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SULPHUR METABOLISM IN *PARACOCCUS DENITRIFICANS*

PURIFICATION, PROPERTIES AND REGULATION OF CYSTEINYL- AND METHIONYL-tRNA SYNTHETASE

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Summary

Cysteinyl- and methionyl-tRNA synthetases (EC 6.1.1.-) were purified 1200- and 1000-fold, respectively, from sonic extracts of *Paracoccus denitrificans* strain 8944, and kinetics, substrate specificity and regulatory properties were determined using the ATP-PP_i exchange reaction. Both enzymes had pH optima of approx. 8 and were inhibited by sulphydryl-group reagents.

Cysteinyl-tRNA synthetase catalysed L-selenocysteine- and α-aminobutyric acid-dependent ATP-PP_i exchange and methionyl-tRNA synthetase catalysed L-homocysteine-, L-selenomethionine- and norleucine-dependent ATP-PP_i exchange. Both enzymes were inhibited by *O*-acetylserine. Cysteinyl-tRNA synthetase activity was stimulated by methionine and methionyl-tRNA synthetase activity was stimulated by sulphide, cysteine, and cysteic acid.

Introduction

The activation and subsequent transfer of amino acids has been shown to be catalysed by specific enzymes, aminoacyl-tRNA synthetases (amino acid : tRNA ligase (AMP-forming), EC 6.1.1.—), which form aminoacyl adenylate intermediates.

Considering the importance of sulphur-containing amino acids in protein synthesis [1], structure and function [2—5], remarkably little work has been conducted on the study of the sulphur amino acid-activating enzymes.

The purpose of this investigation was to study the regulation and control of both cysteinyl- and methionyl-tRNA synthetase from *Paracoccus denitrificans*

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strain 8944. The purification of both enzymes is reported. The ability of amino acid analogues to serve as substrates or to act as inhibitors of aminoacyl adenylate formation was studied to establish which groups of the amino acids were necessary for binding to the enzyme. To provide further insight into the mechanism and control of cysteine and methionine activation the effects of added sulphydryl-group reagents, nucleotides, various metal cations and a variety of intermediates of the sulphur metabolic pathway on aminoacyl adenylate formation were investigated.

Materials and Methods

Purified enzymes. D-Amino acid oxidase (EC 1.4.3.3) and catalase (EC 1.11.1.6) were obtained from The Sigma Chemical Co., Ltd., London, U.K.

Chemicals. Hydroxyapatite and Dowex 1-X2 (Cl^- form), 200—400 mesh, were obtained from BioRad. Labs., Richmond, Calif., U.S.A. $^{32}P_i$, in dilute HCl, was obtained from the Radiochemical Centre, Amersham, Bucks., U.K., and was pyrolysed to $^{32}PP_i$ as described by Lee Peng [6]. Sephadex G-200 was obtained from Pharmacia, Uppsala, Sweden, and DEAE-cellulose 52 from Whatman, Maidstone, Kent, U.K. Streptomycin, cysteine and methionine hydroxamic acid, alumina C_γ, and all amino acids (L-, D- and DL-isomers) were obtained from The Sigma Chemical Co., Ltd., London, U.K. DL-Homocysteine thiolactone · HCl was obtained from Koch Light Labs., U.K. All other chemicals were of analytical reagent grade and were obtained from either The Sigma Chemical Co., Ltd., or British Drug Houses, Dorset, Kent, U.K.

Preparation of L-selenocysteine. Commercially available DL-selenocystine was reduced to DL-selenocysteine with excess dithiothreitol at pH 8.0, treated with D-amino acid oxidase (in the presence of catalase) and the L-selenocysteine isolated and purified by ion-exchange chromatography on Dowex 1-X2 (Cl^- form), 200—400 mesh. The purified L-selenocysteine was concentrated by rotary evaporation and a standard solution of 10 mM L-selenocysteine, in 10 mM Tris · HCl buffer (pH 8.0) and 5 mM dithiothreitol was prepared fresh and used immediately. Homocysteine was prepared by the method of Flavin [7].

Purification of cysteinyl- and methionyl-tRNA synthetase. All operations were conducted at 0—4°C. The buffer used throughout the purification procedure contained 20 mM Tris · HCl (pH 8.0), 5 mM $MgCl_2$, and 1 mM dithiothreitol.

Cells of *P. denitrificans* (strain 8944) were grown, harvested and washed, and crude extracts prepared as described previously [8].

Alumina C_γ was added to the crude extract to give a final concentration of 10 mg/ml. The suspension was stirred for 30 min and then centrifuged at 30 000 × *g* for 40 min. The supernatant was decanted and the precipitate discarded.

Streptomycin (5 g) was added to the alumina C_γ supernatant and the suspension stirred for 6 h. Following centrifugation at 30 000 × *g* for 30 min the supernatant was decanted and the precipitate discarded.

