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# Mechanistic Studies of Ribonucleic Acid Renaturation by a Helix-Destabilizing Protein†

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ABSTRACT: The ability of a nucleic acid helix-destabilizing protein from calf thymus, UP1, to facilitate renaturation of yeast $tRNA_3^{Leu}$ and *Escherichia coli* 5S RNA is shown to be a consequence of the protein's ability to bind stoichiometrically to single-stranded polynucleotide regions. A comparison of the inhibitory effect of different homopolymers on UP1-induced renaturation of $tRNA_3^{Leu}$ does not indicate significant base specificity in UP1 binding, and a 3′-5′ ribose phosphate polymer devoid of heterocyclic bases inhibits as well as the homopolynucleotides. These inhibition studies also show that UP1 requires polynucleotide segments of at least three phosphate residues to bind. $Mg^{2+}$ (which is required for the stabilization of native $tRNA_3^{Leu}$) dissociates complexes of UP1 with inactive tRNA, and since the RNAs in those complexes lack a substantial amount of secondary structure, it can upon dissociation readily refold into the native structure. A semiquantitative treatment of UP1–RNA interaction is developed that suggests that only a small number (approximately six) of protein molecules are bound to $tRNA_3^{Leu}$ in the complex while analysis of the inhibition studies suggests that these UP1 molecules are not bound in a highly cooperative manner.

A number of nucleic acid helix-destabilizing proteins (Karpel et al., 1974, 1975a,b, 1976) (formerly termed unwinding proteins) can accelerate the renaturation of metastable, biologically inactive tRNA (Lindahl et al., 1966; Fresco et al., 1966; Adams et al., 1967; Webb & Fresco, 1973; Ishida & Sueoka, 1967) and 5S RNA conformers (Aubert et al., 1968; Richards et al., 1973). The spontaneous renaturations of yeast $tRNA_3^{Leu}$ and 5S RNA are processes with activation energies of about 60 (T. Lindahl, G. Payne, R. L. Karpel, and J. R. Fresco, unpublished results) and 65 kcal/mol (Richards et al., 1973), respectively. These large barriers suggest that renaturation likely involves the disruption of many "incorrect" base pairs in the inactive conformer to allow correct refolding to the native biologically functional conformer (Fresco et al., 1966; Adams et al., 1967; Webb & Fresco, 1973; Uhlenbeck et al., 1974; Wong et al., 1973). It seems reasonable, therefore, that the accelerating action of nucleic acid helix-destabilizing proteins in these cases is related to their ability to promote disruption of base-paired regions by tightly but transiently binding to segments of the RNA that are single stranded or become so upon interaction with the protein.

Although there is at present no evidence that inactive tRNAs occur in vivo, the process by which helix-destabilizing proteins accelerate RNA renaturation is viewed as a model of the mechanism whereby several different RNA conformational changes occur in the cell (Karpel et al., 1974). For example, the complementary pairing between *Escherichia coli* mRNA and 16S rRNA during protein synthesis (Shine & Delgarno, 1974) may be dependent on the helix-destabilizing activities of proteins S1 and IF3 (VanDieijen et al., 1976). The mechanism of denaturation and formation of *intra*molecular tRNA structure in the presence of a helix-destabilizing protein may thus serve as a model for the analogous melting and formation of *inter*molecular base pairing during initiation of protein synthesis as well as for other processes involving RNA conformational interchange (Karpel, 1981).

This study examines the mechanism by which the calf thymus protein UP1 (Herrick & Alberts, 1976a) brings about the renaturation of RNA, using that of $tRNA_3^{Leu}$ as a model. It explores the binding stoichiometry between protein and RNA, with a view toward understanding the nature of the

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complex formed between them. The inhibition of tRNA renaturation by polynucleotides of different base composition and strandedness by poly(ribosylurea phosphate) (PRUP), which has the same backbone but no bases, and by oligonucleotides of defined lengths is used to help define which structural components of the nucleic acid are required for binding by UP1. The role of $Mg^{2+}$ (which stabilizes the native conformation of tRNA) in modulating RNA–protein complex formation and dissociation is also studied. These various results lead to a semiquantitative picture of UP1–RNA interaction and of UP1-induced RNA renaturation.

## Materials and Methods

*Proteins.* UP1 was prepared according to Herrick & Alberts (1976a). All the experiments with $tRNA_3^{Leu}$ were performed with a high salt eluting fraction of UP1 similar to the "basic" fraction of Herrick & Alberts (1976a). A fraction of UP1 eluting from denatured DNA–cellulose at somewhat lower [NaCl], similar to the "mixed" fraction of Herrick & Alberts, was used in the experiments with 5S RNA. Both protein fractions were at least 90% pure, as judged by NaDodSO$_4$[1]–polyacrylamide gel electrophoretic analysis. UP1 concentration was determined as previously described (Karpel & Burchard, 1980).

*Nucleic Acids.* Yeast $tRNA_3^{Leu}$ (Kowalski et al., 1971) and *E. coli* 5S RNA (Richards et al., 1973) were prepared in this laboratory, as were poly(A) and one sample of poly(C) (Grunberg-Manago et al., 1956). Poly(C) was also obtained from Miles Laboratories, as was poly(U). No significant difference was detected between the abilities of the two poly(C) samples to inhibit UP1-induced renaturation of $tRNA_3^{Leu}$. Poly(A-U) was obtained from Biogenics Research Corp., and oligouridylates were from Collaborative Research, Inc. In the absence of UP1, these polynucleotides had no effect on the aminoacylation of $tRNA_3^{Leu}$.

