RIBOSOME-CATALYZED PEPTIDYL TRANSFER: SUBSTRATE SPECIFICITY AT THE P-SITE

By Robin E. Monro,* Jiriná Cerná,† and Kjeld A. Marcker

MEDICAL RESEARCH COUNCIL, LABORATORY OF MOLECULAR BIOLOGY, CAMBRIDGE, ENGLAND

Communicated by Fritz Lipmann, July 18, 1968

Peptide bond formation in protein biosynthesis takes place by transfer of the growing peptidyl group from the CCA terminus of 1 molecule of transfer RNA (tRNA) to an aminoacyl group attached to the CCA terminus of a second, incoming, molecule of tRNA.[1-4] There is increasing evidence that the reaction is catalyzed by an enzyme, *peptidyl transferase*, which is an integral part of the 50*S* ribosomal subunit.[5-7] The present communication is concerned with substrate specificity at this catalytic center.

The peptidyl transfer reaction is normally linked to other reactions of protein biosynthesis through simultaneous interactions of the tRNA molecules with template, various parts of the ribosome, and, possibly, soluble protein factors (ref. 5: review). By use of substrate analogs which lack certain functional parts of the tRNA molecule but retain others, it is possible to resolve peptidyl transfer from the other reactions. Aminoacyl-tRNA can be replaced by puromycin (an analog of aminoacyl-adenosine) and peptidyl-tRNA by CAACCA-Met-F (a fragment from F-Met-tRNA). The puromycin reaction has been extensively studied and has provided much information on protein biosynthesis.[5, 8] The development of a system in which CAACCA-Met-F acts as a peptidyl donor (with puromycin as acceptor) is a recent development.[9]

The reaction of CAACCA-Met-F with puromycin to give *N'*-formyl-methionyl-puromycin is catalyzed by washed 50*S* ribosomal subunits[6] and is dependent upon the presence of monovalent cations, divalent cations, and alcohol,[9, 10] whereas 30*S* subunits, soluble protein factors, and guanosine 5'-triphosphate are not required.[6] Evidence that the fragment reaction takes place by the same mechanism as peptidyl transfer in protein biosynthesis is provided not only by the involvement of common factors but also by the observation that certain antibiotic inhibitors of protein biosynthesis are very active in the system.[11]

It is widely believed that there are two substrate binding sites on ribosomes,[5, 12-16] one (the *P-site*) for holding the peptidyl-tRNA, the other (the *A-site*) for attachment of the aminoacyl-tRNA. Replacement of the normal substrates of protein biosynthesis by fragments obtained from them, and other small analogs such as puromycin, provides a means to investigate interactions which occur at the two sites in the neighborhood of the catalytic center. Studies with analogs of aminoacyl-tRNA have already provided information on specificity at the A-site.[17-19] In the present work we have investigated the activities of various analogs of peptidyl-tRNA in order to study the specificity at the P-site. Structural features considered are size and base sequence of the oligonucleotide, acylation of the α-amino group, and nature of the aminoacyl residue.

Materials and Methods.—Salt-washed ribosomes, ribosomal subunits, and postribosomal supernatant fraction (S-100) were prepared from *E. coli* MRE 600 by procedures described elsewhere.[7, 10] Sources of standard materials are noted in the same communica-

tions. Ionophoresis was performed as described elsewhere.[20] Radioactivity on ionograms was located by autoradiography or by scanning with a gas-flow strip counter.

