acid by adenylation with the ATP, and secondly the transfer of the aminoacyl moiety to the cognate tRNA.[2-4]

$$H_3\overset{+}{N}\cdot CHR\cdot CO_2^- + MgATP \rightleftarrows H_3\overset{+}{N}\cdot CHR\cdot CO\cdot AMP + PP_i$$

$$H_3\overset{+}{N}\cdot CHR\cdot CO\,AMP + tRNA \rightleftarrows H_3\overset{+}{N}\cdot CHR\cdot CO\text{–}tRNA + AMP$$

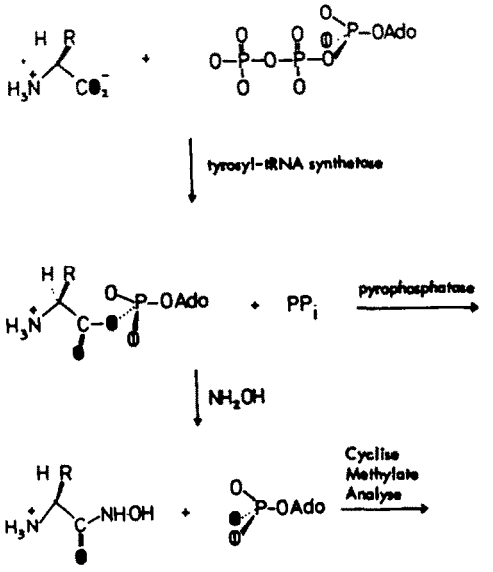
With tyrosyl-tRNA synthetase the activation step takes place in the absence of $tRNA^{Tyr}$, and the enzyme shows half-sites reactivity.[5,6] Tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is an α_2 dimer with a molecular weight of about 90,000.[7,8] The amino acid sequence of two large peptide fragments have been determined[9] and the complete sequence deduced from the nucleotide sequence of the cloned gene.[10,11] The X-ray crystallographic analysis of the enzyme at 2.7 Å resolution has been reported,[12,13] and after soaking the crystals with tyrosinyl-adenylate (a stable analogue of the intermediate tyrosyl-adenylate formed from tyrosinol and AMP) the difference electron density map revealed, the active site of the enzyme and the conformation of the bound tyrosinyl-adenylate.[14] This was confirmed by the difference electron density map formed from the native enzyme and that of the enzyme crystals soaked with tyrosine and ATP (which formed *in situ* the enzyme bound tyrosyl-adenylate intermediate).[14,15]

The aminoacyl-tRNA synthetases show great diversity in their quaternary structures and searches for amino acid sequence homology have been largely inconclusive.[9,16] However, the tertiary structures of tyrosyl-tRNA synthetases from *B. stearothermophilus*[13] and the trypsin treated methionyl-tRNA synthetase from *E. coli*[17] show topological similarities. Moreover, the amino acid sequences of the two enzymes in the region of the nucleotide binding site show a homologous sequence of seven amino acid residues which includes two histidine residues (Ser Ile His^{21} Leu Gly His^{24} Met and Ser Leu His^{45} Ile Gly His^{48} Leu in methionyl-tRNA synthetase and tyrosyl-tRNA synthetase respectively).[18] A conserved cysteine residue (Cys^{11} in methionyl-tRNA synthetase and Cys^{35} in tyrosyl-tRNA synthetase) has also been found and this residue is in contact with the 3′-hydroxyl group of the adenylate moiety in the tyrosyl-tRNA synthetase/tyrosyl-adenylate complex (D. M. Blow personal communication). Replacement of the Cys^{35} by a Ser residue in tyrosyl-tRNA synthetase from *B. Stearothermophilus* by site directed mutagenesis led to a reduction in enzymic activity, but this was largely attributable to a change in K_m for ATP.[19] This conserved Cys residue is not essential therefore for activity.

It has been suggested from model studies that the imidazole group of a histidine residue is directly involved as a nucleophilic catalyst in both the activation and transfer steps catalysed by aminoacyl-tRNA synthetases. In the amino acid activation step an adenylyl-enzyme intermediate and in the transfer step an aminoacyl-enzyme intermediate have been postulated, in each case through the imidazole group of a histidine residue.[20] Evidence from chemical modification studies for the presence of histidine residues in the active site of tryptophanyl-tRNA synthetase (beef pancreas)[21] and phenylalanyl-tRNA synthetase (*E. coli* and yeast)[22,23] has also been provided, but their role, if any, in the mechanism of action is far from clear.

