Crisscross Enzymatic Reaction between the Two Molecules in the Active Dimeric P69 Form of the 2′-5′ Oligoadenylate Synthetase*

Received for publication, July 16, 2002, and in revised form, September 6, 2002
Published, JBC Papers in Press, September 9, 2002 DOI 10.1074/jbc.M207126200

Saumendra N. Sarkar, Srabani Pal, and Ganesh C. Sen‡
From the Department of Molecular Biology, The Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195

2′-5′ oligoadenylate (2′-5′ (A)) synthetases are major components of the antiviral pathways induced by interferons. In the presence of double-stranded RNA, they polymerize ATP to form 2′-5′ (A) oligomers that, in turn, activate the latent ribonuclease RNase L, causing mRNA degradation. These enzymes, unlike other nucleotidyl transferases, catalyze 2′-5′, not 3′-5′, phosphodiester bond formation between substrates bound to the acceptor and donor sites. Moreover, unlike other members of this extended family, the P69 isozyme of 2′-5′ (A) synthetase functions as a homodimer. Here, we report that the need for P69 dimerization is because of a crisscross enzyme reaction joining two substrate molecules bound to two opposite subunits. Consequently, although homodimers of mutants in the previously identified acceptor site, the donor site, or the catalytic site were inactive, selective heterodimers of the mutants were active because of subunit complementation. The catalytic site had to be present in the same subunit that contained the acceptor site, whereas the donor site had to be provided by the other subunit. These results allowed us to design a mutant protein that acted as a dominant-negative inhibitor of wt P69 but not of another isozyme of 2′-5′ (A) synthetase.

Interferon-inducible 2′-5′ oligoadenylate (2′-5′ (A)) synthetases are unique enzymes that catalyze 2′-5′ oligoadenylate formation from ATP in the presence of double-stranded RNA (dsRNA) as co-factor (1, 2). 2′-5′ (A) can activate a latent ribonuclease, RNase L, by causing its dimerization (3). Activation of 2′-5′ (A) synthetase enzymes by viral dsRNA causes the synthesis of 2′-5′ (A), activation of RNase L, and degradation of RNA, thus preventing the replication of certain viruses (4, 5). Three major size classes of 2′-5′ (A) synthetases are found in humans: the large form, P100; the medium form, P69; and the small form, P42. In the medium and the small size classes, different isoforms exist because of alternative splicing. The different isoforms of 2′-5′ (A) synthetase are structurally related with extremely high sequence conservation among similar isozymes from different species (6–8). The oligomeric composition of each form is different: the large form is active as a monomeric protein, the medium form as dimeric protein, and the small forms as tetrmeric proteins. It has been shown that dimerization is necessary for the activity of P69 (9).

We are interested in understanding the structure-function relationship of 2′-5′ (A) synthetases and have focused on the P69 isozyme. In this context, we would like to know why it catalyzes 2′-5′ bond formation, why it needs double-stranded RNA as a co-factor, and why it functions as a dimer. It is the last question that was addressed in the current study. Previously, we have identified the catalytic site, the acceptor binding site, and the donor binding site of P69 (10). These sites are highly conserved in other isozymes as well, and mutations in any of these three sites inactivate the enzyme. Here, we report that heterodimers of different mutant molecules are active, indicating that the two subunits of P69 participate in crisscross reactions, catalyzing the joining of two substrates bound to the opposite P69 monomer molecules. This information allowed us to design a dominant-negative mutant of P69 that was isoyme-specific.

MATERIALS AND METHODS

Production, Purification and Enzymatic Characterization of Wild Type and Mutant P69—Site-directed mutagenesis to generate P69 mutants Y421P, R544A, and D408A/D410A and their expression in insect cells using baculovirus vectors has been described (10). The acceptor site mutant, Y421P (A*), was expressed as N-terminal His-tagged (His-A*) or C-terminal FLAG-tagged (A*-FLAG) protein. The donor site mutant, R544A (D*), was expressed as N-terminal His-tagged protein (His-D*). The catalytic site mutant, D408A, D410A (C*), was expressed as C-terminal FLAG-tagged (C*-FLAG) protein (9). The double mutant of P69 (A*D*) was constructed by combining the Y421P (A*) and R544A (D*) mutants and expressed as N-terminal His-tagged protein. We have used three different types of wt P69 constructs for different experiments: N-terminal His-tagged P69 (His-wt), C-terminal FLAG-tagged P69 (wt-FLAG), and N-terminal His-tagged and C-terminal FLAG-tagged P69 (His-wt-FLAG). The small isozyme of 2′-5′ (A) synthetase, 3′–9, was expressed as a C-terminal FLAG-tagged protein (3′–9-FLAG) (11).

