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Transfer Ribonucleic Acid Deprived of the C-C-A 3′-Extremity Can Interact with Elongation Factor Tu†

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ABSTRACT: In this work we describe that uncharged tRNAs or modified tRNAs lacking all or part of the C-C-A end (i.e., tRNA minus pCpCpA, tRNA minus pA, and tRNA minus A) can still influence the GTPase activity of the elongation factor Tu (EF-Tu), thus showing that, besides the aminoacylated 3′-end, other regions of the aa-tRNA interact with EF-Tu. The existence of an interaction between EF-Tu and truncated tRNAs was also confirmed by examining the dissociation of the EF-Tu·GTP complex: the rate of this reaction is decreased upon addition of $tRNA_1^{Val}$ minus pCpCpA. The effect on the EF-Tu GTPase activity of tRNAs deprived of the C-C-A 3′-end is still evident in the presence of C-C-A-aa. The stimulatory pattern obtained with C-C-A-Val at 5 mM $MgCl_2$ is decreased upon addition of $tRNA_1^{Val}$ minus pCpCpA, $tRNA_1^{Val}$ minus pA, or $tRNA_1^{Val}$ minus A. This shows that the effect of the aminoacylated C-C-A 3′-end can be influenced via EF-Tu by the remaining regions of the tRNA, after cleavage of a bond in the 3′-extremity. However, also with an excess of $tRNA_1^{Val}$ minus pCpCpA over C-C-A-Val, no "aa-tRNA-like" effect, i.e., no inhibition of the EF-Tu GTPase, was obtained, suggesting that, upon binding with EF-Tu, a specific conformational change in the aa-tRNA molecule also takes place, regulating the expression of the GTPase activity. Our results unequivocally show that different regions of the aa-tRNA are needed for a coordinated interaction with EF-Tu.

The C-C-A[1] 3′-terminal region plays a fundamental role in tRNA functions, since it is directly involved in interaction with other cellular components. In fact, the 3′-terminal oligonucleotides of tRNA can still interact with other biological macromolecules, such as synthetases (Renaud et al., 1981), ribosomes (Ringer et al., 1976; Kukhanova et al., 1980), or EF-Tu in the ternary complex (Jõnak et al., 1980). Evidence for the existence of a ternary complex between the C-C-A-aa fragments and EF-Tu also results from the observation that such aminoacylated oligonucleotides can stimulate the GTPase activity of EF-Tu (Campuzano & Modolell, 1980; Bhuta & Chlãdek, 1980; Parlato et al., 1981) and inhibit the dissociation of the EF-Tu·GTP complex (O. Fasano, unpublished results).

Recently, we have reported that ionic conditions, particularly a narrow range of Mg^{2+} concentrations, selectively regulate the aa-tRNA effect but not that of short 3′-aminoacylated fragments (Parlato et al., 1983). This suggests that, besides the 3′-extremity, other domains of the aa-tRNA molecule interact with EF-Tu and are involved in the regulation of the GTPase reaction. A suitable approach to clarify this point may consist in comparing the effects of tRNA molecules that completely or partially lack the C-C-A 3′-terminal sequence with that of aa-tRNA or C-C-A-aa alone.

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[1] Abbreviations: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; GTPase, guanosine-5′-triphosphatase, EC 3.6.1; tRNA, transfer ribonucleic acid; $tRNA^{Arg}$ and $tRNA^{Phe}$, purified arginine- and phenylalanine-accepting tRNA, respectively; $tRNA_1^{Val}$, purified isoacceptor 1 of valine-accepting tRNA; aa-tRNA, aminoacyl-tRNA; Arg-$tRNA^{Arg}$, Phe-$tRNA^{Phe}$, and Val-$tRNA_1^{Val}$, purified specific tRNA charged with Arg, Phe, and Val, respectively; C-C-A, uncharged 3′-terminal trinucleoside diphosphate; C-C-A-aa, 3′-aminoacyl-C-C-A; C-C-A-Val, 3′-terminal trinucleoside diphosphate charged with Val; EDTA, ethylenediaminetetraacetic acid; DTT, 1,4-dithiothreitol; PEP, phosphoenolpyruvate.

0006-2960/83/0422-4400$01.50/0

In this work we show the existence of an interaction between EF-Tu and such modified tRNAs, by examining two typical features of EF-Tu: the GTPase activity and the dissociation of the EF-Tu·GTP complex. Moreover, we have observed that C-C-A-aa-deficient tRNAs are still able to influence the effect of the 3′-aminoacylated extremity on the EF-Tu GTPase activity.