The stereochemical course of the activation step of tyrosyl-tRNA synthetase from *E. coli* has been shown to proceed with inversion of configuration at P_α using adenosine 5′[(*S*)α-thio]triphosphate.[24] Since the in-

volvement of an adenylyl-enzyme intermediate would be expected to lead to retention of configuration at P_α in the enzyme catalysed formation of the aminoacyl-adenylate this provides *prima facie* evidence against the double displacement mechanism.[20,25] However, because of the current intense interest in tyrosyl-tRNA synthetase from *B. Stearothermophilus* we considered it worthwhile to put beyond doubt the stereochemical course of the activation of tyrosine by this enzyme using ATP made chiral at P_α by isotopic substitution. We have also undertaken a positional isotope exchange study[26] with adenosine 5'[β-$^{18}O_2$]triphosphate in order to further delineate the mechanism of activation of tyrosine by tyrosyl-tRNA synthetase.



Scheme 1. The stereochemical course of the activation of [$^{18}O_2$]-tyrosine by adenosine 5'[(*R*)α-^{17}O]triphosphate and tyrosyl-tRNA synthetase from *B. stearothermophilus*. The evidence for the (R_P)-configuration of the 5'[^{16}O, ^{17}O, ^{18}O]AMP is provided in Fig. 1.

EXPERIMENTAL

Tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* was a generous gift from Professor D. M. Blow and Dr. C. J. Bruton (Imperial College, London). It was stored in 50% glycerol containing 10 mM mercaptoethanol and 0.1 mM phenylmethanesulphonyl fluoride at −20° and had an activity of approx. 200 units/mg; 1 unit catalyses the exchange of 1 μmole of $^{32}PP_i$ with ATP in 15 min at pH 8 and 37°. Inorganic pyrophosphatase, hexokinase, L-amino acid oxidase and glucose 6-phosphate dehydrogenase were obtained from Sigma Chemical Co., Ltd. (Poole, Dorset, U.K.).

D_2O (99.8 atom % ^{2}H) was obtained from Fluorochem Ltd. (Glossop, Derbyshire, U.K.). High grade deionised water used in the preparation of all buffers was obtained from a Milli-Q2 water purification system (Millipore Ltd., Harrow, Middlesex, U.K.).

[^{18}O]-Water (99 atm % ^{18}O) was obtained from Prochem Ltd. (London, U.K.).

Adenosine 5'[(R)α-^{17}O]triphosphate. This was prepared by the method of Lowe *et al.*[27,28] and had an isotopic composition of 8.5 at. % ^{16}O, 41 at. % ^{17}O, and 50.5 at. % ^{18}O at the labelled site and consisted of about 95% (*R*)-configuration.

Adenosine 5'[β-$^{18}O_2$]triphosphate. This was prepared by the method of Lowe and Sproat[29] and contained 1% ATP, 12% [β-^{18}O]-ATP and 87% [β-$^{18}O_2$]-ATP. We are grateful to Dr. B. S. Sproat for a gift of this material.

[$^{18}O_2$]-Tyrosine. Dry HCl was bubbled through a soln of tyrosine (50 mg) in [^{18}O]-water (0.5 ml, 99 at. %) for 1 min and then the soln kept at 110° for 72 hr in a sealed tube under N_2. The [^{18}O]-water was recovered by lyophilisation in a vacuum line and the process repeated with [^{18}O]-water (0.5 ml, 99 at. %). The [$^{18}O_2$]-tyrosine (22.5 mg) was shown by mass spectrometry after methylation with diazomethane to contain 79 at. % ^{18}O.

Incubation of [$^{18}O_2$]-tyrosine, adenosine 5'[(R)α-^{17}O]triphosphate and hydroxylamine with tyrosyl-tRNA synthetase

Tyrosyl-tRNA synthetase (100 μL, 200 units/mg, 1 mg/ml) and inorganic pyrophosphatase (0.1 mg, 500 units/mg) were added to Tris HCl buffer (12 mL, 66.7 mM, pH 8) containing [$^{18}O_2$]-tyrosine (5.83 mM, 79 at. % ^{18}O), adenosine 5'[(*R*)α-^{17}O]triphosphate (4.17 mM, 50 μmoles), hydroxylamine hydrochloride (1 M), magnesium acetate (4.17 mM), phenylmethanesulphonyl fluoride (1 mg) and mercaptoethanol (5 μL) and the soln incubated at 37°. The reaction was followed by assaying for residual ATP by the coupled reactions of hexokinase and glucose-6-phosphate dehydrogenase.[30] When the reaction was complete (16 hr), EDTA (70 μmoles) was added and the soln applied to a column (2.6 cm × 30 cm) of DEAE-Sephadex-A-25 which had been equilibrated with triethylammonium bicarbonate buffer (50 mM, pH 7.6) and eluted with a linear gradient of triethylammonium bicarbonate buffer (50–400 mM, pH 7.6). The [^{16}O, ^{17}O, ^{18}O]-AMP (38.5 μmoles) was freed from buffer by addition and evaporation of MeOH (3 × 10 mL) and then cyclized and methylated for analysis by ^{31}P NMR spectroscopy of the chirality at P.[31]