The various proteins were purified by affinity chromatography using Ni-chromatography for His-tagged proteins or anti-FLAG antibody for FLAG-tagged proteins or both in succession for doubly tagged proteins (10). Methods for Western blotting, enzyme activity measurements, and enzyme kinetic parameter calculations have been described before (9, 12).

Preparation and Purification of Heterodimers—Different mutant or wt proteins were expressed by simultaneously infecting high five cells with two recombinant baculoviruses carrying expression cassettes for type and mutant P69—Site-directed mutagenesis to generate P69 mutants Y421P, R544A, and D408A/D410A and their expression in insect cells using baculovirus vectors has been described (10). The acceptor site mutant, Y421P (A*), was expressed as N-terminal His-tagged (His-A*) or C-terminal FLAG-tagged (A*-FLAG) protein. The donor site mutant, R544A (D*), was expressed as N-terminal His-tagged protein (His-D*). The catalytic site mutant, D408A, D410A (C*), was expressed as C-terminal FLAG-tagged (C*-FLAG) protein (9). The double mutant of P69 (A*D*) was constructed by combining the Y421P (A*) and R544A (D*) mutants and expressed as N-terminal His-tagged protein. We have used three different types of wt P69 constructs for different experiments: N-terminal His-tagged P69 (His-wt), C-terminal FLAG-tagged P69 (wt-FLAG), and N-terminal His-tagged and C-terminal FLAG-tagged P69 (His-wt-FLAG). The small isozyme of 2′-5′ (A) synthetase, 3′–9, was expressed as a C-terminal FLAG-tagged protein (3′–9-FLAG) (11).

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Preparation and Purification of Heterodimers—Different mutant or wt proteins were expressed by simultaneously infecting high five cells with two recombinant baculoviruses carrying expression cassettes for different proteins. In the case of single infections, we used virus at 20 multiplicity of infection whereas, for co-infection experiments, 10 multiplicity of infection of each virus was used. Depending on experiments, proteins were either purified by Ni-NTA chromatography or anti-FLAG antibody-agarose immunoprecipitation. Ni-NTA binding was performed as described before (12) except that at the end of the washing, instead of eluting the proteins beads were finally washed and resuspended in 20 mM Tris-HCl (pH 7.5), 20 mM magnesium acetate, 2.5 mM dithiothreitol. Part of the bead suspension was then boiled in SDS–PAGE loading buffer and analyzed by Western blotting with anti-FLAG antibody (Santa Cruz Biotechnology). The quantities of FLAG-tagged proteins were determined by densitometric scanning, normalized in all samples, and 2′-5′ (A) synthetase assay was performed with equivalent amounts of protein.

* This research was supported in part by National Institutes of Health Grants CA68782 and CA62220. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept of Molecular Biology/NCC20, The Lerner Research Inst., The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-444-0636; Fax: 216-444-0513; E-mail: seng@ccf.org.

‡ The abbreviations used are: 2′-5′ (A), 2′-5′-oligoadenylate; wt, wild type; Ni-NTA, nickel-nitritrocetic acid.

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This paper is available on line at http://www.jbc.org

Vol. 277, No. 47, Issue of November 22, pp. 44760–44764, 2002

Printed in U.S.A.
of proteins. Anti-FLAG antibody-agarose (Sigma) binding was done with a protocol described earlier (9).

For enzyme activity analysis, heterodimers were purified by a two-step purification process. First, a standard Ni-NTA purification was done (6). The eluted proteins from this purification were dialyzed against FLAG binding buffer (300 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM betamercaptoethanol, 0.2% Triton X-100, and 10% glycerol). Dia-
lyzed protein samples were then applied to the anti-FLAG antibody-agarose column, followed by washing five times with the same buffer. Proteins were eluted from the beads using synthetic FLAG peptide (Sigma) at a concentration of 0.2 mg/ml in the FLAG binding buffer. Purified proteins were then quantified by Coomassie Blue staining and Bradford assay before use in enzyme assay.