Aminoacyl derivatives: F-Met-tRNA and aminoacyl-tRNA, labeled in the appropriate amino acid, were prepared by standard procedures.[21, 22] Acetylation of the α-amino group of aminoacyl-tRNA was carried out with acetic anhydride.[23] RNase-T_1 fragments were prepared by digestion of tRNA derivatives (20 mg/ml), with 0.25 mg/ml. RNase-T_1 (Calbiochem), 2% NaOAc (pH 5.4), and 2 mM ethylenediaminetetraacetate (EDTA), usually in a total volume of 0.1 ml. After incubation for 15 min at 37°C, the mixture was fractionated by ionophoresis at pH 3.5 on Whatman 52 paper. The appropriate amino acid-containing compounds were eluted with 10^{-4} *M* NaOAc (pH 5), lyophilized, redissolved in water, and stored at −20° or in liquid nitrogen. Alkaline hydrolysis of the products gave rise to the expected amino acid or *N*-acyl-amino acid. The RNase-T_1 fragments containing methionine, leucine, and phenylalanine had ionophoretic mobilities consistent with the known 3′-terminal nucleotide sequences of $tRNA_F$, (ref. 24), $tRNA_M$ (S. Cory, personal communication), $tRNA_1^{Leu}$ and $tRNA_2^{Leu}$ (ref. 25), and $tRNA^{Phe}$ (Barrell, personal communication).

Smaller fragments were obtained from CAACCA-Met-F by limited digestion with spleen phosphodiesterase as described in Figure 1, but with higher initial concentrations of CAACCA-Met-F. Better yields of CA-Met-F were obtained by starting with CCA-Met-F. F-Met-adenosine was also prepared by digestion of F-Met-tRNA with pancreatic RNase.[22]

CCA-Met-F and CCA-Leu-Ac were alternatively prepared by incubation of the appropriate T_1 fragment with concentrated RNase-T_1 (which leads to secondary cleavage at bases other than G (Marcker, unpublished observation)). In a typical preparation, CAACCA-Met-F (0.5 μc) was incubated with 0.5 mg/ml RNase-T_1, 20 μg tRNA (as carrier), 2% NaOAc, pH 5.4, and 2 mM EDTA in a volume of 10 μl. After incubation for 120 min at 37°C, the mixture was fractionated by ionophoresis at pH 3.5 on Whatman 52 paper. The product ionophoresed together with CCA-Met-F obtained by the spleen phosphodiesterase method. CCA-Leu-Ac also ionophoresed at about the same rate.

The identity of CCA-Met-F was confirmed by the following double-label experiment. P^{32}-tRNA (ref. 20) was charged with H^3-methionine and then formylated, digested with RNase-T_1, and ionophoresed at pH 3.5. Material migrating in a position corresponding to CAACCA-Met-F was eluted, digested with concentrated RNase-T_1, and again ionophoresed at pH 3.5. A discrete band was formed in a position corresponding to CCA-Met-F marker, prepared by prolonged digestion of S^{35}-CAACCA-Met-F with RNase-T_1. (An uncharged P^{32}-CCA marker migrated to a different position.) This band was again ionophoresed at pH 1.9 on DEAE-cellulose paper and gave a discrete band migrating at the same rate as the S^{35}-CCA-Met-F marker. The material was eluted and shown by standard methods[20] to contain H^3-*N*′-formyl-methionine and the P^{32}-CpCpA.

Assay of reaction with puromycin: Incubations were carried out under the conditions described in Figure 2. The extent of reaction with the derivatives of *N*′-acetylated methionine, leucine, phenylalanine, and glycine was determined by a modification[10] of the ethyl acetate extraction method developed by Leder and Bursztyn.[26] Over 95% of the puromycin derivatives was recovered, and blanks, incubated without puromycin, amounted to less than 4% of the added radioactivity. In assays with the derivatives of arginine, the extent of reaction was determined by ethanol precipitation, as described elsewhere.[9] When F-Met-adenosine was tested as a substrate, incubated samples were analyzed for the presence of F-Met-puromycin by mild alkaline hydrolysis followed by ionophoresis at pH 3.5. Reaction mixtures from all the other substrates were also checked by ionophoresis at pH 3.5, and the puromycin-dependent formation (or absence of formation) of new compounds with mobilities in the expected ranges was confirmed. In all cases, reaction with puromycin was dependent upon the presence of ribosomes and alcohol; 50*S* subunits were of the same order of activity as 70*S* ribosomes, whereas 30*S* subunits were inactive.

