Phosphonate Analogues of Aminoacyl Adenylates

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Phosphonomethyl analogues of glycyl phosphate and valyl phosphate, i.e. $\text{NH}_2\text{-CHR-CO-CH}_2\text{-PO(OH)}_2$, were synthesized and esterified with adenosine to give analogues of aminoacyl adenylates. The interaction of these adenylate analogues with valyl-tRNA synthetase from *Escherichia coli* was studied by fluorescence titration. The analogue of valyl phosphate has an affinity for the enzyme comparable with that of valine, but that of valyl adenylate is bound much less tightly than either valyl adenylate or the corresponding derivative of valinol. The affinity of the analogue of glycyl adenylate was too low to be measured. We conclude that this enzyme interacts specifically with both the side chain and the anhydride linkage of the adenylate intermediate.

Many of the enzymes that catalyse reactions of esters of phosphate and pyrophosphate also bind phosphonomethyl analogues of their natural substrates in which a methylene group replaces the oxygen that joins the phosphate group to the rest of the molecule. The analogue may act as a substrate or an inhibitor. Such analogues have been extensively tested with the enzymes of glycolysis [Webster *et al.* (1976) and the references they cite] and with ATP-utilizing enzymes (see Yount, 1975). Engel (1977) has reviewed the subject.

In the present paper we report a general procedure for the synthesis of the corresponding analogues of one class of acyl phosphates, the aminoacyl adenylates. There is now considerable evidence that these carboxylic-phosphoric anhydrides, in enzyme-bound form, are genuine intermediates in the aminoacylation of tRNA, the first step in protein biosynthesis (e.g. Fersht & Kaethner, 1976; Midelfort *et al.*, 1975).

We show that, although the analogue of valyl adenylate is not an anhydride but a ketone, it does inhibit the valyl-tRNA synthetase (EC 6.1.1.9) from *Escherichia coli*. The observation that the phosphonomethyl analogue is less tightly bound than the adenylates of valine or valinol suggests that the enzyme interacts favourably with the anhydride oxygen rather than with the carbonyl group.

Materials and Methods

Reagents

N-Benzoyloxycarbonyl-L-valine was made from valine and benzyl chloroformate by the method of

Carter *et al.* (1955). Diazomethane was made without distillation from urea, methylamine hydrochloride and NaNO_2 (see Dixon & Sparkes, 1974). Isopropylidene-adenosine was made from adenosine and 2,2-dimethoxypropane by the method of Fromageot *et al.* (1967) as modified by Webster *et al.* (1978). Other reagents were from commercial sources.

Chromatographic materials

Polystyrene beads (Amberlite XAD-2) were washed with diethyl ether, acetone and methanol until the washes were odourless and showed no absorption at 260 nm. They were then washed thoroughly with water. The sulphonic resin Zerolit 225 SRC 16 had fine particles removed by settling and decantation.

Electrophoresis

Electrophoresis at pH 2.0 and 3.5 was performed at up to 100 V/cm on paper cooled in 'white spirit'. Webster *et al.* (1976) described the system and the methods for detecting the phosphates and phosphonates by their ability to bind Fe^{3+} ions and for detecting reducing sugars. Ketones were detected by reaction with 5 mM-2,4-dinitrophenylhydrazine in 2 M-HCl, and primary amines by reaction with 0.25% ninhydrin in acetone. Nucleosides and their derivatives were detected by their quenching of the fluorescence shown by the paper under u.v. radiation.

Proton magnetic resonance

Spectra were taken on a Varian HA-100 100 MHz spectrometer. All chemical shifts are quoted as δ values in p.p.m. relative to tetramethylsilane.

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Study of valyl-tRNA synthetase

This enzyme (EC 6.1.1.9), prepared from *E. coli* (Mulvey & Fersht, 1977), was kindly given by Professor A. R. Fersht. The rate of reaction it catalysed was measured by the formation of radio-active ATP by exchange with [32 P]PP $_i$ (Fersht & Kaethner, 1976) and by the incorporation of L-[14 C]valine into tRNA precipitable by trichloroacetic acid (R. S. Mulvey & A. R. Fersht, unpublished work).