Positional isotope exchange with adenosine 5'[β-$^{18}O_2$]triphosphate and tyrosyl-tRNA synthetase

In all the experiments Tris HCl (12 mL, 33.3 mM, pH 8), magnesium acetate (1.25 mM), phenylmethanesulphonyl fluoride (1 mg) and 2-mercapto-ethanol (5 μL) in deaerated deionised water were used. Four experiments were performed by adding the following components:

(i) L-Tyrosine (1.8 mM), [β-$^{18}O_2$]ATP (1.25 mM) and tyrosyl-tRNA synthetase (30 μL, 200 units/mg, 1 mg/ml) were added and the solution incubated at 37° for 16.5 hr.

(ii) L-Tyrosinol hydrochloride (10 mM), [β-$^{18}O_2$]ATP (1.25 mM) and tyrosyl-tRNA synthetase (30 μL, 1 mg/ml, 200 units/mg) were added and the solution incubated at 37° for 16.5 hr.

(iii) Tyrosyl-tRNA synthetase (30 μL, 1 mg/ml, 200 units/mg) and L-amino acid oxidase (10 μL, 9.6 mg/ml, 4.7 units/mg) were added and incubated for 1 hr at 37°. [β-$^{18}O_2$]ATP (1.25 mM) was then added and the soln incubated for a further 16.5 hr at 37°. In a control experiment it was shown that the enzyme was still active after incubation with L-amino acid oxidase under these conditions.

(iv) Two solns were made up containing hydroxylamine hydrochloride (1 M), [β-$^{18}O_2$]ATP (1.25 mM) and tyrosyl-tRNA synthetase (30 μL, 1 mg/ml, 200 units/mg) and incubated at 37° for (a) 3 hr and (b) 16.5 hr. In a control experiment it was found that the enzyme was still active after incubation under these conditions for 16.5 hr.

All the above reactions were terminated by the addition of EDTA (20 μmol, 1.7 mM) and vortexing. The soln was diluted and applied to a column (1 cm × 12 cm) of DEAE-Sephadex-A25 which had been equilibrated with triethylammonium bicarbonate buffer (100 mM, pH 7.6) and eluted with a linear gradient of triethylammonium bicarbonate buffer (100–600 mM, pH 7.6). The fractions containing [$^{18}O_2$]ATP were evaporated; addition and evaporation of MeOH (3 × 5 ml) removed residual buffer. The ^{31}P NMR spectra were measured.

^{31}P NMR spectra. ^{31}P NMR spectra were recorded at

121.493 MHz on a Bruker WH-300 WB Fourier transform spectrometer, using quadrature detection. Signal averaging was performed by an Aspect 2000 computer interfaced with the spectrometer. Field frequency locking was provided by the D resonance of D_2O. Parameters used in the figure were offset 2240 Hz, sweep width 2000 Hz, acquisition time 2.05 s, pulse width 16 μs, broadband proton decoupling, gaussian multiplication (line broadening −1.0 Hz, gaussian broadening 0.4) in 8 K, and Fourier transform in 32 K.

RESULTS AND DISCUSSION

The stereochemical course of the activation of tyrosine by tyrosyl-tRNA synthetase

[$^{18}O_2$]-Tyrosine and adenosine 5′[(*R*)α-^{17}O]triphosphate were incubated with tyrosyl-tRNA synthetase from *B. stearothermophilus* in the presence of Mg^{2+} and hydroxylamine. Inorganic pyrophosphatase was also present in the reaction medium to hydrolyse the magnesium pyrophosphate generated and so assist the overall reaction as outlined in the Scheme. The [^{16}O, ^{17}O, ^{18}O]AMP was isolated from the reaction mixture and its chirality at P determined by our established procedure after cyclization and methylation.[31] The ^{31}P NMR spectrum is shown in Fig. 1. From the known isotopic content of the [$^{18}O_2$]-tyrosine and the isotopic content and enantiomeric excess of the adenosine 5′[(*R*)α-^{17}O]-triphosphate used in the reaction, it was possible to calculate the expected relative peak intensities of the ^{31}P NMR resonances of the equatorial and axial triesters for the reaction proceeding with retention and inversion of configuration. Comparison of the observed and calculated relative peak intensities (Table 1). show that the activation of [$^{18}O_2$]-tyrosine by adenosine 5′[(*R*)α-^{17}O]triphosphate occurs stereospecifically (within experimental error)with inversion of configuration at P_α.