Solid $(NH_4)_2SO_4$ was added to the streptomycin-treated supernatant to give 30% saturation. After stirring for 30 min the suspension was centrifuged for 30 min, the precipitate discarded, and the supernatant brought to 50% satura-

tion with solid $(NH_4)_2SO_4$. After stirring and centrifuging, the resultant precipitate was dissolved in a minimal amount of buffer and the solution dialysed against buffer overnight.

The dialysed $(NH_4)_2SO_4$ fraction was loaded onto a column of Sephadex G-200 (85.0 × 3.5 cm), equilibrated with buffer, and the enzyme eluted at a flow rate of 0.45 ml/min. Fractions (5 ml) were collected and assayed for both cysteinyl- and methionyl-tRNA synthetase activity; active fractions were pooled.

The pooled active Sephadex G-200 fractions were applied to a column of DEAE-cellulose (10.0 × 3.5 cm) equilibrated with buffer. The column was washed with 60 ml of buffer, then with 50 ml of 0.2 M KCl (in buffer) and the enzyme was then eluted with a 500 ml linear KCl gradient from 0.2 to 0.5 M at a flow rate of 0.65 ml/min. Fractions (5 ml) were collected, assayed, and the active fractions pooled and dialysed against buffer to remove the KCl. Those fractions containing cysteinyl-tRNA synthetase activity and no methionyl-tRNA synthetase activity were pooled and those fractions containing methionyl- and no cysteinyl-tRNA synthetase activity were pooled.

The active DEAE-cellulose fractions were applied to a column of hydroxyapatite (10 × 1.5 cm) equilibrated with buffer. The enzymes were eluted with a 500 ml linear KCl gradient from 0 to 0.3 M KCl (in buffer) at a flow rate of 0.4 ml/min and those fractions containing the highest specific activity of cysteinyl- (and methionyl)-tRNA synthetase were pooled separately and dialysed against buffer.

The dialysed hydroxyapatite fractions were applied to a small column of DEAE-cellulose (5.0 × 1.0 cm) equilibrated with buffer. The enzyme was adsorbed to the column and then eluted with 10 ml of buffer containing 0.4 M KCl. The active fractions were dialysed against buffer to remove the KCl, and were then stored at −15°C.

Assay of cysteinyl-tRNA synthetase. (1) ATP-$^{32}PP_i$ exchange assay: Reaction mixtures (1 ml) contained 0.1 M Tris · HCl buffer (pH 8.0), 4 mM ATP, 5 mM dithiothreitol, 2 mM $Na_4{}^{32}P_2O_7$ (0.25 Ci/mol), 10 mM $MgCl_2$, 0.1 mM L-cysteine and a limiting amount of enzyme. Reaction mixtures were the same for methionyl-tRNA synthetase except that the $MgCl_2$ concentration was 8 mM and cysteine was replaced with 10 mM L-methionine. Reactions were run for 10 min at 30°C and were stopped with 2 ml of trichloroacetic acid (7.5%, w/v). [^{32}P]ATP was separated from $^{32}PP_i$ by the washing procedure of Berg [9].

(2) Hydroxamate assay: Reaction mixtures (1 ml) contained 0.2 M Tris · HCl buffer (pH 8.0), 10 mM $MgCl_2$, 4 mM ATP, 10 mM L-cysteine (10 mM L-methionine), 5 mM dithiothreitol, 2.5 M hydroxylamine, and a limiting amount of enzyme. After incubation at 30°C for 30 min, reactions were terminated by the addition of 1 ml of 10% (w/v) solution of $FeCl_3$ in 0.2 M trichloroacetic acid and 6.6 M HCl. The mixture was shaken, and after 5 min, the A_{540nm} was determined using a Pye Unicam SP 8000 spectrophotometer. Control tubes contained either no cysteine or no ATP. L-Cysteine (and L-methionine) hydroxamic acid (Sigma Chemical Co., Ltd., London, U.K.) were used as the standard.

Protein determination. Protein in crude extracts, alumina C_γ, streptomycin,

$(NH_4)_2SO_4$, and Sephadex G-200 fractions were determined by the Folin method of Lowry et al. [10]. Purer protein from DEAE-cellulose and hydroxyapatite fractions was measured by the method of Warburg and Christian [11].

Results

Cysteinyl- and methionyl-tRNA synthetase were purified 1200- and 1000-fold, respectively (Tables I and II).

Crude extracts contained a very active inorganic pyrophosphatase which was removed during streptomycin and $(NH_4)_2SO_4$ fractionation. Cysteinyl-tRNA and methionyl-tRNA synthetases were separated by DEAE-cellulose column chromatography (Fig. 1). No evidence for the existence of more than one type of methionyl-tRNA synthetase was obtained during the purification procedure (cf. Allende et al. [12]). The stability of methionyl-tRNA synthetase was increased by the presence of a reducing agent (dithiothreitol); in the presence of 5 mM dithiothreitol both purified enzyme preparations were stable for at least 8 months at −15°C.

Rechromatography of the purified enzymes on Sephadex G-200 gave single, sharp, and symmetrical peaks of enzyme activity with constant specific activity across the peaks.

