Protein Chain Initiation in Wheat Embryo

RESOLUTION AND FUNCTION OF THE SOLUBLE FACTORS*

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SUMMARY

The complete resolution of the initiation and transfer factors of wheat embryo supernatant is described. Two factors function in chain initiation, as ascertained by (a) their requirement for the formation of a ribosome-messenger RNA complex whose capacity for amino acid incorporation is insensitive to aurintricarboxylic acid; and (b) their participation in the tobacco mosaic virus RNA-dependent binding of methionyl transfer RNA to ribosomes. The remaining factors, T1(E) and T2(E), catalyze the poly(U)-dependent polymerization of phenylalanine, establishing their function primarily in chain elongation. All four factors are required for tobacco mosaic virus RNA-dependent amino acid incorporation.

An in vitro system from wheat embryo catalyzes amino acid incorporation into protein in response to natural plant messenger RNA (1-3). The system functions with a rate-limiting step in which a ribosome-messenger complex is formed (4). This reaction requires ATP, GTP, and two supernatant components. In an earlier report (5), we described experiments with aurintricarboxylic acid, a specific inhibitor of protein chain initiation, demonstrating that the supernatant components were participating in chain initiation. The salient observation was that when a ribosome-messenger complex-forming reaction was carried out with supernatant components present, subsequent addition of ATA had no effect on amino acid polymerization. When, however, the supernatant components were omitted from the initial complex-forming reaction, amino acid polymerization was completely inhibited by ATA. These results established the presence of initiating activity in a eucaryotic system (5).

In continuation of these studies, we now report the resolution of wheat embryo supernatant into four soluble factors. Amino acid polymerization catalyzed by TMV-RNA is dependent upon the presence of all four soluble components. Two of these factors, designated C and D, are initiation factors. They are characterized by their absolute requirement for amino acid polymerization catalyzed by natural messenger RNA, their participation in the formation of an initiation complex that is insensitive to ATA, and their catalysis of messenger RNA-dependent binding of methionyl transfer RNA to ribosomes. The other two components catalyze the poly(U)-dependent polymerization of phenylalanine, defining their role as elongation factors (6).

MATERIALS AND METHODS

Resolution of Soluble Factors

All operations are carried out at 0-4° with the column chromatography done under gravitational pressure at a flow rate of approximately 10 to 12 ml per hour.

(1) Separation of Components C and D

Seven milliliters of S100 (23 mg of protein per ml) are chromatographed on a DEAE-cellulose (Whatman DE23) column, 5 × 0.7 cm, equilibrated with Medium II (1 mM Tris acetate, pH 7.0, 2 mM MgAc2, 4 mM mercaptoethanol) containing 0.1 mM KCl. Elution of the column is continued with Medium II, 0.1 M KCl. A void volume of 2 ml is discarded; 7 ml are collected as Fraction C, and a subsequent 5 ml of wash fraction are discarded. The eluant is changed to Medium II, 0.3 M KCl; 1 ml is discarded and 3.8 ml are collected as Fraction D. Both of these fractions are relatively stable when kept at −20° and are used as reagents (0.06 ml of Fraction C and 0.65 ml of Fraction D) in the ATA initiation factor assay for following the purification of the other component.

(2) Purification of Factor C

(a) DEAE-cellulose—Fraction C, 5.5 ml, (10.7 mg of protein per ml) is dialyzed for 45 min against Medium III (1 mM Tris acetate, pH 7.6, 1 mM MgAc2, 4 mM mercaptoethanol, 0.1 mM EDTA) and immediately chromatographed on a DEAE-cellulose column, 9 × 0.9 cm, equilibrated with Medium III. Elution of the column is continued with Medium III, 30 mM KCl, and a
total volume of 10 ml is discarded. The eluant is changed to Medium II, 0.15 mM KCl; 2 ml are discarded and 6 ml are collected. The preparation of Factor C at this point is referred to as C*. It is relatively stable and is free of the other three soluble factors (see Fig. 1 and Table IV).

(b) Phosphocelullose—Four milliliters of the product of Step of medium (2.1 mg of protein per ml) are dialyzed for 30 min against Medium II, 0.1 mM EDTA, 30 mM KCl and immediately applied to a column P11 column, 4.5 x 0.5 cm, previously washed with Medium II, 0.1 mM EDTA, 50 mM KCl. Elution is continued with Medium II, 0.1 mM EDTA, 50 mM KCl, and a total volume of 9.2 ml is discarded. The eluant is changed to a solution containing 20 mM Tris acetate, pH 7.4, 1 mM EDTA, 10% glycerol, 0.5 mM KCl, 0.5 mM dithiothreitol, and 3.5 ml are collected as C-final.

