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# Stereochemical Control of Ribosomal Peptidyltransferase Reaction. Role of Amino Acid Side-Chain Orientation of Acceptor Substrate†

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ABSTRACT: The substrate specificity of the acceptor site of peptidyltransferase of *Escherichia coli* 70S ribosomes was investigated in the fMet-tRNA·A-U-G·70S ribosome and AcPhe-tRNA·poly(U)·70S ribosome systems by using a series of 2′- and 3′-aminoacyldinucleoside phosphates as acceptors. These chemically synthesized compounds are analogues of the 3′ termini of either 2′(3′)-, 2′-, or 3′-aminoacyl transfer ribonucleic acids (AA-tRNAs) of the types C-A-aa, C-2′-dA-aa, C-3′-dA-aa, C-3′-dA-3′-NH-aa, and C-2′-dA-2′-NH-aa (aa = Phe, D-Phe, Lys, Leu, Ala, Glu, Pro, Gly, Asp, Met, and α-aminoisobutyryl). It was found that the 3′-aminoacyl derivatives of optically active amino acids are much better acceptors of *N*-formyl-L-methionine (fMet) or *N*-acetyl-L-phenylalanine (AcPhe) residues than the isomeric 2′-aminoacyl derivatives with affinity constant ratios ($K_M$ 2′/3′) > 100. Likewise, C-A(D-Phe) is a weaker acceptor than the corresponding L derivative C-A-Phe. In contrast, all glycyl derivatives (C-2′-dA-Gly, C-3′-dA-Gly, C-3′-dA-3′-NH-Gly and C-2′-dA-2′-NH-Gly) are good acceptors of the fMet residue, with ratios ($K_M$ 2′/3′) of ~2. On the basis of these results, a model for the stereochemical control of the peptidyltransferase reaction is proposed. It assigns a major role to the orientation of the amino acid side chain in 2′- or 3′-AA-tRNA. A detailed model of the interaction of the acceptor terminus of 3′-AA-tRNA with the acceptor site of peptidyltransferase is also proposed. The model is strikingly similar to those for the active sites of proteolytic enzymes.

AA-tRNA[1] can exist as either a 2′ or 3′ isomer owing to rapid transacylation of the aminoacyl residue within the cis diol grouping of the 3′-terminal adenosine residue (Griffin et al., 1966). The physiological role of 2′- and 3′-AA-tRNA in enzymatic processes involved in protein biosynthesis has recently become the subject of considerable interest (Ofengand, 1977; Sprinzl & Cramer, 1979).

One of the important substeps of protein biosynthesis is peptide bond formation catalyzed by peptidyltransferase. It is, therefore, of considerable interest to study the specificity of this ribosomal enzyme toward isomeric 2′- and 3′-AA-tRNAs. Studies of the peptidyltransferase acceptor site are of particular significance because this is the site where the amino acid becomes incorporated into the growing peptide chain. Thus, the substrate specificity of the acceptor site may be a very important factor influencing selection of the proper AA-tRNAs for incorporation into protein (Sprinzl & Cramer, 1979). Modified AA-tRNAs or their 3′-terminal fragments in which the 2′⇄3′ transacylation cannot occur have recently been used for studies of isomer specificity of the acceptor site of peptidyltransferase. All of these studies used only derivatives of phenylalanine, and most of them concluded that 3′-Phe-tRNA is the acceptor in the peptide bond forming step (Ofengand, 1977; Sprinzl & Cramer, 1979). Quite surprisingly, Chinali et al. (1974) have also observed significant acceptor activity with Phe-tRNA-C-C-3′-dA ("nonisomerizable 2′-Phe-tRNA"). On the basis of these experiments, several important questions have arisen. (1) Is the peptidyltransferase acceptor site really specific for 3′-AA-tRNA, and is this specificity a general feature for all amino acids? (2) Is the peptidyltransferase acceptor site

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[1] Abbreviations used: AA-tRNA, aminoacyl transfer ribonucleic acid; EF-$T_u$, elongation factor $T_u$; Tris, tris(hydroxymethyl)aminomethane; PEP, phosphoenolpyruvate; S-150, the supernatant of the cell extract after centrifugation at 150000*g* (Chládek et al., 1974); AcPhe, *N*-acetyl-L-phenylalanine; fMet, *N*-formyl-L-methionine; $Me_2Gly$, α-aminoisobutyric acid; Z, benzyloxycarbonyl; Bzl, benzyl; Boc, *tert*-butyloxycarbonyl; DCC, dicyclolexylcarbodiimide; $Me_4Si$, tetramethylsilane; DSS, 4,4-dimethyl-4-silapentane-5-sulfonate; tRNA-C-C-3′-dA, tRNA with 3′-deoxyadenosine incorporated at the 3′ end; tRNA-C-C-2′-dA, tRNA with 2′-deoxyadenosine incorporated at the 3′ end; A-Phe, 2′-(3′)-*O*-L-phenylalanyladenosine; 2′-dA-Phe, 2′-deoxy-3′-*O*-L-phenylalanyladenosine, 3′-dA-Phe, 3′-deoxy-2′-*O*-L-phenylalanyladenosine; 3′-dA-3′-NH-Gly, 3′-deoxy-3′-glycylamidoadenosine; 2′-dA-2′-NH-Gly, 2′-deoxy-2′-glycylamidoadenosine; similar abbreviations are used for dinucleotide derivatives and other aminoacyl derivatives; TLC, thin-layer chromatography; RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid.

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Table I: UV and $^1$H NMR Data of Protected 2′(3′)-*O*-Aminoacylnucleosides[a]

| compound | UV spectra in 95% EtOH ($\epsilon$)[d] | $^1$H NMR spectra: H-8 (s, 1 H) | H-2 (s, 1 H) | phenyl (s) | benzyl $CH_2$ (s, 2 H) | H-1′ (d) | amino acid side chain |
|---|---|---|---|---|---|---|---|
| A(Z-Ala)[b] | 14.99 | 8.3 | 8.22 | 7.37 (5) | 5.13 | 6.26 (16%), 6.04 (84%) ($J$ = 7.3 Hz) | 1.55–1.38 (2) |
| 2′-dA(Z-Ala)[b] | 15.55 | 8.27 | 8.21 | 7.36 (5) | 5.12 | 6.45 (m) | 1.50, 1.42 |
| 3′-dA(Z-Ala)[b] | 14.41 | 8.31 | 8.21 | 7.36 (5) | 5.10 | 6.16[e] | 1.47, 1.40 |
| A(Z-$Me_2$Gly)[b] | 14.59 | 8.24 | 8.20 | 7.37 (5) | 5.12 | 6.18 (25%), 5.95 (75%) ($J$ = 7.3 Hz) | 2.1–1.5 (m) |
| A[Z-Gly(Bzl)][c] | 15.65 | 8.38 | 8.17 | 7.37 (10) | 5.12, 5.09 (4) | 5.93 ($J$ = 7.1) | |
| 2′-dA(Z-Pro)[b,f] | 15.13 | 8.22[e] | | 7.37, 7.33 (5) | 5.13 (m) | 6.4 (m) | |
| 3′-dA(Z-Pro)[b,f] | 14.41 | 8.18 (m)[e] | | 7.38, 7.29 (5) | 5.19, 5.09 (2) | 6.00 (m) | |
| A(Boc-Met)[g] | 14.45 | 8.39 | 8.17 | | | 5.19, ($J$ = 7.3 Hz) | 2.08[h] (3) |

