The Nature of the Catalytic Domain of 2′-5′-Oligoadenylate Synthetases*

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2′-5′-Oligoadenylate (2-5(A)) synthetases are a family of interferon-induced enzymes that are activated by double-stranded RNA. To understand why, unlike other DNA and RNA polymerases, they catalyze 2′-5′ instead of 3′-5′ phosphodiester bond formation, we used molecular modeling to compare the structure of the catalytic domain of DNA polymerase β (pol β) to that of a region of the P69 isozyme of 2-5(A) synthetase. Although the primary sequence identity is low, like pol β, P69 can assume an αβαββ structure in this region. Moreover, mutation of the three Asp residues of P69, which correspond to the three catalytic site Asp residues of pol β, inactivated the enzyme without affecting its substrate and activator binding capacity, providing further credence to the concept that this region is the catalytic domain of P69. This domain is highly conserved among all 2-5(A) synthetase isozymes. Biochemical and mutational studies demonstrated that dimerization of the P69 protein is required for its enzyme activity. However, a dimer containing a wild type subunit and an inactive catalytic domain mutant subunit was also active. The rate of catalysis of the heterodimer was half of that of the wild type homodimer, although the two proteins bound double-stranded RNA and ATP equally well.

The 2′-5′-oligoadenylate (2-5(A)) synthetases are a family of enzymes that share many properties. Their synthesis is induced upon interferon treatment of cells, and they participate in mediating the antiviral and antacellular effects of interferons (1). All of these enzymes are inactive, as such, and are activated by an essential co-factor, double-stranded (ds) RNA. The activated enzymes polymerize ATP to produce 2′-5′-linked oligoadenylates (2, 3). The known isozymes of 2-5(A) synthetases are grouped into three size classes: small, medium, and large. Members of the small and the medium classes have been assigned membership in the Class I nucleotidyl transferases. Other members of this class include DNA polymerase β (pol β), terminal deoxynucleotidyl transferase, and poly(A) polymerase. Although the members of this superfamily catalyze the same reaction, namely, the coupling of a nucleoside triphosphate to a free hydroxyl group via elimination of a pyrophosphate, there is only a weak conservation of their amino acid sequences (16). However, an extremely well conserved structural motif present at their catalytic domains became apparent when the crystal structures of a number of polymerases were solved (17, 18). This structural motif consists of four or five β strands packed against two α-helices. In all of them, the catalytic site is created by a triad of aspartates present at the tips of two conserved β strands. These residues coordinate the Mg2+ ions necessary for catalysis (18). Although in some cases the aspartates can be replaced by glutamates, in others any substitution causes inactivation of the enzyme (19–24).

Unlike other members of the superfamily, the 2-5(A) synthetases catalyze the formation of 2′-5′ instead of 3′-5′ phosphodiester bonds. Like poly(A) polymerases and terminal deoxynucleotidyl transferases, they are also template-independent. But unlike the latter enzymes, 2-5(A) synthetases can use mononucleotides instead of DNA or RNA as primers or acceptors for the nucleotidyl transferase reaction. Moreover, unlike other polymerases, 2-5(A) synthetases require dsRNA as a co-factor. In view of the above differences, we investigated the nature of the catalytic domain of 2-5(A) synthetases. Molecular modeling suggested that the putative catalytic domain of the P69 isozyme may have a structure very similar to that of the pol β catalytic domain. Moreover, mutation of the three Asp residues that may constitute the putative catalytic site inactivated P69. However, such a mutant of P69 could bind ATP and dsRNA and, like the wild type (wt) protein, it was a dimer. We used this mutant to demonstrate that although a P69 monomer is inactive, a P69 dimer containing one active and another inactive subunit is enzymatically active.

