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Binding of RNA by the alfalfa mosaic virus movement protein is biphasic

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The movement protein of alfalfa mosaic virus was expressed in *Escherichia coli* and purified by cation exchange chromatography. The purified protein bound single-stranded RNA cooperatively in a biphasic manner. At protein saturation, RNA/protein complexes (designated 'primary complexes') were detected by a nitrocellulose-retention assay within 1 min of mixing, both at 4 and 22°C. In contrast, an incubation of 30 min at 22°C was necessary to obtain electrophoretically retarded complexes ('stabilized complexes'), containing a large number of protein molecules bound stably to each molecule of RNA. Stabilization did not take place at 4°C. The rate of formation of the primary complexes was strongly dependent on protein concentration, and thus appeared limited by a bimolecular interaction. In contrast, the rate of stabilization was independent of protein concentration, suggesting that this process consisted of a rearrangement of the primary complexes without binding of additional protein molecules. In agreement with this suggestion, the amount of complexed RNA at equilibrium was the same when assayed by nitrocellulose retention and by electrophoretic retardation. The possibility that these peculiar kinetics could be caused by the presence of Tween 20 in the incubation media is discussed.

Cell-to-cell movement; Alfalfa mosaic virus; Nucleic acid binding; Protein expression; *Escherichia coli*

1. INTRODUCTION

The alfalfa mosaic virus (AMV) movement protein encoded by RNA3 (P3, 300 amino acids) was shown earlier to bind single-stranded nucleic acids in vitro [1]. Three other viral movement proteins, those of tobacco mosaic virus [2,3], cauliflower mosaic virus [4] and red clover necrotic mosaic virus [5], share this property with the AMV movement protein but show slight variations in the affinity for each type of ligand. We investigated the nucleic acid binding properties of P3 in more detail, using an improved method of preparation based on the expression of the protein in *Escherichia coli* rather than in *Saccharomyces cerevisiae*.

2. MATERIALS AND METHODS

2.1. *Protein expression in* E. coli

We used the expression vector pET3d [6], a kind gift from Dr. W.F. Studier. The complete P3 open reading frame (ORF), obtained from M13mp7.P3S [1] as a *Sal*I–*Bam*HI fragment, was mutagenized with the pSelect system (Promega Biotec) to create an *Nco*I restriction site (5'CCATGG3') at the translational initiation site to facilitate its insertion into the expression vector. The ORF was retrieved from pSelect as an *Nco*I–*Bam*HI fragment (919 bp) and inserted between the *Nco*I and the *Bam*HI sites of pET3d. The recombinant vector was cloned into *E. coli* strain BL21(DE3) in the presence of 50 μg/ml ampicillin. Cloning techniques were as described [7]. Colonies were selected for expression of a P3-related protein after induction with isopropyl-β-D-thiogalacto-pyranoside (IPTG) on the basis of in situ immunochemical detection.

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2.2. *In situ immunochemical detection of expressing clones*

The colonies obtained on selective plates were transferred to a nitrocellulose sheet. The sheet was placed on an agar plate containing Luria-Bertani (LB) medium, 150 μg/ml ampicillin and 0.4 mM IPTG and incubated for 3 h at 37°C. The bacteria were exposed to chloroform vapor for 30 min and lysed in situ in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 5 mM $MgCl_2$, 40 mg/ml lysozyme, 1 μg/ml deoxyribonuclease I and 30 μg/ml bovine serum albumin (technique adapted from [8]). The sheet was then washed twice with PBS (0.15 M NaCl, 1.5 mM KH_2PO_4, 7.9 mM Na_2HPO_4, pH 7.5) containing 1% Tween 20 and colonies synthesizing P3 were visualized as in the immunoblotting procedure.

2.3. *Preparation of the P3-related proteins*

BL21(DE3) cells carrying the recombinant pET3d vectors were grown overnight at 37°C in 20 ml LB medium containing 150 μg/ml ampicillin. The culture was diluted twofold with LB medium and another 150 μg/ml of ampicillin was added to make up for the loss due to the β-lactamase released during overnight growth. IPTG (0.4 mM) was added to induce expression of the chimeric gene and the cells were grown for another 3 h.