[a] Proton chemical shifts in parts per million ($\delta$). [b] $Me_2CO$-$d_6$ + $D_2O$; $Me_4Si$ as an internal standard. [c] $Me_2SO$-$d_6$ + $D_2O$; DSS as an internal standard. [d] $\lambda_{max}$ in 257–261-mm range. [e] Unresolved. [f] Shifting of signals was observed, probably due to anisotropy of amino acid α hydrogen. [g] *t*-Bu group of Boc s, 3 H, 3.54. [h] S-$CH_3$ group in Met.

Chart I

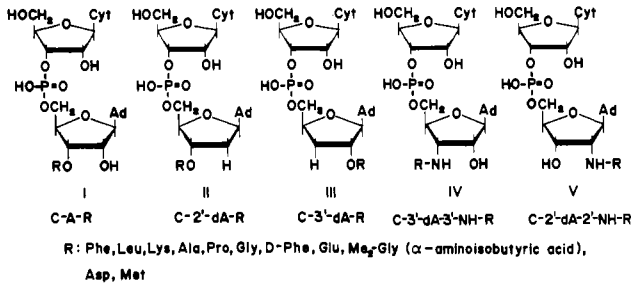
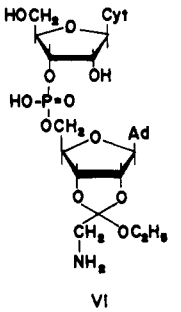



Chart II



VI

specific for L-amino acids? If it is so, can the acceptor site recognize D derivatives, e.g., as inhibitors? (3) If there is isomer specificity and stereospecificity on the peptidyltransferase A site, how is it achieved and what molecular interactions of the AA-tRNA terminus with the enzyme are involved?

Our laboratory has been using 2′(3′)-aminoacyloligonucleotides as analogues of AA-tRNA 3′ termini for the studies of the isomer specificity of peptidyltransferase (Chládek et al., 1974; Ringer et al., 1975; Goldberg et al., 1977; Bhuta & Chládek, 1978). In this report, we address the above questions by using a series of aminoacyloligonucleotide analogues of the 3′ terminus of AA-tRNA derived from the various types of amino acids (aliphatic, aromatic, acidic, basic, and glycine) as acceptors or inhibitors in two peptidyltransferase systems. Preliminary report on portions of this work has appeared (Bhuta & Chládek, 1978), and part of this material was presented at a meeting (Bhuta et al., 1978).

## Materials and Methods

*General Methods.* General methods for chemical synthesis were the same as described in the previous papers of this series (Chládek et al., 1974; Ryu et al., 1977). NMR spectra were recorded on a JEOL FX-100 with the solvents and standards specified in Table I.

Starting materials were as before (Chládek et al., 1974); *N*-benzyloxycarbonyl or *tert*-butyloxycarbonyl amino acids were commercial products (Sigma Chemical Co., St. Louis, MO and Vega Biochemicals, Tucson, AZ). The peptides Met-Phe and Met-D-Phe were products of Bachem, Torrence, CA, and were formylated by treatment with acetic–formic anhydride in pyridine at −20 °C (Greenstein & Winitz, 1961).

*Synthesis of 2′(3′)-Aminoacyldinucleoside Phosphates.* Several 2′(3′)-*O*-aminoacyldinucleoside phosphates (Chart I) used in this study have been described before (Chládek et al., 1974; Ryu et al., 1977; Chládek & Butke, 1980). Cytidyl-(3′→5′)-2′,3′-*O*-[(aminomethyl)ethoxymethylene]adenosine (VI, Chart II) was prepared as described previously (Chládek & Žemlička, 1970).

The 2′(3′)-*O*-aminoacyldinucleoside phosphates I–III were prepared by DCC-mediated condensation of 2′(3′)-*O*-([*N*-(benzyloxycarbonyl)amino]acyl)nucleosides [or 2′(3′)-*O*-[*N*-(*tert*-butyloxycarbonyl)-L-methionyl]adenosine] and *N*-[(dimethylamino)methylene]-2′,5′-di-*O*-tetrahydropyranylcytidine 3′-phosphate as described previously (Chládek et al., 1974). The general method for the synthesis of the appropriate aminoacylnucleosides (derived from adenosine, 2′-deoxyadenosine, or 3′-deoxyadenosine) has been described[2] (Ryu et al., 1977). New components are characterized in Table I. It was observed previously (Ryu et al., 1977) that some degradation of 2′-deoxyadenosine takes place during HCl-catalyzed removal of tetrahydropyranyl groups. Thus, for compounds of general type II, which contain the 2′-deoxyadenosine moiety, *N*-[(dimethylamino)methylene]-2′,5′-di-*O*-(1-ethoxyethyl)cytidine 3′-phosphate was used as the nucleotide component in the condensation step, and the deblocking was effected with dilute acetic acid (Chládek & Žemlička, 1974).

[2] *N*-Benzyloxycarbonyl-α-aminoisobutyric acid reacts very slowly with 5′-monomethoxytrityladenosine in the presence of DCC under standard conditions (Ryu et al., 1977) presumably due to the sterically hindered carboxyl group of the amino acid. The desired deprotected product, 2′(3′)-*O*-(*N*-benzyloxycarbonyl-α-aminoisobutyryl)adenosine [A(Z-$Me_2$Gly)] was obtained in low yield (12%) by using forced conditions: a 2-fold excess of the protected amino acid, a 2.5-fold excess of DCC, and extended reaction time (5 days). A(Z-$Me_2$Gly) partially separates into two spots during TLC on silica gel in the system $CH_2Cl_2$–10% MeOH, which according to NMR (Table I), are the 2′ and 3′ isomers.