Ligand binding by the enzyme was followed by the quenching of its fluorescence, measured with a Perkin-Elmer fluorescence spectrometer (Jakes & Fersht, 1975). The fluorescence was excited at 290 nm, and observed at 338 nm, the wavelength of its maximum emission. The procedure of Mulvey & Fersht (1977) was used, by adding the titrating ligand to the enzyme solution and comparing with a reference solution of enzyme to which the same amount of water was added. Where necessary the quenching was corrected for absorption by the ligand, which was determined in a separate experiment in which the ligand was added to a solution of 26 μ M-Gly-Trp (R. S. Mulvey & A. R. Fersht, unpublished work). In plotting the results the total ligand added was corrected to free ligand concentration by subtracting the concentration bound. For this correction the concentration of the enzyme was taken to be that measured by titration of active sites (Mulvey & Fersht, 1977), which was 31 nM. The values of the dissociation constants quoted in Table 1 are the negative gradients of graphs of the fluorescence change against (fluorescence change)/(concentration of free ligand). The gradients were obtained by the method of least squares and are quoted ± 1 S.D.

Syntheses of analogues of aminoacyl phosphates

Benzylloxycarbonyl derivatives of amino acids were converted into the corresponding *N*-protected chloromethyl ketones by the method of Coggins *et al.* (1974). The product (50 mmol), thoroughly freed from acid by prolonged desiccation over NaOH, was dissolved in ice-cold acetone, and treated with 1.1 mol/mol of NaI, also alkali-dried and dissolved in cold acetone. After incubation for 5 min at 0°C the NaCl formed was filtered off and the filtrate was evaporated to dryness. The crude *N*-protected iodomethyl ketone was then dissolved in chloroform and extracted three times with an equal quantity of water. The organic layer was dried over anhydrous Na $_2$ SO $_4$ and evaporated to dryness.

The iodomethyl ketone was dissolved in a little chloroform and added in small portions to boiling trimethyl phosphite. After boiling under reflux for 1.5 h, most of the unchanged trimethyl phosphite was removed by rotary evaporation (bath at 80°C);

the residue was boiled with 6M-HCl under a stream of N $_2$ for 3 h and again evaporated to dryness.

The analogues of glycyl phosphate and valyl phosphate thus prepared were isolated by chromatography on the sulphonic resin Zerolit 225 SRC 16 in its free acid form by elution with water. The ninhydrin-positive fractions were pooled and evaporated to dryness to give solutions that showed only one spot by electrophoresis at pH 2 and pH 3.5 on staining for primary amines, phosphates and ketones. The analogue of glycyl phosphate was eluted at 4–8 column volumes; that of valyl phosphate was slightly more retarded by the resin. The analogue of glycyl phosphate, 3-amino-2-oxopropylphosphonic acid (Gly-CH $_2$ -P), crystallized from water as white needles of a monohydrate on addition of ethanol. The needles melted with decomposition at 155–157°C. The p.m.r. spectrum of a solution in 2 H $_2$ O showed two signals of equal integration: a singlet at 4.23 (the C-3 protons) and a doublet at 3.19 (J 22 Hz, the C-1 protons). Elemental analysis gave: C, 21.4; H, 5.8; N, 8.5%; C:N ratio, 2.98. C $_3$ H $_5$ NO $_4$ P $_2$ H $_2$ O requires C, 21.1; H, 5.9; N, 8.2%; C:N ratio, 3.