Results.—N′-formyl-methionine derivatives: A series of formyl-methionyl oligonucleotides was prepared from CAACCA-Met-F by limited digestion with

spleen phosphodiesterase followed by ionophoresis (Fig. 1). The compounds were then assayed for reactivity toward puromycin in the presence of ribosomes, salts, and alcohol. Results in Figure 2 show that the reaction with CAACCA-Met-F was 50 per cent completed within approximately 15 minutes at 0°. The reaction

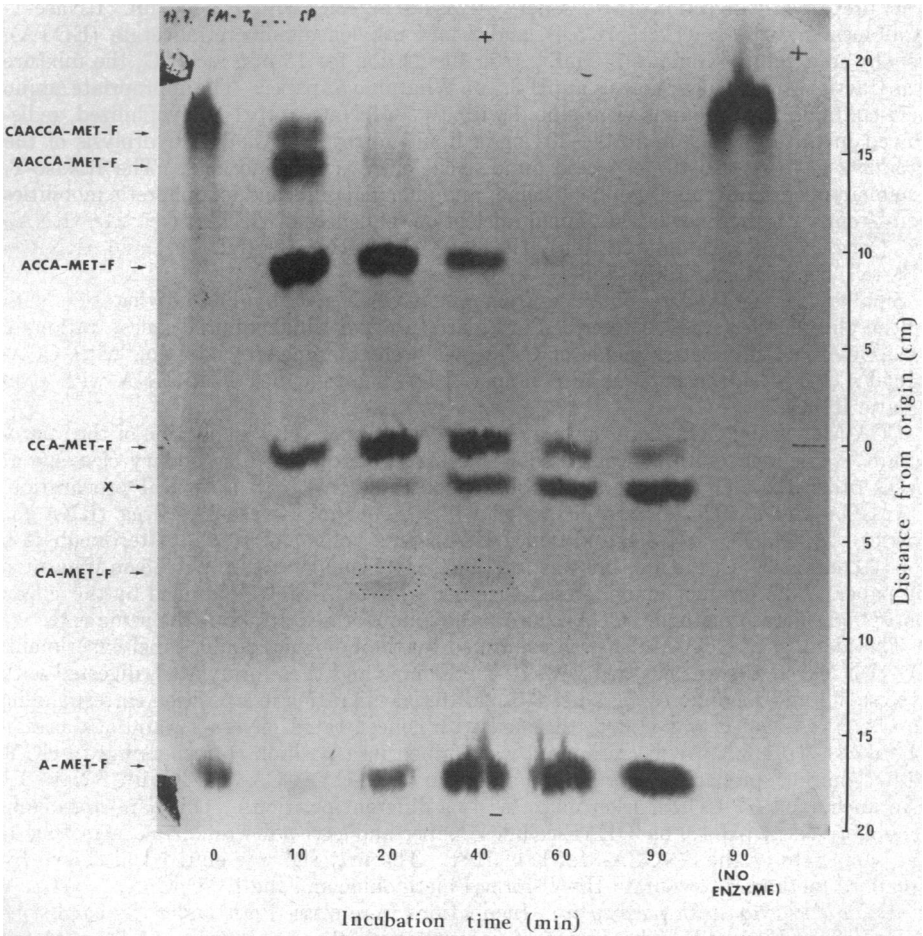

FIG. 1.—Ionogram of CAACCA-Met-F digested with spleen phosphodiesterase for various times.

CAACCA-Met-F, the T_1 fragment from F-Met-$tRNA_F$, was prepared as described in *Materials and Methods*, with S^{35}-methionine of specific activity in the order of 2500 mc/mmole. The fragment (0.02 μc/sample) was incubated with spleen phosphodiesterase (0.3 mg/ml) (Worthington), 20 μg tRNA (as carrier), 0.1 *M* $NH_4(OAc)$(pH 5.7), and 0.02 *M* EDTA. Samples (14 μl) were incubated in capillary tubes at 37°C, and then ionophoresed on Whatman 52 paper for 210 min at 50 v/cm. Times of incubation are indicated on the autoradiograph.