Table II: 2′(3′)-*O*-Aminoacylnucleoside Phosphates

| compound[a] | electrophoric mobility[b] | 2′→5′ (%)[c] | Cp/N | 3′→3′ or 3′→2′ (%)[d] |
|---|---|---|---|---|
| C-A-Ala | 2.55 | 0 | 0.99 | 0 |
| C-2′-dA-Ala | 2.56 | 0 | 0.80 | |
| C-3′-dA-Ala | 2.61 | 0 | 0.82 | |
| C-A-$Me_2$Gly | 2.43 | 0 | 0.83 | 0 |
| C-A-Glu | 3.89 | 0 | 0.88 | 4 |
| C-A-Met | 2.50 | 7.8 | 1.01 | 0 |
| C-2′-dA-Pro | 2.65 | 0 | 0.89 | |
| C-3′-dA-Pro | 2.73 | 0 | 0.93 | |

[a] UV spectra of all compounds (in 0.01 N HCl) were similar to that of C-A. [b] Relative mobility toward cathode; mobility of Cp = 1.00, of A = 3.66, and of C-A = 1.6 (1 M $CH_3COOH$). [c] Determined by degradation with pancreatic ribonuclease. [d] Determined by degradation with snake venom phosphodiesterase.

After acidic removal of protecting groups, 2′(3′)-*O*-([*N*-(benzyloxycarbonyl)amino]acyl)dinucleoside phosphates (or *tert*-butyloxycarbonyl) were isolated on cellulose TLC as described before (Ryu et al., 1977). The yields of these compounds were in the 10–20% range. The N-protecting groups were removed by hydrogenolysis in acetic acid (Chládek et al., 1974) or by trifluoroacetic acid [for C-A-Met (Quiggle et al., 1978)].

The final products, compounds of general structure I, II, and III, were routinely purified by preparative paper electrophoresis in 1 M $CH_3COOH$ (4500 V, 4 h) even though the products were chromatographically uniform (UV detection). The final products were characterized by chromatographic and electrophoretic methods, by enzymic digestion (pancreatic ribonuclease and snake venom phosphodiesterase), and by alkaline hydrolysis to corresponding dinucleoside phosphate and parent amino acid (see Table II).

Ribosomes (3 times $NH_4Cl$ washed) were prepared from *Escherichia coli* MRE-600 (RNase $1^-$) as described previously (Chládek et al., 1974). *E. coli* B tRNA (Gibco) was charged with either [$^{14}$C]phenylalanine (New England Nuclear, specific activity 448 mCi/mmol) or [$^{35}$S]methionine (New England Nuclear, specific activity 594 Ci/mmol). Aminoacylation of tRNA with [$^{14}$C]phenylalanine and subsequent acetylation was carried out as described previously (Chládek et al., 1974). [$^{35}$S]Met-tRNA was prepared as follows. The ribosome-free soluble S-150 fraction was dialyzed overnight against 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 22 mM $NH_4Cl$, and 1 mM dithiothreitol (Sigma). Freshly dialyzed S-150 fraction was used as a source of AA-tRNA synthetase and methionyl-$tRNA_F$ transformylase. The preparative charging reaction mixture contained the following in a final volume of 6 mL: 8 mM Tris-HCl, pH 7.4; 8 mM dithiothreitol; 43 mg of *E. coli* B tRNA; 4.5 mg of S-150 fraction protein; [$^{35}$S]-methionine (~1 mCi); 6 mM $MgCl_2$; 3.2 mM ATP; 1.3 mM PEP; 20 units of pyruvate kinase; 286 μg of calcium leucovorin; 19 amino acids (–methionine) (0.03 mM). The reaction mixture was incubated at 37 °C for 15 min, followed by addition of 0.1 volume of 2 M NaOAc (pH 4.8) and deproteinization with phenol. The aqueous phase was then extracted with diethyl ether to remove phenol, and the traces of ether were removed by $N_2$ bubbling. AA-tRNA was then precipitated with 3 volumes of chilled ethanol, and precipitation was allowed to proceed 3–15 h at –20 °C, after which the precipitate was collected by centrifugation and redissolved and precipitation repeated. At this point, the precipitate was dissolved in cold distilled water and dialyzed against 0.1% potassium acetate, pH 5.0, containing 0.5 mM EDTA and 1 mM dithiothreitol for 5 h, followed by subsequent dialysis against cold distilled water for 2 h at 4 °C. The mixture of aminoacylated $tRNA_M^{Met}$ and $tRNA_F^{Met}$ was purified as in Menninger et al. (1970) followed by phenol extraction as described above. The purity of the *N*-formyl[$^{35}$S]-methionyl-tRNA was checked by paper electrophoresis. A sample of fMet-tRNA was hydrolyzed in 0.4 M $NH_4OH$ at 37 °C for 30 min, and the hydrolyzed sample was applied to Whatman No. 1 paper with appropriate standards. The electrophoresis was carried out in pyridine–acetic acid–water (1:10:189) (2200 V, 2 h), and 1 cm wide strips of the dry electrophoretogram were cut and counted. Nonradioactive methionine and formylmethionine were detected according to Toennies & Kolb (1951). The purified fMet-tRNA contained little (<10%) Met-tRNA. The specific activities of f[$^{35}$S]-Met-tRNA were 26 and 21.6 pmol of [$^{35}$S]methionine/mg of tRNA in two preparations.

*Assays of Peptidyltransferase Activity.* Peptidyltransferase activity was measured in the AcPhe-tRNA·poly(U)·70S ribosome system (system A) essentially as described earlier (Chládek et al., 1974) and in the fMet-tRNA·A-U-G·70S ribosome system (system B) as described previously (Bhuta & Chládek, 1978). The aminoacyldinucleotide acceptors (I–V) were added in the desired concentrations, and the reaction mixtures were incubated for 30 min at 37 °C, unless specified otherwise. The reactions were terminated by addition of 10% $CCl_3COOH$. The results are summarized in Table III and Figures 1, 2, and 5. To measure the actual values of $K_m$ and $V_{max}$ of C-2′-dA-Phe (II, R = Phe) and C-3′-dA-Phe (III, R = Phe) in system B, we first carried out a time course experiment at the highest concentration of substrate tested and, from the linear portion of the graph, chose a suitable reaction time for subsequent experiments to obtain a concentration curve of acceptor activity. Incubation times of 10 and 20 min were used for C-2′-dA-Phe (II, R = Phe) and C-3′-dA-Phe (III, R = Phe), respectively (Table IV).

*Inhibition of Acetyl[$^{14}$C]phenylalanylpuromycin Formation.* The inhibition of Ac[$^{14}$C]Phe-puromycin formation from Ac[$^{14}$C]Phe-tRNA and puromycin ($10^{-4}$ M) on 70S ribosomes by C-A(D-Phe) was performed as reported previously (Ringer et al., 1975) (Figure 3).

*Analysis of Peptidyltransferase Reaction Products.* The analysis was carried out after alkaline hydrolysis of the incubation mixture described for the purity check of fMet-tRNA, with inclusion of, e.g., fMet-Phe and fMet-Gly as nonradioactive markers (data not shown).