EXPERIMENTAL PROCEDURES

Materials—Imidazole, phenylmethylsulfonyl fluoride, β-mercaptoethanol, Triton X-100, P1, P2-diadenosine-5′)-triphosphate (ApppA), guanidine hydrochloride, and the protease inhibitors pepstatin, leupeptin, and aprotinin were purchased from Sigma. The hydrogenated Triton X-100 used for purifying protein for fluorescence spectroscopy was from Calbiochem. Ni-NTA-agarose was from Qiagen. Poly(I)poly(C)-agarose was from Amersham Pharmacia Biotech, and anti-FLAG M2 monoclonal antibody-agarose was from Kodak. Restriction enzymes and

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† The abbreviations used are: 2-5(A); 2′-5′-oligoadenylate; ds, double-stranded; poly(I)poly(C); polyinosinic acid-polycytidylic acid; wt, wild type; ApppA, P1, P2-diadenosine-5′)-triphosphate; pol β, polymerase β; m.o.i., multiplicity of infection.
nucleotide triphosphates were from Roche Molecular Biochemicals, and radioactive ATP and dATP (α-32P]ATP/dATP) were from NEN Life Science Products. Primers used for mutagenesis were from Operon Technologies. The protein cross-linking reagent dimethyl suberimidate was purchased from Pierce.

**Protein Cross-linking and Molecular Modeling of P69 Catalytic Domain—**Amino acid sequences of DNA polymerase β (Swiss-Prot accession no. P06766) and P69 (Swiss-Prot accession no. P29728) were aligned interactively for the polymerase active center using the Homology module of Insight II (Molecular Simulations Inc.). The secondary structure prediction for P69 was done using the PHDsec program at Predictprotein server (25). Molecular modeling of P69 native protein was carried out using Insight II. The catalytic domain of Rat DNA polymerase β (amino acids 148–264) (26) [Protein Data Bank code 1BPB] was taken as a template to construct the backbone conformation of the catalytic domain of P69 (amino acids 360–489). Secondary structure analysis showed that the two α-helices and five β strands were conserved among pol β-related polymerases and 2-5(A) synthetases. We thus treated the predicted α-helix and β strand regions of P69 as structurally conserved regions, and the backbone conformations of these regions were determined by copying the coordinates of the corresponding backbone atoms of pol β. The other parts of the molecule were treated as loops. The structures of these loops were determined by searching the protein conformation data base in the Insight II software. When good candidates were not found in the database, a form-generating procedure, which satisfied all geometric constraints of the other regions of the protein, was used to build the loop structure. The best 10 conformations for each loop were displayed and selected manually to find the most suitable ones. The side chains were adjusted to remove bumps to give a preliminary model. Energy minimizations were carried out to refine this structure.

Amino acid sequences for the proposed polymerase domain of various isoforms of 2-5(A) synthetases (Swiss-Prot accession numbers, with the exception of chicken, are as follows: P69, P29728; mouse L3, P11928; rat, Q05961; human E18, P04820; mouse 92-2, P29081; chicken, GenBank accession no. AB002585; and P900AS, AJ225089) were aligned using the ClustalW program (27) with default parameters. The alignment output was emblabled according to their predicted secondary structure.

**Expression and Purification of wt P69 and Its Mutants—**The expression and purification of wt P69 protein have been described previously (11). Mutants were generated by mega-primer PCR method using appropriate oligonucleotide primers. Mutants were expressed as carboxy-terminal FLAG-tagged proteins and purified using anti-FLAG M2 monoclonal antibody. Beads were washed twice with binding buffer (300 mM NaCl, 20 mM Tris-Cl, pH 7.5, 5 mM β-mercaptoethanol, 0.2% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) and incubated with cell extracts for 4 h at 4 °C on a rotary shaker. After binding, beads were washed five times with the binding buffer for 15 min each. Beads were eluted from the beads with binding buffer containing 20 mM Tris-Cl, pH 7.5, 0.5 mM dithiothreitol for 20 min. Finally, beads were resuspended in 20 mM Tris-Cl, pH 7.5, 20 mM magnesium acetate, 2.5 mM dithiothreitol. One-twelfth of the bead suspension was boiled in SDS-polyacrylamide gel electrophoresis loading buffer and analyzed by Western blot using an antibody against hexahistidine (Santa Cruz Biotechnology). The quantities of hexahistidine-tagged proteins were determined by densitometric scanning, normalized in all samples, and 2-5(A) synthetase assay was performed.