The bacteria were harvested by centrifugation and resuspended in 5 ml buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol, 10 mM EDTA, 10 mM 2-mercaptoethanol) containing 0.4 mg/ml lysozyme and 1 mM phenylmethylsulfonyl fluoride (PMSF). After 20 min of incubation at 37°C, they were submitted to 3 or 4 freeze–thaw cycles. The inclusion bodies and cell debris were pelleted by centrifugation at 5,000 × *g* for 10 min and re-extracted with 1.5 ml buffer A, then with 1.5 ml buffer B (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM 2-mercaptoethanol) containing 1% Nonidet P40 and 1 mM PMSF, then washed twice with 1.5 ml buffer C (50 mM sodium acetate, pH 5.5, 10 mM 2-mercaptoethanol). The recombinant protein was solubilized with 1.5 ml buffer C containing 6 M urea, purified by chromatography on carboxymethyl Trisacryl and renatured as described [1]. It was stored at −80°C in 50% glycerol. The concentration

of the purified protein was determined by SDS-PAGE of an aliquot in parallel with a known amount of bovine serum albumin, and absorption scanning of the Coomassie brillant blue-stained gel at 600 nm.

2.4. *Analysis of proteins by SDS-PAGE and immunoblotting*

The proteins were separated by SDS-PAGE in 9 or 10% gels [9], electrotransferred to nitrocellulose and immunoreacted with a rabbit antiserum against the yeast-expressed protein (anti-P3) [10], diluted 3,000-fold. Bound antibody was visualized with the Immuno-Select kit of Gibco BRL.

2.5. *Nucleic acid binding assays*

A synthetic ^{32}P-labeled RNA3 (2.1 kb) was prepared as in [11]. The mixture of RNA and protein was incubated in buffer D (final concentrations: 12 mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 0.1% Tween 20, 7.5% glycerol) and the binding was assayed either by electrophoretic retardation in a 1% agarose gel prepared in low-salt buffer (6.6 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, 1 mM EDTA), or by nitrocellulose retention, as described in [1]. The gels and the nitrocellulose sheets were autoradiographed. Quantitation of the assays was by absorption scanning of the autoradiograms at 600 nm.

3. RESULTS AND DISCUSSION

3.1. *Expression of P3 in* E. coli

From SDS-PAGE and immunoblotting analysis of the crude extract (not shown), it appeared that the recombinant bacteria expressed a protein which reacted specifically with the anti-P3 serum and amounted to more than 50% of total cellular protein. One hundred-times as much recombinant protein was produced in a given volume of culture medium by *E. coli* as by *S. cerevisiae* [1]. This protein, designated P3-E, precipitated at low speed with inclusion bodies and was not solubilized by buffers containing non-ionic detergents (Tween 20, Triton X-100 or Nonidet P40). It was solubilized by a buffer containing 6 M urea, then purified by cation-exchange chromatography. The resulting sample was at least 95% pure, as shown by SDS-PAGE and photodensitometry of the Coomassie brillant blue-stained gel (Fig. 1, lane 1). Some of the faster-migrating contaminants may be degradation products of P3-E because they reacted with the anti-P3 serum after being transferred to nitrocellulose (lane 2). In addition, the immunoblot revealed that P3-E migrated slightly more slowly than the movement protein induced in tobacco by viral infection (lane 3) or than the yeast-expressed P3 (lane 4; this protein is designated P3-Y). The very slight difference of mobility, which corresponded to an apparent molecular mass difference of less than 0.5 kDa, was observed repeatedly. It might be an indication that the protein made by *E. coli* was slightly different from that made by yeast or tobacco. However, the two recombinant proteins had the same ion-exchange behavior (not shown), suggesting that no charge difference was involved. One of the possible explanations would be that the N-terminus of the protein was processed differently in *E. coli* and in the two eukaryotes. Since the penultimate residue of P3 is glutamic acid, the initiator methionine is probably not removed in *E. coli* [12]. In contrast, in eukaryotes the methionine could be acetylated, then cleaved off by an acyl amino acid hydrolase [13]. The nature of the N-termini of P3-E and P3-Y is being investigated.