(3) Purification of Factor D

Fraction D, 3.8 ml, (10.2 mg of protein per ml) is dialyzed for 60 min against 1 mM Tris 7.6, 1 mM MgAc2, 4 mM mercaptoethanol, 0.15 mM KCl, and chromatographed on a DEAE-cellulose column, 5 x 0.7 cm, equilibrated with the same solution. Elution of the column is continued with the same solution and a total volume of 19 ml is discarded. The eluant is changed to Medium II, 0.3 mM KCl; 1 ml is discarded and 3 ml are collected as D-final.

(4) Factor T1(E)

The 12 ml (10 ml and 2 ml) normally discarded in Step 2(a) of the purification of Factor C are collected and 3.8 g of solid (NH4)2SO4 are added (45% saturation). The pellet obtained after centrifugation is discarded and an additional 0.85 g of (NH4)2SO4 (55% saturation) are added. After overnight storage at 4°C, the suspension is centrifuged and the pellet is dissolved in 0.7 ml of Medium II, 50 mM KCl; dialyzed for 24 hours against 500 ml of 1 mM Tris acetate, pH 7.3, 50 mM KCl, 4 mM mercaptoethanol with a change of the dialysis solution after 1 hour, and diluted with an equal volume of Medium II, 50 mM KCl, 60% glycerol.

(5) Factor T2(E)

The first 15 ml of the 19 ml) normally discarded in the purification of Factor D are collected and 3.71 g of solid (NH4)2SO4 are added (35% saturation). The pellet obtained after centrifugation is discarded and an additional 1.59 g of (NH4)2SO4 are added (50% saturation). After overnight storage at 4°C, the pellet is collected by centrifugation and processed as described for T1 (E).

The amino acid incorporation assays used for the various factors are sensitive to monovalent cation concentration (7). Consequently, the factor solutions are routinely dialyzed for 14 to 2 hours against 1 mM Tris acetate, pH 7.3, 50 mM KCl, 4 mM mercaptoethanol just prior to use and their K+ concentrations are taken to be 50 mM. With C-final after an initial 1 hour dialysis, the protein solution is transferred to a second dialysis bag and dialyzed against a new solution of the same buffer for an additional hour. With T1(E) and T2(E), dialysis is carried out in the usual manner prior to use, in addition to the dialysis already performed as the final preparatory step. The designations T1(E) and T2(E) maintain the accepted nomenclature for the transfer factors (Reference 6), at the same time designating the source of these factors as the embryo (in contrast to T1 and T2 which are obtained from wheat germ).

ATA Assay for Initiation Factor Activity

A preliminary incubation containing in a volume of 0.35 ml, 10 μmoles of Tris acetate, pH 8.1, 0.4 μmole of ATP, 3.2 μmoles of disodium creatine phosphate, 16 μg of creatine phosphokinase, 0.01 μmole of GTP, 10 μg of TMV-RNA, 0.9 μmole of dithiothreitol, ribosomes (once washed, 280 øg of RNA), 0.84 μmole of MgAc2, 17.5 μmoles of KCl, 400 μmoles of each of eight 14C-amino acids (corresponding to the 14C-amino acids, see below) and soluble factors, is carried out for 6 min at 30°. Thereafter, 1.2 μmoles of ATA (final concentration 8 x 10^-6 M), 0.6 μmoles of MgAc2, 60 μmoles of a mixture of eight 14C-aminoacyl-tRNA's (leucine, serine, valine, isoleu, threonine, proline, phenylalanine, and glutamic acid) (1 pmol = 450 cpm) and 0.03 ml of S100 (undialyzed) are added to a final volume of 0.41 ml; the incubation is continued for 9 min at 30°, and the radioactive material insoluble in hot trichloroacetic acid is determined (8). In the standard assay, crude components C and D (prepared as in Step 1) are used as reagents; 0.06 ml of C (0.64 mg of protein) with fractions to be tested for Factor D activity and 0.05 μl of D (0.51 mg of protein) with fractions to be tested for Factor C activity.