Since there is accumulating evidence that enzyme catalysed displacement reactions at phosphate esters and anhydrides occur with inversion of configuration at P,[32-34] this observation is most simply interpreted in terms of a direct "in line" displacement of pyrophosphate from P_α of ATP by tyrosine at the active site of the enzyme.

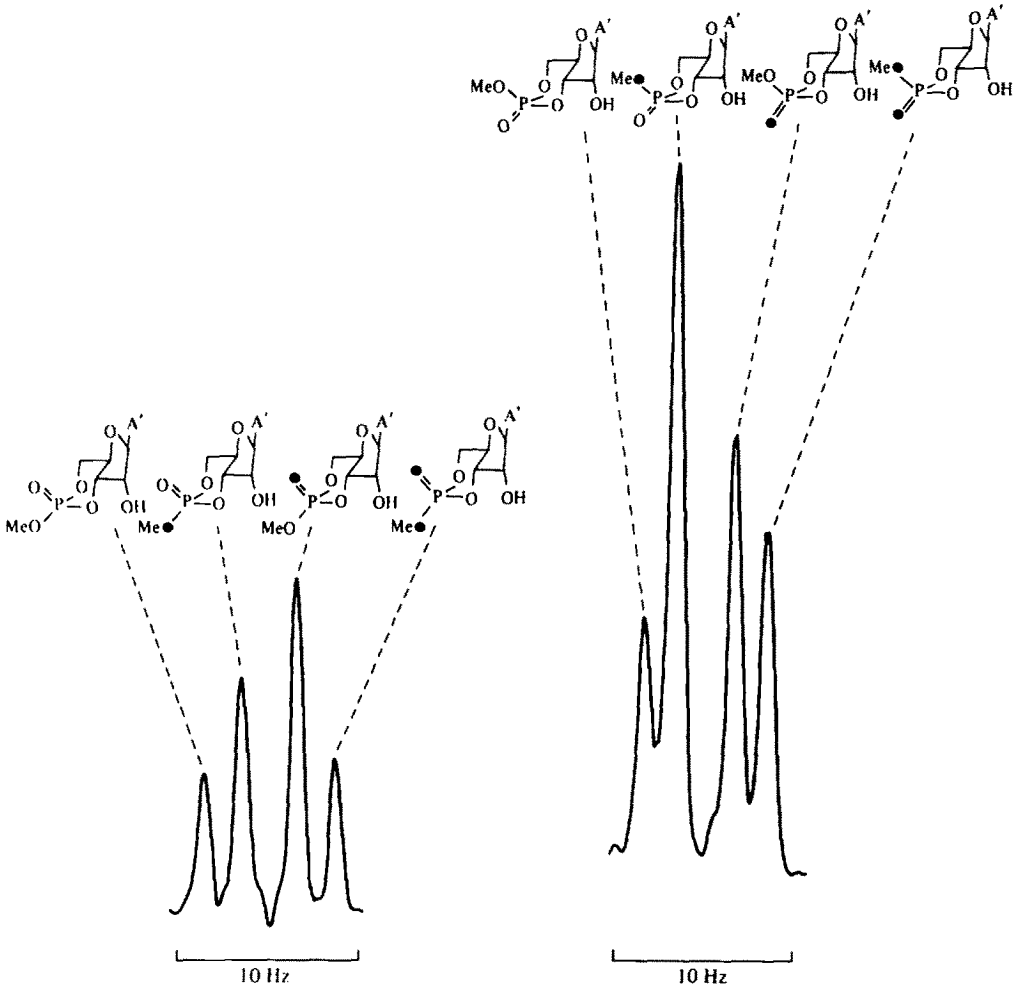


Fig. 1. The ^{31}P NMR spectrum (121.5 MHz) of the equatorial and axial triesters derived by cyclization and methylation of the 5′[^{16}O, ^{17}O, ^{18}O]AMP obtained by incubating [$^{18}O_2$]-tyrosine, adenosine 5′[(*R*)α-^{17}O]triphosphate and hydroxylamine with tyrosyl-tRNA synthetase. The ratio of the [$^{16}O_{ax}$, $^{18}O_{eq}$]- to [$^{18}O_{ax}$, $^{16}O_{eq}$]-triesters shows that the 5′[^{16}O, ^{17}O, ^{18}O]AMP has the (R_p)-configuration and hence the reaction has proceeded with inversion of configuration at P_α of ATP as indicated in Scheme 1. • = ^{18}O, A′ = N^1-methyladenine.