Materials and Methods

The materials, methods, and biological components not quoted in this section were as previously described (Sander et al., 1980). Crystalline, electrophoretically pure EF-Tu was from *Escherichia coli* B or A19 (Parmeggiani & Sander, 1981). A 1-μg aliquot of EF-Tu is equivalent to 23.1 pmol (Arai et al., 1980). Protein concentration was measured by the method by Lowry et al. (1951). The 30S and 50S ribosomal subunits were isolated from 70S "tight couples" obtained from NH_4Cl-washed ribosomes (Parmeggiani & Sander, 1981). Equimolar amounts of 30S and 50S displayed a very low GTPase activity in the absence of EF-Tu (less than 0.5 pmol of GTP hydrolyzed/pmol of ribosomes in 1 h at 30 °C). A total of 1 A_{260} unit of 50S and 30S ribosomes was taken to represent 39 and 67 pmol, respectively (Sander et al., 1975).

The different *E. coli* tRNAs (70–100% pure) were isolated from unfractionated tRNA obtained from Schwarz/Mann or from the pilot plant of the Institute de Chimie des Substances Naturelles (C.N.R.S., Gif-sur-Yvette, France). The purification procedure included successive chromatography on BD-cellulose (Gillam et al., 1967) and Sepharose 4B and HPLC on RPC-5 (Colantuoni et al., 1979). Aminoacylation was carried out with ^{14}C-labeled amino acids (24–50 cpm/pmol), by using partially purified aa-tRNA synthetases from *E. coli* (Chinali & Parmeggiani, 1973).

Preparation of tRNAs Shortened in Their C-C-A Extremity. Full digestion of the C-C-A 3′-end was accomplished with cobra venom phosphodiesterase at pH 9 (Miller et al., 1970). In order to obtain tRNA minus A and tRNA minus pA, the terminal adenosine was removed from intact tRNA by periodate oxidation followed by lysine-catalyzed β-elimination (Tal et al., 1972). In a few cases, this tRNA was further treated with alkaline phosphatase to remove exclusively the exposed 3′-phosphate group (Tal et al., 1972). To verify the absence of internal cuts, we analyzed these modified tRNAs by 20% polyacrylamide gel electrophoresis in 7 M urea. The residual aminoacylation was <1%.

Preparation of C-C-A-Val. This fragment was obtained by enzymatic digestion with RNase U_2 from *Ustilago sphaerogena* (Boehringer) (Parlato et al., 1983). [^{14}C]Val-tRNA$_1^{Val}$ was incubated for 45 min at 30 °C in 50 mM ammonium acetate, pH 4.5, 1 mM EDTA, and 2 mM β-mercaptoethanol, in presence of 1 unit of RNase U_2/40 A_{260} units of tRNA. The reaction mixture was applied onto a DEAE-Sephadex A-25 (0.5 cm × 2 cm) column equilibrated with 10 mM ammonium acetate, pH 4.5, and the retained C-C-A-Val eluted with a 10–600 mM ammonium acetate gradient (2 × 20 mL). The radioactive fractions, pooled and lyophilized, were suspended in 10 mM ammonium acetate, pH 3.5, and applied onto a sulfopropyl- (SP) Sephadex C-25 (0.5 × 2 cm) column equilibrated and thoroughly washed with the same buffer until the 254 nm absorbing material was no longer detectable. After equilibration with 10 mM ammonium acetate, pH 4.7, a gradient from 10 to 100 mM ammonium acetate, pH 4.7 (2 × 20 mL) was applied to elute the retained C-C-A-Val.

The purity of the fragment, which was characterized by high-voltage electrophoresis (Gilson Electrophorator) on Whatman 3MM paper at pH 3.5, was estimated spectrometrically (ϵ_M = 28 900) and by specific activity of the ^{14}C-labeled amino acid; it was found to be >90%. The C-C-A-Val was subdivided in small batches of 1000–2000 pmol each and stored, either lyophilized or in 1 mM acetic acid, at −70 °C.

Assay for GTP Hydrolysis. The GTPase activity was measured as liberation of [^{32}P]P_i under conditions in which the GTP hydrolysis is the rate-limiting step of the EF-Tu GTPase and its kinetics is linear with respect to time (Parmeggiani & Sander, 1981). For a 10-μL reaction mixture, the following procedure was applied: 3 μL of a solution containing 167 mM imidazolium acetate, pH 7.5, 3.3 mM DTT, 17 mM $MgCl_2$, 267 mM NH_4Cl, 5 pmol of EF-Tu·GDP, and either 167 μM kirromycin (50 μM final) or 5 pmol of EF-Ts, 4 mM PEP, 0.5 μg of pyruvate kinase, 6 pmol of 30S, and 6 pmol of 50S was added to 5 μL containing the aa-tRNA or the modified tRNA and/or the C-C-A-aa at the required concentrations. Thus, the final concentrations were 50 mM imidazolium acetate, pH 7.5, 1 mM DTT, 5 mM $MgCl_2$, 80 mM NH_4Cl, 0.5 μM EF-Tu, and, when present, 0.5 μM EF-Ts, 1.2 mM phosphoenolpyruvate, 0.05 mg/mL pyruvate kinase, and 0.6 μM ribosomes.