Properties

The kinetic properties of the enzymes were determined from ATP-$^{32}PP_i$ exchange data with the use of the purified enzymes. For the evaluation of kinetic constants it was assumed that the initial rate of $^{32}PP_i$ incorporated into ATP was equivalent to the initial rate of aminoacyl adenylate formed.

Both cysteine- and methionine-dependent ATP-PP_i exchange was proportional to enzyme concentration and was linear with time for at least 60 min at 30°C.

When a series of buffers was used to study the pH optimum of the enzymes, the pH optimum of cysteinyl-tRNA synthetase was found to lie between 7.5

TABLE I

PURIFICATION OF CYSTEINYL-tRNA SYNTHETASE FROM *P. DENITRIFICANS*

Purification step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (-fold)
Crude extract	13 259	30 496	2.3	100	0
Alumina C_γ fractionation	8 295	29 865	3.6	97	1.6
Streptomycin fractionation	2 293	28 438	12.4	93	5.4
$(NH_4)_2SO_4$ fractionation	430	27 362	63.6	90	27
Sephadex G-200 gel filtration	105	25 945	247.5	85	107
DEAE-cellulose column chromatography	11.5	22 780	1976	75	860
Hydroxyapatite column chromatography	6.5	18 430	2849	60	1239

TABLE II

PURIFICATION OF METHIONYL-tRNA SYNTHETASE FROM *P. DENITRIFICANS*

Fraction No.	Purification step	Protein (mg)	Enzyme activity		Recovery (%)	Purification (-fold)
			Total units	Specific activity (units/mg)		
1	Crude extract	13 259	26 380	1.98	100	0
2	Alumina C_γ fractionation	8 295	25 845	3.1	98	1.6
3	Streptomycin fractionation	2 293	24 764	10.8	94	5.5
4	$(NH_4)_2SO_4$ fractionation	430	23 521	54.7	89	28
5	Sephadex G-200 gel filtration	104	22 259	212.4	84	107
6	DEAE-cellulose column chromatography	14.7	17 080	1162	65	587
7	Hydroxyapatite column chromatography	8.04	15 675	1950	59	985

and 8.5 and between pH 8.0 and 8.5 for methionyl-tRNA synthetase. The fact that endogenous ATP-PP_i exchange remained low in assays conducted in glycine/NaOH buffer for both enzymes indicated that no glycyl-tRNA synthetase activity was present in either synthetase preparation.

Kinetic constants for both cysteinyl- and methionyl-tRNA synthetase were calculated from double reciprocal plots and are summarised in Tables III and IV.

Excess dithiothreitol was added to reaction mixtures to ensure the mainte-

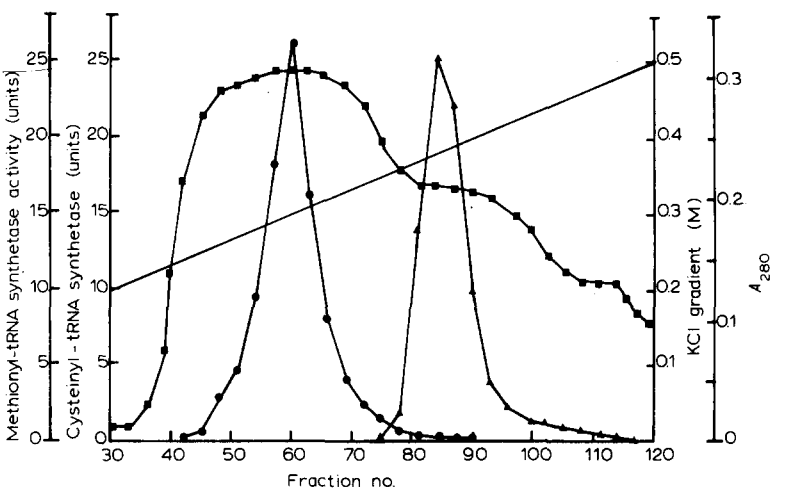

Fig. 1. Separation of cysteinyl- and methionyl-tRNA synthetase activities by column chromatography on DEAE-cellulose. The activities were separated by ion-exchange chromatography following Sephadex G-200 gel filtration, as described in Materials and Methods. Cysteinyl-tRNA synthetase and methionyl-tRNA synthetase activities were measured as cysteine- and methionine-dependent ATP-PP_i exchange, respectively. ■, protein; ▲, cysteinyl-tRNA synthetase; ●, methionyl-tRNA synthetase.

TABLE III

Km AND K_m VALUES FOR VARIOUS SUBSTRATES AND INHIBITORS OF PURIFIED CYSTEINYL-tRNA SYNTHETASE IN THE ATP-PP_i EXCHANGE ASSAY

Double reciprocal plots were used to determine the K_m and the K_i values. Conditions for each assay were as described in Materials and Methods except that the concentration of substrate was varied and inhibitors were added at various concentrations. K_m values are the means of at least three series of experiments; K_i values are the means of at least two series of experiments.

