Effects of Mutating Specific Residues Present Near the Amino Terminus of 2′–5′-Oligoadenylate Synthetase*

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In this study, we investigated the role of specific amino acid residues present near the amino terminus of the 9–2 isozyme of 2′–5′-oligoadenylate synthetase. In vitro expression of deletion mutants showed that residues 1–9 are required for enzyme activity. Within this region, residues 3, 7, and 8 were found to be conserved among all known isozymes of 2′–5′-oligoadenylate synthetase. Mutation of these residues singly or in combination resulted in partial or total loss of enzyme activity. Substitution of the proline residue at position 7 by different residues caused a partial or complete loss of activity. The properties of the inactive P7Q mutant were further explored by expressing the protein in bacteria. The bacterially expressed protein was also enzymatically inactive. The mutant protein could bind the substrate ATP and the activator double-stranded RNA normally. Oligomerization properties of the protein were examined by an affinity-based interaction assay and by glycerol gradient centrifugation; there was no detectable difference between the wild type and the P7Q mutant. These results demonstrated the importance of the proline residue at position 7 in conferring enzyme activity to the protein without affecting its other properties.

2′–5′-Oligoadenylate (2′–5′A) synthetases are a family of enzymes that share several properties (1, 2). Their synthesis is induced by interferon (IFN), and they all polymerize ATP into 2′–5′-linked oligoadenylates that activate the latent ribonuclease RNase L. The 2–5(A) synthetase/RNase L pathway is responsible for mediating IFNs antiviral action against specific groups of viruses, and it has also been implicated in the anti-growth effects of IFN. For manifesting their enzyme activity, the 2–5(A) synthetases require a co-factor, namely double-stranded (ds) RNA. Another IFN-induced enzyme that requires dsRNA as a co-factor is the protein kinase, PKR, which, when activated, can phosphorylate the translation initiation factor eIF-2 and the transcriptional inhibitory factor IκB (1, 3, 4). The dsRNA-binding domain of PKR has been defined, and essential residues within this domain have been identified (5–9). Recently, it has been shown that this domain overlaps with a dimerization domain of PKR (10). The dsRNA binding motif of PKR is shared by a number of dsRNA-binding proteins from a variety of organisms (11). 2–5(A) synthetases, however, do not have this motif, and the nature of their interaction with dsRNA is quite distinct from that of PKR.

The different isozymes of 2–5(A) synthetase are structurally related. There is also extremely high sequence conservation among similar isozymes from different species (12). These enzymes are classified into three families: large, medium, and small (2). The large 2–5(A) synthetase has a molecular mass of about 100 kDa; the corresponding cDNA and the gene have not yet been cloned. The medium human 2–5(A) synthetase has a molecular mass of about 69 kDa. Two isozymes of this family are derived from alternatively spliced mRNAs encoded by one gene. This gene appears to have arisen by duplication of a gene encoding a small synthetase (13). The small synthetases have molecular masses ranging from 40 to 46 kDa. Two human small synthetases, E16 and E18, are the products of alternatively spliced mRNAs of the same gene (14). The same is true for the murine 9–2 and 3–9 synthetases (12). However, in mouse there is an additional small synthetase, L-3, which is encoded by a separate gene (15). The different isozymes partition differently to various subcellular compartments, which could be caused by the different post-translational modifications they carry (16).

Although the primary structures of at least six 2–5(A) synthetase isozymes are known, the structural features responsible for their dsRNA binding activity and their enzyme activity remain to be identified. We have previously reported that truncation of 94 residues, but not of 70 residues, from the carboxyl terminus of the 9–2 protein causes a loss of its enzyme activity (12). Here we report that the loss of even nine residues from the amino terminus of the 9–2 protein also causes a complete loss of enzyme activity. Sequence comparison and site-directed mutagenesis within this region revealed the importance of the residues at positions 3, 7, and 8 in determining the enzyme activity of the protein. Detailed analysis of the properties of one mutant, P7Q, is presented here.