The reaction was then started by adding 2 μL of a solution containing 80 μM [γ-^{32}P]GTP (sp act. 2000–4000 cpm/pmol) and by transferring the assay tubes kept in ice to a 30 °C incubation bath. After 15 min, the reaction was stopped with 100 μL of ice-cold 0.6 M $HClO_4$, containing 0.6 mM KH_2PO_4.

The nonhydrolyzed [γ-^{32}P]GTP was sequestered by the addition of 250 μL of a 6% suspension of activated charcoal in 1 M HCl (Donner et al., 1978), and the mixture was centrifuged for 15 min at 5000 rpm. To an aliquot of the supernatant was added Aquasol 2 (New England Nuclear), and the radioactivity of [^{32}P]P_i was counted in an Intertechnique liquid scintillation spectrometer Model SL 4000.

Dissociation of EF-Tu·GTP. The EF-Tu·GTP complex was obtained starting from GDP-free EF-Tu, which was prepared as described by Fasano et al. (1982). The reaction mixture contained in a volume of 300 μL 13 mM $MgCl_2$, 133 mM NH_4Cl, 67 mM imidazolium acetate, pH 7.5, 1.3 mM DTT, ~1000 pmol of [γ-^{32}P]GTP (sp act. ~2000 cpm/pmol), and 200 pmol of GDP-free EF-Tu. After 20 min at 0 °C to allow formation of the EF-Tu·GTP complex, aliquots of 30 μL were added to 10 μL containing the tRNA$_1^{Val}$ minus pCpCpA at the required concentration. Following further incubation for 15 min at 0 °C, either 1 μL of water (control) or 1 μL of unlabeled GTP (100 nmol) was added to the mixture.

The excess (1000×) of unlabeled GTP added allowed us to neglect the backward reaction:

$$\text{EF-Tu}\cdot[\gamma\text{-}^{32}\text{P}]\text{GTP} \rightleftharpoons \text{EF-Tu} + [\gamma\text{-}^{32}\text{P}]\text{GTP}$$

Aliquots of 5 μL were filtered at the indicated times on nitrocellulose filters previously rinsed in 50 mM imidazolium acetate, pH 7.5, 10 mM $MgCl_2$, and 100 mM NH_4Cl. After being washed twice with 2 mL of the same buffer at 0 °C, the filters were dried, and the radioactivity was counted.

Results

Uncharged tRNAs or tRNAs minus pCpCpA Influence the GTPase Activity of EF-Tu. In the presence of ribosomes and in the absence of poly(U), aa-tRNA inhibits the GTPase activity of EF-Tu at Mg^{2+} concentrations ≤10 mM (Parlato et al., 1983). When we examined the influence on this reaction of either uncharged tRNA or tRNA lacking the C-C-A 3′-terminal region in place of aa-tRNA, we observed similar results. Figure 1 shows that at 5 mM $MgCl_2$, tRNA^{Arg} minus pCpCpA, tRNA$_1^{Val}$ minus pCpCpA, and tRNA^{Phe} minus pCpCpA, as well as tRNA^{Arg}, tRNA$_1^{Val}$, and tRNA^{Phe}, play

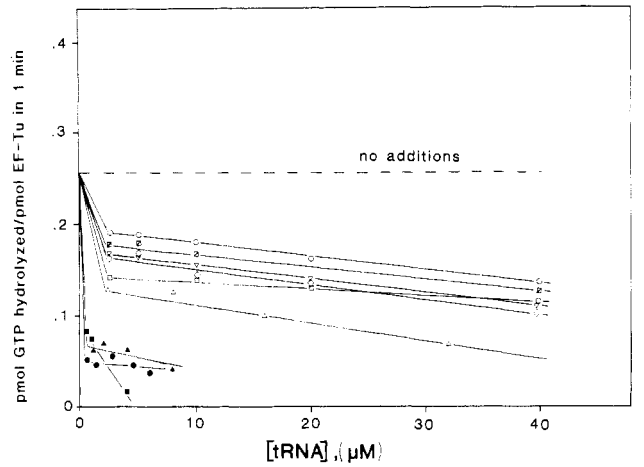

FIGURE 1: Effect on the EF-Tu GTPase of various uncharged tRNAs or tRNAs lacking the C-C-A end and their corresponding aa-tRNAs in the presence of ribosomes. The reaction mixture was as indicated under Materials and Methods. Val-tRNA$_1^{Val}$ (●), tRNA$_1^{Val}$ (◇), tRNA$_1^{Val}$ minus pCpCpA (○), Phe-tRNA^{Phe} (▲), tRNA^{Phe} (△), tRNA^{Phe} minus pCpCpA (▽), Arg-tRNA^{Arg} (■), tRNA^{Arg} (□), or tRNA^{Arg} minus pCpCpA (slashed box) at the concentrations indicated. The dashed line indicates the level of the [γ-^{32}P]GTP hydrolyzed by EF-Tu and ribosomes. Blanks of [γ-^{32}P]GTP nonenzymatically hydrolyzed (0.09 pmol of GTP/pmol of EF-Tu in 1 min) were subtracted.