The various bands in the ionogram were identified by consideration of their temporal order of formation and degradation, and by their ionophoretic mobilities, with CAACCA-Met-F and F-Met-adenosine as reference points. The identity of CCA-Met-F was confirmed by ionophoresis along with an authentic CCA-Met-F marker (*Materials and Methods*). CA-Met-F was formed in only small amounts (its positions on the ionogram are marked by dotted lines, since the corresponding bands, though visible on the original autoradiograph, were too weak to be apparent on the reproduction). For unknown reasons, the yield of this fragment was always low. The compound marked *X* was not identified, but other experiments indicate that it arises from F-Met-adenosine by a nonenzymic reaction.

took place at about the same rate with formyl-methionyl derivatives of AACCA, ACCA, and CCA, but CA-Met-F and F-Met-adenosine were inactive. Intact F-Met-tRNA gave an initial rate of reaction more than twice that with the active fragments.

The approximately equivalent activity of fragments in the range from CCA to CAACCA was confirmed in other experiments. Moreover, a comparative study with CCA-Met-F and CAACCA-Met-F, under varied ionic conditions, revealed only minor differences in response between the two fragments.[10] The enhanced activity of F-Met-tRNA and the inactivity of CA-Met-F and F-Met-adenosine were also repeatedly observed. F-Met-adenosine was also tested for activity with a variety of other conditions in addition to those described in Figure 2: incubations were carried out at 30°C as well as at 0°C, and various concentrations of K^+, Mg^{2+}, and alcohol were used. Under none of these conditions could any reaction with puromycin be detected.

N'-acetyl-aminoacyl derivatives: CCACCA-Met-Ac, the RNase-T_1 fragment from N'-acetyl-methionyl $tRNA_M$, reacted at a rate similar to the F-Met-oligonucleotides (Fig. 2). This shows, in agreement with previous work,[12] that N'-acetyl and N'-formyl derivatives are approximately equivalent as substrates for ribosomal peptidyl transferase.

The activities of acetylated derivatives from other aminoacyl-tRNA species were also examined. Results with H^3 leucine derivatives are shown in Figure 3. CACCA-Leu-Ac and UACCA-Leu-Ac reacted with puromycin at about the same rate—approximately 50 per cent of the rate with CAACCA-Met-F. CCA-Leu-Ac was also active. Intact Ac-Leu-tRNA reacted more than three times as rapidly as the acetyl-leucyl-pentanucleotides. Other experiments showed that the initial rate with Ac-Leu-tRNA was not as great as with F-Met-tRNA. The product of the reaction with the N'-acetyl-leucine derivatives had an ionophoretic

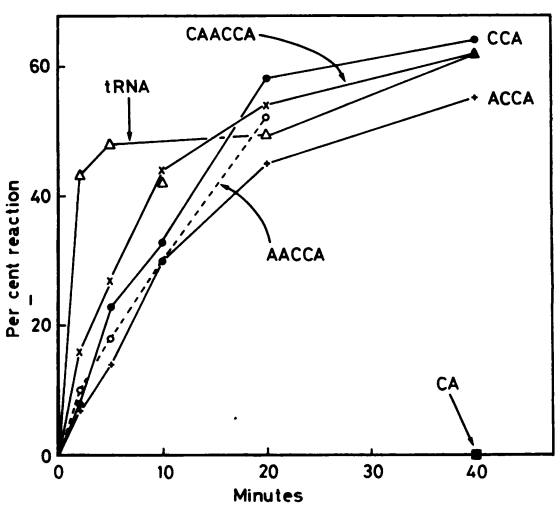

FIG. 2.—Reaction of N'-formyl-methionine derivatives with puromycin. The derivatives were prepared as described under *Materials and Methods*. Reaction mixtures contained (prior to alcohol addition) 0.05 *M* Tris-HCl buffer (pH 7.4, giving *ca.* pH 7.8 under conditions of assay), 0.4 *M* KCl, 0.02 *M* $Mg(OAc)_2$, 1 mg/ml ribosomes, 1 mM puromycin, and S^{35}-methionine derivative (in the order of 10^{-8} *M*). Incubation was at 0°C. The reaction was initiated by addition of 50 μl of methanol (ethanol was used in earlier work but it has subsequently been observed that methanol is more effective (Monro, unpublished observations)). The extent of reaction was assayed as described under *Materials and Methods*.

