Identification of the Substrate-binding Sites of 2′-5′-Oligoadenylate Synthetase*

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2′-5′-Oligoadenylate synthetases are interferon-induced enzymes that upon activation by double-stranded RNA polymerize ATP to 2′-5′-linked oligo(adenosine-5′-O)-oligoadenylates. In our continuing effort to understand the mechanism of catalysis by these enzymes, we used photo affinity cross-linking and peptide mapping to identify the substrate-binding sites of the P69 isozyme of human 2′-5′-oligoadenylate synthetases. Radiolabeled azido 2′-5′-oligoadenylate dimers were enzymatically synthesized and used as ligands for cross-linking to the P69 protein by exposure to ultraviolet light. The radiolabeled protein was digested with trypsin, and two ligand-cross-linked peptides were purified by immobilized aluminum affinity chromatography followed by reverse phase high pressure liquid chromatography. The peptides were identified by mass spectrometry and peptide sequencing and were found to correspond to residues 420–425 and 539–547 of P69. To examine the functional importance of the cross-linking sites, specific residues in the two peptides were mutated. When residues in the two sites were mutated individually, ligand cross-linking was selectively eliminated at the mutated site, and the enzyme activity was lost almost completely. Using substrates that can serve either as a donor or as an acceptor but not both, we could identify one of the sites as the acceptor and the other as the donor site.

Interferons are potent cytokines with anti-viral and cell growth modulating properties (1, 2). These effects of interferons are mediated by the interferon-induced proteins (3). Among them are the family of enzymes called 2′-5′-oligoadenylate (2′-5′A) synthetases (4, 5). 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. The 2′-5′A synthetases, in turn, activate a latent ribonuclease, RNase L. Activated RNase L can degrade cellular and viral RNAs and inhibit protein synthesis (6). Three sets of interferon-induced genes encode three size classes of these proteins: small, medium, and large. Members of all of the size classes have been cloned, and their enzymatic properties have been characterized (5, 7). Within each size class, multiple members arise as a result of alternate splicing of the primary transcript. Recently it has been shown that one of the alternatively spliced isoforms of small synthetase can act as a pro-apoptotic protein of the Bcl-2 family (8). The enzymatic properties between the members of three size classes vary considerably in terms of the length of the 2′-5′(A) oligomers synthesized. The small isoforms synthesize only up to hexamers of 2′-5′A (9), whereas the medium isozyme, P69, can synthesize up to 30-mers of 2′-5′A (10). But the large isozyme, P100, makes mostly dimers of 2′-5′A (11). Because dimeric 2′-5′A synthetases cannot activate RNase L, the biological role of P100 remains elusive. Another difference between the three size classes of isozymes is their oligomeric protein compositions. The small isoforms are functional only as tetrameric proteins, whereas the medium isozyme must dimerize for enzymatic activity, and the large isozyme functions as a monomer. We have previously identified specific residues in the carboxyl termini of the small and the medium synthetases, which are required for their oligomerization (12, 13). These residues are absent from P100, which does not require oligomerization for activity.

Previously, we have used sequence comparison, molecular modeling, and site-directed mutagenesis to identify the three aspartic acid residues that constitute the catalytic center of these enzymes (13). In the current study, we have used photo affinity cross-linking of a 2′-5′A analogue followed by peptide mapping in conjunction with point mutagenesis to identify two substrate-binding sites of the P69 isozyme. These sites are conserved in other isoforms as well.