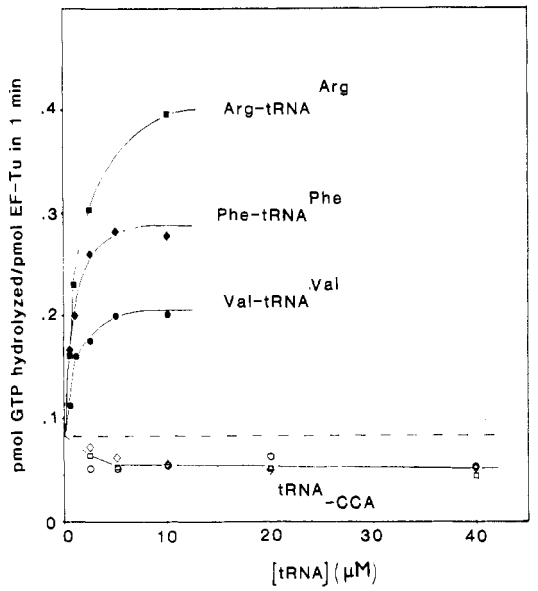

FIGURE 2: Effect on the EF-Tu GTPase of various tRNAs lacking the C-C-A end and their corresponding aa-tRNAs in the presence of kyrromicin. Val-tRNA$_1^{Val}$ (●), tRNA$_1^{Val}$ minus pCpCpA (○), Phe-tRNA^{Phe} (♦), tRNA^{Phe} minus pCpCpA (◇), Arg-tRNA^{Arg} (■), and tRNA^{Arg} minus pCpCpA (□) at the concentrations indicated. The dashed line indicates the level of the [γ-^{32}P]GTP hydrolyzed by EF-Tu and kyrromicin. Blanks of [γ-^{32}P]GTP nonenzymatically hydrolyzed (0.06 pmol of GTP/pmol of EF-Tu in 1 min) were subtracted.

an inhibitory role on the GTPase activity, their effects being however smaller than those of aa-tRNAs. The inhibition is likely to result from a direct interaction between EF-Tu and such uncharged or modified tRNAs, since a similar pattern is obtained in the absence of ribosomes when kirromycin, which activates the EF-Tu GTPase (Parmeggiani & Sander, 1980, 1981), is present (Figure 2): the effect displayed by the tRNAs deprived of the C-C-A 3′-end or by uncharged tRNAs is still inhibitory, while the one displayed by aa-tRNAs is stimulatory. However, in the presence of ribosomes, the level of the GTPase activity of EF-Tu, with or without tRNA, is considerably increased [cf. also Parlato et al. (1981, 1983) and Guesnet et al. (1983)]. This effect is also evident by comparing the experiments in Figures 1 and 2.

Table I: Effect of tRNA$_1^{Val}$ Deprived of the pCpCpA Sequence on the Retention of the EF-Tu·[γ-^{32}P]GTP Complex on Nitrocellulose Filters

EF-Tu·[γ-^{32}P]GTP (pmol)	additions	[γ-^{32}P]GTP retained on the filter (cpm)
2.5	none	4585
2.5	tRNA$_1^{Val}$ minus pCpCpA, 50 pmol	4415
2.5	tRNA$_1^{Val}$ minus pCpCpA, 100 pmol	4612
2.5	tRNA$_1^{Val}$ minus pCpCpA, 200 pmol	4681
2.5	Val-tRNA$_1^{Val}$, 18 pmol	1003
2.5	tRNA$_1^{Val}$, 50 pmol	4726
	[γ-^{32}P]GTP, 14 pmol	250

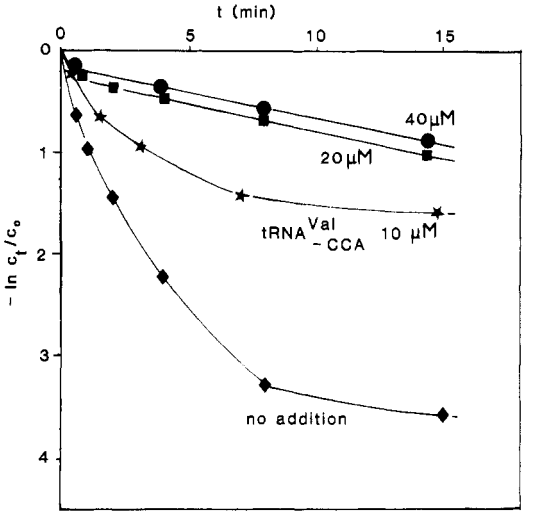

FIGURE 3: Effect of tRNA$_1^{Val}$ minus pCpCpA on dissociation of the EF-Tu·GTP complex at 0 °C. The concentration of tRNA$_1^{Val}$ minus pCpCpA was 10 (solid star), 20 (■), and 40 μM (●). EF-Tu·GTP alone (♦). For other details, see Materials and Methods.