Poly[(3′–5′)-ribosylurea phosphate] was prepared by permanganate oxidation of poly(C) (Chatamra & Jones, 1963; Holbrook et al., 1965). A 0.6-mL solution containing 3.2 mg of $KMnO_4$ was slowly added to a stirred 0.6-mL solution containing 3.45 mg (11 μmol of residues) of poly(C) and 30 mg of sodium bicarbonate (pH 9) and incubated at 37 °C for 19 h. The reaction was monitored from the loss of $A_{270}$ and the simultaneous rise of acid-labile 1′-substituted ribose, measured by the orcinol reaction (Mejbaum, 1939), for which the standard was adenosine. The brown flocculent $MnO_2$, which settled out of the colorless solution, was removed by filtration. The filtrate was exhaustively dialyzed against 200 mL of 0.1 M EDTA, pH 7.5, then 1 M NaCl, and finally doubly distilled water. The dialyzate (3.5 mL) was lyophilized and brought to 0.5 mL with doubly distilled water. Ribose polymer concentration was measured by the orcinol reaction. The yield of nondialyzable PRUP was 46%, 5.1 μmol. This material showed no residual absorbance between 230 and 300 nm and provided a positive color test for urea on a thin-layer chromatogram with a dimethylaminobenzaldehyde–HCl fume staining procedure (Waldi, 1965), confirming that cytosine-moiety oxidation was complete and that a urea moiety was a significant end product.

*Assays.* The $tRNA_3^{Leu}$ aminoacylation and 5S RNA polyacrylamide gel electrophoresis assays were performed as previously described (Lindahl et al., 1966; Richards et al., 1973).

[1] Abbreviations: NaDodSO$_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol.

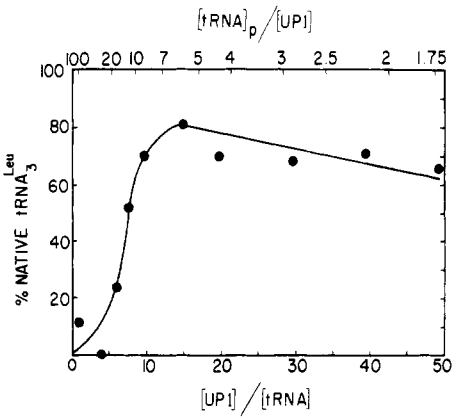


FIGURE 1: Dependence of yeast $tRNA_3^{Leu}$ renaturation on [UP1]:[tRNA]. Each preassay mixture contained $1.33 \times 10^{-11}$ mol of tRNA (0.39 μg) in 65 μL ($2.05 \times 10^{-7}$ M or $1.74 \times 10^{-5}$ M for $tRNA_p$) and varying amounts of UP1 as indicated. The solvent was 3.8 mM Tris, 1.5 mM cacodylate, 0.78 mM $Na_2EDTA$, 0.077 mM DTT, 0.035 M KCl, and 7.7% (v/v) glycerol, pH 8.2 ± 0.2. After incubation of the mixtures for 60 min at 0 °C, $MgCl_2$ was added to 0.016 M, and the aminoacylation assay was performed. It has been observed that the [UP1]:[tRNA] ratio giving the maximum level of renaturation varies somewhat with pH and the absolute [tRNA].

## Results

*UP1 Facilitates RNA Renaturation Only When in Stoichiometric Excess.* Those proteins that bring about renaturation of inactive tRNAs do so only when in sufficient stoichiometric excess (Karpel et al., 1974). This is illustrated by a titration experiment in which varying amounts of UP1 were incubated with a constant amount of inactive $tRNA_3^{Leu}$ at 0 °C for 1 h prior to the aminoacylation assay at 25 °C. It can be seen in Figure 1 that significant renaturation occurs only when the protein:RNA molar ratio > 4 (weight ratio > 3). So, unlike a typical enzyme, UP1 is active only in stoichiometric amounts, although the process is formally catalytic since the protein does not appear to be consumed in the process (see below). If an intermediate of this renaturation process is a protein–RNA complex, as has been observed on polyacrylamide gels (Karpel et al., 1976), the need for stoichiometric amounts of protein becomes clear.

This effect of UP1 is not restricted to $tRNA_3^{Leu}$ renaturation. An analogous titration of 5S RNA incubated with increasing amounts of UP1 in the absence of $Mg^{2+}$ is shown in Figure 2. Like the aminoacylation assay used for $tRNA_3^{Leu}$ renaturation, electrophoresis at room temperature of the protein–5S RNA mixtures was performed under renaturing conditions, i.e., in the presence of $Mg^{2+}$. However, the amounts of 5S RNA (4 μg/gel, of which $^2/_3$ was inactive) are 1 order of magnitude greater. It is seen that an excess of protein is required for conversion of the inactive conformer to renatured 5S RNA. Above a protein:RNA molar ratio of 8, complex formation predominates, coinciding with the disappearance of *both* free 5S RNA bands. Thus, under the conditions employed, UP1 may have the capacity to bind the renatured as well as the inactive 5S RNA conformer. At high protein concentrations, it appears that the UP1–RNA complex(es) is (are) more stable than free renatured RNA. Since UP1 can facilitate the renaturation of inactive tRNA as well as inactive 5S RNA, two molecules with different tertiary structure, there must be some common structural element accessible in both; this is presumably single-stranded segments, present or induced by UP1 binding.

