Interferon, Double-stranded RNA, and RNA Degradation

ISOLATION OF HOMOGENEOUS pppA(2'5')A - synthetase from Ehrlich ascites tumor cells

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pppA(2'5')A - synthetase (2'5')(A), synthetase is one of the mediators of interferon action. On activation by double-stranded RNA, it converts ATP into (2'5')(A); in turn, (2'5')(A), activates an endonuclease (RNase L) which cleaves single-stranded RNA. We report a simple procedure for the isolation of pure (2'5')(A), synthetase from interferon-treated Ehrlich ascites tumor cells. The procedure involves differential precipitation of the ribosomal salt wash fraction with ammonium sulfate and chromatography on DEAE-cellulose and CM-cellulose. The apparent molecular weight of the enzyme is 105,000 as determined by gel electrophoresis in sodium dodecyl sulfate and about 85,000 when determined by centrifugation through a glycerol gradient. The size range of the (2'5')(A), produced by the enzyme extends from the dimer to at least the pentadecamer.

Interferons are glycoproteins produced by a large variety of vertebrate cells in response to viral infection or some other stimuli. They are secreted, interact with other cells, and alter the biochemical and immunological characteristics of these in various ways (1, 2). Exposure of cells to interferon results in the enhancement of the accumulation of particular mRNAs and proteins (3-7). Moreover, as determined in experiments with cell extracts, the level of certain enzyme activities is enhanced in cells treated with interferons. At least some of these enzymes (e.g., an endonuclease system and a protein kinase system) remain latent unless activated by dsRNA and ATP (8-12). Earlier, the endonuclease system (13) was divided into two complementary fractions (DE1,NT and DE2,NT) by differential precipitation with ammonium sulfate and chromatography on DEAE-cellulose. When incubated with dsRNA and ATP, DE1,NT was found to give rise to a thermostable product of low molecular weight. This in turn was found to activate a latent endonuclease (now designated RNase L) in DE2,NT (Ref. 14, see also Refs. 15 and 16). The thermostable product was identified as (2'5')(A), a series of compounds originally discovered as inhibitors of protein synthesis formed in extracts of interferon-treated cells in the presence of dsRNA and ATP (17). (2'5')(A), synthetases have been found in extracts of interferon-treated Ehrlich ascites tumor, L, HeLa, and chicken cells and rabbit reticulocytes (not exposed to exogenous interferon) (14, 15, 18-21). The enzyme from L cells has been partially purified by affinity chromatography on dsRNA (10). Exogenous (2'5')(A), has been introduced into cells and was found to cause a transient nuclease activation, a transient decrease of protein synthesis, and an impairment of virus replication (22-24).

Here we present a simple procedure for the purification to homogeneity of (2'5')(A), synthetase from Ehrlich ascites tumor cells treated with interferon and report on some of the characteristics of the enzyme and its products.

EXPERIMENTAL PROCEDURES

Assay for (2'5')(A), Synthetase

In Solution Assay—The reaction mixtures contained 17 mM Hepes (pH 7.5), 100 mM KCl, 4 mM Mg(OAc)2, 30 mM 2-mercaptoethanol, 1 mM EDTA, 10% (v/v) glycerol, 1 mM [32P]ATP (specific activity, 10 to 40 Ci/mmol), 5 µg/ml of poly(I).poly(C) (Miles), as well as the fraction to be assayed, and were incubated at 30°C for 0 to 18 h. Thereafter, the reaction mixtures were heated at 95°C for 3 min to inactivate the enzymes, cooled, and clarified by a 15-s centrifugation in the microfuge. To convert unreacted ATP to ADP, aliquots of the clarified solutions were supplemented with an equal volume of a second solution to give a final concentration of 0.1 µg/ml of hexokinase (EC 2.7.1.1, Sigma, type VI, 75 units/mg), 10 mM glucose, and 5 mM Mg(OAc)2 and were incubated at 30°C for 30 min. Aliquots of 2 µl were then chromatographed on PEI-cellulose thin layer plates (Polygram Cel 300 PEI, Brinkmann) in 0.75 M potassium phosphate (pH 3.5). The radioactive spots corresponding to (2'5')(A), were located by autoradiography, eluted with 1 M HCl, and counted in a liquid scintillation counter.