Met-tRNA Binding to Ribosomes

The reaction mixture in a total volume of 0.34 ml contains: 1.1 mM methionine, 30 mM Tris acetate, pH 8, 1.1 mM ATP, 60 μM GTP, 10 μM of TMV-RNA, 2.6 mM dithiothreitol, 1.3 mM MgAc2, 51 mM KCl, ribosomes (twice washed, 220 pg of RNA), 38 pmoles of unresolved [35S]Met-tRNA or 34 pmoles of [35S]Met-tRNA, or [35S]Met-tRNAm (1 pmole = 330 cpm) and soluble factors as indicated. After incubating for 10 min at 20°, 4 ml of ice-cold diluting buffer (10 mM Tris acetate, pH 7.6, 70 mM KCl, 5 mM MgAc2) are added and the solution is filtered under gentle suction through nitrocellulose filters (previously washed with diluting buffer). The filter is then washed twice with ice-cold diluting buffer, dried, and counted.

The assay for elongation factors is the poly(U)-directed polymerization of phenylalanine with deoxycholate-washed ribosomes as previously described (6). The specific activities of factors T1(E) and T2(E), as assayed in the poly(U)-catalyzed elongation factor assay (6), are 331 and 497 pmols per mg of protein, respectively. Poly(U)-dependent binding of Phe-tRNA to ribosomes was assayed at 4.5 mM Mg++ (Reference 6, Table IV).

Procedures for preparing deoxycholate-washed ribosomes (6), once washed (1) and twice washed (2) standard ribosomes, eight aminoacyl-tRNA's (2), methionyl-tRNA (9, 10), and S100 (1), have been described previously. The only significant modification is an increase in the KCl concentration to 100 mM of the homogenization medium used in the preparation of ribosomes and S100. In addition, if the pH of the initial homogenate was below 6.7, sufficient 1 M HC104 was added to bring the pH to this point.

Ribosomal 40 and 60 S subunits were prepared by dissociation of once-washed ribosomes and were provided by Donald Weeks. A manuscript describing the dissociation method as well as the functional activity of the subunits is in preparation. tRNA_{35S} and tRNA_{m35S} were prepared by resolution of total tRNA on...
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The conditions were those of the initiation factor assay with the addition of 29.7 µg of T1(E) plus 20.7 µg of T2(E) where indicated. In other experiments, T1(E) and T2(E) were tested as single additives with either C or D. No activity was obtained.

<table>
<thead>
<tr>
<th>Components added to preliminary incubation</th>
<th>Amino acid incorporation (p moles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C + D</td>
<td>15.5</td>
</tr>
<tr>
<td>C + T1(E) + T2(E)</td>
<td>0.1</td>
</tr>
<tr>
<td>D + T1(E) + T2(E)</td>
<td>0.1</td>
</tr>
<tr>
<td>C + D + T1(E) + T2(E)</td>
<td>15.5</td>
</tr>
</tbody>
</table>

benzyalted DEAE-cellulose (9) and were a gift of Dr. Elizabeth Keller.

RESULTS

Factor Requirement for TMV-RNA-dependent Amino Acid Incorporation—Fractionation of wheat embryo supernatant on DEAE-cellulose results in four resolved fractions, all of which are required for TMV-RNA-dependent amino acid incorporation (Fig. 1). In the presence of an excess of any three of these components, incorporation is linearly dependent upon the concentration of the limiting fourth factor.

Components C and D Are Initiation Factors—As discussed earlier, the initiation-inhibiting effect of ATA can be used to recognize initiation factors. The reagent can also be utilized to provide an assay allowing the quantitative determination of the initiation factors. The assay involves a preliminary incubation in which an excess of one of the crude components, C or D (see under “Materials and Methods,” “Resolution of Soluble Factors,” Step 1), is added as a reagent, together with a limiting amount of the component being analyzed as a source of the second factor. Subsequently, ATA, aminoacyl-tRNA, and excess supernatant are added and amino acid polymerization is determined. As shown in Fig. 2, with the two resolved factors, C-final and D-final, the extent of amino acid polymerization is linearly dependent upon the concentration of the limiting factor. The remaining resolved factors, T1(E) and T2(E), although absolutely required for messenger-dependent amino acid incorporation (Fig. 1), are completely inactive in the initiation factor assay (Table I). These observations indicate clearly that resolved Factors C and D are indeed initiation factors and suggest, in addition, that Components T1(E) and T2(E) are probably functioning as elongation factors.