*Single-Stranded Polynucleotides Inhibit UP1-Induced $tRNA_3^{Leu}$ Renaturation.* The relationship between the affinity of helix-destabilizing proteins for single-stranded nucleic acids

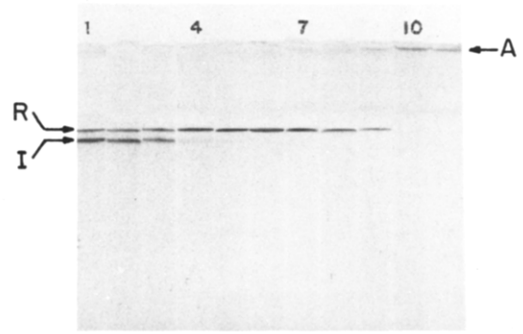


FIGURE 2: Effect of increasing [UP1] on *E. coli* 5S RNA renaturation. Electrophoresis was performed on 15% polyacrylamide gels, 5 × 0.5 cm, at room temperature for 5 h at 65 V with 0.065 M Tris and 1 mM $MgCl_2$, pH 7.5, as the buffer. Samples contained 10 μL of 5S RNA, $1.0 \times 10^{-10}$ mol (4 μg), in 0.065 M Tris, pH 7.5, 20 μL of 0.001% bromophenol blue, 10% sucrose, and 100 μL of varying amounts of UP1 in 5 mM Tris, pH 8.8, 1 mM $Na_2EDTA$, 0.1 mM DTT, and 10% (v/v) glycerol. After electrophoresis gels were stained with 1% pyronin Y in 15% acetic acid. Inactive and renatured bands are designated I and R, respectively, and the protein–RNA complex [which can also be stained with Coomassie blue (Karpel et al., 1974)] is designated A. [UP1]:[RNA] was as follows, from left to right: gel 1, 0; gel 2, 0.65; gel 3, 1.3; gel 4, 2.6; gel 5, 3.2; gel 6, 3.9; gel 7, 5.2; gel 8, 6.5; gel 9, 7.8; gel 10, 10; gel 11, 13.

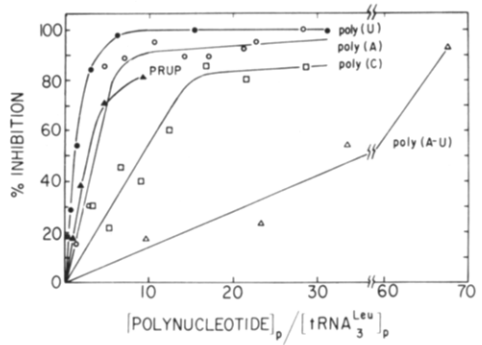


FIGURE 3: Inhibition of UP1-facilitated renaturation of $tRNA_3^{Leu}$ by polynucleotides. Each data point represents a mixture containing $1.33 \times 10^{-11}$ mol of inactive tRNA in 75 μL ($1.78 \times 10^{-7}$ M or $1.49 \times 10^{-5}$ M for $tRNA_p$), polynucleotide in the indicated ratio, and UP1 ($1.92 \times 10^{-6}$ M) that was incubated at 0 °C for 20 min in 3.3 mM Tris, 2.0 mM cacodylate, 0.68 mM $Na_2EDTA$, 0.067 mM DTT, 0.030 M KCl, and 6.7% (v/v) glycerol, pH 8.1, and then subjected to aminoacylation assay in 0.016 M $MgCl_2$ at 25 °C for 18 min.

and their ability to accelerate RNA renaturation was explored by monitoring the inhibitory effect of single-stranded polynucleotides on UP1-induced renaturation of $tRNA_3^{Leu}$. These effects were studied at 0 °C in the absence of $Mg^{2+}$ by adding the polynucleotides to the inactive tRNA *prior* to the addition of UP1. Figure 3 shows that poly(U), whose residues do not stack (Richards et al., 1963), is the most effective inhibitor, reducing the action of UP1 by half when present at a residue concentration equal to that of the tRNA. It can also be seen that poly(A) and poly(C), with residues largely stacked at 0 °C (Fresco & Klemperer, 1959; Brahms et al., 1967), are somewhat less effective inhibitors, while double-helical poly(A-U), with double-helical hairpin turns (Spatz & Baldwin, 1965), inhibits very much less at comparable concentrations.

*Binding of Single-Stranded Polynucleotides by UP1 Does Not Require Base Substituents.* For determination of whether the presence of the heterocyclic bases linked to the polynucleotide backbone is required for binding by UP1, PRUP was also tested for its ability to inhibit UP1-induced renaturation of $tRNA_3^{Leu}$. As seen in Figure 3, it is nearly as effective an inhibitor as poly(U) and better than the poly(C) from which it was derived. The base is therefore not required for binding.

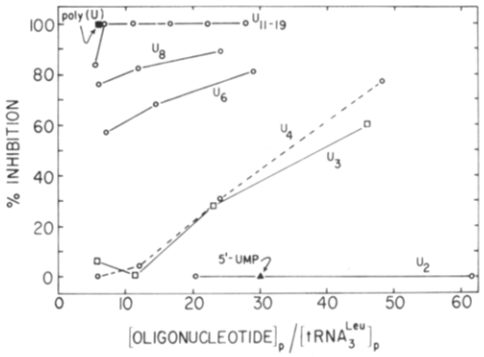


FIGURE 4: Inhibition of UP1-facilitated renaturation of $tRNA_3^{Leu}$ by oligonucleotides. Conditions were as in Figure 3 except that [UP1] = $2.62 \times 10^{-6}$ M.