**Protein Cross-linking—**Purified P69 was chemically cross-linked by performing a protein–DNA cross-linking procedure as follows. Twelve ng of purified P69 was cross-linked in 100-, 50-, 20-, and 10-μl volumes with 1 mM dimethyl suberimidate in cross-linking buffer (10 mM HEPES (pH 8.0), 125 mM NaCl, 0.5 mM dithiothreitol) at 25 °C for 15 min. At the end of incubation, the reaction was stopped by adding 1 mM glycine to a concentration of 100 mM. Samples were then denatured by boiling in Laemmli buffer for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using anti-(His)₆ antibody. The amounts of dimer and monomer were quantified by densitometric scanning of the autoradiogram.

**Size Fractionation Analysis—**The molecular mass values of purified native P69 and DADA mutant proteins were determined by gel filtration chromatography as described earlier (11).

**RESULTS**

**A Model for the Structure of the Putative Catalytic Domain of 2-5(A) Synthetase—**The three-dimensional crystal structures of several DNA and RNA polymerases have been solved; among them is DNA pol β (26). Its active center is in the palm domain, a discrete domain that consists of two α-helices and five β strands. The primary sequence of this region of pol β (residues 148–264) was compared with that of the P69 isozyme of 2-5(A) synthetase to find a region in the latter protein that has the maximum structural homology (Fig. 1A). Residues 360–489 of the P69 aligned optimally. Although the primary sequences of the two proteins were conserved by only about 25%, the predicted secondary structures were highly conserved. Moreover, three aspartate residues (Asp-190, Asp-192, and Asp-256) of pol β, which form its active site, were perfectly conserved in the P69 protein at positions 408, 410, and 481. To examine whether the three-dimensional structure of this region of P69 mirrors that of the pol β palm domain, molecular modeling studies were done. The structure of the palm domain of pol β was used as the template, and the corresponding P69 structure was built. As shown in Fig. 1B, both contained αβαβαββ structures that were similarly spaced. The two α-helices were antiparallel, and β strands 1 and 4 pointed in one direction, whereas β strands 2, 3, and 5 were antiparallel to them. The three aspartate residues that form the catalytic triad in pol β were placed at very similar positions in P69; Asp-408 and Asp-410 were directly before and directly after the beginning of strand 5. The excitation and emission wavelengths were 290 and 322 nm, and the excitation and emission slit widths were 3 and 5 nm, respectively. ATP, dATP, or ApppA were added to the sample and incubated at room temperature for 5 min, and the fluorescence intensity was measured. Fluorescence intensities were corrected for inner filter effect due to ATP, dATP, or ApppA using the following equation:

\[ F = F_{\text{obs}} - F_{\text{ads}} \times \text{extinction} \]

Preparation and Characterization of Heterodimer—Heterodimers of wt and DADA mutant proteins were expressed by infecting high (H5) cells with recombinant baculovirus carrying wt P69 (m.o.i. 20), wt P69, and DADA mutant (m.o.i. 10 each), DADA mutant (m.o.i. 20) or aprotinin. The H5 cells were infected with recombinant baculovirus carrying wt P69 (m.o.i. 20), wt P69, and DADA mutant (m.o.i. 20) or aprotinin. 

Infection of the primary structures of other isoforms of 2-5(A) synthetase revealed that the sequence of the putative catalytic domain of P69 is considerably conserved among them (Fig. 2). The arrangements of the α-helices and the β strands are conserved even more so. More importantly, the Asp residues that may form the putative active site of P69 were conserved in identical positions in all isoenzymes suggesting that the same catalytic domain may be used by all members of this family of
enzymes. There has been speculation that the P69 gene arose by duplication of a small synthetase gene, and hence the P69 protein may contain two catalytic domains (6). However, comparison of the sequence of the carboxyl-terminal catalytic domain of P69 with that of the corresponding amino-terminal domain revealed that, despite strong sequence conservation, two Asp residues of the putative active site of the carboxyl-terminal catalytic domain are not present in the amino-terminal domain. Thus, it appears from this analysis that P69 contains one, not two, catalytic domains. All of the small isozymes also contain one catalytic domain. In contrast, the 2-5(A) synthetase-related protein P59 (29, 30) does not contain the critical Asp residues, thus accounting for its lack of activity.