*Determination of Racemization in* D- *and* L-*Amino Acids.* The amino acid residue of C-A-Phe and C-A(D-Phe) was removed by alkaline hydrolysis, and the amino acid was separated by paper electrophoresis under conditions used for purity check of fMet-tRNA. The amounts of L- and D enantiomers in each sample were measured by assay with L- and D-amino acid oxidases (Sigma, St. Louis MO or Worthington Biochemical Corp., Freehold, NJ), respectively, by using the method of Malmstadt & Hadjiioannou (1953) except that D-amino acid oxidase assays were run at pH 7.5 with 2.2 mg/mL of enzyme. Optical purities of the starting materials (Z-Phe and Z-D-Phe) for the synthesis of the aminoacyldinucleoside phosphates were similarly determined after removal of the benzyloxycarbonyl group by HBr in nitromethane (Albertson & McKay, 1953). Dipeptides formed from C-A-Phe or C-A(D-Phe) and f[$^{35}$S]Met-tRNA by peptidyltransferase were purified by paper electrophoresis as above following alkaline hydrolysis of the dipeptidyl dinucleotide. The dipeptide was then subjected to digestion by carboxypeptidase A for 6 h in 0.1 M $NH_4HCO_3$ at 37 °C (Ambler,

Table III: $K_m^{app}$ of Various Acceptor Substrates in the Peptidyltransferase Reaction[a]

| compound | $K_m^{app}$ (system A) (M)[b] | $K_m^{app}$ (system B) (M)[b] | $K_m(2')/K_m(3')$ |
|---|---|---|---|
| C-A-Phe | $1 \times 10^{-7}$[c] | $1.1 \times 10^{-7}$ | |
| C-3'-dA-Phe | no activity | $2.0 \times 10^{-5}$ | 182 |
| C-2'-dA-Phe | $7.5 \times 10^{-7}$[c] | $1.1 \times 10^{-7}$ | |
| C-3'-dA-3'-NH-Phe | $1.1 \times 10^{-7}$ | $4.4 \times 10^{-8}$ | |
| C-A-Leu | $3.6 \times 10^{-6}$ | $1.0 \times 10^{-6}$ | |
| C-3'-dA-Leu | no activity | no activity | |
| C-2'-dA-Leu | $4 \times 10^{-5}$ | $9.5 \times 10^{-6}$ | very large |
| C-A-Lys | $5 \times 10^{-7}$ | $3.4 \times 10^{-7}$ | |
| C-3'-dA-Lys | no activity | no activity | |
| C-2'-dA-Lys | $1.6 \times 10^{-6}$ | $3 \times 10^{-7}$ | very large |
| C-3'-dA-3'-NH-Lys | $2.5 \times 10^{-7}$ | $1.1 \times 10^{-7}$ | |
| C-A-Glu | $1.5 \times 10^{-6}$ | $2.4 \times 10^{-6}$ | |
| C-2'-dA-2'-NH-Glu | no activity | no activity | |
| C-3'-dA-3'-NH-Glu | $1.5 \times 10^{-5}$ | $1.3 \times 10^{-5}$ | large |
| C-A-Ala | | $9 \times 10^{-7}$ | |
| C-3'-dA-Ala | | no activity | |
| C-2'-dA-Ala | | $1 \times 10^{-5}$ | large |
| C-A-Gly | $1.9 \times 10^{-5}$ | $5 \times 10^{-6}$ | |
| C-3'-dA-Gly | low activity | $6.4 \times 10^{-5}$ | 1.6 |
| C-2'-dA-Gly | low activity | $4.0 \times 10^{-5}$ | |
| C-2'-dA-2'-NH-Gly | low activity | $2.8 \times 10^{-5}$ | 2.3 |
| C-3'-dA-3'-NH-Gly | | $1.2 \times 10^{-5}$ | |
| C-A-Met | $1.1 \times 10^{-6}$ | $1.1 \times 10^{-7}$ | |
| C-A-Pro | $4.0 \times 10^{-6}$ | $4.0 \times 10^{-6}$ | |
| C-A-Asp | $2.0 \times 10^{-5}$ | $1 \times 10^{-5}$ | |

[a] $K_m^{app}$ is defined as the concentration of substrate at half of the maximum activity (Glick et al., 1979). [b] System A: AcPhe-tRNA·poly(U)·70S ribosomes; system B: fMet-tRNA·A-U-G·70S ribosomes. [c] Data from Chládek et al. (1974).

1967). Electrophoresis was carried out to separate fMet from unreacted fMet-Phe. The amounts of fMet and fMet-Phe were determined by measuring the radioactivity of f[$^{35}$S]Met (Figure 4) with a Packard radiochromatogram scanner (Model 7201).

## Results

Acceptor activity in the peptidyltransferase reaction with 25 different 2'(3')-aminoacyldinucleoside phosphates I–V was measured in two systems. In system A, the transfer of an AcPhe residue from the complex AcPhe-tRNA·poly(U)·70S ribosomes to an acceptor is measured (Chládek et al., 1974). In system B, the transfer of an fMet residue from the complex fMet-tRNA·A-U-G·70S ribosomes to an acceptor is determined (Glick et al., 1979). The results obtained with good acceptors are similar in both systems. However, fMet-tRNA (the natural initiator) is a better donor than the "unnatural" AcPhe-tRNA, which allows detection of the peptidyltransferase activity in system B with acceptors which are weakly active in system A (e.g., compounds II–V, R = Gly).

Some illustrative acceptor activities of the aminoacyl dinucleoside phosphates investigated in system A and B can be seen in Figures 1 and 2; all data are summarized in Table III. The electrophoretic analyses of products of some of the peptidyltransferase reactions were performed after saponification of the peptidyl–dinucleotide linkage. The expected dipeptides, e.g., fMet-Phe or fMet-Gly are formed from the appropriate acceptors upon reaction with fMet-tRNA (data not shown).

Comparison of the acceptor activities of the five isomeric pairs of aminoacyldinucleoside phosphates derived from the optically active amino acids (Phe, Lys, Leu, Ala, and Glu) clearly shows a strong preference for the 3' isomers as acceptors. We were able to detect acceptor activity with a 2' derivative, C-3'-dA-Phe [see also Bhuta & Chládek (1978)], by using system B [in system A, this compound is completely

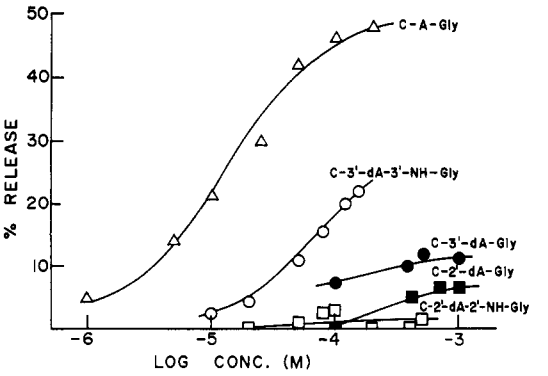



FIGURE 1: Aminoacyldinucleoside phosphate dependent release of Ac[$^{14}$C]Phe from Ac[$^{14}$C]Phe-tRNA in peptidyltransferase reaction. Percent release represents the acceptor-dependent decrease in $CCl_3COOH$-precipitated counts trapped by a Millipore membrane. For other details see text. (△) C-A-Gly; (○) C-3'-dA-3'-NH-Gly; (□) C-2'-dA-2'-NH-Gly; (■) C-2'-dA-Gly; (●) C-3'-dA-Gly.