The greater affinity of PRUP for UP1 is probably due to its lack of base stacking. It is unlikely that the cytosine moieties in poly(C) per se reduce its binding to UP1, since the protein binds somewhat better to the same backbone with adenine residues and still better when the residues are uracil. PRUP is also an effective inhibitor of UP1-induced $T_m$ depression of poly[d(A-T)] (Karpel et al., 1981).

*Oligonucleotides with Three or More Phosphates Inhibit UP1-Induced RNA Renaturation.* The effectiveness with which poly(U) inhibits UP1-facilitated renaturation of $tRNA_3^{Leu}$ suggested the use of oligouridylates of defined length in this assay to determine the minimum chain length that can bind effectively to UP1. The results of such experiments performed in the same manner as the polynucleotide inhibition studies are shown in Figure 4. Given the finding that the bases do not markedly influence UP1 binding and since these oligomers lack both 3′- and 5′-terminal phosphates, the size-indicating subscript for an oligomer refers to its number of phosphates. It can be seen that $(U)_{11-19}$ is about as effective as poly(U). While $(U)_8$ is almost as effective as $(U)_{11-19}$, the degree of inhibition (at the same phosphate residue concentration) drops rapidly as oligomer chain length is further reduced. Below the limit of three backbone phosphates, effective inhibition does not occur, indicating the minimal backbone segment for an effective ligand.

*Oligo- and Polynucleotides Reverse RNA Renaturation.* If the UP1–tRNA association is readily reversible, agents that inhibit complex formation should dissociate preformed complexes. So that this possibility could be tested, poly(U) was added in the absence of $Mg^{2+}$ to a UP1–$tRNA_3^{Leu}$ mixture (17:1 molar ratio) that had been preincubated at 0 °C for 20 min. The level of renaturation at that temperature slowly decreased to zero in a first-order decay process, with $t_{1/2}$ = 35 min at $[poly(U)]_p$:$[tRNA]_p$ = 46 (Figure 5). This rate was essentially the same when the $[poly(U)]_p$:$[tRNA]_p$ was varied between 23 and 390. $Oligo(U)_{11-19}$ was found to analogously reverse UP1-induced renaturation. These low rates of reversal, which are representative of complex dissociation, are in contrast to the rapid kinetics of complex formation, where $t_{1/2} \lesssim 0.5$ min at 0 °C (Karpel et al., 1974).

*Renaturation Spontaneously Follows the $Mg^{2+}$-Induced Dissociation of the UP1–$tRNA_3^{Leu}$ Complex.* Many of the foregoing results indicate formation of a complex between UP1 and inactive $tRNA_3^{Leu}$ in the renaturation process. It is reasonable to expect that subsequent to complex formation, the renaturation mechanism requires the tRNA-bound UP1 to be displaced in order for the aminoacyl-tRNA synthetase to interact with its recognition sites on the tRNA molecule. However, a ternary complex of UP1, tRNA, and synthetase is conceivable. The level of aminoacylation of tRNA does not

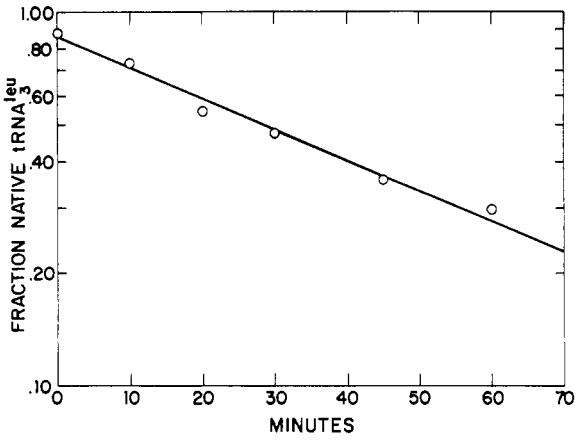


FIGURE 5: Kinetics of reversal by poly(U) of UP1-facilitated renaturation of $tRNA_3^{Leu}$. 60-$\mu$L samples of inactive RNA ($2.23 \times 10^{-7}$ M or $1.86 \times 10^{-5}$ M for $tRNA_p$) were preincubated with protein ($2.57 \times 10^{-6}$ M) for 20 min at 0 °C in the buffer described in Figure 3, and then poly(U) was added (to $8.56 \times 10^{-4}$ M for polynucleotide$_p$) to each. After incubation at 0 °C for the times shown, $MgCl_2$ was added to 0.016 M, and the aminoacylation assay was performed.

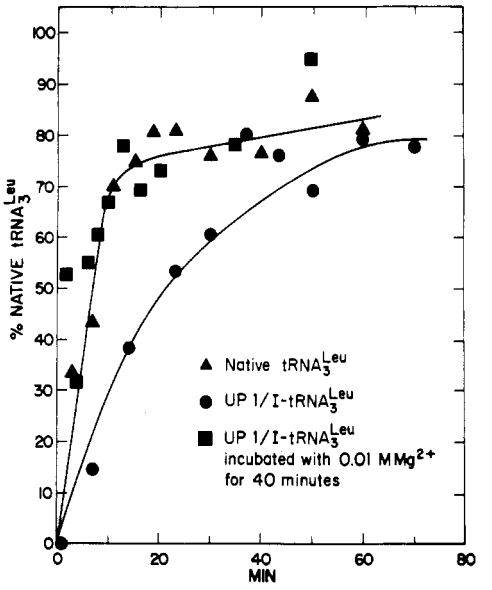


FIGURE 6: Effect of $Mg^{2+}$ on UP1-facilitated renaturation at 0 °C, as measured by aminoacylation kinetics. Mixtures of inactive $tRNA_3^{Leu}$ ($2.05 \times 10^{-7}$ M) and UP1 ($3.02 \times 10^{-6}$ M) were prepared in the buffer described in Figure 1 and either subjected immediately to the aminoacylation assay in 0.016 M $Mg^{2+}$ for the times shown (●) or else incubated with 0.010 M $MgCl_2$ for an additional 40 min prior to aminoacylation in 0.016 M $Mg^{2+}$ (■). For comparison, the time course of aminoacylation of heat-renatured $tRNA_3^{Leu}$ under the same buffer conditions is also shown (▲). Since the plateau level of aminoacylation is somewhat dependent on temperature, the 100% level was arbitrarily set as that obtained at 25 °C.