The analogue of valyl phosphate, 3-amino-4-methyl-2-oxopentylphosphonic acid (Val-CH $_2$ -P), did not crystallize. The solid formed from evaporation of column fractions had the following p.m.r. spectrum in [2 H $_4$]methanol: a doublet at 4.36 (J 4 Hz, the C-3 proton), a doublet at 2.90 (J_{HP} 21 Hz, the C-1 protons), a multiplet at 2.30–2.65 (the C-4 proton) and doublets at 1.15 and 0.95 (J 7 Hz, the methyl groups). The integrations of these signals were in the proportion 1:0.2:1:3:3, suggesting that reversible enolization results in extensive deuteration at the 1-position. The elemental analysis suggested that the solid contained some water: C, 34.6; H, 7.3; N, 6.7%; C:N ratio, 6.0. C $_6$ H $_{14}$ NO $_4$ P $_2$ H $_2$ O requires C, 33.8; H, 7.5; N, 6.6%; C:N ratio, 6.

Synthesis of analogues of glycyl adenylate and valyl adenylate

To the 3-amino-2-oxoalkylphosphonic acid (2 mmol), prepared as described above, were added 2 equiv. of isopropylidene-adenosine and 10 equiv. of 2,4,6-tri-isopropylbenzenesulphonyl chloride. The mixture was stirred in freshly distilled dimethylformamide at room temperature for 4–5 days. It was then diluted threefold with 0.1M-sodium hydrogen maleate, pH 6.0, and extracted ten times with an equal volume of ethyl acetate. The aqueous layer was loaded on to a column of Zerolit 225 SRC (H $^+$ form) and the column was washed with 10 vol. of water over 4 h. This resulted in deprotection of the 2'- and 3'-groups (cf. Tener, 1961). The analogue was then eluted with 2M-HCl, the material in the major peak absorbing at 260 nm being pooled. This material was rechromatographed on a column of the same resin in the

ammonium form, equilibrated with a solution of 0.25M-ammonium formate and 0.25M-formic acid. The major u.v.-absorbing peak eluted was desalted on a long column (1 cm $\times$ 80 cm) of Amberlite XAD-2, eluted with water. Salt was unretarded and the valine analogue emerged between 4 and 18 column volumes. The glycine analogue, however, emerged between 0.75 and 5 column volumes, so part overlapped with the (unretarded) salt and the run had to be repeated a few times to obtain a good yield of the salt-free material. The u.v.-absorbing fractions were freeze-dried.

The analogue of valyl adenylate gave only one spot on electrophoresis at pH2.0 and 3.5, the paper being inspected for nucleosides and stained for primary amines, phosphonates and sugars. The analogue migrated to the negative electrode slightly more slowly than adenosine at pH2.0. Elemental analysis of the freeze-dried solid gave: C, 39.0; H, 6.2; N, 17.0%; C:N ratio, 2.68; $C_{16}H_{25}N_6O_7P \cdot 3H_2O$ requires C, 38.5; H, 6.2; N, 16.9%; C:N ratio 2.67. The p.m.r. spectrum in 2H_2O was complex between 4 and 5 p.p.m.; the resonances outside this region were characteristic of adenine and of the analogue of valyl phosphate (see above). The u.v. spectrum of the analogue in 50 mM-KCl/13 mM-HCl, pH2.0, was essentially identical with that of AMP. In view of the difficulty of weighing accurately a hygroscopic solid the concentration of the analogue in solution was estimated from its A_{257} when diluted into buffer of pH2.0. The absorption coefficient was assumed to be that of AMP, $1.50 \times 10^4 M^{-1} \cdot cm^{-1}$ (Bock *et al.*, 1956). By using this estimate of concentration, the analogue was titrated for 1,2-diols by the method of Fields & Dixon (1968), and found to consume 0.9–1.1 equiv. of periodate (three determinations).

The analogue of glycyl adenylate was found to be electrophoretically homogeneous, except for a trace of ninhydrin-positive sugar-positive material, which was almost neutral at pH2.0 and did not absorb in the u.v. This may have been the depurinated analogue. The elemental analysis was C, 34.6; H, 5.3; N, 17.1%; $C_{13}H_{19}N_6O_7P \cdot 3H_2O$ requires C, 34.2; H, 5.5; N, 18.4%. The analysis fits the analogue contaminated by 3.4% of depurinated product and 2.55 molecules of water. We think the compound is pure enough for preliminary tests of binding to enzymes, particularly as the p.m.r. and u.v. spectra and the periodate titration gave results analogous to those described above for the valyl derivative. In fact, the analogue of glycyl adenylate did not bind significantly to valyl-tRNA synthetase even at 1 mM (the highest concentration that could be tested); the presence of a trace of impurity could not therefore affect the result of the experiment.