Functional Testing of the Putative Active Site—Three Asp residues in the catalytic domain of pol β form the active site. These residues are thought to coordinate two Mg ions and catalyze the nucleotidyl transferase activity. Mutation of any of these Asp residues causes drastic inactivation of the enzyme (21, 23). To test whether the corresponding Asp residues in P69, Asp-408, Asp-410, and Asp-481 form the active site of the catalytic triad, we carried out site-directed mutagenesis to replace the relevant Asp residues in P69 with Ala. Two mutants were generated: DADA and DADA mutant of 9-2, expressed in insect cells, was also inactive (Fig. 3B). These results suggest that, like pol β, the three mutated Asp residues form the catalytic triad of P69. To extend this conclusion to other isozymes of 2-5(A) synthetase, two of the three corresponding Asp residues in the small isozyme 9-2, Asp-75 and Asp-77 (see Fig. 2), were replaced by Ala. As anticipated, the resultant mutant to catalyze the 2-5(A) formation was expressed in insect cells using the baculovirus system (10, 11), and the expressed proteins were purified to apparent homogeneity using affinity matrices (Fig. 3A). When the purified proteins were tested for enzyme activity, the wt protein was highly active but the mutants were completely inactive (Fig. 3B). The basis of the inability of these mutants to bind dsRNA was assessed in the experiments (Fig. 4A). The ability of the two proteins to bind dsRNA was assessed in the experiments shown in Fig. 4B, and both proteins were found to bind equally...
well to poly(I)-poly(C)-agarose. ATP can serve as both the acceptor and the donor of AMP residues polymerized by the enzyme reaction, and the ability of the wt and DADA proteins to bind ATP was measured in the experiment shown in Fig. 5A. The assay measured ATP-mediated fluorescence quenching of the two proteins at different ATP concentrations. It should be noted that no dsRNA was present in the reaction mixtures; thus, no enzymatic turnover of ATP took place during the measurements. From the data presented in Fig. 5A, the dissociation constants for ATP were calculated to be 5.6 mM for the wt protein and 8.2 mM for the mutant. To distinguish the binding of the acceptor from that of the donor, we did similar analyses using ApppA and dATP in place of ATP. Because ApppA can serve as the acceptor, but not the donor, of the reaction catalyzed by the 2-5(A) synthetases (7, 31), we assumed that ApppA-binding would reflect the substrate binding to the acceptor site only. This analysis showed that the dissociation constant of ApppA was 2.8 mM for the wt protein and 7.1 mM for the mutant protein (Fig. 5B). Reciprocally, dATP can serve as the donor but not as the acceptor because of the absence of the 2'-OH group (see Fig. 9 below). The dissociation constants of dATP was 6.17 mM for the wt protein and 6.81 mM for the mutant protein (Fig. 5C). These experiments showed that there was only a modest effect of the DADA mutation on the binding affinity of the substrate at either the acceptor or the donor site.

Effects of Subunit Composition of P69 on Its Enzyme Activity—We have previously shown that mutation of three specific residues of the 9-2 isozyme affects its oligomerization and consequently its enzyme activity (13). In the experiment shown in Fig. 6A, we investigated the effects of similar mutation in P69. As shown in Fig. 6A, the relevant residues of 9-2, Cys-331, Phe-332, and Lys-332 are conserved in the carboxyl-terminal half of P69 in positions 668, 669, and 670 but not in the amino-terminal half. When the CFK residues in P69 were replaced by Ala, the resulting mutant protein, CAFAKA, was
primarily a monomer (Fig. 6B). Thus, the same three residues mediate oligomerization of both the 9-2 and P69 isozymes. As anticipated, the P69 CAFAKA mutant was enzymatically inactive (Fig. 6C), suggesting the need of dimerization for enzyme activity of the protein.