distinguish between these two possibilities since it is not necessarily a direct probe of *free* native tRNA. An experiment was therefore designed, predicated on the notion that the kinetics of aminoacylation of $tRNA_3^{Leu}$ *complexed* to UP1 will be slower than those of the *free* renatured conformer. Thus, the kinetics of aminoacylation at 0 °C (to slow down the reaction sufficiently for quantitation) were compared for inactive $tRNA_3^{Leu}$ samples subjected to treatment with UP1 or to renaturation by heat in the presence of $Mg^{2+}$. Since $Mg^{2+}$ inhibits the renaturing action of UP1 when added to inactive $tRNA_3^{Leu}$ prior to addition of the protein but is required for the aminoacylation assay, its potential to induce release of the bound tRNA and at the same time to stabilize it in the renatured form was also examined.

When $Mg^{2+}$ was absent from UP1-inactive $tRNA_3^{Leu}$ mixtures prior to aminoacylation but included in the assay reaction mixture, the kinetics of aminoacylation were significantly slower than those of heat-renatured tRNA (Figure 6). But, when inactive tRNA and UP1 were mixed in the absence of $Mg^{2+}$ and *then* brought immediately to 0.01 M $Mg^{2+}$ and incubated for 40 min at 0 °C (the time required for complete aminoacylation of the UP1-renatured tRNA) prior to charging, the aminoacylation kinetics were identical with those of the heat-renatured tRNA (Figure 6). Apparently, when $Mg^{2+}$ is absent from the preaminoacylation medium, dissociation of the complex in the assay medium must precede charging; but, with $Mg^{2+}$ in the preassay medium, this cation, like poly(U), can induce dissociation of the UP1–tRNA complex and allow refolding to aminoacylatable tRNA prior to assay. Either of these $Mg^{2+}$-dependent processes could be the rate-determining step in the observed kinetics. Whatever, in vitro UP1-facilitated renaturation of $tRNA_3^{Leu}$ is a $Mg^{2+}$-modulated event: the level of the cation must be sufficiently *low* initially to permit complex formation and then sufficiently *high* to promote dissociation of the complex and refolding of the tRNA to the native conformer.

Conceivably, the dissociation of the UP1–$tRNA_3^{Leu}$ complex might also be facilitated by the binding of the synthetase to the tRNA. So that this could be tested, a UP1–$tRNA_3^{Leu}$ mixture was preincubated at 0 °C for 20 min in the absence of $Mg^{2+}$ and then incubated for 40 min longer with the assay level of synthetase. Then, cofactors and $Mg^{2+}$ were added, and aminoacylation was allowed to proceed at 25 °C for 25 min (assay conditions). This protocol failed to alter the level of UP1-induced renatured tRNA, indicating that the synthetase, unlike poly(U) or $Mg^{2+}$, does not displace the tRNA from the complex. Had displacement occurred, *all* the tRNA would have remained inactive, since the displacement would have taken place in the absence of $Mg^{2+}$, conditions favoring the inactive conformer. Since the effect of synthetase necessarily had to be assessed *prior* to aminoacylation, neither $Mg^{2+}$, ATP, nor amino acid was present. Conceivably, the synthetase could make a contribution to the dissociation of the UP1–tRNA complex when it is functioning as a catalyst in the presence of all the required cofactors, but this could not be readily tested.

## Discussion

*General Features of UP1-Facilitated RNA Renaturation.* The results of this and other studies enable us to develop a general mechanism of UP1-facilitated renaturation of RNA. The salient observations are as follows: (1) UP1 lowers the $T_m$ of double-helical polyribonucleotides (Herrick & Alberts, 1976b); (2) UP1 has strong, preferential affinity for the single-stranded nucleic acid backbone in as much as poly(U), PRUP, poly(A), and poly(C) are effective inhibitors of UP1-facilitated renaturation of $tRNA_3^{Leu}$; (3) UP1 interaction with tRNA and poly(A) results in both cases in significant loss of secondary structure of these polynucleotides (Karpel & Burchard, 1980); (4) UP1 forms a complex with 5S RNA and tRNA as intermediates in the renaturation processes. All these aspects of RNA–UP1 interaction are consistent with the notion that UP1 helps overcome the high activation energy barrier associated with the spontaneous renaturation of $tRNA_3^{Leu}$ and 5S RNA.