MATERIALS AND METHODS

Preparation of Radiolabeled Azido 2′-5′A—We used purified P69 (10) to prepare a radiolabeled azido 2′-5′A dimer using 8-azido ATP and radioactive dATP. The incubations were done in 50-μl aliquots for 3 h at 30 °C in the presence of 0.3 mg/ml P69, 5 μm 8-azido ATP (ICN), 5 μm dATP (Roche Molecular Biochemicals), 50 μg/ml poly(I)poly(C) (American Biochemicals), 200 μCi of [α-32P]dATP (3000 Ci/mmol, 4 μCi/ml; PerkinElmer Life Sciences). After 3 h, the reaction mixture was heated for 90 s and centrifuged, and the supernatant was collected. HPLC Purification of 2′-5′A—We used pure 2′-5′A (14–16) generated by a P69 reaction were separated on a HPLC using an ion exchange column (Rainin Pure-Gel SAX column; 7-μm particle size; 500-A pore size; 10 mm × 10 cm) and a flow rate of 1 ml/min. HPLC Solvent A was 20 mM HEPES, pH 8.0; Solvent B was the same HEPES buffer containing 800 mM NaCl. The gradient used was: t = 2 min, Solvent B = 0%; t = 2.2 min, Solvent B = 10%; t = 30 min, Solvent B = 40%; and t = 32 min, Solvent B = 100%. 2′-5′A dimer peaks from five HPLC runs (see Fig. 1A) were collected. During the peak elution, the UV lamp was turned off to avoid photo activation of the azido group. Pooled peaks were dialyzed against 20 mM HEPES, pH 8.0, using Spectropor CE Float-A-Lyzer (3000 molecular weight cut off overnight at 4 °C. The dialyzed sample was concentrated in a SpeedVac (Savant Instrument Inc.) followed by another round of dialysis for 4 h. The concentration of radiolabeled azido 2′-5′A was determined by measuring the A250; assuming the ε of 2′-5′A dimer was same as that of ApppA.

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§ The abbreviations used are: 2′-5′A, 2′-5′-oligoadenylate; ds, double-stranded; ApppA, P3′,5′di(adenosine-5′-O)-triphosphate; IAAC, immobilized aluminum affinity chromatography; HPLC, high pressure liquid chromatography; Wt, wild type.

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Radiolabeled Azido 2–5(A) Dimer Cross-linking to P69—The standard cross-linking protocol used 0.83 mM radiolabeled azido 2–5(A) dimer, 0.1 mg/ml P69, 20 mM Tris–Cl, pH 7.5, and 20 mM magnesium acetate, pH 7.5. The reaction mixtures were incubated on ice for 30 min and aliquoted in 25 μl of volume in a Nunc 96-well mini tray. The tray was placed in a dark chamber and the samples were photolyzed for 2 min with 254-nm radiation from a hand-held UV Mineralight lamp (UVP Inc., San Gabriel, CA) at a distance of 4 cm. Following the cross-linking, the samples were boiled for 1 min in SDS-PAGE sample loading buffer and electrophoresed. The protein bands were visualized by Coomassie Blue staining. For analytical experiments, the extent of cross-linking was determined by exposing dried gels to a PhosphorImager screen followed by scanning and quantification by Imagequant software. In the Aппра competition experiment, increasing concentrations of Aппра were present in the reaction mixture during the incubation prior to cross-linking.

Purification of Cross-linked Peptides—For preparative scale cross-linking, 80–100 μg of purified P69 was used in the standard cross-linking reaction. The samples were then electrophoresed in a preparative scale SDS-PAGE followed by Coomassie Blue staining. The radioactive cross-linked protein bands were excised, washed with water, followed by 0.1% trifluoroacetic acid, 60% acetonitrile, cut into small pieces (1 mm³), and immersed in 100 mM NH₄HCO₃ containing 10 mM dithiothreitol. Following reduction for 45 min at 55 °C, dithiothreitol was removed, and the sulfhydryl groups were alkylated with 55 mM iodoacetamide in 10 mM NH₄HCO₃ for 30 min at room temperature. The alkylated samples were washed sequentially with 50% acetonitrile containing 50 mM NH₄HCO₃ and then 10 mM NH₄HCO₃ for 30 min each. Following the wash, the gel pieces were vacuum dried and rehydrated in 10 mM NH₄HCO₃ containing 10 μg/ml trypsin (Promega) and incubated overnight at 37 °C. The peptides were extracted from gels with 0.1% trifluoroacetic acid, 60% acetonitrile (more than 80% of the initial radioactivity was recovered from the gel pieces). The peptide solution was then partially dried to remove acetonitrile and diluted with 1 mM NaCl, 1% ammonium acetate, pH 5.8, for aluminum affinity chromatography.