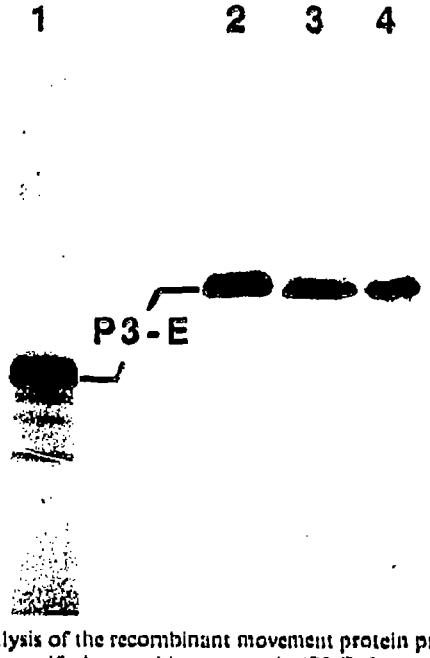

Fig. 1. Analysis of the recombinant movement protein prepared from *E. coli*. The purified recombinant protein (P3-E; 3 μg) was analysed by SDS-PAGE in a 9% gel and stained with Coomassie brillant blue (lane 1). In another experiment, 5 ng of P3-E were immunoblotted after SDS-PAGE in a 10% gel (lane 2), in parallel with the 30,000-*g* pellet prepared as described [16] from 20 mg of AMV-infected tobacco leaves (lane 3), and with 2 ng of purified P3-Y [1] (lane 4).

3.2. *Nucleic acid binding properties of the recombinant movement protein*

Fig. 2 shows the electrophoretic retardation of a fixed amount of ^{32}P-labeled RNA by increasing amounts of P3-E. The fact that the radioactive material migrated either as free or as completely retarded RNA (without any intermediate) indicates that the binding was highly cooperative. This is at variance with a similar experi-

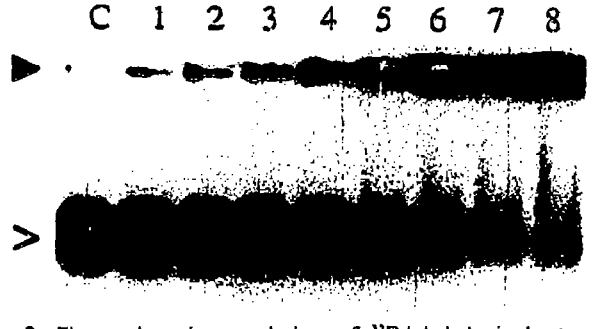

Fig. 2. Electrophoretic retardation of ^{32}P-labeled single-stranded RNA by increasing amounts of P3-E. The ^{32}P-labeled RNA probe (1 ng in 10 μl) was incubated for 1 h at 22°C with 0, 0.4, 0.8, 1.6, 3.1, 6.2, 12.5, 25 or 50 ng P3-E (respectively in lanes C, 1–8) and submitted to agarose gel electrophoresis, then the gel was autoradiographed. The open and closed triangles indicate, respectively, the positions of free and completely retarded RNA.

ment done with P3-Y [1], in which complexes of intermediate mobility were detected. The variation in apparent degree of cooperativity is not due to the 10-fold difference in RNA concentration between the two experiments (1 ng/μl in [1] vs. 0.1 ng/μl in Fig. 2) because a similar experiment (not shown) done with P3-E and 1 ng/μl of RNA revealed the same degree of cooperativity as seen in Fig. 2. Rather, the variation may be related to the physical or chemical difference between P3-E and P3-Y which causes the slight difference of electrophoretic mobility. The highly cooperative complexes made by P3 are likely to be metastable, as are those made by the single-strand binding protein of *E. coli* [14], and their rate of evolution toward equilibrium complexes may depend in a subtle way on the conformation of the protein. It is conceivable that this evolution was faster with P3-Y than with P3-E, explaining why such complexes were not detected with the former.

P3-E had the same preference for single-stranded nucleic acids and the same lack of sequence specificity as P3-Y [1] when tested by competition, using the nitrocellulose retention assay (not shown).

The kinetics of binding of P3-E to single-stranded RNA were analysed by electrophoretic retardation and by nitrocellulose retention, on ice (4°C) and at room temperature (22°C). Interestingly, these two techniques gave quite different results at high protein concentration (5 ng/μl; Fig. 3a and b). At 4 as well as 22°C, the labeled RNA was completely retained by nitrocellulose within 1 min of mixing with the protein. In contrast, an incubation of 30 min was required to obtain complete