In experiments not illustrated, we have also examined the dependence on ionic conditions and found a similar regulation pattern in both systems (with ribosomes or kirromycin): the effect is almost unaltered at 5 and 10 mM $MgCl_2$, whereas the inhibition is changed into a slight stimulation at 30 mM $MgCl_2$, as in the case of aa-tRNA in the presence of ribosomes (Parlato et al., 1983).

Dissociation of the EF-Tu·GTP Complex Is Retarded by tRNA$_1^{Val}$ minus pCpCpA. As shown in Table I, tRNAs deprived of the 3′-terminal region or uncharged tRNAs do not induce the passage of the EF-Tu·GTP complex through nitrocellulose filters, in contrast to aa-tRNAs (Gordon, 1968). Thus, this technique is adequate to study the influence of tRNA deprived of the C-C-A 3′-end on the EF-Tu·GTP interaction. It is known that aa-tRNA decreases the dissociation rate of the EF-Tu·GTP complex, without affecting the association rate (Fasano et al., 1978). Here we show that also tRNA$_1^{Val}$ minus pCpCpA inhibits this dissociation, the effect increasing with increasing concentration of this modified tRNA (Figure 3). This result confirms the existence of an interaction between EF-Tu·GTP and tRNA lacking the C-C-A 3′-end, in the absence of any further addition such as kirromycin or ribosomes.

tRNA Lacking the 3′-Terminal Region Influences the GTPase Stimulated by C-C-A-Val. We have already shown

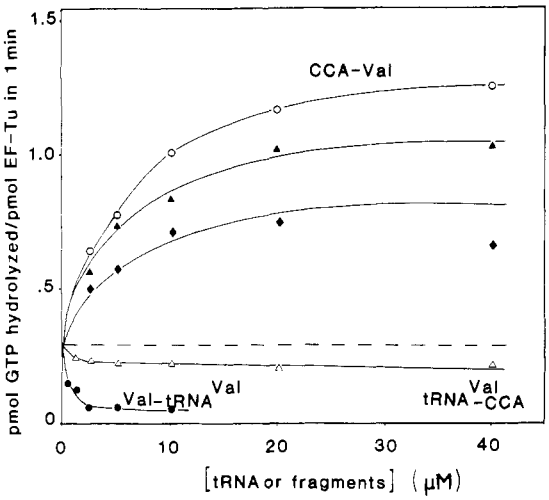

FIGURE 4: Influence of $tRNA_1^{Val}$ minus pCpCpA on the EF-Tu GTPase stimulated by C-C-A-Val in the presence of ribosomes. C-C-A-Val as indicated alone (○) or plus equimolar amounts of $tRNA_1^{Val}$ minus pCpCpA (▲) or plus 4-fold excess of the same tRNA (◇). Val-$tRNA_1^{Val}$ (●) and $tRNA_1^{Val}$ minus pCpCpA (△) at the indicated concentration. The dashed line indicates the level of the [γ-^{32}P]GTP hydrolyzed by EF-Tu and ribosomes. Blanks of non-enzymatically hydrolyzed [γ-^{32}P]GTP (0.07 pmol of GTP/pmol of EF-Tu in 1 min) were subtracted.

that tRNA minus pCpCpA is still able to interact with EF-Tu, influencing both GTPase and dissociation of GTP. It was previously reported that tRNAs lacking all or part of the C-C-A 3′-terminal region can still bind to their cognate and noncognate synthetases (Bacha et al., 1982), inducing a conformational alteration of the enzyme that results in the aminoacylation of the C-C-A fragment or of adenosine.

To verify if such a mechanism also occurs in our system, we have examined the effect on the C-C-A-Val-stimulated GTPase by addition of $tRNA_1^{Val}$ minus pCpCpA, $tRNA_1^{Val}$ minus pA, or $tRNA_1^{Val}$ minus A. These experiments were carried out under different conditions: in the presence of fixed concentrations of one of the components, either modified $tRNA_1^{Val}$ or C-C-A-Val, and of increasing concentrations of the other or by keeping the ratio of these two components constant.