Immobilized Aluminum Affinity Chromatography was done using metal chelating Sepharose (Amersham Biosciences) loaded with aluminum (14). 2.5 ml of metal chelating Sepharose was packed in a 1.5 × 12-cm disposable column and washed several times with water. The column was charged with 30 μl of 50 mM AlCl₃. Before loading the peptides, the column was equilibrated with 30 μl of IAAAC running buffer (1 mM NaCl, 1% ammonium acetate, pH 5.8). Samples diluted in 5 μl of IAAAC running buffer were loaded on the column, and the unbound peptides were washed with 30 μl of IAAAC running buffer. A brief, 5-μl wash of 0.1% ammonium acetate was applied to reduce the NaCl concentration in the eluted peptides. The bound peptides were eluted from the column with 15 μl of 10 mM K₂HPO₄, pH 7.3. Throughout the chromatography, all of the eluents from the column were collected in 2-ml fractions and monitored for radioactivity by Cerenkov counting. The peptides eluted in fractions 20–22 (see Fig. 2A) were pooled and concentrated in the Speedvac for further purification by reverse phase HPLC.

Reverse phase HPLC purification of cross-linked peptides was performed with an Applied Biosystems model 120A HPLC system using a Vydac C18 column (5-μm particle size; 300-A pore size; 1 mm × 250 mm), aqueous trifluoroacetic acid/acetonitrile solvents, and a 50 μl/min flow rate. The gradient was: t = 2 min, Solvent B = 5%; t = 66 min, Solvent B = 100%; and t = 78 min, Solvent B = 100%. The column flow was split with 30% directed to the mass spectrometer and the remainder collected automatically in a fraction collector (1 min/fraction). The fractions were analyzed for radioactivity by measuring Cerenkov radiation. The regions of the total ion current corresponding to the radioactive fractions were analyzed for modified peptide mass. The radioactive fractions were also used for peptide sequencing by Edman degradation.

Liquid Chromatography Electrospray Mass Spectrometry—Liquid chromatography electrospray mass spectrometry was performed with a PerkinElmer Sciex API 3000 triple quadrupole mass spectrometer equipped with an ion spray source (15). Nitrogen was used as the nebulization gas (at 40 psi) and curtain gas and was supplied from a nitrogen Dewar. A scan range of 300–2000 in the positive ion mode was used with 0.5-s atomic mass unit steps, 4.4-μs dwell time/step, 40-V orifice potential, and 5000-V ion spray.

Peptide Sequencing—Peptide sequencing was done by the Cleveland Clinic Molecular Biotechnology core service using an Applied Biosystems model 492 Procise automated protein sequencing.

Production and Purification of P69 and Its Mutants—Site-directed mutagenesis by the mega primer polymerase chain reaction method was used for mutating Tyr⁴⁴⁴, Arg⁴⁵⁴, and Lys⁵⁴⁷ of P69. Individual mutant proteins carrying an amino-terminal histidine tag were expressed in insect cells using a baculovirus vector and purified using nickel-nitriobriaic acid affinity chromatography as described before for the production and purification of wild type P69 (7).

RESULTS

Preparation of the Ligand—A photoactivable radiolabeled substrate of 2–5(A) synthetases is an ideal ligand for cross-linking. One inherent problem in selecting such a ligand is the fact that the affinity for ATP or other substrates for these enzymes is low (10, 13). Consequently, a high concentration of the ligand is required to obtain a substantial amount of the ligand-bound protein. These considerations ruled out the possibility of using commercially available radiolabeled azido ATP as a ligand, because of prohibitive costs. Instead we decided to synthesize our own ligand, a 2–5(A) dimer, pp₃azidoA₅pp₃A, using purified P69, 8-azido ATP, and α₁⁵²P-labeled dATP. Because dATP does not have an acceptor 2' OH group, the desired dimer was the exclusive radiolabeled product. The conditions were developed using a high enzyme concentration and a short incubation time to ensure that almost all input substrates had been converted to dimers containing both pp₃-azidoa₅-azidoA and pp₃-azidoa₅-dA molecules. The dimers were purified by HPLC (Fig. 1A) and used as ligands in subsequent experiments.