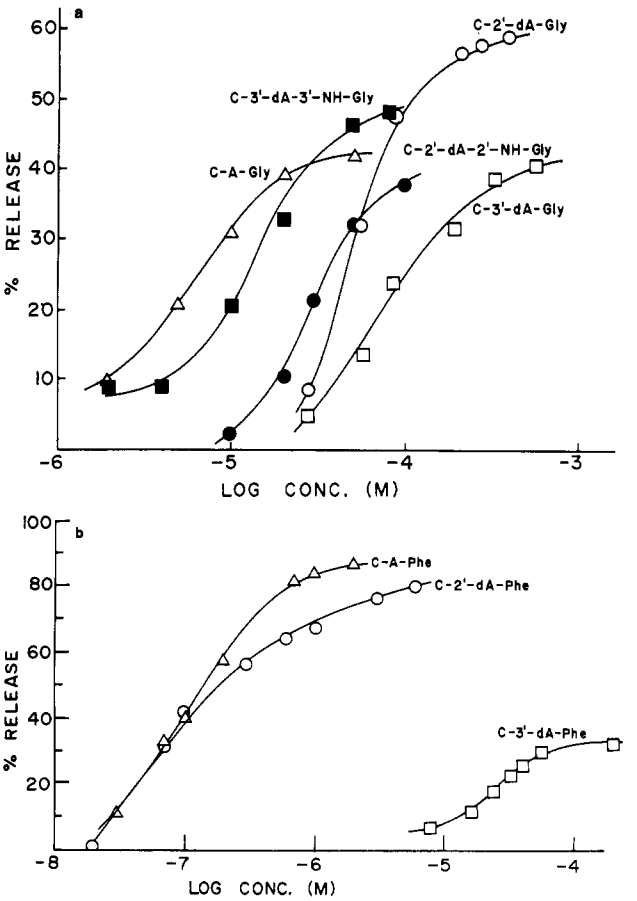



FIGURE 2: Aminoacyldinucleoside phosphate dependent release of f[$^{35}$S]Met residue from f[$^{35}$S]Met-tRNA in the peptidyltransferase reaction. For other details see text. (a) (△) C-A-Gly; (■) C-3'-dA-3'-NH-Gly; (●) C-2'-dA-2'-NH-Gly; (○) C-2'-dA-Gly; (□) C-3'-dA-Gly (Bhuta & Chládek, 1978). (b) (△) C-A-Phe; (○) C-2'-dA-Phe; (□) C-3'-dA-Phe. Incubation time was 20 min.

inactive (Chládek et al., 1974)]. Nevertheless, the activity of the 2' ester (C-3'-dA-Phe) is much lower than that of the parent 3' isomer C-A-dA-Phe, the ratio of $K_m^{app}$ 2'/3' being ~200. The 2' derivatives of Ala, Leu, Lys, and Glu are practically inactive in both systems in the concentration range used, showing a great preference for the 3' isomer. When two isomeric proline derivatives C-2'-dA-Pro and C-3'-dA-Pro were investigated, no activity was observed with either compound in the usual concentration range (results not shown). Eadie–Hofstee plots for C-2'-dA-Phe and C-3'-dA-Phe were

Table IV: Kinetic Parameters of C-2′-dA-Phe and C-3′-dA-Phe[a]

| compound | $K_m$ (M) | $V_{max}$ (fmol min$^{-1}$) | $K_m(2')/K_m(3')$ | $V_{max}(3')/V_{max}(2')$ |
|---|---|---|---|---|
| C-2′-dA-Phe | $1.28 \times 10^{-7}$ | 0.96 | 250 | 2.3 |
| C-3′-dA-Phe | $3.2 \times 10^{-5}$ | 0.42 | | |

[a] Kinetic parameters were determined in system B as described in the text.

Table V: Ratios of $K_m^{app}$ of Compounds II and I[a]

| II/I | $K_m^{app}$(II)/ $K_m^{app}$/(I) |
|---|---|
| C-2′-dA-Phe/C-A-Phe | 1.0 |
| C-2′-dA-Lys/C-A-Lys | 0.9 |
| C-2′-dA-Leu/C-A-Leu | 9.0 |
| C-2′-dA-Ala/C-A-Ala | 11.0 |
| C-2′-dA-Gly/C-A-Gly | 8.0 |

[a] Based on data from Table III (system B).

drawn to obtain kinetic parameters ($K_m$ and $V_{max}$) (plots not shown). The values obtained from Eadie–Hofstee plots (Table IV) show the ratio of $K_m$'s 2′/3′ as 250 and ratio of $V_{max}$'s 3′/2′ as 2. The former number is in very good agreement with the ratio obtained on the basis of $K_m^{app}$ (Table III), and thus $K_m^{app}$'s were used throughout this paper. It can further be seen that the activity of C-3′-dA-Phe is ~200 times lower than that of C-A-Phe (Table III) which is the terminus of native Phe-tRNA. Thus, these results are in complete agreement with those of Chinali et al., 1974, who observed the acceptor activity of Phe-tRNA-C-C-3′-dA in the peptidyltransferase reaction. However, the activity of the 2′-Phe-tRNA was considerably lower than that of the natural isomerizable Phe-tRNA, although an exact kinetic comparison was not made at the time. The point to be made here is that the "fragment assays" used in this report or in the previous reports of this series are in good agreement with assays using intact AA-tRNA (Chinali et al., 1974).

As can be seen from Table III, 2′- and 3′-glycyldinucleoside phosphates [see also Bhuta & Chládek (1978)] behave differently than the other aminoacyl derivatives with an $\alpha$ substituent. The testing of glycyl derivatives II, III, IV, and V (R = Gly) relies more heavily on system B, since the activities are much lower in system A (Figure 1 and 2a).

In system B, there clearly is no significant difference between isomeric pairs of glycine derivatives; in both pairs of compounds the difference of $K_m^{app}$ 2′/3′ could be characterized by a factor of ~2. Significantly, almost the same results were obtained in comparing the acceptor activities of either deoxy derivatives (II and III, R = Gly) or aminodeoxy derivatives IV and V (R = Gly, Figure 2a).[3] In summary, it could be concluded from these results that there is an enormous difference in acceptor activities of 2′- and 3′-aminoacyl derivatives of optically active amino acids but only small differences between 2′- and 3′-glycyl derivatives.