This requirement was confirmed by a protein cross-linking experiment done at different concentrations of the wt protein. As shown in Fig. 7A, the amount of dimeric protein increased with increasing concentrations of P69. The dissociation constant for dimerization, as determined from this experiment, was 5.1 nM. Enzyme activities were also determined at the same concentrations of protein (Fig. 7B). The enzyme activity increased linearly with the dimer concentration. As a result, the specific activity expressed as enzyme activity per unit of dimer remained relatively constant over the concentration range tested (Table I). Once we established that dimeric, but not monomeric, P69 is active, we wondered whether both subunits of P69 required functional catalytic domains to maintain the activity. To address this question, we prepared a heterodimer of wt and DADA P69 and assayed its enzyme activity (Fig. 8). For this purpose, insect cells were co-infected with viruses encoding His-tagged wt P69 and FLAG-tagged DADA P69, and proteins were immunoprecipitated from the extracts using anti-FLAG antibody. As shown in Fig. 8A, no P69 was precipitated from cells producing only His-tagged wt P69 (lane 1), whereas a wt P69 tagged with both His and FLAG precipitated very well (lane 4). From cells co-infected with viruses encoding His-tagged wt protein and FLAG-tagged DADA protein, the FLAG-antibody immunoprecipitated a complex containing the His-tagged wt protein (lane 2). If cells were infected only with the virus encoding the FLAG-tagged DADA mutant, as expected, no wt protein co-precipitated (lane 3). Thus, the immunoprecipitates from the four experiments analyzed in lanes 1-4 (Fig. 8A) contained no wt or mutant P69 (lane 1), heterodimers of the wt and the mutant protein plus homodimers of the mutant protein (lane 2), only homodimers of the mutant protein (lane 3) and only homodimers of the wt protein (lane 4). The enzyme activities of these precipitates are shown in Fig. 8B. As expected, no activity was observed in lanes 1 and 3, and the precipitate in lane 4 was active. Surprisingly, the precipitate in lane 2 was active as well, thus demonstrating that a P69 dimer containing only one active subunit is enzymatically active. In the experiment shown in Fig. 8C, we compared the activities of the wt homodimer and the wt/DADA heterodimer at different concentrations. The two proteins were quantitated by Western blot analysis, similar to the one shown in Fig. 8A, and increasing amounts of the two proteins were tested for enzyme activity.
using conditions under which the reaction velocities were proportional to enzyme concentrations and the initial reaction rates were measured (11). Using several concentrations of each protein, we observed that the activity was similarly dependent on the concentrations of both. But the heterodimer was only half as active as the homodimer.

The properties of the wt/DADA heterodimer were more rigorously compared with those of the wt homodimer in experiments shown in Fig. 9. Using the same amounts of the two proteins, the dependence of enzyme activity on ATP concentration was measured (Fig. 9A). The results showed that they had similar \( K_m \) values, but the \( V_{\text{max}} \) and \( k_{\text{cat}} \) values were about one-half of those for the wt homodimer (Table II). To discriminate between the acceptor site and the donor site, both of which binds ATP, we resorted to using two different substrates in combination. \( \text{ApppA} \) can serve as an acceptor and receive 2'–5'–A residues from ATP (7, 31). We observed that 2'-deoxyATP can substitute for ATP as the donor (Fig. 9B, lane 2). As expected, because of the lack of a 2'–OH group, dATP cannot, however, be an acceptor, and unlike ATP, it cannot by itself be polymerized by the enzyme (Fig. 9B, lane 1). Thus, a combination of ApppA and dATP can serve as substrate but neither is sufficient alone. Two products were obtained under these conditions; one presumably contain three adenosine residues and the other, four. When we determined the kinetic constants of the enzyme using the new assay with increasing concentrations of ApppA and a fixed amount of radiolabeled dATP, it was apparent that the acceptor site binding affinities of the substrate were very similar for the wt homodimer and the wt/DADA heterodimer proteins (Table II). Again, the heterodimer was only half as active in catalyzing the polymerization reaction compared with the wt protein. Note that ATP is a better substrate than the ApppA plus dATP combination for both enzymes, as manifested by the 3-fold higher \( k_{\text{cat}} \) values.