The secondary and tertiary structural impediments to renaturation of $tRNA_3^{Leu}$ should not require disruption of the entire higher order structure of the inactive conformer. The large activation energy for spontaneous renaturation (60 kcal/mol) corresponds to the disruption of about 10 base pairs,

but this does not necessarily mean that UP1 must bind to all 20 residues for renaturation to proceed readily. Various studies of the inactive conformer suggest that the DHU loop and stem are denatured and fully accessible to solvent and the anticodon loop, which is free in the native conformer, is inaccessible and probably bound to the TψC loop and/or stem (Webb & Fresco, 1973; Uhlenbeck et al., 1974; Wong et al., 1973). Renaturation may therefore only require the disruption of this "incorrect" tertiary structural interaction between the anticodon and TψC loops. The steric consequences of only one or two UP1 molecules bound to the DHU arm (17 residues) could have a strong destabilizing effect on the interaction between the anticodon loop and the TψC loop, lowering the barrier enough for renaturation to proceed rapidly. On the other hand, protein molecules bound to the other major single-stranded region of the molecule, the ACCA-3′-OH terminus, would probably have little effect on the barrier to renaturation.

If UP1 disrupts double-helical segments of inactive tRNA, it is the addition of $Mg^{2+}$ to 0.016 M at 0 °C just prior to aminoacylation that must facilitate refolding to the native state. First, $Mg^{2+}$ probably weakens the protein–tRNA complex, as has been observed for other protein–nucleic acid interactions (Record et al., 1978). Specifically, if UP1 molecules are bound to the accessible single-stranded DHU arm of inactive $tRNA_3^{Leu}$, then renaturation would require dissociation because this region of the tRNA is not accessible in the native conformer (Uhlenbeck et al., 1974), as it participates in important tertiary structural interactions [cf. Kim (1976)]. The second consequence of adding $Mg^{2+}$ is a marked increase in the $T_m$ of the tRNA. If UP1 molecules bound to single-stranded regions of the tRNA destabilize adjacent helical segments, then the addition of $Mg^{2+}$ should counteract this effect. In fact, the partial disruption of $tRNA_3^{Leu}$ secondary structure by UP1 is reversed on the addition of $Mg^{2+}$ (Karpel & Burchard, 1980). Finally, it should be noted that there are conditions under which UP1 or other helix-destabilizing proteins effectively facilitate tRNA renaturation in the presence of $Mg^{2+}$ (Karpel et al., 1974; N. S. Miller, J. R. Fresco, and C. Long, unpublished observations).

The stoichiometry of UP1–inactive $tRNA_3^{Leu}$ interaction can be estimated in the following way. UP1 occludes 7 residues in single-stranded nucleic acids (Karpel & Burchard, 1980), so that the 85 residues of $tRNA_3^{Leu}$ would be saturated with 12 protein molecules. A quarter of the residues of the uncomplexed inactive $tRNA_3^{Leu}$ conformer (DHU loop and stem and CCA terminus) are already accessible as single-stranded segments (Uhlenbeck et al., 1974), and upon interaction with UP1 a third of the residues heretofore in base-paired structure become unpaired (Karpel & Burchard, 1980). Consequently, half the residues of an inactive $tRNA_3^{Leu}$ molecule are available to bind as many as six UP1 molecules.

Consideration of a simple model of UP1–tRNA interaction leads to the same stoichiometry and provides also an estimate of the association constant between the two macromolecules [see Karpel et al. (1975a,b) for an analogous derivation and calculation of the stoichiometries and association constants for the interaction of metal complexes with $tRNA_3^{Leu}$]. With the assumption that all renatured tRNA molecules are derived from UP1–RNA complexes and that all complexes are productive, leading to native tRNA in the $Mg^{2+}$-containing aminoacylation medium, we find that an average of about six protein molecules are bound per tRNA. Although this approach ignores the statistical contribution brought about by the presence of overlapping binding sites on the nucleic acid lattice (McGhee & von Hippel, 1974) and assumes no significant binding cooperativity [which appears to be the case (Herrick et al., 1976; R. L. Karpel and S. H. Kim, unpublished observations], the calculated value of approximately six protein molecules bound to a tRNA is consistent with the fraction of tRNA residues we estimated above to be available for binding. The association constant of UP1 for its tRNA binding site, $K_{site}$, is estimated to be $\sim 1 \times 10^6$ $M^{-1}$.

Table I: Affinities of UP1 for Polynucleotides

| poly-nucleotide | $[poly(X)]_p$: $[tRNA_3^{Leu}]_p$ at 50% renaturation | $K_X/K_{site}$ | $K_X$ ($M^{-1}$) | |
|---|---|---|---|---|
| | | | *a* | *b* |
| poly(U) | 1.0 | 0.38 | $4 \times 10^5$ | $2 \times 10^6$ |
| PRUP[c] | 2.1 | 0.15 | $2 \times 10^5$ | $8 \times 10^5$ |
| poly(A) | 2.9 | 0.11 | $1 \times 10^5$ | $4 \times 10^5$ |
| poly(C) | 7.5 | 0.038 | $4 \times 10^4$ | $2 \times 10^5$ |
| poly(A-U) | 29 | 0.0096 | $1 \times 10^4$ | $4 \times 10^4$ |

[a] Based on $K_{site} = 1 \times 10^6$ $M^{-1}$. [b] Based on $K_A = 4 \times 10^5$ $M^{-1}$. [c] Poly(ribosylurea phosphate).

*Evaluation of UP1 Inhibition by Polynucleotides.* The ability of single-stranded polynucleotides to inhibit UP1 renaturation of inactive $tRNA_3^{Leu}$ strongly suggests that they effectively compete for the protein. The polynucleotide inhibition data make it possible to assess the affinities of the polynucleotides and tRNA for UP1, which can be defined as

$$K_X = \frac{[XP]}{[X][P]} \tag{1}$$

where [X] is the concentration of free polynucleotide binding sites, [P] is the concentration of free protein, and [XP] is the concentration of protein bound to polynucleotide. [XP] is calculated by subtracting from the total [UP1] at 50% renaturation (41% inhibition of maximal UP1-induced renaturation) in the presence of competing polynucleotide, the corresponding [UP1] which effects the same level of renaturation in the absence of competing inhibitor. It can readily be shown that, at 50% renaturation

$$\frac{[XP]}{[X]} = \frac{K_X}{K_{site}} \tag{2}$$

Polynucleotide concentrations (relative to $[tRNA]_p$) effecting a 41% level of inhibition are listed in Table I.