Characterization of Cross-linking—In our experimental conditions, maximal photo-cross-linking was obtained after 5 min of exposure to UV light (data not shown). For structural analysis we used a subsaturating UV exposure of 2 min to avoid nonspecific cross-linking. The cross-linking of azido 2–5(A) dimer to P69 was saturable with increasing concentrations of the ligand. The dissociation constant (K₈) for the ligand as determined from this experiment was 0.88 nM (Fig. 1C), which agrees well with the binding constant of Aппра to P69 as determined earlier by fluorescence quenching assays (0.82 mM). To establish the specificity of azido 2–5(A) dimer cross-linking to the P69, we used an unrelated protein, alcohol dehydrogenase, under the same cross-linking conditions. As shown in Fig. 1B, alcohol dehydrogenase did not cross-link to the ligand. The specificity of the photo-cross-linking of P69 with the ligand was also tested by competition experiments. Aппра is known to serve as an acceptor and can be elongated with one or two adenine moieties to produce ApAппра or ApAппаP (13). We used increasing concentrations of Aппра to compete out the cross-linking of the radiolabeled ligand. Almost 75% of the cross-linking could be competed out with excess Aппра (Fig. 1D). Because dsRNA is a co-factor for all of the 2–5(A) synthetases, we tested the effect of dsRNA on the ligand cross-linking. Poly(I)poly(C) did not affect the extent of cross-linking (data not shown).

Purification of Radiolabeled Peptides—Once we had established the specificity of cross-linking, we used the same methodology to generate ligand-cross-linked P69 for structural analysis. Approximately 80–100 μg of purified P69 was cross-linked and subjected to SDS-PAGE, radiolabeled bands were excised, and radioactivity was measured. Based on radioactivity measurements and estimating protein amounts by Coomassie Blue staining, the approximate stoichiometry of the cross-linking was determined to be 0.8:1 (ligand:protein) for 2–5(A) synthetases.
linking was 0.45 mol of ligand dimer/mol of P69. This is in good agreement with typical azido ATP cross-linking efficiency found with other polymerases (17). Following in-gel tryptic digestion of cross-linked P69, IAAC and reverse phase HPLC were used to purify the radiolabeled peptides. IAAC has been successfully used by others to partially purify ATP cross-linked peptides (14). In the present study, about 90% of the applied radioactivity was bound to the aluminum column, and about 42% was recovered in fractions 18–26, specifically eluted with 10 mM phosphate (Fig. 2A). Peptides in IAAC fractions 20–22 were pooled, concentrated, and further purified on reverse phase HPLC. Two major radioactive peaks were observed by reverse phase HPLC and are marked as peaks A and B in Fig. 2B.