**DISCUSSION**

Our molecular modeling studies suggest a strong similarity in the structures of the catalytic domains of 2-5(A) synthetases and pol \( \beta \). Unlike several other polymerases, in these enzymes, the two \( \beta \) strands containing the active site are parallel, not antiparallel (18). Moreover, the single Asp residue of the active site is carboxyl-terminal to the other two Asp residues in the primary structures of both proteins, and in both of them the other two Asp residues are separated by one residue, although in many other enzymes of this class they are adjacent (discussed in detail in Ref. 32). Studies with pol \( \beta \) and other members of the nucleotidyl transference superfAMILY have revealed that the three carboxylates of the catalytic site coordinate two \( \text{Mg}^{2+} \) ions, which in turn coordinate the \( \alpha \)-phosphate and the 3'-hydroxyl group. The activated hydroxyl group acts as the nucleophile in the phosphodiester bond formation causing an inversion of the stereochemistry at the \( \alpha \)-phosphorous of the donor nucleotide (33, 34). It remains to be seen whether the same mechanism applies to 2-5(A) synthesis. Even if the general mechanism of catalysis is the same, it remains to be explained why the 2'-hydroxyl, but not the 3'-hydroxyl, of the acceptor molecule participates in the reaction catalyzed by these enzymes. The determination of a high resolution structure of the catalytic domain of P69 may reveal specific structural features that distinguish it from other polymerases and thus provide a structural basis for favoring the 2'-5' bond formation.

We have developed several new assays, as reported here, for monitoring the substrate binding and the catalytic activity of

**TABLE I**

| Protein concentration | Percent dimer | Dissociation constant | Enzyme activity | Specific activity |
|-----------------------|--------------|-----------------------|-----------------|------------------|
| \( \text{nM} \)       | \( \text{nm} \) |                        | \( \text{nm} \) |                  |
| 15.0                  | 66           | 5.2                   | 0.457           | 92               |
| 7.5                   | 56           | 4.9                   | 0.181           | 85               |
| 3.0                   | 38           | 5.9                   | 0.068           | 118              |
| 1.5                   | 31           | 4.5                   | 0.022           | 95               |
| 0.75                  | 19           | 5.12                  | 0.008           | 114              |

* a Expressed in terms of monomeric concentration using a molecular mass of 80 kDa.
* b All are experimental values except for the value at 0.75 nm concentration, which is calculated assuming a 5.12 nm dissociation constant.
* c The top four values are calculated using dimer percentages obtained experimentally. The mean of the four values is 5.12 nm, which is used for the lowest concentration.
* d All are experimental values, expressed as \( \mu \text{mol} \) of ATP polymerized/ml.
* e Specific activity expressed as pmol of ATP polymerized/pmol of dimer.