CCACCA-Met-Ac was also tested in this assay, but, to avoid confusion, the results are not shown. The progress curve was close to that of CCA-Met-F.

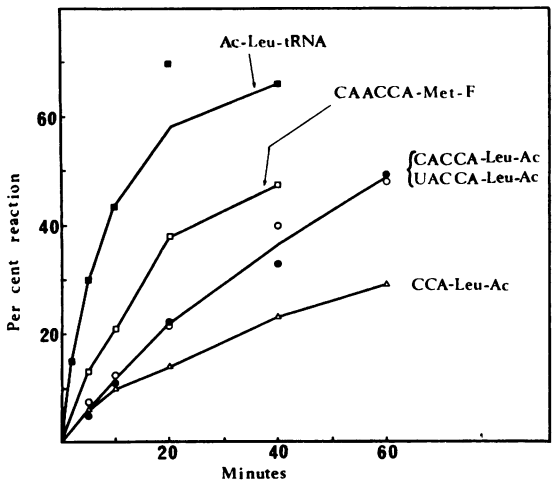

FIG. 3.—Reaction of N'-acetyl-leucine derivatives with puromycin. The derivatives were prepared as described under *Materials and Methods*, with H^3-leucine of specific activity 18 c/mmole. Conditions of incubation and assay were as in Fig. 2. Concentrations of leucine derivatives (prior to methanol addition) were about 10^{-8} M (in the range where rate of reaction is proportional to fragment concentration). The ribosome preparation used in this assay was less active than in the assays described in Figs. 2 and 4. ■, Ac-Leu-tRNA; O, CACCA-Leu-Ac; ●, UACCA-Leu-Ac; △, CCA-Leu-Ac; □, CAACCA-Met-F.

mobility at pH 3.5 which was in the range expected for N'-acetyl-leucyl-puromycin.

Results in Figure 4 show that CACCA-Phe-Ac, the RNase-T_1 fragment from N'-acetyl-phenylalanyl-tRNA, reacted at a significantly lower rate than CACCA-Leu-Ac. Since the oligonucleotide moiety is identical in these two fragments, it follows that the nature of the side group of the aminoacyl residue has a marked influence on the effectiveness of substrates as peptidyl donors. This conclusion is supported by results with derivatives of other N-acetyl-amino acids.

RNase-T_1 fragments of unknown sequence were prepared from N'-acetyl-H^3-arginyl-tRNA, N'-acetyl-H^3-glycyl-tRNA and N'-acetyl-H^3-aspartyl-tRNA. The N'acetyl-arginyl-oligonucleotides (two were obtained) showed activity in the same order as CAACCA-Met-F, whereas the acetyl-glycine derivative was nearly inactive. No evidence for reaction with puromycin could be obtained with the acetyl-aspartic derivatives. In every case where the RNase-T_1 fragment was active, the corresponding derivative of intact tRNA was found to react two to four times as rapidly (cf. Figs. 2 and 3). The relative activities of the different N'-acetyl-aminoacyl tRNA derivatives correlated with the relative activities of the corresponding RNase-T_1 fragments. These results favor the conclusion, drawn from the behavior of the leucine and phenylalanine derivatives, that the amino acid group has a marked influence on peptidyl donor activity. However, the possibility cannot be excluded at present that the low activities of the glycyl and aspartyl derivatives are related to their base sequence rather than to their amino acid moieties.