The yields of the analogues were both about 10% on the starting phosphonic acid. Electrophoresis of the crude esterification mixture suggested much

higher yields, about 50%, so material may have been lost on adsorption on the sulphonic resin and elution by HCl, possibly by depurination. It may be possible to avoid this by eluting directly with the chromatographic buffer. Both analogues were stored well-stoppered in the dark at 0°C, in which condition they were stable for at least 3 months.

Results and Discussion

Preparation of the analogues

The synthetic route to the analogues of glycyl adenylate and valyl adenylate is shown in Scheme 1. An amino acid is converted into the corresponding 3-amino-2-oxoalkylphosphonic acid by making the *N*-benzyloxycarbonyl iodomethyl ketone (I in Scheme 1), treating it with trimethyl phosphite, and de-esterifying the product with HCl. The chloromethyl ketone was converted into the iodomethyl ketone to minimize the danger of a Perkow reaction (Perkow *et al.*, 1952; Lichtenthaler, 1961). The chiral centre of the amino acid becomes an enolizable position in the product; hence de-esterification in acid results in racemization.

Scheme 1. *Synthesis of a phosphonomethyl analogue of an aminoacyl adenylate from the corresponding amino acid*
Abbreviations: Z, benzyloxycarbonyl; Ado, 5'-adenosyl.

The synthesis was performed for glycine and valine and should be general for amino acids, subject to the requirement for protection of reactive side chains. A possible improvement to the synthesis would be the use of $P(O-SiMe_3)_3$ in the Arbuzov reaction, as suggested by Rosenthal *et al.* (1975). The resulting diester is readily hydrolysed by water, so this modification would be particularly useful for making derivatives of potentially acid-labile amino acids, such as tryptophan and serine, and possibly for retaining stereospecificity.

The resulting 3-amino-2-oxoalkylphosphonic acid (II) was esterified with isopropylidene-adenosine, the amino group being left unprotected (apart from protonation). This esterification appears to require an acidic incubation, since addition of even small amounts of pyridine completely abolishes product formation. This may reflect a requirement for the amino group to remain protonated during the reaction.

The analogues of aminoacyl adenylates (III) were isolated by chromatography on a strongly acidic resin (resulting in removal of the isopropylidene groups) followed by desalting on polystyrene beads. This method was better than gel filtration, because purine derivatives are somewhat retarded by dextran gels, and so not well separated from salt.

Stability of the analogues in solution

Although the phosphonomethyl analogues are not capable of hydrolysis at the bonds substituted, they are, being α -aminoketones, prone to self-condensation when unprotonated. We found that this dimerization

was not appreciable at pH 8, either in rate or extent, at concentrations below 1 mM, but that solutions more concentrated than this developed a yellow colour if incubated at neutral pH. This limits any biochemical applications. Although they inhibit protein biosynthesis in rabbit reticulocyte lysates (P. J. Farrell & T. Hunt, unpublished work), their instability makes them less suitable than aminoalkyl adenylates for this application.

For investigations of their interaction with valyl-tRNA synthetase, the analogues were dissolved in water at their own pH and at concentrations up to 10 mM, kept cold, and stored in liquid N_2 when not in use. Such solutions were completely stable, as judged by electrophoresis at pH 2.0 and 3.5, for at least 1 month.