In an earlier communication (10) we reported the participation of Met-tRNA in the initiation reaction and demonstrated that such participation could be monitored by the ribosomal binding of Met-tRNA. Table II demonstrates that the latter reaction is messenger-dependent and is quite specific, requiring both initiation factors. No ribosomal binding of Met-tRNA is obtained with Factors T1(E) and T2(E), either alone or in combination with Factor C. In combination with Factor D, some Met-tRNA binding is obtained with Factors T1(E) and T2(E). The nature of this reaction is currently under investigation. It may be noted, however, that the amount of Met-tRNA bound in these incubations (Factor D with elongation factors) is never more than 50% of that obtained with the combination of purified initiation factors.

Fig. 1. Soluble factor requirement for TMV-RNA-dependent amino acid incorporation. The reaction mixture in a final volume of 0.4 ml contains: 25 mM Tris acetate, pH 8, 1 mM ATP, 8 mM disodium creatine phosphate, 16 µM of creatine phosphokinase, 25 µM GTP, 10 µg of TMV-RNA, 2 mM dithiothreitol, ribosomes (once washed, 280 µg of RNA), 3.6 mM MgAc2, 4 mM KCl, 1 mM eight [14C]-amino acids, 60 pmoles of eight [14C]-aminoacyl-tRNA's (1 pmoles = 450 cpm) and soluble factors. In each of the experiments three of the soluble factors were added in excess (C-final, 50 µg; D-final, 114 µg; T1(E), 29.7 µg; T2(E), 20.7 µg) while one of the factors was varied as indicated. After incubating for 15 min at 30°, the radioactive material insoluble in hot trichloroacetic acid was determined as previously described (8). The specific designation of the various factors is explained under “Materials and Methods.” Substituting C* (170 µg) for C-final, results in similar data, i.e. a complete requirement for each of the three factors.

Fig. 2. Dependence of the initiation reaction on factors C and D. The ATA initiation factor assay (see under “Materials and Methods”) was used with the purified factor varied as indicated and the other crude component added in excess.
Initiation factor requirement for Met-tRNA binding to ribosomes

Met-tRNA binding to ribosomes was assayed as under "Materials and Methods" with 170 μg of C* (purified through the second DEAE step, see under "Materials and Methods"). 125 μg of D-final, 29.7 μg of T1(E), and 20.7 μg of T2(E) added as indicated. Unresolved Met-tRNA was used throughout. Control incubations with ribosomes omitted, bound approximately 0.1 pmole of Met-tRNA under all conditions. Since the primary analysis is that of mRNA-dependent Met-tRNA binding, the nonribosomal blank is not subtracted and the data obtained are presented as of Met-tRNA.

Factor specificity in elongation factor assay

The incubation was the standard elongation factor assay with 157 μg of C*, 110 μg of D-final, 29.7 μg of T1(E), and 20.7 μg of T2(E) added where indicated. The assay was performed at 4.5 mM Mg2+ with the addition of either 176 μg of C*, 115 μg of D-final, 29.7 μg of T1(E), or 20.7 μg of T2(E). The data are corrected for nonenzymatic binding (0.4 pmole).

<table>
<thead>
<tr>
<th>Soluble factors added</th>
<th>Phenylalanine polymerised</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1(E) + T2(E)</td>
<td>7.3</td>
</tr>
<tr>
<td>T1(E)</td>
<td>0.1</td>
</tr>
<tr>
<td>T2(E)</td>
<td>0.2</td>
</tr>
<tr>
<td>T1(E) + C*</td>
<td>0.3</td>
</tr>
<tr>
<td>T1(E) + D-final</td>
<td>0.2</td>
</tr>
<tr>
<td>T2(E) + C*</td>
<td>0.3</td>
</tr>
<tr>
<td>T2(E) + D-final</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Factor requirement for ribosome binding of Met-tRNA and Met-tRNA

The experimental conditions are identical with Table II with 31 pmol of Met-tRNA or Met-tRNA, or 51 pmol of eight aminoacyl-tRNA's added as indicated. Aurintricarboxylic acid, when added, was 3 x 10⁻⁶ M.