The results further show that isomerizable parent ribo derivatives of type I are more active than 3′ isomers of type II (Table V). This is understandable, since the modified compounds II are lacking a 2′-hydroxyl group, which has been implicated in the binding of substrate to the acceptor site (Rychlík et al., 1969). It is seen that the contribution of 2′-OH group to overall activity is much larger in the less active

[3] It should be noted that both compounds C-2′-dA-2′-NH-Gly and C-3′-dA-3′-NH-Gly might possibly be contaminated with 3′→3′ or 3′→2′ isomers which cannot be reliably detected by enzymic degradation (Chládek & Butke, 1980). Contaminations with these presumably inactive compounds can, in a minor degree, influence the peptidyltransferase activities of C-2′-dA-2′-NH-Gly and C-3′-dA-3′-NH-Gly.

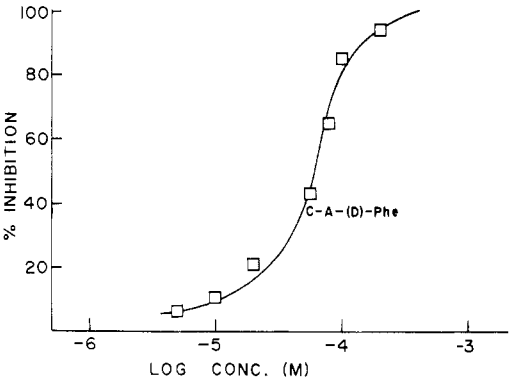



FIGURE 3: Inhibition of Ac[$^{14}$C]Phe-puromycin formation in the presence of C-A(D-Phe) (□). The puromycin concentration was $1.0 \times 10^{-4}$ M. For other details, see Ringer et al. (1975).

substrates (e.g., C-A-Gly) and less critical in substrates with high acceptor activity (e.g., C-A-Phe).

It was further found that C-A($Me_2$Gly), the dinucleotide derived from "unnatural" $\alpha$-aminoisobutyric acid, in inactive in both systems. Also, the ortho ester derivative of C-A-Gly VI (Chart II) shows no activity in either system A or B (results not shown).

The properties of the D-amino acid derivative C-A(D-Phe) were investigated next. We found that C-A(D-Phe) does not function as an acceptor in system A (data not shown) but does inhibit strongly the peptidyltransferase-catalyzed transfer of AcPhe residue to puromycin in system A (Figure 3). In system B, however, C-A(D-Phe) does show considerable activity. It was necessary to determine whether the observed activity was due to contamination with the highly active C-A(L-Phe), which could be produced by partial racemization of the amino acid in the course of chemical synthesis. To our knowledge, it was never investigated whether the synthetic pathway chosen for C-A-Phe were completely racemization free. Assays with L-amino acid oxidase showed no detectable L enantiomer in *N*-benzyloxycarbonyl-D-phenylalanine (Sigma) used as a starting material. When D-Phe obtained by saponification of C-A(D-Phe) was assayed with the amino acid oxidases, it was found to be contaminated with ~0.6% of L-Phe, an amount that could account for much of the acceptor activity. Similarly, when the dipeptide fMet-Phe is digested with carboxypeptidase A, only fMet-L-Phe should be hydrolyzed (Hansen & Smith, 1949). It can be seen from Figure 4 that the dipeptide formed during peptidyltransferase reaction between C-A(L-Phe) and f[$^{35}$S]Met-tRNA is completely digested by carboxypeptidase A as expected. However, when the dipeptide generated from C-A(D-Phe) was digested, 31% of the radioactivity remained in undigested fMet-Phe, presumably as fMet-D-Phe. This indicates that C-A(D-Phe) itself has some acceptor activity. When the peptidyltransferase acceptor activity of a C-A(D-Phe) sample is measured in the entire concentration range and the data are corrected for the C-A(L-Phe) contamination, the curve shown in Figure 5 is obtained. It is seen that the acceptor ($1/K_m^{app}$) activity of C-A(D-Phe) is between 1 and 2 orders of magnitude lower than that of C-A(L-Phe), while the $V_{max}$ for C-A(D-Phe) is ~2 times lower than that of C-A(L-Phe). At higher concentra-

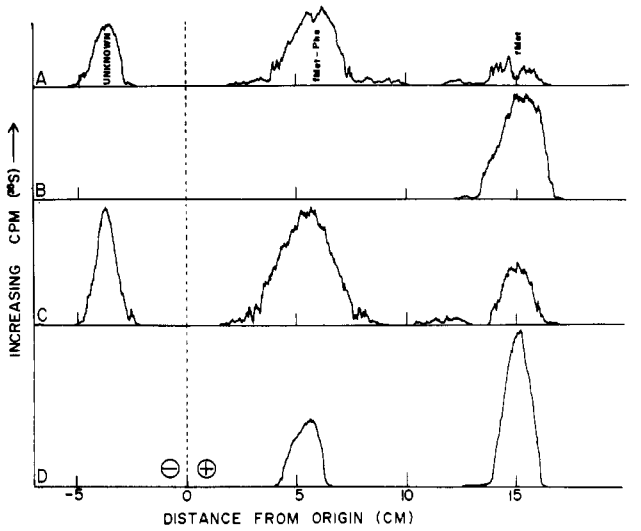



FIGURE 4: Radiochromatographic scan of paper electrophoretograms of dipeptides from C-A-Phe or C-A(D-Phe) and f[$^{35}$S]Met-tRNA. (A) Electrophoretogram of the alkaline hydrolysate of fMet-Phe from C-A(L-Phe). The incubation mixture contained 28 $\mu$g of f[$^{35}$S]-Met-tRNA (~59 000 cpm), 18.5 $A_{260}$ units of ribosomes, 0.76 $A_{260}$ units of A-U-G, and $5 \times 10^{-5}$ M C-A(L-Phe) in 307.5 $\mu$L of 0.05 M Tris-HCl (pH 7.4), 0.10 M $NH_4Cl$, and 0.01 M $MgCl_2$. Identification of radioactive compounds following electrophoresis was made by comparison with standards located by platinic iodide reagent (Toennies & Kolb, 1951). (B) Electrophoretogram of the products of carboxypeptides A digestion of fMet-Phe which was obtained by elution from paper with 0.1 M $NH_4HCO_3$ of the fMet-Phe purified by electrophoresis as illustrated in (A). (C) Electrophoretogram of the alkaline hydrolyzate of fMet-Phe from C-A(D-Phe). The incubation mixture contained 56.6 $\mu$g of f[$^{35}$S]Met-tRNA (~120 000 cpm), 37 $A_{260}$ units of ribosomes, 1.5 $A_{260}$ units of A-U-G, and $10^{-4}$ M C-A-(D-Phe) in 800 $\mu$L of 0.05 M Tris-HCl (pH 7.4), 0.10 M $NH_4Cl$, and 0.01 M $MgCl_2$. Identification of the radioactive compounds following electrophoresis was made by comparison of the standards located by platinic iodide reagent. (D) Electrophoretogram of the product of carboxypeptidase digestion of fMet-Phe which was obtained by elution from paper of the fMet-Phe purified by electrophoresis as illustrated in (C).