Substrate	Inhibitors	K_m (M)	K_i (M)	Type of inhibition
L-Cysteine		$1.25 \cdot 10^{-5}$		
L-Selenocysteine		$1.59 \cdot 10^{-6}$		
α-L-Aminobutyric acid		$1.00 \cdot 10^{-2}$		
ATP		$1.33 \cdot 10^{-3}$		
PP_i		$3.00 \cdot 10^{-4}$		
L-Cysteine	L-Selenocysteine		$1.25 \cdot 10^{-5}$	Competitive
L-Cysteine	α-L-Aminobutyric acid		$1.35 \cdot 10^{-3}$	Competitive
L-Cysteine	Sulphite		$8.0 \cdot 10^{-3}$	Competitive
L-Cysteine	Sulphide		$6.5 \cdot 10^{-4}$	Competitive
L-Cysteine	D-Cysteine		$2.3 \cdot 10^{-3}$	Competitive
L-Cysteine	Gluthathione		$2.1 \cdot 10^{-3}$	Competitive
L-Cysteine	L-Cysteic acid		$2.5 \cdot 10^{-2}$	Competitive
L-Cysteine	L-Alanine		$9.0 \cdot 10^{-3}$	Competitive
L-Cysteine	L-Homocysteine		$2.2 \cdot 10^{-3}$	Non-competitive
L-Cysteine	*O*-Acetylserine		$3.2 \cdot 10^{-2}$	Non-competitive
L-Cysteine	Cysteamine		$3.0 \cdot 10^{-4}$	Non-competitive
L-Cysteine	Phosphate		$1.6 \cdot 10^{-2}$	Non-competitive
ATP	ADP		$4.3 \cdot 10^{-3}$	Non-competitive
ATP	5′-AMP		$5.8 \cdot 10^{-3}$	Un-competitive
ATP	3′-AMP		$4.8 \cdot 10^{-3}$	Competitive

nance of the amino acid in the free thiol form; dithiothreitol is not a substrate for either cysteinyl- or methionyl-tRNA synthetase activity.

Concentrations of ATP, PP_i and Mg^{2+} from 0.1 to 10 mM were examined by varying the concentrations of each of these compounds separately in ATP-PP_i

TABLE IV

K_m AND K_i VALUES FOR METHIONYL-tRNA SYNTHETASE FROM *P. DENITRIFICANS*

Substrate	Inhibitor	K_m (M)	K_i (M)	Type of inhibition
ATP		$8.7 \cdot 10^{-4}$		
L-Methionine		$2.6 \cdot 10^{-4}$		
L-Homocysteine		$3.83 \cdot 10^{-4}$		
Norleucine		$2.86 \cdot 10^{-2}$		
L-Selenomethionine		$3.1 \cdot 10^{-4}$		
L-Methionine	*O*-Acetylserine		$2.17 \cdot 10^{-2}$	Non-competitive
L-Methionine	Glutathione		$2.66 \cdot 10^{-2}$	Non-competitive
L-Methionine	D-Methionine		$6.4 \cdot 10^{-4}$	Uncompetitive
ATP	ADP		$4.0 \cdot 10^{-3}$	Uncompetitive
ATP	3′-AMP		$5.5 \cdot 10^{-4}$	Non-competitive
ATP	5′-AMP		$9.0 \cdot 10^{-4}$	Competitive
L-Methionine	Sulphide	Stimulation	K_m constant	*V* variable
L-Methionine	Cysteine	Stimulation	K_m lowered	*V* constant
L-Methionine	Cysteic acid	Stimulation	K_m lowered	*V* constant

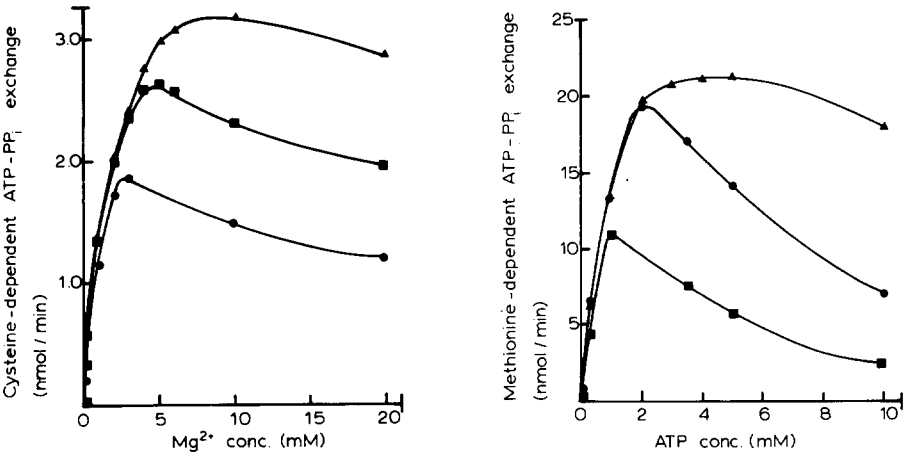

Fig. 2. Effect of Mg^{2+} concentration on cysteine-dependent ATP-PP_i exchange at varying concentrations of ATP. Reaction mixtures were as described in Materials and Methods except that the Mg^{2+} concentrations were as indicated; ●, 1 mM ATP; ■, 2 mM ATP; ▲, 4 mM ATP.