Poly(I).Poly(C) Paper Assay—Poly(I).poly(C) paper was prepared as described by Stark et al. (21). (2'5')(A), synthetase in 150 µl of Buffer A (10 mM Hepes (pH 7.5), 1.5 mM Mg(OAc)2, 7 mM 2-mercaptoethanol, 20% (v/v) glycerol) was bound to a piece (0.8 × 0.8 cm) of poly(I).poly(C) paper by incubation at 25°C for 1 h. The paper was washed three times with Buffer A supplemented with 90 mM KCl and soaked in Buffer A supplemented with 50 mM KCl at room temperature for 30 min. The paper-bound enzyme was incubated in a 700-µl reaction mixture containing 10 mM Hepes (pH 7.5), 8.5 mM Mg(OAc)2, 50 mM KCl, 7 mM 2-mercaptoethanol, 20% (v/v) glycerol, and 1 mM [32P]ATP (specific activity, 10 to 40 Ci/mmol) at 30°C for 10 to 20 h. Aliquots of the incubated reaction mixtures were treated with hexokinase and tested for (2'5')(A), as described under “In Solution Assay.”

Using pure (2'5')(A), synthetase, the rate of (2'5')(A), synthesis is about 30-fold faster in the “in solution assay” than in the “poly(I).poly(C) paper assay” (not shown). However, impure enzyme preparations may contain (2'5')(A), cleaving enzyme and other nucleases cleaving (2'5')(A),. In the “in solution assay,” all these remain present during the incubation. Thus, the assay registers the balance between (2'5')(A), synthesis and cleavage. In the “poly(I).poly(C) paper assay,” the (2'5')(A), synthetase is first bound to dsRNA, immobilized on paper. Subsequently, by washing the paper, the proteins with little or no affinity to dsRNA (including (2'5')(A), cleaving enzymes) are removed. Consequently, this is the preferred method of assay.

These findings were presented at the Conference on Regulatory Function of Interferons of the New York Academy of Sciences, New York, October, 1979.
Isolation of (2'-5')(A)ₙ, Synthetase

In the course of both assays, the products of the reactions catalyzed by (2'-5')(A)ₙ synthetase are treated with hexokinase and glucose to convert residual ATP to ADP. This is necessary, because in the chromatographic procedure used, (2'-5')(A)ₙ is separated from ADP and the residual ATP is converted to ADP. This is necessary, as in the cleaving (2'-5')(A)ₙ, the activity of the S-30 fraction is taken as 100%.

The increase in total activity upon differential precipitation with ammonium sulfate is thought to be due to the removal of inhibitors of (2'-5')(A)ₙ synthetase, enzymes degrading (2'-5')(A)ₙ, etc.

The 0 to 35% ammonium sulfate fraction is a side product in the purification of another protein (25). Since we have much of this fraction available, we used it as a starting material for isolating (2'-5')(A)ₙ. If this had been unavailable, we would have directly prepared the 30 to 40% ammonium sulfate fraction from the ribosomal wash fraction.

The activity in the S-30 fraction is taken as 100%. The purity of the S-30 fraction is taken as 1.

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**Purification of (2'-5')(A)ₙ, Synthetase**