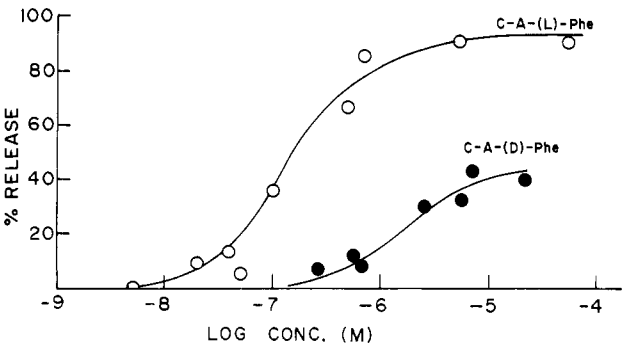



FIGURE 5: Acceptor activity of C-A(D-Phe) (●) and of C-A(L-Phe) (○) in peptidyltransferase reaction with f[$^{35}$S]Met-tRNA. The activity of C-A(D-Phe) has been corrected for activity attributable to the 0.6% impurity of C-A(L-Phe) (the 0.6% impurity of C-A(D-Phe) in C-A-(L-Phe) has a negligible effect on the activity of C-A(L-Phe).

tions, where release of the fMet residue due to contaminating C-A(L-Phe) alone is nearly quantitative, the acceptor activity of C-A(D-Phe) could not be determined.

## Discussion

Results presented here clearly show the strong preference for the 3′-aminoacyl derivatives of optically active amino acids as acceptors in the peptidyltransferase reaction. We therefore conclude that 3′-AA-tRNA is the strongly favored acceptor in the peptidyltransferase reaction, this conclusion being valid for probably all $\alpha$-substituted amino acids. On the other hand, results with glycine derivatives indicate that glycine can perhaps be incorporated into proteins from either the 2′ or 3′ positions of tRNA with similar efficiency. Consequently, it was of interest to study the acceptor properties of the ortho ester glycine derivative VI, which is an analogue of a hypothetical intermediate of 2′⇄3′ transacylation (Wolfenden et al., 1964; Griffin et al., 1966). Unfortunately, the derivative VI was completely devoid of acceptor activity in both peptidyltransferase systems, most probably due to steric hindrance by the ethoxyl group of the ortho ester.

What determines the stereospecificity of the peptidyltransferase reaction? From a comparison of results with optically active amino acids and those with glycine derivatives, it appears that the orientation of the amino acid side chain must be involved. Is the chirality of the aminoacyl residue involved in recognition? Cytidylyl-(3′–5′)-2′(3′)-*O*-($\alpha$-aminoisobutyryl)adenosine (I, R = $\alpha$-aminoisobutyryl, $Me_2Gly$) was thought to be a model compound to help to answer this question since the aminoacyl residue has a side chain and is not chiral. Unfortunately, this compound was also completely inactive as an acceptor in the peptidyltransferase reaction; therefore, it was pointless to study its nonisomerizable derivatives. Nevertheless, this negative result has an important implication: the peptidyltransferase acceptor site probably cannot tolerate a second substituent on the $\alpha$ carbon of the amino acid (vide infra). Another model which helped to decide the question of the stereospecificity of peptidyltransferase reaction was the D-amino acid derivative C-A(D-Phe). The literature on the question of the stereospecificity of peptidyltransferase reaction was somewhat contradictory. Most of the investigators (Rychlík et al., 1970; Nathans & Neidle, 1963; Symons et al., 1969; Eckermann et al., 1974) concluded that the L configuration of the amino acid in aminoacylnucleosides is necessary for the acceptor or inhibitory activity in the peptidyltransferase reaction, but see also Harris et al. (1971). Our results indicate that the aforementioned inconsistency has been probably caused by partial racemization of D-aminoacyl derivatives. We have shown that even though C-A(D-Phe) does not accept an AcPhe residue in Ac-Phe-tRNA·poly(U)·70S ribosomes (system A), it is nevertheless recognized by the peptidyltransferase A site as an inhibitor. On the other hand, the results with C-A(D-Phe) in system B show that the requirements for stereospecificity in peptidyltransferase reaction are not absolute; the acceptor activity ($1/K_m^{app}$) of C-A(D-Phe) is, however, at least 50 times lower than that of the parent C-A(L-Phe).

It is reasonable to assume that specific positioning of the $\alpha$-amino group of the acceptor is required for peptide bond formation at the peptidyltransferase catalytic center (Chinali et al., 1974). If both 2′- and 3′-aminoacyl derivatives react, presumably their respective amino groups must adopt the same spatial position. It is clear that the side chains of the amino acids of the 2′ and 3′ isomers are then pointing in different directions (Chinali et al., 1974; Ringer & Chládek, 1975). Thus, the low reactivity of the 2′-AA derivatives is probably due to the inability of their side chains to be properly accommodated by the peptidyltransferase A site. This should be true for all optically active amino acids. The 2′- and 3′-glycyl derivatives lacking side chains evidently both fit into the peptidyltransferase A site and thus react efficiently as acceptors. The low reactivity of C-A(D-Phe), which can exist as both 2′ and 3′ isomers, further shows that the carbonyl group of the acceptor is also involved in interaction with the peptidyltransferase A site since C-A(2′-D-Phe) also cannot react efficiently as an acceptor. As the findings with the

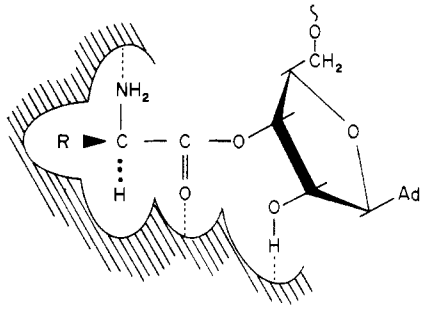



FIGURE 6: Schematic drawing of the 3′ region of the peptidyltransferase A site showing interaction with moieties of the 3′ terminus of 3′-AA-tRNA. Additional binding points are probably provided by the Ad and Cp residue(s). Similar models have been suggested for chymotrypsin (Schultz et al., 1977).

isomeric pairs of glycine derivatives show, the requirement for the definite position of the carbonyl is perhaps less stringent thant the requirement for the proper orientation of the side chain. Presumably, the carbonyl groups in the glycine derivatives have more freedom due to the absence of a side chain rigidly oriented in a certain direction by interaction with the peptidyltransferase A site locus [Rychlík et al. (1970) and vide infra]. It is thus apparent that the peptidyltransferase A site is well able to distinguish between 2′ and 3′ (or D and L) isomers of AA-tRNA so as to exercise stereochemical control of the peptidyltransferase reaction.