Fig. 3. Effect of ATP concentration on methionine-dependent ATP-PP_i exchange in the presence and absence of Mg^{2+}. Reaction mixtures were as described in Materials and Methods except that ATP and Mg^{2+} were added at the concentration specified: ■, 2 mM Mg^{2+}; ●, 4 mM Mg^{2+}; ▲, 10 mM Mg^{2+}.

exchange assays. Both cysteine- and methionine-dependent ATP-PP_i exchange was negligible in the absence of Mg^{2+} (Figs. 2 and 3). At a fixed concentration of ATP the rate of cysteine- and methionine-dependent ATP-PP_i exchange increased with increase in the Mg^{2+} concentration up to a maximum; further increase in the Mg^{2+} concentration caused inhibition of enzyme activity. Maximum enzyme activity was observed at a Mg^{2+}/ATP ratio of 2.5 : 1 for cysteinyl-tRNA synthetase and at 2 : 1 for methionyl-tRNA synthetase. In a reaction mixture containing 10 mM $MgCl_2$, 4 mM ATP and 2 mM PP_i, the ATP and the PP_i will be in the form of $Mg \cdot ATP^{2-}$ and $Mg \cdot PP_i^{2-}$, respectively. Using the method of Perrin and Sayce [13] to calculate the forms of Mg^{2+}, ATP and PP_i at various concentrations of $MgCl_2$ in the reaction mixture, the concentration of free ATP did not correlate with the rate of exchange; the concentration of $Mg \cdot ATP^{2-}$ did, however, correlate with the rate of exchange. Therefore, Mg^{2+} is required, presumably, to form $Mg \cdot ATP^{2-}$ and $Mg \cdot PP_i^{2-}$, which are probably the active substrates for both cysteinyl- and methionyl-tRNA synthetases. (Cole and Schimmel [14] reached a similar conclusion as regards the substrates for the isoleucyl-tRNA synthetase of *Escherichia coli*.) Both enzymes were inhibited by free ATP as well as free Mg^{2+}.

The rate of both cysteine- and methionine-dependent ATP-PP_i exchange was essentially independent of $^{32}PP_i$ concentration above 0.15 mM.

Both cysteine- and methionine-dependent ATP-PP_i exchange was negligible in the absence of Mg^{2+}; only Co^{2+} (13%) and Zn^{2+} (6%) supported cysteine-dependent ATP-PP_i exchange and only Mn^{2+} (15%), Co^{2+} (20%) and Ca^{2+} (14%) supported methionine-dependent ATP-PP_i exchange. Other divalent cations tested included Ni^{2+}, Ba^{2+}, Fe^{2+}, and Cu^{2+}.

Neither purified enzyme catalysed $^{32}PP_i$ exchange when ATP was replaced

with either ADP or AMP. Similarly neither enzyme catalysed the incorporation of P_i into ATP, ADP or AMP when $^{32}PP_i$ was replaced with $^{32}P_i$, and neither purified enzyme catalysed ATP-PP_i exchange when cysteine (or methionine) was replaced with sulphate or selenate, indicating that the enzyme was free of ATP sulphurylase.

Only L-cysteine (or L-methionine) of the 20 amino acids commonly found in proteins could act as substrate for ATP-PP_i exchange, indicating that the purified cysteinyl- (and methionyl-) tRNA synthetase was not contaminated with other aminoacyl-tRNA synthetases and that both enzymes were specific for the L-isomer of the respective amino acid substrate (cf. Calender and Berg [15]).

The only true alternative substrates of L-cysteine in the exchange assay were L-selenocysteine and α-L-aminobutyric acid. The only true alternative substrates of L-methionine in the exchange assay were L-selenomethionine, L-homocysteine and norleucine. Kinetic constants are summarised in Tables III and IV. Whilst the affinity of cysteinyl-tRNA synthetase for selenocysteine ($K_m = 1.59 \cdot 10^{-6}$ M) is greater than the affinity for cysteine ($K_m = 1.29 \cdot 10^{-5}$ M) the V(selenocysteine) is approx. the same as V(cysteine). The affinity of the enzyme for α-aminobutyric acid ($K_m = 1.00 \cdot 10^{-2}$ M) is much less than that for cysteine, but the V(α-aminobutyric acid) is three times the V(cysteine).