Effect of free α-amino group: The experiments described above were carried out with derivatives formylated or acetylated at the α-amino group. The effect of having a free α-amino group was examined with the two RNase-T_1 fragments from Leu-tRNA—CACCA-Leu and UACCA-Leu. Samples were incubated under standard conditions (Fig. 2) and then ionophoresed at pH 3.5. No evidence could be obtained either for the formation of leucyl-puromycin or for the stimulation of leucyl-oligonucleotide breakdown by puromycin. Under similar condi-

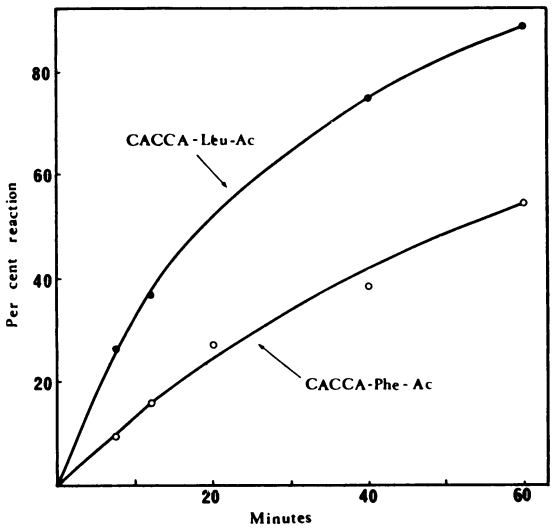

FIG. 4.—Comparison of CACCA-Leu-Ac and CACCA-Phe-Ac reactions with puromycin. The CACCA-Leu-Ac preparation was similar to that in Fig. 3. The CACCA-Phe-Ac was prepared by the same procedure, with H^3-phenylalanine of specific activity 4.3 c/mmole. Conditions of incubation and assay were as in Fig. 2. Concentrations of fragments (prior to methanol addition) were in the order of 10^{-3} M.

tions the corresponding acetylated derivatives reacted readily with puromycin (Fig. 3). We conclude that substrates with a free α-amino group are much less active as peptidyl donors than those with an acylated α-amino group.

Discussion.—The present experiments do not distinguish between effects of substrate structure on (*a*) affinity for peptidyl transferase and (*b*) rate of reaction once bound. It is reasonable to suppose, *a priori*, that affinity would be the relevant factor in cases where different derivatives of the same N'-acyl amino acid are concerned (such as the F-Met series). Further experiments are required to confirm this interpretation and also to determine which factors are responsible for the effects of amino acid side group on reactivity. The possibility must also be considered that the presence of alcohol in our system modifies specificity toward substrates, although there is no evidence that this is the case. With these qualifications in mind, we draw a number of tentative conclusions.

The observation that CCA-Met-F (and CCA-Leu-Ac) is active, whereas CA-Met-F and A-Met-F are inactive, suggests that the CCA grouping of nucleotides must be intact for interaction at the *P-site* to occur. The nucleotides in the next three positions appear to be of little importance for the interaction, since fragments in the formyl-methionine series are of nearly constant activity in the range from CCA to CAACCA; and CCA-Leu-Ac is of the same order of activity as CACCA-Leu-Ac. Moreover, changes of base in the fifth position do not affect activity, as shown by the similar responses with (*a*) CAACCA-Met-F and CCACCA-Met-Ac and (*b*) CACCA-Leu-Ac and UACCA-Leu-Ac. Systematic base substitutions in each position would be required to establish the degree of specificity with which the ribosome interacts with CCA and to confirm the absence of specific interaction with bases in the next three positions.

The enhanced activities of N'-acylated aminoacyl derivatives of intact tRNA over those of the corresponding fragments suggest that other moieties of the tRNA molecule, besides the terminal CCA, interact with the 50*S* ribosomal subunit. The region of base pairing close to the CCA, or the GTψC-containing

loop, might be involved. Such possibilities could be tested by assay of larger fragments than those employed in the present experiments.