Identification of the Cross-linked Peptides—Ligand cross-linking to a peptide would produce a mass addition of 834. The mass spectra obtained from radioactive reverse phase HPLC peak A (Fig. 2B) included one major signal at m/z 773.8 (Fig. 3A). The doubly charged m/z 773.8 corresponded to the P69 peptide SYTSQK containing the ligand modification. A weaker singly charged ion from this peptide could also be seen at m/z 1547.4 (MH+ calculated = 1547.3). Another peak observed was the singly charged m/z 815, which corresponds to the unmodified P69 tryptic peptide FCLFTK (MH+ calculated = 815.4). The identity of the cross-linked peptide was confirmed by sequencing the peptide present in the fraction 46 of Fig. 2B by Edman degradation. The sequences obtained in the two experiments were SXXTSQK and SXXTSQKNER, respectively (Fig. 3D). The latter peptide is an extension of the former, produced by incomplete trypsin digestion. In both analyses, no amino acid was identifiable in the second cycle of degradation, suggesting that the ligand was cross-linked to this residue. Indeed, the eluate from this cycle of degradation contained most of the peptide-associated radioactivity, thus confirming that the Tyr residue in SYTSQK (Fig. 3D) was the site of cross-linking. In the peptide sequencing experiments, a minor contamination of the peptide, FCLFTK, was also observed. The high intensity of this contaminating peptide signal in the mass spectrum (Fig.
FIG. 2. Purification of cross-linked peptides. Purified P69 was cross-linked with 0.83 mM radiolabeled azido 2–5(A) dimer. Cross-linked protein was electrophoresed and digested with trypsin. The cross-linked peptides were purified by two successive purifications. A, immobilized aluminum affinity chromatography. Tryptic digested peptides from cross-linked P69 were separated by aluminum affinity column chromatography. Radioactivity from each column fractions was plotted. Fractions 1–15 were washed with 1 M NaCl, 1% ammonium acetate, fractions 16 and 17 were washed with 0.1% ammonium acetate, and fractions 18–26 were elution of peptides with 10 mM K$_2$HPO$_4$. B, reverse phase high pressure liquid chromatography. Fractions 20–22 from A were pooled and analyzed by HPLC. The top panel shows the UV absorption profile, whereas the bottom panel shows the radioactivity in HPLC fractions. The two major UV peaks corresponding to the radioactive fractions are marked as peaks A and B and were further analyzed by sequencing and mass spectroscopy.
3A) could be explained by the higher ionizibility of this unmodified peptide compared with the highly negatively charged modified peptide. We concluded that SYTSQK was the authentic cross-linked peptide and FCLFTK was a contaminant because of the following reasons. In the mass spectrum, only the SYTSQK peptide signal contained the ligand as well, and in the Edman sequencing experiment radioactivity was released in the second cycle in which, because of the modification, no Tyr could be detected, although Cys from FCLFTK was detected. The above conclusion was unequivocally validated by the mutational analysis described below.

The mass spectra from reverse phase HPLC peak B (retention time, 57–60 min) (Fig. 4B), contained several peptides but only one possibly modified peptide (Fig. 3B). A strong doubly charged m/z 797.2 and a weaker triply charged m/z 531.4 corresponded to the P69 peptide DLIRLVK plus a mass addition of 834, contributed by the ligand. Upon repeating the complete photoaffinity labeling and purification procedure, very similar results were obtained. In the second set of labeling and peptide characterization results, the mass spectra of peak B indicated the modified peptide to be DLIRLVK (Fig. 3C). In this experiment, the level of contaminating peptides was much lower. The two peptides identified in Fig. 3 (B and C) are overlapping peptides generated by incomplete tryptic digestions at two sets of alternate sites (Fig. 3D). Their common region, DLIR, probably contained the site to which the adduct was cross-linked.
Properties of P69 with Mutations in the Substrate-binding Sites

To confirm the substrate binding functions of the identified regions, we mutated several residues in these regions of P69 and studied the 2–5(A) cross-linking and enzyme activity of the mutant protein. Because the modification site in peak A was tentatively identified as Tyr421, this residue was mutated. For the peak B region, we were unable to specifically identify amino acid residues that cross-linked to the ligand. Two residues, Arg544 and Lys547, were selected for mutation, because they were positively charged residues and could be the docking sites for the negatively charged ligand. A triple mutant P69, containing mutations at Tyr421, Arg544, and Lys547, was expressed in insect cells using the baculovirus vector, and the mutant protein was purified to homogeneity. The triple mutant protein could not cross-link the ligand and was enzymatically inactive (Fig. 4, A, B, and D), although it could dimerize and bind dsRNA (data not shown). These results indicated that the identified substrate-binding sites are functionally important and that they are required for maintaining enzyme activity of the protein.