The low speed (30,000 x g) supernatant fraction (S30) from the extract of Ehrlich ascites tumor cells grown in suspension culture was prepared by published procedures (13) except that the growth medium contained 3% calf serum (instead of 7% fetal calf serum) and 0.1% glucose and the cells were treated with 500 NIH mouse reference standard units of interferon (specific activity, 2 x 10⁸ units/mg of protein/ml of medium). The ribosomal wash fraction was prepared from the S30, further fractionated by precipitation with 38% ammonium sulfate, and the resulting precipitate was stored in liquid nitrogen according to published procedures (25) (619 mg of protein, 27.5 ml). Upon thawing, this was found to contain some flocculent precipitate. The latter was removed by centrifugation at 9000 x g for 10 min. The clarified solution was further fractionated with ammonium sulfate at pH 8.2. The material precipitating at 30% saturation was sedimented and discarded. The material precipitating at 40% saturation was sedimented, dissolved in Buffer B (10 mM Tris-Cl (pH 8.5), 30 mM 2-mercaptoethanol, 1 mM EDTA, 30 mM PMSF, 10% (v/v) glycerol), and dialyzed against Buffer B. The resulting solution (145 mg of protein and 18.1 ml) was applied to a DEAE-cellulose (Whatman DE52) column (20 x 2.4 cm) which had been equilibrated with Buffer B. After being washed with Buffer B, the column was eluted with a 1.1-liter linear KCl gradient (0 to 200 mM) in Buffer B. Fractions of 9 ml were collected and tested using the "poly(I)-poly(C) paper assay." Fractions containing the enzyme (peak eluting at 40% KCl) were pooled (6.7 mg of protein in 190 ml) and the pH was adjusted to 7.5 by adding 88 ml of 20 mM Hepes (pH 6.8). The resulting solution was applied to a CM-cellulose column (5 x 1.8 cm) which had been equilibrated with Buffer C (17 mM Hepes (pH 7.5), 30 mM KCl, 4 mM Mg(OAc)₂, 30 mM 2-mercaptoethanol, 1 mM EDTA, 10 mM PMSF, and 10% (v/v) glycerol). After being washed with Buffer C, the column was eluted with a 130-mL linear KCl gradient (30 mM to 250 mM) in Buffer C. Fractions of 2.5 ml were collected and tested by the "poly(I)-poly(C) paper assay" and the "in solution assay." The peak fractions containing the purified enzyme (eluting at 150 mM KCl) were pooled (0.4 mg of protein in 22 ml). A portion of this material was concentrated by binding to a small CM-cellulose column and elution with 200 mM KCl. Protein was determined according to Lowry et al. (26).

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Specific activity*</th>
<th>Yield of activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>nmol AMP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-30</td>
<td>12,300 mg</td>
<td>0.96</td>
<td>100%</td>
<td>1'</td>
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<td>Ribosomal wash</td>
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<td>5.1</td>
<td>121*</td>
<td>5.3</td>
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<tr>
<td>(NH₄)₂SO₄ 0-40%</td>
<td>610 mg</td>
<td>29</td>
<td>150*</td>
<td>30</td>
</tr>
<tr>
<td>28% (NH₄)₂SO₄</td>
<td>145 mg</td>
<td>53</td>
<td>65</td>
<td>55</td>
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<td>490</td>
<td>28</td>
<td>510</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>0.44 mg</td>
<td>2,400</td>
<td>2</td>
<td>2,500</td>
</tr>
</tbody>
</table>

"The "poly(I)-poly(C) paper assay" was used to determine enzyme activity.

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The purity of the S-30 fraction is taken as 1.

**Characteristics of (2'-5')(A)ₙ, Produced by Purified (2'-5')(A)ₙ, Synthetase**

**Fig. 1. Analysis of the purified (2'-5')(A)ₙ, synthetase by polyacrylamide gel electrophoresis in the presence of SDS.**

**Track 1, 0.2 µg of the purified enzyme was analyzed according to published procedures (27) by electrophoresis in polyacrylamide gel (containing 12.5% acrylamide) in the presence of SDS and the gel was stained with Coomassie brilliant blue R250. Track 2, a second aliquot of the purified enzyme was labeled with ³²P. For this purpose, 40 µl of ³²P-Bolton-Hunter reagent (28; Anbersham; 2 µCi/ml, 1600 Ci/mmol) was evaporated to dryness in a stream of air, dissolved immediately in 5 µl of 0.2 M sodium borate (pH 8.5), and supplemented with a 10-µl aliquot of the purified enzyme. The reaction mixture was incubated at 0°C for 15 min, supplemented with 5 µl of 20 mM Tris-Cl (pH 7.5), further incubated at 0°C for 10 min, and finally supplemented with 50 µl of SDS gel sample buffer (80 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 10% (v/v) glycerol, and 0.001% bromphenol blue). After electrophoresis as above, the labeled protein was visualized by radioautography using Kodak XR-1 film.

**Characteristics of (2'-5')(A)ₙ, Produced by Purified (2'-5')(A)ₙ, Synthetase: Cleavage with Various Enzymes and Alkali (to Fig. 4)**

(2'-5')(A)ₙ, was prepared by incubating 0.2 µg of purified (2'-5')(A)ₙ, synthetase with [³²P]-ATP and dsRNA in a 100-µl reaction mixture for 17 h. The reaction mixture was treated with hexokinase and glucose and was fractionated by chromatography on PEI-cellulose plates in 0.75 M potassium phosphate (pH 3.5). (2'-5')(A)ₙ, was eluted with 30% triethylamine carbonate (pH 4.5). The activities of (2'-5')(A)ₙ, and RNase T₁ were demonstrated by showing that the enzymes cleaved 3'-5' linked ApApApApAp; not shown).
for 19 h and neutralized with HCl. The chromatographic analyses of the products of the above treatments are shown in Fig. 4.