The influence of amino acid side chain on peptidyl donor activity, though unexpected, is reminiscent of results with peptidyl *acceptor* substrates: there are striking variations in activity according to the nature of the aminoacyl residue both in puromycin analogs[17] and in derivatives of adenosine.[18, 19] There is no evidence for correlation of amino acid specificities at the P-site with those at the A-site (thus, leucine and phenylalanine derivatives are in reverse orders of activity at the two sites), and our present interpretation is that discrimination between amino acids by peptidyl transferase is fortuitous and is enhanced in systems employing fragments of the normal substrates. The enzyme must clearly be able to accept any of the common aminoacyl residues at either site during normal protein synthesis, though it is possible that there are rate-limiting steps in the synthesis of polypeptides at positions where less acceptable amino acids occur.

It is reasonable to suppose that the function of peptidyl transferase would be favored by specificity at the A-site toward substrates with a free α-amino group and by specificity at the P-site toward substrates with an amide group in that position. Our present results on the enhancement of peptidyl donor activity by formylation or acetylation favor such a model. They may also explain, at least partly, the stimulation of chain initiator activity and of reactivity toward puromycin, which occurs upon formylation of Met-tRNA$_F$ or N'-acylation of phenylalanyl-tRNA.[12, 13, 27–32]

At one time it seemed possible that the terminal CCA of tRNA underwent a metabolic change in protein synthesis, since CCA was observed to turn over *in vivo* and there is a specific enzyme[1, 25] which adds CCA to degraded tRNA. However, the demonstration that CCA turnover does not necessarily accompany protein synthesis[33] disfavors such an hypothesis, and it is much more probable that the primary function of CCA is to act as a recognition site for the peptide bond-forming enzyme. By this means the enzyme would be enabled to orientate the various species of aminoacyl-tRNA in spite of the variations which occur in the other parts of their structures. Our present results on specificity toward peptidyl donor substrates, as well as the results of Rychlík and co-workers[19] (which indicate specificity toward both bases in the terminal CA of a peptidyl acceptor substrate) favor such a model. It is reasonable to suppose that CCA is recognized at both the P- and the A-site and that the two substrates are normally distinguished by whether or not their α-amino group is acylated as well as by the sequence of events during each cycle of peptide bond formation. There is no *a priori* reason why the nature of the CCA interaction should be the same at both sites. Apparent differences are already emerging, such as activity of A and CA derivatives as peptidyl acceptors but not as peptidyl donors. The results and tentative conclusions of the present study should help to design experiments for the more detailed characterization of substrate binding sites on ribosomes.

Summary.—Structural factors affecting the association between peptidyl donor substrate and ribosomal peptidyl transferase were investigated. Evidence is presented that CpCpA, the 3′-terminal grouping of nucleotides common to all species of tRNA, is necessary for effective interaction of substrate with enzyme.

Interaction is also favored by acylation (at the α-amino group) of the aminoacyl residue attached to adenosine and is influenced by the nature of the amino acid side group.

The experiment described in Figure 4 was performed by Miss Maria Luisa Celma at the Instituto de Biología Celular, Madrid. We (J. C. and R. E. M.) thank the European Molecular Biology Organization for the award of fellowships during different stages of this work.

Abbreviations used in this paper include: F-Met or Met-F, *N'*-formyl-methionyl; Ac- or -Ac, *N'*-acetyl; P-site and A-site, the respective sites on the ribosome with which peptidyl-tRNA and aminoacyl-tRNA are associated at the moment of the peptidyl transfer reaction.

* Present address: Instituto de Biología Celular, Centro de Investigaciones Biológicas, Velázquez 144, Madrid-6, Spain.

† Present address: Czechoslovak Academy of Sciences, Institute of Organic Chemistry and Biochemistry, Flemingovo námestí 2, Praha 6, Czechoslovakia.

[1] Hecht, L. I., P. C. Zamecnik, M. L. Stephenson, and J. F. Scott, *J. Biol. Chem.*, **233**, 954 (1958).