Evidence that the activation of cysteine, selenocysteine and α-aminobutyric acid are catalysed by the one enzyme and that methionine, selenomethionine and norleucine are activated by the one enzyme, is provided by the following observations; (1) the similarity in the structure of the respective compounds, (2) the ratio of cysteine-, selenocysteine- and α-aminobutyric acid-dependent ATP-PP_i exchange activities (and methionine-, selenomethionine-, homocysteine- and norleucine-dependent ATP-PP_i exchange) was constant during purification, (3) the activities for different substrates were not separated by any of the purification procedures, and (4) double reciprocal plots obtained from competition experiments with various concentrations of substrates. The kinetics of the competition experiments were similar to those obtained for the competition between sulphate and selenate for ATP sulphurylase [16] indicating competition between two substrates for one enzyme [17].

The ^{32}P-labelled product formed in the presence of purified enzyme, and of the alternative amino acid substrates, ATP and $^{32}PP_i$ as substrates, and which adsorbed to charcoal, was eluted and identified by high voltage paper electrophoresis as [^{32}P]ATP; neither [^{32}P]ADP nor unlabelled AMP were detected.

A study of the effect of some common inhibitors on cysteinyl-tRNA synthetase activity was confused by the reaction of cysteine with a number of the commonly used thiol-group reagents. The sensitivity of purified cysteinyl-tRNA synthetase to some common inhibitors was studied using the alternative substrate, α-aminobutyric acid, which does not react directly with the inhibitors. The effect of inhibitors on cysteinyl- and methionyl-tRNA synthetase activities is summarised in Table V. Both cysteinyl- and methionyl-tRNA synthetase are sensitive to thiol-group reagents. The enzymes were inhibited by EDTA; this was due, presumably, to the chelation of Mg^{2+} by EDTA.

A variety of nucleotides, sulphur-containing anions, amino acids and

TABLE V

EFFECT OF SOME COMMON INHIBITORS UPON CYSTEINE-, α-AMINOBUTYRIC- ACID AND METHIONINE-DEPENDENT ATP-PP_i EXCHANGE

The compounds were incubated with the enzyme for 1 min before the assay was initiated by the addition of cysteine, α-aminobutyric acid or methionine.

Inhibitor	Cysteinyl-tRNA synthetase Inhibition (%)		Methionyl-tRNA synthetase
	Cysteine	α-Aminobutyric acid	
Nil (control)	0	0	0
Phenylhydrazine (10 mM)	8	5	2
N-Ethylmaleimide (10 mM)	100	18	60
p-Chloromercuribenzoate (8 μM)	100	100	98
Iodoacetamide (10 mM)	100	87	20
EDTA (10 mM)	97	95	97
NaF (10 mM)	11	7	5

peptides were surveyed as possible regulators of cysteinyl- and methionyl-tRNA synthetase activity; enzyme activities were measured by the ATP-PP_i exchange method. The results are summarised in Tables III and IV.

Selenocysteine was the most effective competitive inhibitor tested. Sulphide and cysteamine (the decarboxylated form of cysteine) were also inhibitory. D-Cysteine also competitively inhibited enzyme activity. Phosphate, considered an end product of amino acid activation, competitively inhibited enzyme activity. Strong inhibition of purified phenylalanyl-tRNA synthetase from *E. coli* by phosphate has been reported [18]; similarly phosphate inhibits seryl-tRNA synthetase from yeast [19]. L-Alanine, the amino acid which is one methyl group shorter than α-aminobutyric acid (which was capable of acting as a substrate for cysteinyl-tRNA synthetase) competitively inhibited cysteine-dependent ATP-PP_i exchange. In contrast to the other sulphur-containing amino acids, addition of methionine to reaction mixtures stimulated cysteine-dependent ATP-PP_i exchange (Fig. 4). Sulphate and sulphite had no effect upon the rate of methionine-dependent ATP-PP_i exchange at high or low concentrations of L-methionine. Addition of sulphide to reaction mixtures increased the rate of methionine-dependent ATP-PP_i exchange; sulphide increased the V without altering the K_m of the enzyme for L-methionine. This contrasted to the stimulation of enzyme activity caused by cysteine; L-cysteine had no effect upon the V, but lowered the K_m of the enzyme for L-methionine. Cysteic acid stimulated methionyl-tRNA synthetase in a way similar to cysteine. *O*-Acetyl-serine and reduced glutathione non-competitively inhibited methionine-dependent ATP-PP_i exchange with respect to methionine. Other compounds investigated as possible regulators, but which had no effect upon methionyl-tRNA synthetase activity, included phosphate, methionine sulphoxide, methionine sulphone, cystine and L-serine.