**FIG. 8.** Purification of the wt/DADA heterodimer and its activity assay. A, H5 cells were infected with different recombinant baculoviruses singly or in combinations as described under “Experimental Procedures.” Cell extracts were immunoprecipitated with anti-FLAG antibody and Western blotted with anti-(His)_6 antibody. Lane 1, (His)_6 wt; lane 2, (His)_6 wt and DADA-FLAG; lane 3, DADA-FLAG; and lane 4, (His)_6 wt-FLAG. The co-immunoprecipitation of (His)_6 wt in lane 2 shows that in vivo it forms heterodimers with the DADA mutant protein. B, activity assay of the immunoprecipitated samples. The 2-5(A) production in lane 2 shows that the heterodimer of the wt and DADA mutant proteins is as active as the wt homodimer protein (lane 4). C, concentration dependence of the wt and the heterodimer activities. An activity assay of the immunoprecipitated wt P69 ((His)_6 wt-FLAG) and heterodimer ((His)_6 wt and DADA-FLAG) was done at several concentrations as described under “Experimental Procedures.” Radioactive products were analyzed by gel electrophoresis, quantified, and expressed as the amount of ATP polymerized.
FIG. 9. Enzymatic characterization of the wt/DADA heterodimer. A. Determination of ATP $K_m$ for wt and heterodimer. Activity assays of of wt homodimer (2 μg/ml) and wt/DADA heterodimer (2 μg/ml) were done for 4 h in the presence of increasing concentrations of ATP as described earlier (35). Products were analyzed by electrophoresis, quantified, and plotted as μmol of ATP polymerized/ml. B. Use of ApppA and dATP as substrates for P69 reaction. For each lane, 2 μg/ml of P69 was incubated for 4 h in a standard reaction condition with 5 mM dATP, 5 μCi of [α-32P]dATP, and varying amounts of ApppA (lane 1, 0; lane 2, 1 μM). Products were analyzed by electrophoresis. C. Determination of ApppA $K_m$ for wt and heterodimer. Activity assays of of wt homodimer (2 μg/ml) and wt/DADA heterodimer (2 μg/ml) were done for 4 h in the presence of 5 mM dATP and increasing concentrations of ApppA as described above. Products were analyzed by electrophoresis, quantified, and plotted as μmol of dATP polymerized/ml.

| Table II | Properties of wt homodimer and wt/DADA heterodimer |
|----------|-----------------------------------------------------|
|          | wt homodimer                                      | wt/DADA heterodimer |
|          | $K_m$ μM | $V_{max}$ μmol/ml | $k_{cat}$ s$^{-1}$ | $K_m$ μM | $V_{max}$ μmol/ml | $k_{cat}$ s$^{-1}$ |
| ATP      | 2.18    | 1.2             | 5                | 2.09    | 0.54             | 2.2             |
| ApppA    | 0.75    | 0.48            | 2                | 0.8     | 0.25             | 1               |

* Enzyme concentration was calculated with a monomeric molecular mass value of 80 kDa.

P69. In the conventional assay for 2-5(A) synthetase enzyme activity, ATP is used both as the initial acceptor and the universal donor for the 2′-5′ phosphodiester bond formation. For obvious experimental reasons, we wanted to use two different nucleotides as the donor and the acceptor so that they could be manipulated independently. ApppA was selected as the acceptor because its 5′-OH groups are blocked and it cannot serve as a donor. Conversely, 2′-dATP was a good donor of the phosphate group, but it could not accept the same because of the absence of a 2′-OH group. Thus, ApppA and 2′-deoxyATP were a good pair of substrates for the enzyme reaction. An added advantage was that the products were restricted to only two kinds of molecules with one or two AMP moieties attached to ApppA. Moreover, it seems that ApppA not only fails to serve as a donor but also does not compete with dATP to serve as a donor because from the concentration curve of ApppA (Fig. 9C and data not shown), it appears that even at high concentrations of ApppA the reaction was not inhibited. In contrast, when a fixed amount of ApppA was used, excess dATP (more than 5 mM) inhibited the reaction (data not shown) indicating that dATP may have a low affinity for the acceptor site as well. However, at 5 mM and lower concentrations of ApppA and dATP, the two reagents did not interfere with each other’s functions, although the rate of reaction was three times lower than that observed with ATP alone.

For monitoring substrates binding to the P69 protein, we have developed a new assay using fluorescence quenching. Although an indirect assay, it is fast, convenient, and quantitative. This assay was used to determine the affinity constants of ATP for P69. The relatively weak affinity, with a dissociation constant of 5.6 mM, was predictable from the need of a high ATP concentration for the optimum enzyme activity (11). The affinity of dATP, presumably for the donor site, was very similar to that of ATP (Fig. 5), but the affinity of ApppA for the acceptor site was slightly higher. It should be noted, however, that in coming to the above conclusions, we assumed that the binding of the substrates at the acceptor and the donor sites of the protein generates the same degree of fluorescence quenching. The fluorescence quenching assay, although convenient, is not amenable for studying competitive binding. For example, we could not use this assay to directly demonstrate that dATP does not interfere with the binding of ApppA and vice versa.