For examining the contributions of each of the two sites in ligand cross-linking and enzyme activity, two additional mutants were generated. The mutant Y421P had a mutation only in the peptide in peak A, and the mutant R544Y/K547A had mutations only in the peptide in peak B. Each mutant retained partial ability to cross-link the ligand (Fig. 4 C). Analysis of the cross-linked peptides generated from the mutants showed that peak A was lost in the Y421P mutant and peak B was reduced in the R544Y/K547A mutant (Fig. 4 C). This result confirmed that the introduced mutations had selectively dis-
ruptured binding of the ligand to the mutated sites. Moreover, because the binding and the cross-linking of the ligand to the nonmutated sites of these mutants remained unaffected, it can be concluded that the two sites can bind substrates relatively independently and that they are not part of the same site. Binding to each site was, however, required for enzyme activity; the mutants had less than 2% enzyme activity as compared with the wild type protein (Fig. 4D). These results demonstrated that both of the ligand-cross-linked sites of P69 individually contribute to the enzyme activity of the protein. In the experiments shown in Fig. 5, we continued this mutational analysis by testing the properties of additional point mutants (Fig. 5A). As expected, all five mutants were partially defective in ligand cross-linking (Fig. 5B). Similar to the Y421P mutant, the Y421A mutant had a very low enzyme activity (Fig. 5C, bar 3). Point mutants R544A and R544Y were also equally inactive (Fig. 5C, bars 4 and 5), indicating that the Arg residue at position 544 is the crucial functional determinant of this domain. In contrast, point mutant K547A retained about 8% of enzyme activity (Fig. 5C, bar 6). Thus, for maintaining enzyme activity, Tyr\(^{421}\) and Arg\(^{544}\) were identified as the most important residues at the two substrate-binding sites.

**Evidence Regarding the Nature of the Two Substrate-binding Sites**—Between the two substrate-binding sites, one presumably serves as the acceptor-binding site and the other as the donor-binding site. We wanted to identify them; however, there is no absolute binding specificity for these sites, as reflected by the observed binding of the cross-linking ligand to both sites, although it could not serve as a donor. For this reason, we took advantage of the low residual activities of the single site mutants. We examined the kinetic properties of appropriate mutants, using as substrates, dATP, which can only donate dAMP residues, and A\(^5\)ppp\(^5\)A, which can only accept them. Keeping the concentration of one substrate high, we changed the concentration of the other substrate and measured the rates of the enzyme reactions. Examples of the results of such experiments are shown in Fig. 6. This series of investigations enabled us to determine the \(K_m\) values for different substrates for each mutant. As reported previously, the Wt protein has a lower \(K_m\) for ApppA and a higher \(K_m\) for dATP as compared with that for ATP (13). For both Y421P and Y421A, the ATP \(K_m\) was increased 4-fold; similarly the \(K_m\) for ApppA was also increased, but that for dATP remained relatively unchanged. In contrast, for the R544A and R544Y pair, it was the \(K_m\) for dATP that was 2.5-fold higher. For Y421P mutant, as expected from its retention of considerable activity, the \(K_m\) for dATP was only slightly elevated (Table I). The above results indicated that mutations of Tyr\(^{421}\) affected ApppA binding, whereas those of Arg\(^{544}\) affected dATP binding. We, therefore, tentatively identified the substrate-binding site at Tyr\(^{421}\) as the acceptor site and that at Arg\(^{544}\) as the donor site.

**DISCUSSION**

2–5(A) synthetases are a unique family of enzymes because of their ability to catalyze 2′–5′ phosphodiester bond formation. Previously we identified the catalytic center of these enzymes (13). In the present study, we have used photo affinity cross-linking followed by peptide mapping to identify putative substrate-binding sites in P69, one isozyme of this family. P69 is a nonprocessive nuclease 5′ phosphodiesterase, and it is expected to bind two substrate molecules simultaneously to join them by a 2′–5′ phosphodiester bond. One site should bind the acceptor molecule whose 2′ OH will be linked to the 5′ PO\(_4\) of the other molecule bound to the donor site. In addition to ATP and 2–5(A), many other nucleic acids containing a penultimate adenine can serve as the acceptor. Similarly, ATP and other nucleotide triphosphates can serve as donors. By virtue of their specific chemical structures, some of these molecules can only accept or only donate. For example, dATP can only be a donor in the reaction because of the lack of a 2′ OH residue. Similarly, A\(^5\)ppp\(^5\)A is used as a specific acceptor because its 5′ phosphate groups are blocked. Although such compounds can function selectively as donors or acceptors, their binding to the two sites is not as selective. Thus, no diagnostic compound is known that specifically binds to one site but not the other. However, previous studies by us and others (13, 18, 19) have shown the existence of the two substrate-binding sites whose affinities toward specific ligands are different.