The influence of the ionic environment was investigated, under a broad range of Mg^{2+} and NH_4^+ concentrations. In all conditions tested we found essentially the same picture: $tRNA_1^{Val}$ lacking the 3′-extremity lowered the stimulatory effect of C-C-A-Val, as shown in Figure 4. The change in GTPase stimulation by C-C-A-Val is illustrated by the addition of equimolar amounts or of a 4-fold excess of $tRNA_1^{Val}$ minus pCpCpA: in both cases the stimulation by C-C-A-Val was decreased, the extent of this effect depending on the excess of added modified $tRNA_1^{Val}$. This phenomenon was not caused by a lowering of the level of GTPase activity of EF-Tu, since in the presence of Val-$tRNA_1^{Val}$, no effect was displayed by $tRNA_1^{Val}$ minus pCpCpA (not shown).

When we examined the effect of increasing concentrations of Val-$tRNA_1^{Val}$ on the C-C-A-Val-stimulated GTPase, we found a progressive reduction of the GTPase activity down to the same level obtained with Val-$tRNA_1^{Val}$ alone (Figure 5). Thus, this effect appears to be competitive.

In order to investigate the possible role played by the single bases of the 3′-terminal C-C-A sequence, we also studied the effect of $tRNA_1^{Val}$ minus A and $tRNA_1^{Val}$ minus pA in the same conditions used with $tRNA_1^{Val}$ minus pCpCpA. No differences were detectable. In Figure 6, panel A, is shown the EF-Tu GTPase in the presence of increasing concentrations

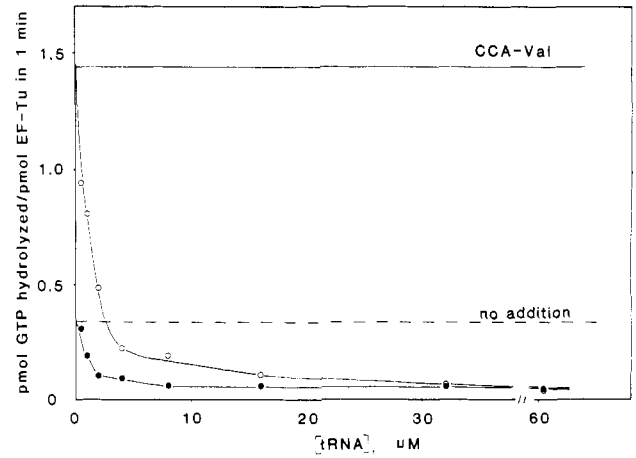

FIGURE 5: Influence of Val-$tRNA_1^{Val}$ on the EF-Tu GTPase stimulated by C-C-A-Val in the presence of ribosomes. Val-$tRNA_1^{Val}$ alone (●) or plus 330 pmol of C-C-A-Val (○). The dashed line indicates the level of the [γ-^{32}P]GTP hydrolyzed by EF-Tu and ribosomes. Blanks of [γ-^{32}P]GTP nonenzymatically hydrolyzed (0.08 pmol of GTP/pmol of EF-Tu in 1 min) were subtracted.

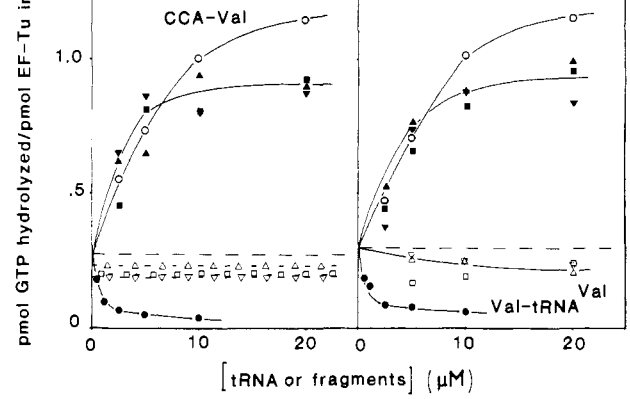

FIGURE 6: Influence of different modified $tRNA_1^{Val}$s on the EF-Tu GTPase stimulated by C-C-A-Val in the presence of ribosomes. Val-$tRNA_1^{Val}$ was as indicated (●). (Panel A) 100 pmol of $tRNA_1^{Val}$ minus pCpCpA (△), $tRNA_1^{Val}$ minus pA (▽), or $tRNA_1^{Val}$ minus A (□). C-C-A-Val as indicated, alone (○) or plus 100 pmol of $tRNA_1^{Val}$ minus pCpCpA (▲), 100 pmol of $tRNA_1^{Val}$ minus pA (▼), or 100 pmol of $tRNA_1^{Val}$ minus A (■). (Panel B) $tRNA_1^{Val}$ minus pCpCpA (△), minus pA (▽), or minus A (□) at the indicated concentrations. C-C-A-Val at the indicated concentration, alone (○) or plus 2-fold excess of $tRNA_1^{Val}$ minus pCpCpA (▲), minus pA (▼), or minus A (■). The dashed line indicates the level of the [γ-^{32}P]GTP hydrolyzed by EF-Tu and ribosomes. Blanks of [γ-^{32}P]GTP nonenzymatically hydrolyzed were subtracted.

of C-C-A-Val and 100 pmol of $tRNA_1^{Val}$ minus A, $tRNA_1^{Val}$ minus pA, or $tRNA_1^{Val}$ minus pCpCpA. In all cases, the same effect is obtained with the different modified tRNAs, as confirmed in panel B, where a 2-fold excess of each tRNA is used.