The effectiveness with which these polymers inhibit UP1-induced renaturation of $tRNA_3^{Leu}$ varies as poly(U) > PRUP > poly(A) > poly(C) ≫ poly(A-U). The high relative affinity of PRUP for UP1 suggests that this order of affinities is not due to base recognition per se by the protein; rather, it probably reflects the influence of base stacking on backbone conformation (see below). Hence, it is assumed that each UP1 molecule binds to the same number of residues on each polynucleotide. Setting $K_{site} = 1 \times 10^6$ $M^{-1}$ (see above), we calculated $K_X$ values and tabulated them in Table I along with the relative affinities, $K_X/K_{site}$.

In principle, this method of calculating [XP] and [X] can be used to determine $K_X$ at any data point in Figure 3 below 100% inhibition. In general, it can be shown that

$$\frac{K_X}{K_{site}} = \frac{[XP][I]}{[X][N]} \tag{3}$$

where [I] and [N] are the concentrations of inactive and native tRNA, respectively. The $K_U$ [$K_U$ is $K_X$ for poly(U)] values so calculated from all the data points all fell within a factor

of 2 of the value at 50% renaturation, indicating the validity of the analysis.

The calculated values of $K_X$ are dependent on $K_{site}$ and thus are not directly obtained from the inhibition data. The accuracy of these numbers rests not only on this experimental data but also on the $tRNA_3^{Leu}$–UP1 titration data. However, the relative values of $K_X$ are independent of $K_{site}$ and $i$ (the number of bound UP1 molecules per tRNA molecule) and should directly reflect the relative affinities of the polynucleotide for UP1.

$K_X$ for poly(A) association with UP1, i.e., $K_A$, can be obtained from the diminution of this polynucleotide's circular dichroism upon interaction with the protein (Karpel & Burchard, 1980). With the assumption that the interaction is noncooperative, the approach of McGhee & von Hippel (1974) was applied to the dependence of the interaction on $[Na^+]$, and a linear log $K_A$ vs. log $[Na^+]$ plot was obtained. At 0.035 M $Na^+$, the same concentration of monovalent cations as that of this study ($K^+$), the interpolated value for $K_A = 4.0 \times 10^5\ M^{-1}$. When no significant difference between the effect of $Na^+$ and $K^+$ on the association is assumed, this value is well within 1 order of magnitude of that based on $K_{site}$. $K_X$ values calculated from the relative affinities of UP1 for poly(A) and other polynucleotides, with $K_A = 4 \times 10^5\ M^{-1}$, are tabulated in Table I.[2]

The calculated relative affinities of polyribonucleotides for UP1 are similar to those for the *E. coli* helix-destabilizing protein (Molineux et al., 1975). Poly(U) bound the *E. coli* protein 17 times more tightly than poly(A) and 63 times more tightly than poly(C). Although no definitive data are available for the binding of helix-destabilizing protein to double-stranded RNA, Molineux et al. (1975) did find the affinity of the *E. coli* binding protein for double-stranded DNA to be 1000 times lower than for single-stranded DNA. On the basis of the effect of native *Micrococcus luteus* DNA on the UP1-induced $T_m$ depression of *Clostridium perfringens* DNA, Herrick & Alberts (1976b) concluded that UP1 has a low but appreciable affinity for native DNA. The 30-fold lower affinity of UP1 for poly(A-U) relative to poly(U) could reflect a weak affinity for double-stranded RNA; however, there is also likely to be a large contribution from single-stranded hairpin loops in this polymer of alternating sequence, which is difficult to anneal to a perfect double helix.

*Nonrecognition of Polynucleotide Bases by UP1.* The affinity of UP1 for single-stranded polynucleotides is greatest for that with no base stacking [poly(U)], although the preference for poly(A) over poly(C) indicates that other factors also play a role. When the cytosine residues of poly(C), which stack well, are converted to nonheterocyclic urea moieties, the resultant poly(ribosylurea phosphate) has a significantly greater affinity for UP1 than its precursor, that is only slightly less than that of poly(U). These and other results (see below) indicate that UP1 binding involves minimal if any direct interactions between the protein and the bases.

A likely explanation for the graded affinity of UP1 for different single-stranded homopolymers is, instead, that some particular distorted orientation of the ribose–phosphate backbone enables it to interact most favorably with basic residues and other substituents on the protein. Base-stacking interactions or other substituents on carbon 1′ probably constrain the polynucleotide backbone in a less favorable orientation. Hence, any backbone reorientation required for UP1 binding must involve an input of energy (to disrupt the constraints), the extent of which results in correspondingly lower affinity of the protein.

In fact, a decrease in base stacking (Karpel & Burchard, 1980) and an increase in chain length (Herrick et al., 1976) have been observed upon UP1 binding to single-stranded nucleic acid chains. Location of UP1 on the ribose–phosphate chain is also consistent with the observed stimulation of DNA replication catalyzed by DNA polymerase $\alpha$ (Herrick et al., 1976); if UP1 bound, instead, to the bases, their accessibility for template function might be impaired. That the bases are not obscured upon UP1 binding has been confirmed by the observation that the reactivity of adenine residues toward chloroacetaldehyde is comparable in UP1-denatured DNA complexes and in free denatured DNA (Kohwi-Shigematsu et al., 1978). In view of all these observations indicating the single-stranded polynucleotide backbone as the primary source of interactions between UP1 and nucleic acids, the great sensitivity of UP1 affinity to the nature and concentration of cations (Karpel et al., 1974; Karpel & Burchard, 1980) is readily understandable.