[2] Nathans, D., and F. Lipmann, these PROCEEDINGS, **47**, 497 (1961).

[3] Gilbert, W., *J. Mol. Biol.*, **6**, 389 (1963).

[4] Bretscher, M. S., *J. Mol. Biol.*, **12**, 913 (1965).

[5] Monro, R. E., B. E. H. Maden, and R. R. Traut, in *Symposium of the Federation of European Biochemical Societies*, ed. D. Shugar (London: Academic Press, 1967), p. 179.

[6] Monro, R. E., *J. Mol. Biol.*, **26**, 147 (1967).

[7] Maden, B. E. H., R. R. Traut, and R. E. Monro, *J. Mol. Biol.*, **35**, 333 (1968).

[8] Nathans, D., in *Antibiotics*, ed. D. Gottlieb and P. Shaw (Berlin: Springer-Verlag, 1967), vol. 1, p. 259.

[9] Monro, R. E., and K. A. Marcker, *J. Mol. Biol.*, **24**, 347 (1967).

[10] Maden, B. E. H., and R. E. Monro, *European J. Biochem.* in press.

[11] Monro, R. E., and D. Vazquez, *J. Mol. Biol.*, **28**, 161 (1967).

[12] Bretscher, M. S., and K. A. Marcker, *Nature*, **211**, 380 (1966).

[13] Bretscher, M. S., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 31 (1966), p. 289.

[14] Nomura, M., and C. V. Lowry, these PROCEEDINGS, **58**, 946 (1967).

[15] Ghosh, H. P., and H. G. Khorana, these PROCEEDINGS, **58**, 2455 (1967).

[16] Mukundan, M. A., J. W. B. Hershey, K. F. Dewey, and R. E. Thatch, *Nature*, **217**, 1013 (1968).

[17] Nathans, D., and A. Neidle, *Nature*, **197**, 1076 (1963).

[18] Waller, J. P., F. L. Erdös, S. Guttmann, and E. Sandrin, *Biochim. Biophys. Acta*, **119**, 566 (1966).

[19] Rychlík, I., S. Chládek, and J. Žemlička, *Biochim. Biophys. Acta*, **138**, 640 (1967).

[20] Sanger, F., G. G. Brownlee, and B. G. Barrell, *J. Mol. Biol.*, **13**, 373 (1965).

[21] Clark, B. F. C., and K. A. Marcker, *J. Mol. Biol.*, **17**, 394 (1966).

[22] Marcker, K. A., *J. Mol. Biol.*, **14**, 63 (1965).

[23] Haenni, A. L., and F. Chapeville, *Biochim. Biophys. Acta*, **114**, 135 (1966).

[24] Dube, S. K., K. A. Marcker, B. F. C. Clark, and S. Cory, *Nature*, **218**, 232 (1968).

[25] Berg, P., I. Lagerkvist, and M. Diekmann, *J. Mol. Biol.*, **5**, 159 (1962).

[26] Leder, P., and H. Bursztyn, *Biochem. Biophys. Res. Commun.*, **25**, 233 (1966).

[27] Lucas-Lenard, J., and F. Lipmann, these PROCEEDINGS, **57**, 1050 (1967).

[28] Salas, M., M. J. Miller, A. J. Wahba, and S. Ochoa, these PROCEEDINGS, **57**, 1865 (1967).

[29] Leder, P., and M. M. Nau, these PROCEEDINGS, **58**, 774 (1967).

[30] Economon, A. E., and T. Nakamoto, these PROCEEDINGS, **58**, 1033 (1967).

[31] Anderson, J. S., M. S. Bretscher, B. F. C. Clark, and K. A. Marcker, *Nature*, **215**, 490 (1967).

[32] Anderson, J. S., J. E. Dahlberg, M. S. Bretscher, M. Revel, and B. F. C. Clark, *Nature*, **216**, 1072 (1967).

[33] Cannon, M., *Biochim. Biophys. Acta*, **129**, 221 (1966).