Table 1. Observed and calculated relative ^{31}P NMR intensities. Observed relative peak intensities of the ^{31}P NMR resonances (from Fig. 1) of the ^{18}O-labelled diastereoisomeric triesters derived by cyclization followed by methylation of the 5'[^{16}O, ^{17}O, ^{18}O]AMP, and the calculated values expected from the known isotopic composition of the [$^{18}O_2$]-tyrosine and adenosine 5'[(*R*)α-^{17}O]triphosphate, for the tyrosyl-tRNA synthetase catalysed nucleotidyl transfer with retention and inversion of configuration. • = ^{18}O.

	Equatorial triester			Axial triester		
	Observed	Calculated		Observed	Calculated	
		Retention	Inversion		Retention	Inversion
MeO-P=O	0.43	0.43	0.43	0.39	0.43	0.43
Me•-P=O	0.71	1.00	0.71	1.00	0.71	1.00
MeO-P=•	1.00	0.71	1.00	0.64	1.00	0.71
Me•-P=•	0.47	0.45	0.45	0.50	0.45	0.45

Positional isotope exchange with adenosine 5'[β-$^{18}O_2$] triphosphate

Activation of tyrosine by tyrosyl-tRNA synthetase with inversion of configuration at P_α of ATP actually implies that an odd number of nucleotidyl transfer steps have occurred. In view of the evidence for an adenylyl-enzyme being formed when tryptophanyl-tRNA synthetase (from beef pancreas) is incubated with MgATP,[35] and the proposal that adenylyl-enzyme intermediates may be on the reaction pathway for all aminoacyl-tRNA synthetases,[20,25] we undertook experiments with tyrosyl-tRNA synthetase to explore the possibility that several adenylyl-enzyme intermediates (any even number would be consistent with inversion of configuration at phosphorus) are on the reaction pathway.

Incubation of tyrosine and [β-$^{18}O_2$]ATP with tyrosyl-tRNA synthetase in the presence of Mg^{2+} for 16.5 hr at 37° led as expected to complete positional isotope exchange into the P_α–O–P_β bridge and into P_γ of recovered [$^{18}O_2$]ATP. Incubation of [β-$^{18}O_2$]ATP and Mg^{2+} with tyrosyl-tRNA synthetase in the absence of added tyrosine for 16.5 h under the same experimental conditions led to partial scrambling, but this was due to traces of endogeneous tyrosine present in the enzyme preparation since if the enzyme was pre-incubated with L-amino acid oxidase (the tyrosyl-tRNA synthetase was still active after this treatment) prior to the addition of the [β-$^{18}O_2$]ATP, no positional isotope exchange occurred after 16.5 hr at 37°. In a further control experiment it was found that if hydroxylamine was added to tyrosyl-tRNA synthetase prior to the [β-$^{18}O_2$]ATP partial scrambling occurred initially (investigated after 4.5 hr) but thereafter no further scrambling occurred up to 16.5 hr (the enzyme was shown to be active after this period). The initial scrambling was expected since activation of the endogenous tyrosine is necessary before it can react with hydroxylamine to form tyrosyl-hydroxamic acid. Finally, in the absence of added tyrosine but in the presence of the competitive inhibitor, tyrosinol, no positional isotope exchange occurred in [β-$^{18}O_2$]ATP in 16.5 hr at 37°.

These results show that an adenylyl-enzyme is not formed between ATP and tyrosyl-tRNA synthetase in the absence of tyrosine or in the presence of tyrosinol. Thus multiple odd numbered displacements at P_α of ATP can be excluded leaving the direct 'in-line' displacement of pyrophosphate from ATP by tyrosine as the only acceptable interpretation of the stereochemical course of the reaction. The lack of positional isotope exchange in the absence of tyrosine and in the presence of tyrosinol also provides evidence against a dissociative mechanism. We therefore conclude that tyrosyl-tRNA synthetase from *B. stearothermophilus* catalyses the activation of tyrosine by a direct associative "in line" displacement at P_α of ATP.

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