In conclusion, no independent role can be attributed to the single bases of the 3′-terminal pCpCp sequence in the EF-Tu–tRNA interaction; these bases probably induce the correct positioning of the (aminoacyl)adenosine moiety in the EF-Tu·aa-tRNA complex.

Discussion

The C-C-A-aa 3′-terminal sequence represents the principal site of interaction between aa-tRNA and EF-Tu [for a review, see Miller & Weissbach (1977)], but a number of recent reports point to the importance of other regions of the tRNA

molecule (Boutorin et al., 1981; Bhuta et al., 1982; Wikman et al., 1982; Parlato et al., 1983; Guesnet et al., 1983).

In the present work, we have tried to define these interactions by the use of uncharged tRNAs or of tRNAs lacking the 3′-terminal region. The results obtained in both GTPase and EF-Tu·GTP dissociation experiments show that EF-Tu can still recognize tRNAs in the absence of aminoacylation or of the pCpCpA sequence. However, with such modified tRNAs, the resulting effects on the GTPase of the factor are weaker than with intact aa-tRNA, thus confirming the important role played per se by aminoacylation of the 3′-terminal OH (Gordon, 1967; Kruse et al., 1980).

We observed already (see Results) that no difference exists between the interactions with $tRNA_1^{Val}$ minus pCpCpA, $tRNA_1^{Val}$ minus pA, or $tRNA_1^{Val}$ minus A, suggesting that in the pCpCp sequence neither the cytidine 5′-phosphate residues nor the 3′-terminal phosphate interacts individually with EF-Tu. Their action may concern the orientation of the (aminoacyl)adenosine. This is also supported by the observation that free cytidine 3′-phosphate does not influence the action of (aminoacyl)adenosine on the EF-Tu GTPase, whereas cytidylyl(aminoacyl)adenosine displays a much greater effect (Parlato et al., 1981). It is also possible that the 3′-terminal adenosine exerts a specific role in the interaction with EF-Tu since uncharged tRNA was found to have an intermediary behavior between aa-tRNA and tRNA minus pCpCpA (see Figure 1).

Interestingly, there were slight differences in the effect of the tRNA minus pCpCpA depending on the various species. This indicates that not only the nature of the aminoacyl side chain but also other sequences of the tRNA molecule influence the interaction with EF-Tu. In line with this conclusion is the observation that the role of the amino acid is much more evident with short aminoacylated oligonucleotides than with their corresponding aa-tRNAs (Parlato et al., 1983).

The stimulation by C-C-A-Val disappears in the presence of increasing concentrations of Val-$tRNA_1^{Val}$, in a way that indicates that both components compete for the same site of EF-Tu (Figure 5). The effect produced by $tRNA_1^{Val}$ deprived of its C-C-A 3′-end also disappears upon addition of Val-$tRNA_1^{Val}$, i.e., in the presence of all structural features required for optimal interaction with EF-Tu. The same does not occur when tRNAs deprived of the 3′-extremity are added to C-C-A-Val, which lacks the regulatory regions and thus allows other domains of the protein to interact with the specific tRNA sequences.

The reduction observed in the C-C-A-Val-induced GTPase activity upon addition of $tRNA_1^{Val}$ minus pCpCpA, $tRNA_1^{Val}$ minus pA, or $tRNA_1^{Val}$ minus A indicates that a conformational change of EF-Tu, induced by the regulatory regions of these modified tRNAs, takes place such that it is able to influence the effect of the C-C-A 3′-end on the GTPase center of EF-Tu. The cleavage of the bond between A-74 and C-75 (or C-76 and A-77) in the aa-tRNA molecule prevents the regulatory regions of the tRNA to exert any direct influence on the orientation of the C-C-A-aa extremity, whose effect on the EF-Tu GTPase can be therefore solely modified by the conformational changes occurring in the protein moiety. Our findings that, in the experiments carried out in the presence of both C-C-A-Val and the remaining part of the $tRNA_1^{Val}$ molecule (with complementary or overlapping sequences), no "aa-tRNA-like" effect was revealed, i.e., no inhibition occurs, suggests that, upon binding with EF-Tu, a specific conformational change in the intact aa-tRNA molecule also takes place, influencing the EF-Tu GTPase. Therefore, EF-Tu and aa-tRNA appear to be involved in a reciprocal adaptation process that regulates the expression of the catalytic activity.

Acknowledgments

We are grateful to Drs. G. Parlato and R. Pizzano for carrying out preliminary experiments concerning the preparation of tRNA deprived of the C-C-A 3′-end.