<table>
<thead>
<tr>
<th>Factor added</th>
<th>Met-tRNA bound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ TMV-tRNA</td>
</tr>
<tr>
<td>None</td>
<td>0.2</td>
</tr>
<tr>
<td>C* + D-final</td>
<td>0.20</td>
</tr>
<tr>
<td>C*, C* + T1(E), C* + T2(E)</td>
<td>0.17-0.22</td>
</tr>
<tr>
<td>T1(E) + T2(E)</td>
<td>0.17</td>
</tr>
<tr>
<td>D-final + T1(E)</td>
<td>0.79</td>
</tr>
<tr>
<td>D-final + T2(E)</td>
<td>0.51</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Factor added</th>
<th>Met-tRNA bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Met-tRNA</td>
<td>C* + D-final</td>
<td>1.45</td>
</tr>
<tr>
<td>Met-tRNA</td>
<td>C*, C* + T1(E), C* + T2(E)</td>
<td>0.19-0.28</td>
</tr>
<tr>
<td>Met-tRNA</td>
<td>D-final</td>
<td>0.35</td>
</tr>
<tr>
<td>Met-tRNA</td>
<td>ATP</td>
<td>0.32</td>
</tr>
<tr>
<td>Met-tRNA (ATA)</td>
<td>C* + D-final</td>
<td>0.23</td>
</tr>
<tr>
<td>Met-tRNA</td>
<td>C* + D-final</td>
<td>0.42</td>
</tr>
<tr>
<td>Met-tRNA</td>
<td>C*, C* + T1(E), C* + T2(E)</td>
<td>0.14-0.17</td>
</tr>
<tr>
<td>Met-tRNA (no ATP)</td>
<td>C* + D-final</td>
<td>0.18</td>
</tr>
<tr>
<td>Met-tRNA (ATA)</td>
<td>C* + D-final</td>
<td>0.24</td>
</tr>
<tr>
<td>Eight aminoacyl-tRNA's</td>
<td>C* + D-final</td>
<td>0.50</td>
</tr>
</tbody>
</table>

When the Met-tRNA ribosome binding reaction was tested with the two separated species, the initiating Met-tRNA, and the internal Met-tRNA, the TMV-RNA-dependent binding reaction occurred predominantly with the initiating species (Table III), again requiring both of the soluble initiation factors, C and D. There was, however, also a small but reproducible TMV-RNA-dependent binding of the internal species of Met-tRNA.
factor activity. As may be noted in Fig. 3, the supernatant is also apparent that at least an appreciable fraction of the initiation factors have been found primarily, if not exclusively, with the lines present endogenously in the S100 fraction.

In preliminary experiments, however, we have observed the existence of a third initiation factor which, in contrast to C and D, is associated primarily with the ribosomes.

Methionyl-tRNA binding to ribosomes at low Mg\textsuperscript{2+} concentration appears to be a primary criterion of initiation factor activity. This reaction has also been reported for the reticulocyte system (25), although the requirement for the simultaneous presence of both factors in this latter system is not as complete as with wheat embryo. In the bacterial reaction analogous to eucaryotic Met-tRNA binding, Factor F2 alone catalyzes considerable fMet-tRNA binding to ribosomes particularly at low temperature (26, 27). We have examined the wheat embryo Met-tRNA-binding reaction under a variety of conditions, finding in all cases an absolute requirement for both initiation factors. The wheat embryo reaction differs from the bacterial reaction in one other regard, the requirement for ATP in addition to GTP (2, 10).

W. Klein and J. Clark\textsuperscript{a} have recently found that in\textit{vitro} translation of the RNA of satellite tobacco necrosis virus in the wheat embryo system results in the synthesis of satellite virus coat, thereby establishing the translational fidelity of the system. Experiments in our laboratory\textsuperscript{b} have shown that the formation of the messenger-ribosome initiation complex and the binding of Met-tRNA take place on the 40 S ribosomal subunit. Subsequent peptide synthesis is totally dependent on the 60 S subunit. Taken together with the present publication which defines the function of the soluble factors, it would appear that the basic requirements for protein chain initiation in the wheat embryo system have now been established. Elucidation of the mechanisms of the partial reactions as well as the analysis of sites of regulatory influences remain as future problems.

**Acknowledgment**—The authors acknowledge Donald Weeks for his valuable suggestions and discussions.

**REFERENCES**


\textsuperscript{a} Personal communication.

\textsuperscript{b} D. P. Weeks, and S. N. Seal, manuscript in preparation.
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