Both 2′- and 3′-AA-tRNA isomers of their terminal fragments can interact with the peptidyltransferase A site [for review, see Sprinzl & Cramer (1979)], and it was postulated that this site is composed of two subsites which are able to bind the aminoacyl termini of either 2′- or 3′-AA-tRNA (2′ and 3′ subsites) (Ringer et al., 1975, 1976; Sprinzl & Cramer, 1979). The findings presented here allow a determination of the interacting functionalities of the AA-tRNA termini, most importantly for the 3′ subsite. The obvious function of these interactions is to orient the 3′ terminus of AA-tRNA properly for acceptance of the peptide residue. As can be seen from Figure 6, the aminoacyl terminus of 3′-AA-tRNA interacts with the enzyme in at least two points (side chain and carbonyl group). A "pocket" for the $\alpha$ hydrogen should be understood to mean that a space is available just for the $\alpha$ hydrogen and not for the substituent (see the case of $\alpha$-aminoisobutyryl derivative of C-A, vide supra). In all these respects, the peptidyltransferase A site appears to be strikingly similar to the active sites of such proteolytic enzymes as chymotrypsin (Blow, 1976; Schlutz et al., 1977). Recall that the stereospecificity of proteolytic enzymes is not achieved by preferential binding of L isomers but by selective positioning of configurational isomers with respect to the catalytic functionalities of the enzyme (Bosshard, 1974). This is the case for peptidyltransferase as well. The side-chain pockets of the active sites of chymotrypsin or trypsin show considerable specificity for certain amino acids (Steitz et al., 1969; Blow, 1976). The peptidyltransferase A site must, on the other hand, be able to accommodate all the amino acid residues of the different AA-tRNAs, although not all AA-tRNAs need necessarily react at the same rate (Goldberg et al., 1977; Glick et al., 1979). In fact, all 2′(3′)-*O*-aminoacyl derivatives of C-A (I) tested in this report are active as acceptors, although their $K_m^{app}$'s (in system B) range from $10^{-7}$ to $10^{-5}$ M. It is probable that differences between various aminoacyl acceptors are most pronounced in fragment assays and probably level off somewhat with complete AA-tRNA molecules, where additional interactions of the tRNA molecules with ribosomes make a contribution [for discussion, see also Goldberg et al. (1977)].

It is easy to understand that the stereospecificity of the peptidyltransferase A site, together with the stereospecificity of EF-$T_u$ (Pingoud & Urbanke, 1980), will prevent incorporation of D-amino acids into proteins.[4] It might be also reasoned that the isomer specificity developed as a consequence of the primary task of rejection of D-amino acids. We feel that this suggestion is not tenable, since the isomer specificity of the peptidyltransferase A site is probably more pronounced than its stereospecificity.

The rationale for the peptidyltransferase isomer specificity has been recently discussed by Sprinzl & Cramer (1979). It was previously suggested that transacylation of the aminoacyl residue of AA-tRNA from the 2′ to 3′ position may be occurring in a step preceding the peptide bond formation on the ribosome (Ringer et al., 1976). If this is the case, the peptidyltransferase specificity for 3′-AA-tRNA becomes the final check before irreversible peptide bond formation occurs (Sprinzl & Cramer, 1979).

The findings presented in this report document clearly a very strong preference for 3′-AA-tRNA of optically active amino acids in the peptidyltransferase reaction as acceptors. It is not yet clear why Gly-tRNA, which can probably function as an acceptor with the glycine residue in either the 2′ or 3′ position, should be exempted from this control process.

Acknowledgments

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# Calorimetric Evaluation of the Existence of Separate Domains in Bovine Prothrombin†

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ABSTRACT: At least two endothermal (denaturing) transitions, at 53.1 ± 1.5 °C and 58.2 ± 1.5 °C, of a total $\Delta H$ of 324 ± 12 kcal mol$^{-1}$, are observed, by differential scanning calorimetry (DSC), for solutions of bovine prothrombin. Studies with the appropriate isolated fragments show that the low-temperature transition arises from the prethrombin 1 portion of the molecule and the higher temperature transition originates from the fragment 1 region. We have compared the transition temperature midpoints, $T_m$, the total $\Delta H$ values of the thermal transitions, and the ratio of the calorimetric to van't Hoff enthalpies of these isolated regions of prothrombin with those of the prothrombin molecule. The data obtained strongly suggest that they exist as independent domains in the intact bovine prothrombin molecule. On the other hand, when prethrombin 1 is digested to fragment 2 and prethrombin 2, DSC experiments performed on the isolated components, in the presence or absence of $Ca^{2+}$, strongly support the conclusion that these regions contribute in a cooperative fashion to the structure of intact prethrombin 1 (and prothrombin). Addition of saturating (5 mM) levels of $Ca^{2+}$ to intact bovine prothrombin results in essentially no change in the $T_m$ of the prethrombin 1 domain, an increase in the $T_m$ of the fragment 1 domain, and a decrease in the total $\Delta H$ of the thermal transitions. Further, the endotherm for the prethrombin 1 region is markedly sharpened in the presence of $Ca^{2+}$. Addition of $Ca^{2+}$ causes no alteration in the $T_m$ of 52.6 ± 1.5 °C or the $\Delta H$ of 200 ± 10 kcal mol$^{-1}$ of isolated prethrombin 1, and the sharpening effect induced by $Ca^{2+}$ in the prethrombin 1 region of bovine prothrombin was not observed with isolated prethrombin 1. The $T_m$ of the fragment 1 region of intact bovine prothrombin is increased to 64.8 ± 1.5 °C upon addition of 5 mM $Ca^{2+}$ to the solution. Similar changes are also noted in isolated fragment 1 as a result of addition of $Ca^{2+}$. Here, the $T_m$ of fragment 1 of 58.5 ± 1.5 °C and the $\Delta H$ of fragment 1 of 134 ± 10 kcal mol$^{-1}$ are altered to 64.5 ± 1.5 °C and 102 ± 10 kcal mol$^{-1}$, respectively, as a result of addition of 5 mM $Ca^{2+}$. Other metal ions such as $Mg^{2+}$ and $Mn^{2+}$ induce similar alterations in the thermal transitions of prothrombin and isolated fragment 1.

The conversion of fibrinogen to fibrin is catalyzed by thrombin, a serine protease derived from a plasma precursor, prothrombin. Bovine prothrombin is a 72000 molecular weight glycoprotein (Heldebrant et al., 1973a) whose structure (Magnusson et al., 1975), function, and activation mechanism (Esmon et al., 1974; Esmon & Jackson, 1974a,b; Bajaj et al., 1975; Heldebrant et al., 1973a) have been extensively investigated. The efficient activation of prothrombin to thrombin requires the cooperative effect of two proteins, factor Xa and factor Va, in addition to phospholipid and calcium (Nesheim et al., 1979b).

Several proteolytic cleavages occur in the prothrombin molecule upon activation, giving rise to various intermediate fragments (Esmon et al., 1974; Esmon & Jackson, 1974a,b;

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