Effect of sulphur source during growth on the specific activity of cysteinyl- and methionyl-tRNA synthetase

Cells were grown in the presence of a variety of sulphur sources including

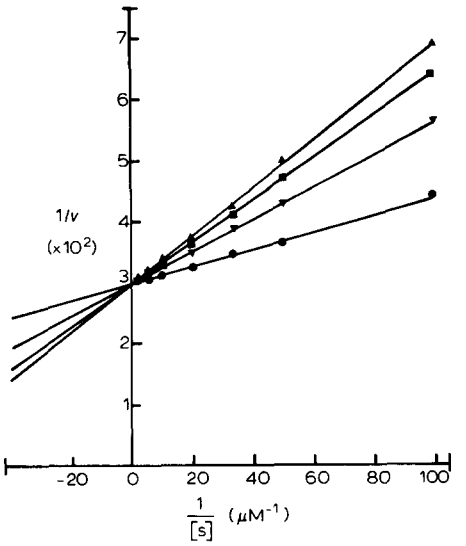

Fig. 4. Double reciprocal plots of cysteine concentration versus the rate of cysteine-dependent ATP-PP_i exchange in the presence or absence of methionine. Reaction mixtures were as described in Materials and Methods except that cysteine and methionine were added to the reaction mixtures at the concentrations specified: ▲, no methionine; ■, 1 mM methionine; ▲, 5 mM methionine; ●, 10 mM methionine.

sulphite, sulphate, sulphide, cystine, sulphate plus L-methionine and sulphate plus L-homocysteine (*P. denitrificans* cannot utilise cysteine, methionine or homocysteine as sole sulphur sources for growth (unpublished results)). No differences in the specific activity of either cysteinyl- or methionyl-tRNA synthetase were detected between extracts of cells grown in the presence of different sulphur sources.

Synthesis of cysteine and methionine hydroxamate

The synthesis of both cysteine and methionine hydroxamate was linear with time up to 60 min, and was dependent on the presence of ATP, Mg^{2+}, cysteine (methionine), and a high concentration of hydroxylamine and enzyme (Table VI). The rate of amino acid hydroxamate synthesis was dependent on enzyme concentration. Under the standard reaction mixture conditions, the formation of amino acid hydroxamate was accompanied by the liberation of equivalent amounts of AMP and PP_i (Table VI). Cysteinyl-tRNA synthetase also catalysed the synthesis of selenocysteine hydroxamate; the rate of selenocysteine hydroxamate synthesis was slightly less than that for cysteine hydroxamate synthesis; selenomethionine hydroxamate and methionine hydroxamate were formed at almost equivalent rates (Table VI).

No ethionine or α-methyl methionine hydroxamate was formed when L-methionine was replaced with ethionine or α-methyl methionine. The rates of norleucine and homocysteine hydroxamate formation were low as compared with the rate of methionine or selenomethionine hydroxamate formation.

When L-methionine was replaced with D-methionine at concentrations between 0.1 and 50 mM, no D-methionine hydroxamate synthesis was detected.

TABLE VI

STOICHIOMETRY OF PRODUCTS FORMED IN ATP-CYSTEINE (ATP-METHIONINE) REACTIONS IN THE PRESENCE OF HYDROXYLAMINE AND PURIFIED CYSTEINYL-tRNA SYNTHETASE AND METHIONYL-tRNA SYNTHETASE

Six duplicate reaction mixtures were conducted for each treatment of which two were assayed for each compound. Cysteine (and methionine) hydroxamate was assayed as the ferric complex as described in Materials and Methods; PP_i was measured as P_i after treatment with purified inorganic pyrophosphatase; AMP was measured using an ADP-AMP test kit obtained from Boeringer und Soehne G.m.b.H., Mannheim, Germany. The results are expressed in μmol/h.

Components	Amino acid hydroxamate	PP_i	AMP
Complete (with cysteine)	0.47	0,45	0.45
Complete minus ATP	<0.01	<0.01	<0.01
Complete minus Mg^{2+}	<0.01	<0.01	<0.01
Complete minus cysteine	0.03	0.02	0.04
Complete (with selenocysteine)	0.38	0.37	0.39
Complete minus ATP	<0.01	<0.01	<0.01
Complete minus Mg^{2+}	<0.01	<0.01	<0.01
Complete minus selenocysteine	0.03	0.03	0.04
Complete (with methionine)	0.68	0.64	0.67
Complete minus ATP	<0.01	<0.01	<0.01
Complete minus Mg^{2+}	<0.01	<0.01	<0.01
Complete minus methionine	0.05	0.04	0.05
Complete (with selenomethionine)	0.62	0.64	0.64
Complete minus ATP	<0.01	<0.01	<0.01
Complete minus Mg^{2+}	<0.01	<0.01	<0.01
Complete minus selenomethionine	0.06	0.04	0.04

Discussion

A comparison of the aminoacyl-tRNA synthetases from *P. denitrificans* with the synthetases from other bacteria, and a comparison of the cysteinyl- and methionyl-tRNA synthetases of *P. denitrificans*, shows that the synthetases differ widely in their K_m values for amino acids, ATP, and Mg^{2+}, and are especially sensitive to Mg^{2+}/ATP ratio. Although the optimal Mg^{2+}/ATP ratio for methionyl-tRNA synthetase, as measured by the ATP-PP_i exchange method is 2 for both *P. denitrificans* and *E. coli* [20], other aminoacyl-tRNA synthetases differ drastically with respect to their Mg^{2+}/ATP ratios; the leucyl-tRNA synthetase from *E. coli* has an optimal Mg^{2+}/ATP ratio of 10, while for prolyl-tRNA synthetase it is 30 [21].