RESULTS AND DISCUSSION

Purification and Characterization of (2'-5')(A)n, Synthetase—The bulk (at least 79%) of the (2'-5')(A)n synthetase activity in an extract of interferon-treated Ehrlich ascites tumor cells is in the ribosomal wash fraction in our conditions. The enzyme in this fraction was further purified by differential precipitation with ammonium sulfate, chromatography on DEAE-cellulose, and finally chromatography on CM-cellulose (Table I). The enzyme eluted from CM-cellulose is apparently homogeneous: both gel electrophoresis in SDS followed by staining with Coomassie brilliant blue (Fig. 1, Track 1) and labeling with [32P]I followed by gel electrophoresis in SDS and radioautography (Fig. 1, Track 2) reveal a single protein of Mr = 105,000. The identity of (2'-5')(A)n synthetase with the Mr = 105,000 protein is supported by the fact that the enzyme activity and the Mr = 105,000 protein co-chromatograph on CM-cellulose (Fig. 2, left panel) and co-sediment in a glycerol density gradient (Fig. 2, right panel).

To establish the apparent Mr of the enzyme under non-denaturing conditions, its sedimentation velocity (through the glycerol gradient) was compared to those of standard proteins. The results indicate an apparent Mr of 85,000. This, taken together with the result of the Mr determination by gel electrophoresis in SDS probably means that at least before binding to dsRNA, the native enzyme is a monomer.

The purified (2'-5')(A)n synthetase is dependent on dsRNA for activity. This is demonstrated by the data in Fig. 3 which also illustrate the assay procedure. The enzyme is incubated in the "in solution assay" with [α-32P]ATP in the presence or absence of dsRNA. Thereafter, aliquots are treated (or not treated) with hexokinase and glucose. This treatment converts ATP to ADP but has no effect on (2'-5')(A)n. Finally, the reaction mixtures are analyzed by thin layer chromatography. It can be seen that only when dsRNA is present together with the (2'-5')(A)n synthetase is (2'-5')(A)n produced. The results also indicate that the bulk of the ATP (in this experiment over 95%) can be converted into (2'-5')(A)n.

Characterization of the Product of the Purified (2'-5')(A)n, Synthetase—To verify that the purified enzyme makes authentic (2'-5')(A)n, we subjected the products of the enzyme to various tests. First we established that, as would be expected of (2'-5')(A)n, the products activate RNase L (not shown).

Furthermore, as expected, the products yield 5'-AMP on digestion with snake venom diesterase (Fig. 4, Track 2) but are resistant to RNase P1 and T1, enzymes which do not cleave (2'-5') phosphodiester linkages (Tracks 3 and 4) (29). Treatment with bacterial alkaline phosphatase yields inorganic phosphate and a material migrating in the position expected for dephosphorylated (2'-5')(A)n (Core, Track 5). Alkaline digestion results in the formation of 2'-AMP, 3'-AMP, ppAp, and a small amount of ppAp. This indicates that while most
Isolation of (2'-5') (A)n, Synthetase

of the (2'-5')(A)n, does indeed have a 5'-terminal triphosphate, a small proportion has the 5'-terminal diphosphate on DEAE-cellulose plates (Cell HR/DEAE, Inc.). We are still investigating how the diphosphate-terminated products of these treatments, together with markers if so indicated, were analyzed by chromatography on PEI-cellulose plates in 0.75 M potassium phosphate (pH 3.5) (Tracks 1 to 6) or 4 M acetic acid (Track 7) or homochromatography on DEAE-cellulose plates (Cel HR/DEAE, Inc.) (Tracks 8 to 12). The locations of other markers in the chromatograms are indicated. The numbers to the left of the spots in Track 7 and Tracks 8 to 12 indicate the presumed number of adenylate residues in the adjacent (2'-5')(A)n spots. For further details see "Experimental Procedures." It is of interest that the size of the (2'-5')(A)n synthetase from mouse cells (i.e., 105,000 daltons) is different from that (56,000 daltons) reported for a partially purified (2'-5')(A)n synthetase from chicken cells.

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REFERENCES