RESULTS

Enzyme Activity of P69 Heterodimers Containing a Wild Type Subunit and a Mutant Subunit
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Monomeric wt P69 does not have enzymatic activity, but its association with an inactive P69 molecule carrying a mutation in the catalytic (C) domain restores enzyme activity (9). We wondered whether similar restoration could be achieved by acceptor (A) site and donor (D) site mutants. For this purpose, the wt protein and the test mutant proteins were tagged with different epitopes, and the two proteins were co-expressed in insect cells by infecting them with the respective baculoviral vectors. In the co-infected cells, wt homodimers, mutant homodimers, and wt/mutant heterodimers were formed (Fig. 1A). The heterodimers were segregated from the active wt homodimers by affinity chromatography (Fig. 1B, lanes 2 and 6). A wt homodimer carrying both epitope tags was purified efficiently (Fig. 1B, lanes 4 and 8) and was highly active (Fig. 1C, lanes 4 and 8). In contrast, similarly purified A site mutant (A*) homodimers (Fig. 1B, lane 3) and D site mutant (D*) homodimers (Fig. 1B, lane 7) were enzymatically inactive (Fig. 1C, lanes 3 and 7). The heterodimers of the wt and the mutant proteins, wt/A* (Fig. 1C, lane 2) and wt/D* (Fig. 1C, lane 6) were both active. The compositions of the products produced by the wt/mutant heterodimer (lanes 2 and 6) and the wt/wt homodimer (lanes 4 and 8) were slightly different because the substrate to active enzyme ratios, which affect the 2–5 (A) profile, were different (12). To characterize these heterodimers further, they were purified away from the mutant homodimers, and their enzyme activity in the presence of increasing ATP concentrations was measured. These measurements allowed us to compare the $k_{\text{cat}}$ values of different proteins (Fig. 3). The $k_{\text{cat}}$ values of the different wt/mutant heterodimers were approximately half that of the wt ho-
modimer, indicating that only one catalytic unit was functional in the heterodimers. Under the conditions of the assays, there was no dissociation of the wt subunit from the heterodimers to form wt homodimers. This was tested by immunoprecipitation of the mutant from the purified heterodimer preparation after the assay and testing the supernatant for the presence of wt molecule by activity assay and Western blot (data not shown).

Restoration of Enzyme Activity by Complementation of Inactive Mutant Subunits—The observed enzyme activity of wt/mutant heterodimers can be explained in at least two ways. It is possible that binding of the mutant molecule changes the active site of the wt molecule. This was tested by immunoprecipitation of the mutant from the purified heterodimer preparation after the assay and testing the supernatant for the presence of wt molecule by activity assay and Western blot (data not shown).

Different heterodimers containing two mutant subunits had different properties: A*/D* (lane 4) and C*/D* (lane 5) were active, whereas A*/C* was inactive (Fig. 2B, lane 3). Thus, in two of three combinations, there was restoration of enzyme activity by complementation of two inactive molecules. To further characterize the properties of the A*/D* and C*/D* proteins, these heterodimers were purified away from the homodimers by successive affinity chromatography using anti-FLAG antibody-agarose and Ni-agarose, and their activities were compared with that of similarly purified doubly tagged wt protein. The specific activities ($k_{cat}$) of these heterodimers were about half that of the wt protein, indicating that, as anticipated from the crisscross model, only one catalytic unit was functional (Fig. 3). The product profiles and the kinetics of reaction by the two active heterodimers and the wt protein were identical (data not shown).

**Generation of a Dominant-negative Mutant of P69—** Although P69 functions as a dimer, the single site inactive mutants of P69 failed to act as dominant-negative inhibitors of the wt protein (Fig. 1). The reason for this failure became apparent from the results shown in Fig. 2: for producing enzyme activity, the unmutated sites in the inactive molecule acted in conjunction with the site in the wt molecule that was mutated in the inactive molecule. These results, in turn, led to the successful design of a dominant-negative mutant of P69. Our results suggested that if both the acceptor and the donor sites are mutated in one subunit, the mutant protein should act as a dominant-negative inhibitor of the wt protein. This was indeed the case, as shown in Fig. 4. A hexahistidine-tagged double mutant of P69 (A*/D*) was co-expressed with a FLAG-tagged wt protein. The heterodimer was purified by successive affinity chromatography (Fig. 4A, lane 1, inset). As expected, the heterodimer was completely inactive, whereas the wt homodimer was highly active (Fig. 4A). The ability of A*/D* to inhibit the activity of wt P69 in vivo was tested by expressing a fixed amount of the wt protein and increasing amounts of A*/D* and measuring the enzyme activity in cell extracts. Cells expressing the wt protein had a high activity (Fig. 4, B and C, lane 1), whereas there was no activity in cells expressing only A*/D* (Fig. 4, B and C, lane 2). In the cells co-expressing the two proteins, comparable amounts of the wt protein were present (Fig. 4B, lanes 3–5). As increasing amounts of A*/D* were expressed, the enzyme activity diminished gradually (Fig. 4C, lane 2–4).
His-A*D* was purified by Ni-affinity chromatography. Purified proteins were Western blotted with anti-FLAG antibody. His-wt-FLAG and His-A*D* at the ratio of 1:2 plaque-forming units (PFU); wt-FLAG and His-wt-FLAG at the ratio of 1:3 PLFUs; His-A*D* at a ratio of 1:5 (A) synthetase isoform. A*D* was co-expressed with wt P69 or wt 3–9, and enzyme activity in the extracts was measured (Fig. 5). Although, as expected, A*D* inhibited the activity of wt P69 (Fig. 5B, lane 2), it could not inhibit 3–9 activity (Fig. 5B, lanes 3 and 4). This was because A*D* could not interact with 3–9, as revealed by the lack of their co-immunoprecipitation (Fig. 5A, lanes 3 and 4). Thus, although 3–9 and P69 have a high sequence homology (8), their activities could be selectively blocked by A*D* P69.