Different assays using radioligands and differentially mutated proteins need to be developed for this purpose.

The DADA mutant of P69 was found to be completely inactive enzymatically. It, however, dimerized and bound dsRNA and the substrates almost as efficiently as the wt protein. The D481A mutant was also practically inactive, but when Mg$^{2+}$ was replaced by Mn$^{2+}$ in the reaction mixture, it displayed a very low level of activity (data not shown). Similar observations have been documented for active site point mutants of other polymerases (23, 24). Once we established that the dimerization of P69 is necessary for its enzyme activity, identification of the DADA mutant as an enzymatically inactive protein allowed us to investigate whether both subunits of P69 require functional catalytic domains. The key finding was that the wt/DADA heterodimer was enzymatically active. We were concerned that the observation could be misleading because of the wt subunit dissociating from the DADA subunit bound to the matrix and forming wt homodimer in solution. To rule out this possibility, the matrix-bound proteins were centrifuged down after the reaction, and the supernatant was tested for the
presence of wt P69 by Western blotting and enzyme activity assays. Both assays showed that no P69 protein was present in the solution (data not shown). Given these experimental data and the fact that the wt protein cannot bind to the FLAG-antibody matrix, we concluded that the wt/DADA heterodimer was indeed active. Our results also clearly show that the P69 protein has only one functional catalytic site per subunit. Because the two halves of the protein have strong sequence homology, it has been suggested that each P69 molecule may be composed of two functional subunits (6). The lack of activity of the DADA and the D481A mutant, however, is not compatible with the presence of another catalytic site in the amino-terminal half. Moreover, the Asp triad is not conserved in the corresponding putative active center of the amino-terminal half (see Fig. 2). For pol β, mutational studies have shown that these Asp residues are absolutely required for activity and they cannot be replaced even by Glu residues. Thus, the experimental data strongly indicate that each P69 chain contains only one catalytic site. Similarly, the newly cloned large P100 isozyme appears to have three catalytic domains (35). But homology comparison revealed that only the domain closest to the carboxyl terminus contains the Asp triad (data not shown). Future mutational studies will determine the number of catalytic sites of the large isozyme in a definitive fashion.

Characterization of the enzyme activity of the wt/DADA heterodimer using different substrates allowed us to conclude that it has half of the catalytic activity of the wt homodimer. The simplest interpretation of this result is that the wt protein contains two functional catalytic domains in the two subunits and both of these are simultaneously engaged in 2-5(A) synthesis. Moreover, the two catalytic domains can function independently of each other because the wt/DADA heterodimer is active. Then why is dimerization of P69 necessary for enzyme activity? Although the real reason remains to be determined, there are several possibilities. First, the dimerization process may bring about a conformational change in each subunit that is required for the catalytic domain to be functional. Second, the two subunits may participate in criss-cross reactions in which the substrates bound to one subunit are used by the catalytic domain of the other subunit for polymerization. The latter model, however, will not fit the paradigm of other polymerases such as pol β, which function as monomers and whose catalytic domains use substrates bound to the same protein subunit. Future studies using new P69 mutants defective in acceptor or donor binding sites for complementation of the catalytic domain mutant will be needed for critical testing of the second model. Information generated from such studies will also be useful to the understanding of the mechanism of catalysis by small synthetases, which are tetramers, and the large synthetases, which are monomers. We anticipate that a knowledge of these mechanisms will eventually enable us to explain why the small and large families of 2-5(A) synthetases generate shorter oligomers, primarily dimers, of 2-5(A), whereas the medium isozymes, such as P69, synthesize long chains of 2-5(A) quite efficiently (9–11).