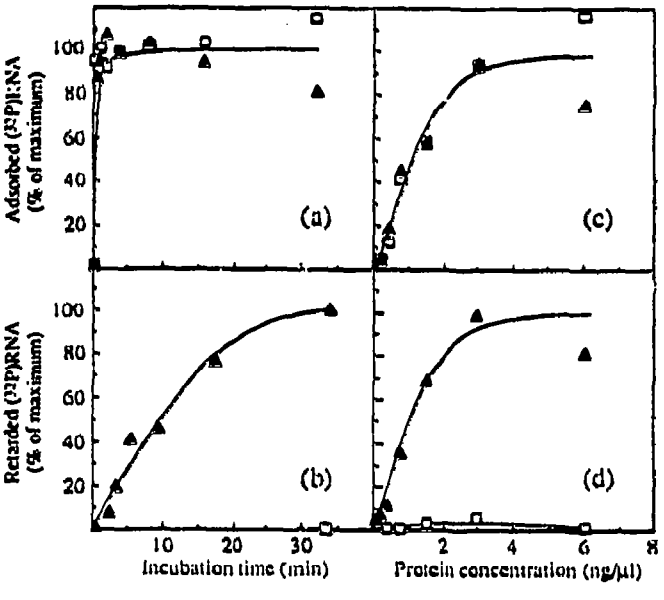

Fig. 3. Kinetics and protein saturation of the binding of P3-E to RNA, assayed by nitrocellulose retention and by retardation electrophoresis. The ^{32}P-labelled RNA3 (0.4 ng in 10 μl) was incubated with purified P3-E at 22°C (▲) or 4°C (□), and the binding was assayed by nitrocellulose retention (a and c) and by retardation electrophoresis (b and d). In (a) and (b), the protein concentration was 5 ng/μl; in (c) and (d), the incubation time was 1 h.

retardation of the labeled RNA at 22°C, and no retardation was detected at 4°C even after 2 h incubation. It is thus clear that the binding of P3-E to RNA is biphasic. We designate the interaction detected by the nitrocellulose-retention assay as 'primary interaction', and the process leading to the formation of completely retarded complexes as 'stabilization'. The primary interaction between protein and RNA could be made slower by lowering the protein concentration, showing that it was kinetically limited by a bimolecular process. Indeed, with 0.6 ng/μl P3-E, its half-time was about 4 min at 22°C and 15 min at 4°C (not shown). In contrast, the rate of stabilization appeared independent of protein concentration. At 22°C in the presence of 0.6 ng/μl protein, its half-time (measured after a pre-incubation of 1 h at 4°C) was about 10 min, i.e. was nearly the same as in the presence of 5 ng/μl protein (Fig. 3b). Thus the primary interaction seems to consist of a simple collision-limited process which does not require a large activation energy, whereas the stabilization process appears as an energy-requiring evolution of the preformed complexes.

The amount of complexed RNA was measured as a function of protein concentration by the two methods, after a 1 h incubation period at 22 or 4°C. At 22°C, half-maximal binding was observed at the same concentration (ca. 1 ng/μl) with the two methods, and the saturation curves obtained at the two temperatures with the nitrocellulose retention assay were almost superimposable (Fig. 3c and d). The similarity of the saturation curves obtained at 22°C indicates that the two methods detected the same type of complexes, i.e. the completely retarded, highly cooperative complexes of Fig. 2. If non-cooperative complexes were present at this temperature they were not stable enough to survive either assay. The identity of the titration curves obtained at 4 and 22°C by the nitrocellulose retention method suggests that, even though the complexes made at 4°C were less stable than those made at 22°C, they contained the same proportion of protein and RNA. This result is in agreement with the fact that the rate of stabilization did not depend on protein concentration.

Any physical interpretation of these kinetics must take into account the fact that the incubation media contained 0.1% Tween 20. Since the critical micellar concentration of this detergent is 0.006% at room temperature [15] (and probably much less at 4°C), most of the protein must have been associated with detergent micelles. Thus we suspect that a large part of the primary interaction was between RNA molecules and micelles carrying several protein molecules. The resulting primary complexes did not consist of RNA bound to single protein molecules, but rather of RNA molecules wrapped around protein-containing micelles. Stabilization of those complexes required the breakage of hydrophobic bonds between protein and detergent as a prerequisite to the establishment of the proper protein-

protein interactions, which explains the large effect of temperature.

It can of course be argued that what is observed in vitro in the presence of detergent is not relevant to the in vivo function of the protein. However, it should be remembered that the movement protein of AMV has never been detected in the soluble fraction of cell extracts, but rather is associated with membranes and cell walls [10,16,17]. It is therefore possible that the exchange of protein between a micellar (or membrane-associated) state and an RNA-bound state happens in vivo as well as in vitro.

In contrast with our results with the AMV movement protein, the binding of single-stranded nucleic acids by the TMV movement protein was shown to be cooperative at 4°C, both in the absence [2] and in the presence of non-ionic detergent [3]. Thus, even though both proteins make cooperative complexes with single-stranded nucleic acids, their kinetics of interaction with these ligands are significantly different.

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