**DISCUSSION**

Previously, we had identified four functionally important sites of P69, a protein of 687 residues, that are required for its enzyme activity; mutations in any of these sites inactivated the protein. One site, containing the residues 668–670, mediates dimerization of P69 that is essential for its activity (9). Three Asp residues at 408, 410, and 481 are also essential because they form the ‘C’ site, the catalytic triad (9). Recently, using photoaffinity cross-linking of substrates followed by appropriate mutational studies, we have identified two substrate binding sites of this protein. The residue Tyr-421 is essential for binding the acceptor 2–5 (A) or ATP at the acceptor (A) site and the residue Arg-544 is essential for binding the donor ATP molecules at the donor (D) site (10).

Homodimers of P69 containing point mutations in any of the above sites are enzymatically inactive (10). However, heterodimers with one wt molecule and one mutant molecule were active. Moreover, these heterodimers had approximately half the specific activity of that of the wt homodimer (Fig. 3). These observations, along with the mutant complementation results (Fig. 2), led us to propose a model for P69 action (Fig. 6). The central feature of this model is that a dimeric protein is essential for enzyme activity because of the crisscross nature of the enzyme reaction. This implies that one active dimer of the protein simultaneously catalyzes two units of catalysis. In each of these reactions, the catalytic center (C) of the first subunit catalyzes the transfers of an AMP molecule from the donor (D) site to the acceptor (A) site of the second subunit (Fig. 6). In other words, the reaction involves the A and the C sites of one subunit and the D site of the other. In a normal wt/wt heterodimer, two reciprocal reactions proceed, but in the wt/wt or wt/wt mutant heterodimers only one reaction occurs. Consequently, these heterodimers had half the specific activity of the wt protein.
Fig. 6. Crisscross actions of P69 subunits. A model for P69 action. A, C, and D designate the acceptor, catalytic, and donor sites. For clarity, only one of the two 2’-5’ bond formations is shown. However, the model predicts that two sets of crisscross reactions are catalyzed by the two subunits.

(Fig. 3). Note that the model does not permit the A site and the C site to be contributed by the opposite subunits. This restriction reflects the observed failure of the A* and the C* mutants to complement (Fig. 2). It is conceivable that because of the physical proximity of the two sites (residues 408, 410, and 421), a mutation in one site also affects the structure of the other site to make it nonfunctional. In other words, both the A* and the C* mutants were functionally A*C* double mutants. In that case, according to our model, a homodimer containing two A*C* molecules is not expected to be active. In support of the view that mutations in the A site or the C site affect the unmutated site as well, we observed reduced cross-linking of the acceptor to the C* mutant protein (data not shown). Similar observations for DNA polymerases, that mutations in the catalytic site affect substrate binding to the protein, have been made (15). Similar models have also been proposed for ornithine decarboxylase, diacylglycerol kinase, and thymidylate synthetases (15–17). However, a similar mechanism of catalysis has not been noted before for any other member of the class I nucleotidyl transferase superfamily to which P69 belongs (18). It remains to be seen whether this unique feature of P69 contributes to its property of catalyzing the 2’-5’ bond formation. It also remains to be examined whether the general feature of this model holds true for the other size classes of 2’-5’ (A) synthetases. The small isozymes, which appear to be active as tetramers, can easily accommodate some variations of the crisscross model. In contrast, the large isozymes function as a monomer (19, 20). However, because it contains three repeated units of the monomeric sequence (21), it is conceivable that the crisscross reaction in its case is intra-subunit. Future investigations along this line may also reveal the reason for the inability of the large isozyme to synthesize anything longer than a 2’-5’ (A) dimer.

Once the nature of the crisscross reaction became apparent, it was clear that an A*D* mutant should inhibit the activity of the wt protein. As expected, the wt/A*D* heterodimer was completely inactive (Fig. 4A). Moreover, when co-expressed, the mutant was able to inhibit the activity of the wt protein in the cell extract in a dominant-negative fashion. The degree of inhibition increased with increased expressions of the mutant, although the level of the wt protein remained constant (Fig. 4, B and C). This effect was isozyme-specific; the activity of 3’-5’ A*D* because the two proteins did not interact (Fig. 5). These observations now provide us with tools to investigate isozyme-specific functions of 2’-5’ (A) synthetases both in cultured cells and in experimental animals.

Acknowledgment—We thank Karen Toil for secretarial assistance.

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J. Biol. Chem. 2002, 277:44760-44764. doi: 10.1074/jbc.M207126200 originally published online September 9, 2002

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