Binding of the analogue of valyl adenylate to valyl-tRNA synthetase

Preliminary kinetic experiments showed that the analogue of valyl adenylate inhibited both ATP-PP_i exchange and valylation of tRNA catalysed by this enzyme. From the concentration of analogue required to give 50% inhibition, rough K_i values were obtained (Table 1). Kinetic analysis was rendered difficult by the likelihood that the analogue competed not only with valine but with ATP, as found by Cassio *et al.* (1967) for the adenylate of L-valinol. If so, the apparent K_i values found with ATP at a concentration that normally saturated the enzyme will be much higher than the true dissociation constants.

Since we wished to compare tightness of binding of the analogue with that of the adenylates of valine and

Table 1. Measures of the affinity of valyl-tRNA synthetase for amino acid derivatives

Ligand		K_s (M)	Apparent K_i (M)*
L-Valine	$Me_2CH-CH(NH_2)-CO-O-H$	$1.28 \times 10^{-4} \dagger$	
Phosphonomethyl analogue of DL-valyl phosphate	$Me_2CH-CH(NH_2)-CO-CH_2-PO(OH)-O-H$	$2.5 \times 10^{-4} \pm 1.0 \times 10^{-4}$	
L-Valyl adenylate	$Me_2CH-CH(NH_2)-CO-O-PO(OH)-O-Ado$	$10^{-11}-10^{-9} \ddagger$	
L-Valinol adenylate	$Me_2CH-CH(NH_2)-CH_2-O-PO(OH)-O-Ado$		$2.9 \times 10^{-8} \S$
Methylene analogue of DL-valyl adenylate	$Me_2CH-CH(NH_2)-CO-CH_2-PO(OH)-O-Ado$	$5.2 \times 10^{-7} \pm 0.4 \times 10^{-7}$	$3 \times 10^{-6} \pm 1 \times 10^{-6} $
Methylene analogue of glycyl adenylate	$H-CH(NH_2)-CO-CH_2-PO(OH)-O-Ado$	$>10^{-3} \P$	

* The K_i values quoted were obtained from assay of ATP-PP_i exchange at a high concentration of ATP. They are therefore considerably above the true dissociation constant.

† Mulvey & Fersht (1977). The K_m values in the ATP-PP_i exchange reaction are 0.83×10^{-4} M for L-valine and 2.8×10^{-4} M for ATP (R. S. Mulvey & A. R. Fersht, unpublished work).

‡ Unpublished work by A. R. Fersht, based on estimates of the rates of association and dissociation of the enzyme-ligand complex.

§ Cassio *et al.* (1967).

|| This is the value (with worst-cases error) of half the concentration needed to give 50% inhibition when valine was at its K_m ; no detailed study was made.

¶ No sign of saturation was observed up to a concentration of 1 mM; higher concentrations could not be tested (see the section on stability).

valinol, we determined the dissociation constant directly by a non-kinetic method, fluorescence titration. In order to investigate the contributions to binding of the various parts of the valyl adenylate molecule, we also titrated the analogues of glycyl adenylate and valyl phosphate. The dissociation constants obtained (the slopes of apparently linear Scatchard plots, calculated by the method of least squares) are shown in Table 1.

These results show that, although the methylene analogue of valyl adenylate is bound considerably more tightly than either L-valine or ATP, it is not nearly as tightly bound as the natural intermediate valyl adenylate, or indeed as the superficially less similar L-valinol adenylate tested by Cassio *et al.* (1967). As it is probably mainly the L-isomers of the analogues that are bound by the enzyme (see Owens & Bell, 1970), the dissociation constants of these isomers may be as low as half those given in Table 1. The affinity of the enzyme for the analogue of valyl phosphate is therefore comparable with that for valine. Not surprisingly, the side chain of valine makes a great difference to the binding, at least 35 kJ/mol [Flossdorf *et al.* (1977) report a discrimination by isoleucyl-tRNA synthetase of over 25 kJ/mol for L-isoleucinol-AMP over glycyl-AMP].