Since L-cysteine not only inhibits a number of reactions including leucine, isoleucine, threonine and valine biosynthesis [22] and respiration (ref. 22 and unpublished results) but is also the substrate for a number of biosynthetic reactions, cysteinyl-tRNA synthetase must not only have a high affinity for its substrate, L-cysteine, but it must also be strictly controlled.

Cysteinyl-tRNA synthetase has a high affinity for its true substrate, L-cysteine. The K_m (cysteine) in the exchange reaction is almost 1/50th of the value obtained under comparable conditions for the cysteinyl-tRNA synthetase from yeast [23]. The K_m (methionine) as measured by ATP-PP_i exchange is approximately the same as that obtained for the *E. coli* enzyme [24], and approximately half that obtained for the *Sarcina lutea* enzyme [25] under

comparable assay conditions. Cysteinyl-tRNA synthetase also has a high affinity for selenocysteine.

Unlike the cysteinyl-tRNA synthetase from yeast [23] and *S. lutea* [26] the enzyme from *P. denitrificans* is sulphydryl-group reagent sensitive (like almost all other aminoacyl-tRNA synthetases [21]).

The substrate specificity of the methionyl-tRNA synthetase from *P. denitrificans* differs from that of *E. coli* [27,28], *S. lutea* [25] and yeast [9]. The *P. denitrificans* methionyl-tRNA synthetase catalyses homocysteine-dependent ATP-PP_i exchange, but not ethionine- or L-cystathionine-dependent ATP-PP_i exchange, whereas the *S. lutea* enzyme does not catalyse L-homocysteine-dependent ATP-PP_i exchange but does catalyse both ethionine- and cystathionine-dependent ATP-PP_i exchange. Methionyl-tRNA synthetase from *P. denitrificans*, *E. coli* and *S. lutea* catalyses selenomethionine- and norleucine-dependent ATP-PP_i exchange.

The relatively low affinity of cysteinyl-tRNA synthetase for α-aminobutyric acid, and of methionyl-tRNA synthetase for norleucine, supports Novelli's [21] proposal that the K_m of an aminoacyl-tRNA synthetase for the "wrong" amino acid, or the analogue, is several orders of magnitude larger than the K_m of the natural substrate. It appears that for *P. denitrificans*, *E. coli*, [27,28], *S. lutea* [25] and yeast [9] selenomethionine is a more satisfactory substitute than is norleucine. The similarity of the K_m values of methionyl-tRNA synthetase for methionine and selenomethionine and of cysteinyl-tRNA synthetase for cysteine and selenocysteine in the amino acid activation reactions present major exceptions to the safeguard against incorporating an amino acid analogue into protein [21] whereby the K_m for the "wrong" amino acid, or the analogue, is several orders of magnitude larger than the K_m for the natural substrate.

So the amount of selenocysteine or selenomethionine in proteins in *P. denitrificans* would be governed by the ratio of selenocysteine to cysteine or selenomethionine to methionine, respectively, in the cell.

Since *P. denitrificans* is unable to grow in culture media containing high concentrations of selenate, although it is able to incorporate selenide into selenocysteine [21] and is able to catalyse the activation of selenocysteine in the initial steps of its incorporation into proteins, it is reasonable to suppose that the toxicity of selenate to *P. denitrificans* may be due to its ultimate incorporation into proteins.

The effects of ethionine in biological systems have been extensively studied [29] and ethionine has been found to inhibit protein synthesis in a number of organisms [30—32]. Yoshida and Yamasaki [33] found that ethionine can be incorporated, in place of methionine, into the normal peptide sequence of α-amylase without altering the physiochemical properties, or the enzyme activity, of the normal proteins. On the contrary, incorporation of norleucine into proteins by *E. coli* apparently in place of methionine [34,35] results in the loss of enzyme activity of that protein. Huber and Criddle [36] isolated β-galactosidase from *E. coli* grown on selenate; approx. 50% of the methionine residues of the enzyme were replaced by selenomethionine. Although the stability of the enzyme was less for the selenium-substituted enzyme the catalytic parameter (K_m and V) were not changed.

Cysteinyl- and methionyl-tRNA synthetases in addition to β-cystathionase

[37] are inhibited by *O*-acetylserine. This may represent an important mechanism in the control of the metabolic pathway of sulphur metabolism in *P. denitrificans*. An increase in the intracellular concentration of *O*-acetylserine might be expected if there were a sulphur deficiency, especially a deficiency in sulphide. This would, in turn, lead to a decrease in the rate of cysteine synthesis. Therefore, when there is an *O*-acetylserine surplus, and a sulphide deficiency, incorporation of cysteine into protein, lysis of cystathionine (by β-cystathionase) and incorporation of methionine into proteins would be inhibited. Conversely, when the intracellular levels of sulphide and cysteine are increased, methionyl-tRNA synthetase activity would be stimulated.

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