*Evaluation of Chain Length Dependence of Oligo(U) Inhibition of UP1.* The data on the inhibition of UP1-induced renaturation of $tRNA_3^{Leu}$ by oligouridylates of defined length (Figure 4) are insufficient for the calculation of reliable binding constants. It is possible, nevertheless, to show that the variation of inhibitor activity with chain length is qualitatively consistent with statistical considerations arising from the presence of overlapping binding sites on an oligonucleotide. Kelly et al. (1976) have emphasized that an oligonucleotide of length $l$, exceeding the minimum number of (phosphate) residues $m$ required for interaction with a protein, can bind to the protein in several different ways. For example, since $m = 3$ for UP1 binding [oligo$(U)_2$ is ineffective as an inhibitor even at 62 times the residue concentration of $tRNA_3^{Leu}$], oligo$(U)_4$ can bind UP1 either through phosphates 1–3 or 2–4. In the general case, with $l > m$

$$K_l = (l - m + 1)K_{int} \qquad (4)$$

(Kelly et al., 1976) where $K_l$ is the observed association constant for binding a protein to an oligonucleotide of length $l$ that is capable of accommodating only one protein molecule in more than one way ($l > m$ but less than $2m$) and $K_{int}$ is the intrinsic binding constant of the protein to an oligonucleotide of length $m$. From this relationship, and the experimentally determined value of $m = 3$, we obtain $K_4 = 2K_3$ and $K_5 = 4K_3$.

Now, when [X] ≫ [XP], as it is for the oligonucleotides, from eq 3, it can be shown that at a given level of inhibition of renaturation $K_l/K_m \simeq [\text{oligo}(U)_m]/[\text{oligo}(U)_l]$. Thus, from Figure 4, at 60% inhibition of renaturation, $K_4 \simeq 1.2K_3$ and $K_6 \simeq 5K_3$, in approximate agreement with the predictions of eq 4.

A comparison of $K_3/K_{site}$ [calculated from eq 3 assuming that $[\text{oligo}(U)_3] = [\text{oligo}(U)_3]_{total}$] and $K_U/K_{site}$ indicates that $K_{poly(U)} \simeq 32K_3$. In contrast, the affinity of $T_4$ gene-32 protein for single-stranded poly(A) and poly[d(A)] is about 1000 times greater than it is for ApA [oligo$(A)_m$ for this protein] and dApA [oligo d$(A)_m$], respectively (Kelly et al., 1976). For

[2] The approach of McGhee & von Hippel (1974) could theoretically be applied directly to the inhibition data in order to calculate $K_X$. Since $[X]_{total}$ is a known quantity, the degree of protein saturation of polynucleotide binding sites, $[XP]/[X] \equiv \nu$, can be determined for all data points below 100% saturation. Unbound protein, [P], can be determined by subtracting ($[XP] + i[IP_i]$) from $[P]_{total}$ for each data point (where $IP_i$ is the UP1–tRNA complex with the assumed value of $i$). $\nu/[P]$ vs. $\nu$ could then be plotted and $K_X$ determined from eq 10 of McGhee & von Hippel. However, we calculate only a 2-fold variation in $\nu$, and any error in $i$ would lead to a corresponding error in $K_X$. For the poly(U) data, values of $K$ calculated in this way are about 1 order of magnitude lower than those reported in Table I. For these reasons the McGhee–von Hippel analysis could not be usefully employed.

highly cooperatively binding ligands such as gene-32 protein, this 30-fold greater affinity for polymers than for oligomers exhibited by UP1 is largely attributed to the highly cooperative interaction between gene-32 protein molecules binding adjacently to the polynucleotide chains. We have noted that UP1 has not been observed to bind to single-stranded nucleic acids in a highly cooperative manner, and these results are in keeping with those findings. Hence, although the data presented here do not exclude the possibility of some cooperativity in UP1–nucleic acid interaction, it is clear that if there is any, it is considerably less than that observed for gene-32 protein. Whatever, cooperativity is not likely to be a significant factor in the ability of UP1 to effect tRNA renaturation since only a small number of protein molecules are bound in the process.

*Conclusions.* The foregoing analysis sheds further light on our understanding of the acceleration of RNA renaturation induced by calf thymus UP1 and delineates several general aspects of the interaction of this protein with single-stranded segments of nucleic acids. A consideration of the dependence of $tRNA_3^{Leu}$ renaturation on [UP1]:[tRNA] leads to the conclusion that only a small number (five to six) of protein molecules are (temporarily) bound to the tRNA molecule during this process. Evaluation of the inhibition of UP1-induced $tRNA_3^{Leu}$ renaturation by polynucleotides yields relative and absolute binding constants. The oligonucleotide inhibition data indicate that UP1 binds to three phosphate residues on a single-stranded chain, and the increase in affinity with chain length is consistent with the statistical effects arising from overlapping binding sites on the oligonucleotide. Comparison of the differential binding to oligomers and polymers by gene-32 protein and UP1 indicates that UP1 does not interact with its adjacently bound neighbors in the highly cooperative manner characteristic of gene-32 protein binding to single-stranded nucleic acids.

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