We decided to use radiolabeled azido 2–5(A) dimers as ligands for cross-linking for several reasons. 2–5(A)s have a higher affinity for the enzyme than ATP; thus the cross-linking would be more specific, and the yield of cross-linked protein

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**Fig. 5. Ligand cross-linking and enzyme activity of substrate-binding sites mutants.** A, sequences of the two cross-linking sites in Wt and mutants. Mutated residues are shown in bold. B, cross-linking activities of mutants. Purified proteins were cross-linked with radiolabeled ligand and electrophoresed. Radioactive cross-linked protein bands were quantified and expressed as percentages of binding to Wt P69. Bar 1, Wt P69; bar 2, Y421P mutant; bar 3, Y421A mutant; bar 4, R544A mutant; bar 5, R544Y mutant; bar 6, K547A mutant. C, enzyme activities of purified Wt and mutant proteins. The activities are expressed as percentages of Wt activity, in the same order as in B.
5(A) dimerization. The incorporation of dATP acted as a chain terminator. This dimeric 2–5(A) mixture also had azido 2–5(A) dimers in which two azido ATP dimerized. They were not separated from the radiolabeled ligand but could not be detected because of the absence of radioactivity. When the ligand was purified and used for cross-linking with P69, it showed specific labeling of the P69 protein. The labeling followed a hyperbolic saturation with an apparent dissociation constant ($K_D$) of 0.88 mM, compared with the 0.82 mM $K_D$ obtained from fluorescence quenching experiment. The cross-linking could also be specifically competed by another substrate, ApppA, indicating that the sites of cross-linking were authentic. The co-factor dsRNA did not affect cross-linking, which was in agreement with our earlier observation that dsRNA does not affect substrate binding, as judged by fluorescence quenching of the protein, in response to substrate binding.

The total tryptic digest of cross-linked P69, when separated on a reverse phase HPLC column, always gave two distinct radioactive peaks (data not shown). But the complexity of the chromatogram made it impossible to identify the cross-linked peptides. When an extra IAAC purification step was introduced before HPLC, the basic two-peak characteristic remained. As mentioned in the results, we were able to identify and confirm the peptide present in the peak A region. But the peak B region of the chromatogram was always broad, indicating heterogeneity. Our efforts to identify the cross-linked peptides in this region by Edman sequencing were not successful because the yield of the cross-linked peptide in this region was low, and there were other unmodified peptides present in this region. However, the modified peptides identified in this region by mass spectrometry were consistent. To confirm our identification of the substrate-binding sites and to functionally test their roles in substrate binding, we mutated three residues in the two putative substrate-binding sites. The mutant showed no cross-linking and was enzymatically inactive. This provided evidence that these residues are involved in substrate binding, as judged by fluorescence quenching of the protein, in response to substrate binding.

The reaction sequence of 2–5(A) synthetases enzymes can be visualized as a two-step reaction. In the first step, the acceptor 2–5(A) or ATP, which will accept the incoming phosphate group at its 2′ OH, and the donor ATP, which will be donating its 5′

### Table I

| Protein  | $K_m$ (mM) | ApppA $K_m$ (mM) | dATP $K_m$ (mM) |
|----------|-----------|-----------------|----------------|
| Wild type | 1.9       | 0.8             | 2.7            |
| Y421P    | 7.9       | 2.2             | 3.1            |
| Y421A    | 5.8       | 2.0             | 2.5            |
| R544A    | 8.2       | 0.9             | 7.4            |
| R544Y    | 8.1       | 0.8             | 7.0            |
| K547A    | 2.2       | 0.7             | 3.7            |