Several explanations could be advanced for the great discrimination in favour of the $-\text{CO}-\text{O}-\text{PO}_2^-$ group over the $-\text{CO}-\text{CH}_2-\text{PO}_2^-$ group. The most obvious is that the very tight binding of the adenylate depends on a favourable interaction of the enzyme with anhydride oxygen. Other factors that could contribute to the discrimination include differences in the electron distribution and hydration between the carbonyl groups of substrate and analogue (unlikely because of the tight binding of the valinol derivative), the bulk of the methylene protons, and the differences in bond lengths and angles caused by the substitution of a methylene group for the oxygen atom of the natural substrate.

Further insight into the nature of the interaction of the enzyme with valyl adenylate could be obtained by synthesizing an analogue with the $-\text{CO}-\text{O}-$ group replaced by $-\text{CH}_2-\text{CH}_2-$. This analogue would complement the adenylates already studied, and permit an evaluation of individual contributions to binding made by the carbonyl group and the central oxygen atom.

We conclude that for valyl adenylate the 2-oxo-alkylphosphonate analogue of the natural acyl phosphate does mimic the natural compound in binding to the enzyme, and has the predicted inhibitory effect. The affinity of the enzyme for this analogue, however, does not approach that for the

natural intermediate. This confirms that the very tight binding of valyl adenylate by valyl-tRNA synthetase is also very specific. It would be interesting if poor enzymic binding of such analogues of acyl phosphates is general, e.g. binding by phosphoglycerate kinase (EC 2.7.2.3) of an analogue of 3-phosphoglycerol phosphate.

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References

- Bock, R. M., Ling, N.-S., Morell, S. A. & Lipton, S. H. (1956) *Arch. Biochem. Biophys.* **62**, 253–264
- Carter, H. E., Frank, R. L. & Johnston, H. W. (1955) *Org. Synth. Collect. Vol.* **3**, 167–169
- Cassio, D., Lemoine, F., Waller, J.-P., Sandrin, E. & Boissonnas, R. A. (1967) *Biochemistry* **6**, 827–836
- Coggins, J. R., Kray, W. & Shaw, E. (1974) *Biochem. J.* **137**, 579–585
- Dixon, H. B. F. & Sparkes, M. J. (1974) *Biochem. J.* **141**, 715–719
- Engel, R. (1977) *Chem. Rev.* **77**, 349–367
- Fersht, A. R. & Kaethner, M. M. (1976) *Biochemistry* **15**, 818–823
- Fields, R. & Dixon, H. B. F. (1968) *Biochem. J.* **108**, 883–887
- Flossdorf, J., Marutzky, R., Messer, K. & Kula, M.-R. (1977) *Nucleic Acid Res.* **4**, 673–683
- Fromageot, H. P. M., Griffin, B. E., Reese, C. B. & Sulston, J. E. (1967) *Tetrahedron* **23**, 2315–2331
- Jakes, R. & Fersht, A. R. (1975) *Biochemistry* **14**, 3344–3350
- Lichtenthaler, F. W. (1961) *Chem. Rev.* **61**, 607–649
- Midelfort, C. F., Chakraborty, K., Steinschneider, A. & Mehler, A. H. (1975) *J. Biol. Chem.* **250**, 3866–3873
- Mulvey, R. S. & Fersht, A. R. (1977) *Biochemistry* **16**, 4005–4013
- Owens, S. L. & Bell, F. E. (1970) *J. Biol. Chem.* **245**, 5515–5523
- Perkow, W., Ullerich, K. & Meyer, F. (1952) *Naturwissenschaften* **39**, 353
- Rosenthal, A. F., Vargas, L. A., Isaacson, Y. A. & Bittman, R. (1975) *Tetrahedron Lett.* 977–980
- Tener, G. M. (1961) *J. Am. Chem. Soc.* **83**, 159–168
- Webster, D., Jondorf, W. R. & Dixon, H. B. F. (1976) *Biochem. J.* **155**, 433–440
- Webster, D., Sparkes, M. J. & Dixon, H. B. F. (1978) *Biochem. J.* **169**, 239–244
- Yount, R. G. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* **43**, 1–56