Registry No. pCpCpA, 6899-53-2; GTPase, 9059-32-9; C-C-A-Val, 85956-70-3.

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Arrangement of Subunit IV in Beef Heart Cytochrome *c* Oxidase Probed by Chemical Labeling and Protease Digestion Experiments†

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ABSTRACT: The arrangement of subunit IV in beef heart cytochrome *c* oxidase has been explored by chemical labeling and protease digestion studies. This subunit has been purified from four samples of cytochrome *c* oxidase that had been reacted with *N*-(4-azido-2-nitrophenyl)-2-aminoethyl[^{35}S]-sulfonate (NAP-taurine), diazobenzene[^{35}S]sulfonate, 1-myristoyl-2-[12-[(4-azido-2-nitrophenyl)amino]lauroyl]-*sn*-glycero-3-[^{14}C]phosphocholine (I), and 1-palmitoyl-2-(2-azido-4-nitrobenzoyl)-*sn*-glycero-3-[^{3}H]phosphocholine (II), respectively. The labeled polypeptide was then fragmented by cyanogen bromide, at arginyl side chains with trypsin (after maleylation), and the distribution of the labeling within the sequence was analyzed. The N-terminal part of subunit IV (residues 1–71) was shown to be heavily labeled by water-soluble, lipid-insoluble reagents but not by the phospholipid derivatives. These latter reagents labeled only in the region of residues 62–122, containing the long hydrophobic and putative membrane-spanning stretch. Trypsin cleavage of native cytochrome *c* oxidase complex at pH 8.2 was shown to clip the first seven amino acids from subunit IV. This cleavage was found to occur in submitochondrial particles but not in mitochondria or mitoplasts. These results are interpreted to show that subunit IV is oriented with its N terminus on the matrix side of the mitochondrial inner membrane and spans the membrane with the extended sequence of hydrophobic lipid residues 79–98 buried in the bilayer.

Cytochrome *c* oxidase, the terminal part of the electron-transport chain, is a redox-linked proton pump containing two heme moieties (*a* and a_3) and two copper atoms as electron acceptors (Azzi, 1980; Capaldi et al., 1983). The protein is Y shaped with two arms of the Y, called the M_1 and M_2 domains, each spanning the mitochondrial inner membrane (Deatherage et al., 1982; Fuller et al., 1979, Henderson et al., 1977).

Beef heart cytochrome *c* oxidase is isolated as a complex of at least 7 and up to 13 different polypeptides (Steffens & Buse, 1976; Downer et al., 1976; Kadenbach & Merle, 1981; Verheul et al., 1979), the three largest of which are coded for on mitochondrial DNA (mtDNA)[1] (Anderson et al., 1982). Labeling studies with diazobenzene[^{35}S]sulfonate (Ludwig et al., 1979; Prochaska et al., 1980) have established that all of the subunits are at least partly exposed to water in both the detergent-dispersed and the membrane-bound enzyme. Subunits II and III were found to be reactive to DABS in intact mitochondria, showing that these components are on the cytoplasmic side of the mitochondrial inner membrane and thus contribute to the stalk of the Y or C domain (Eytan et al., 1975; Ludwig et al., 1979). Subunits II, III, IV, and VII were reactive to DABS in inverted membranes or submitochondrial particles, and therefore, these components must be a part of the two M domains (Ludwig et al., 1979).

The arrangement of protein with respect to the lipid bilayer has also been examined by labeling with radioactive aryl-azidophospholipids (Bisson et al., 1979; Prochaska et al., 1980), iodonaphthyl azide (Cerletti & Schatz, 1979), and adamantane diazirine (Georgevich & Capaldi, 1982). Subunits I and III were heavily labeled by each of these hydrophobic reagents and must contribute the major portion of the protein in contact with lipid. Subunits II, IV, and VII were also labeled by the lipid analogues but not subunits V and VI. The above studies along with the results of cross-linking experiments (Briggs & Capaldi, 1977, 1978) have been used to derive a model for the arrangement of subunits in the cytochrome *c* oxidase complex (Capaldi et al., 1983). However, they do not locate the positions of the N and C termini of each polypeptide in the complex and do not establish which segments of the polypeptide are within the lipid bilayer. This information is critical to an understanding of the assembly of the protein, as well as to our understanding of how a membrane protein is stable when partly exposed to water and partly buried in a hydrocarbon environment.

Recently, the sequence of all of the subunits of beef heart cytochrome *c* oxidase has been obtained either from the protein

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[1] Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; DABS, diazobenzenesulfonate; mtDNA, mitochondrial DNA; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; TPCK, tosylphenylalanyl chloromethyl ketone; STI, soybean trypsin inhibitor; IgG, immunoglobulin G; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

0006-2960/83/0422-4405$01.50/0