* Wild type and mutant proteins were purified as His-tagged proteins. 0.01 mg/ml of each protein was used for assays. 
* ApppA $K_m$ values were determined with constant dATP concentrations (4 mM) and increasing ApppA concentrations. 
* dATP $K_m$ values were determined in the presence of constant ApppA (4 mM) and increasing dATP concentrations.
a phosphate, bind to two respective sites. In the second step, the catalysis of the 2'-5' phosphodiester bond takes place between the donor and the acceptor, resulting in a one nucleotide longer 2'–5(A) molecule. Thus, we can theoretically expect two substrate-binding sites in the enzyme defined as the acceptor-binding site and the donor-binding site. In the literature there has also been evidence for two substrate-binding sites for these enzymes with different affinities (13, 18, 19). The two sites of P69 identified in this study most probably do not constitute two parts of the same site, because mutations in one did not abolish ligand cross-linking to the other (Fig. 4C), and mutants in those two sites had distinctly different affinities for acceptors and donors (Fig. 6 and Table I). Having identified the two substrate-binding sites in P69, we wanted to see which one of these two sites is the acceptor-binding site and which one is the donor-binding site. In the absence of any substrate that can bind to only the acceptor site or only the donor site of this enzyme, we used an indirect approach to identify them. Because of their chemical nature, ApppA can only be used by P69 as an acceptor in an enzyme reaction and dATP only as donor. Although ApppA and dATP can bind to both the acceptor and the donor sites, the successful reaction will take place only when they bind to the acceptor and the donor sites, respectively. Thus, when we monitor the formation of 2'–5(A) between these two substrates, the $K_m$ for ApppA will indirectly reflect the binding efficiency of ApppA to the acceptor site, and similarly the dATP $K_m$ will represent its binding efficiency to the donor site. We measured the ApppA and dATP $K_m$ for several mutants to identify which mutations had affected the acceptor and the donor binding. The Tyr421 mutants showed an increased ApppA $K_m$ compared with the Wt enzyme. However, the dATP $K_m$ for these mutants remained almost unchanged. This indicated that Tyr421 mutations affect the acceptor binding properties of P69. On the other hand, when we tested the Arg544 mutants, the dATP $K_m$ was increased, but the ApppA $K_m$ was not substantially affected, signifying that this region is involved in the donor binding.

When the primary structures of the peptides present in peaks A and B were compared with the corresponding regions of other isozymes, a strong sequence conservation was observed (Fig. 7A). The sequences in the putative donor site were almost totally conserved including the critical Arg544 and Lys547 residues. Arg544 was replaced by a Leu in the amino-terminal half of P69, providing further evidence that this half is functionally inert (13), although there is strong sequence homology between the two halves of the protein. The sequence conservation in the acceptor site was less pronounced (Fig. 7A). The Tyr residue, to which the ligand was cross-linked in P69, was replaced by another aromatic residue, Phe, in most of the other isozymes. This suggests the possibility of a base stacking interaction between the adenine moieties of the acceptor and the aromatic residue at the site. In contrast, the critical interaction at the other site could be ionic between the acidic phosphate residues of the donor and the basic amino acid residues Arg544 and Lys547. Both of the above sites are located in the same region of P69 where the catalytic triad of three Asp residues is located (Fig. 7B). In the modeled structure of this region (13), Tyr421 will be located at the end of the second $\beta$-sheet, very close to the catalytic center of the protein. The region in peak B, located further downstream (Fig. 7B), can form a long $\alpha$-helix, similar to the nucleotide-binding helix region identified in poly(A) polymerase (21). In that enzyme, residues present in this region were shown to interact with the $\gamma$-phosphate group of ATP (22). It is clear that the characteristics of the catalytic
center and the two substrate-binding sites of P69 conform to
the general structure of organizations of the nucleotidyl
transferases, the large enzyme family to which the 2–5(A)
synthetase belong (23).

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