MATERIALS AND METHODS

Reagents—Poly(I)poly(C)-agarose and m7GppGm were from Pharmacia Biotech Inc. Polyethyleneimine cellulose plates were from EM Science. Restriction enzymes, RNase inhibitor, and other fine chemicals were from Boehringer Mannheim. [α-32P]ATP (specific activity, 800 Ci/mmol) and [35S]methionine (specific activity, 1000 Ci/mmol) were from DuPont NEN. Plasmid pGEM4 and rabbit reticulocyte lysate were from Promega. Anti-FLAG M2 monoclonal antibody Sepharose was purchased from Eastman Kodak Co.

2–5(A) Synthetase Mutants—The substitution mutants were generated by polymerase chain reaction (PCR) using appropriate primers carrying the desired mutations. cDNA products of PCR were digested

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The abbreviations used are: 2–5(A), 2′–5′-oligoadenylate; IFN, interferon; dsRNA, double-stranded RNA; PKR, protein kinase, RNA-activated; poly(I)-poly(C), polyinosinic acid-polycytidylic acid; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; WT, wild-type; bp, base pair.
with the appropriate enzymes and joined to the coding region of wild type synthetase. The portion of the cDNA generated by PCR was completely sequenced to confirm the presence of the desired mutation and the absence of any other mutation. The mutant cDNAs were expressed in vitro from a NS-9–2 hybrid cDNA clone (12) or expressed in Escherichia coli using the pET15b vector as described (18). For generating the deletion mutants, the expression vectors pGEM39T-5' or -3' were used (5). The +2 mutant encoded a protein with Met-Gly-Pro-Asp (residue 2 of authentic 9–2), α9 encoded a protein with Met-Gly-Pro-Ser (residue 10 of authentic 9–2), and ΔΔ23 encoded a protein with Met-Ala-Pro-Cys (residue 24 of authentic 9–2) at the amino terminus.

In Vitro Transcription of 2–5(A) Synthetase mRNA—All the cDNAs to be transcribed were cloned in pGEM4 in an orientation appropriate for the synthesis of sense strand RNA by SP6 RNA polymerase. The capped mRNAs were synthesized from NS-9–2 hybrid cDNAs linearized by digestion with HinfI at amino acid 344. A typical transcription mixture (40-μl volume) contained 2 μg of DNA template and 20 units of SP6 RNA polymerase in a buffer containing 40 mM Tris-HCl, pH 7.9, 6 mM MgCl2, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 40 units of RNase inhibitor, 500 μM each of ATP, CTP, UTP, 50 μM GTP, and 500 μM 32P to 200 cpm. After 1 h at 30 °C followed by digestion of the template with 5 units of DNase I at 37 °C for 10 min. The reaction products were analyzed by SDS-PAGE. After drying, the gels were exposed to x-ray film.

In Vitro Translation—The respective mRNAs were translated in a mRNAd-dependent protein-synthesizing system prepared from nuclease-treated rabbit reticulocyte lysate. Typically 500 ng of mRNA was translated in a 25-μl reaction mixture containing 17.5 μl of reticulocyte lysate, 20 units of RNase inhibitor, 12.5 μM of [35S]methionine, and 1 μl of amino acid mixture without methionine. Incubations were at 30 °C for 90 min. The reaction products were analyzed by SDS-PAGE. Alternatively, the plasmid DNA was used directly in a coupled transcription–translation system.

2–5(A) Synthetase Assay of in Vitro Translation Product—Synthetase assay was according to the method of Mory et al. (18) with minor modifications. In vitro translated protein products were separated on SDS-PAGE and quantitated by PhosphorImager analysis. For enzyme assay, equal quantities of translated proteins were used in the same volume of translation mixture. If required, translation mixture without any added mRNA was used as a dilutant. Bacterially produced synthetase was purified and used directly for enzyme assay. Unless otherwise specified, 10 μl of reaction mixture containing the enzyme, 20 μM Tris-HCl, pH 7.5, 20 mM magnesium acetate, 2.5 mM dithiothreitol, 5 mM ATP, 5 μCi of [α-32P]ATP, and 50 μg/ml poly(I)-poly(C) (21) was incubated for 2 h at 30 °C. The reaction was stopped by boiling for 3 min and centrifuged at 14,000 × g for 10 min, and 8 μl of the supernatant was incubated for 3 h at 37 °C with 3 μl of 1 unit/ml calf intestine alkaline phosphatase to convert the unreacted [α-32P]ATP to inorganic phosphate. Two μl of the sample were then spotted on a polyethyleneimine-cellulose thin layer chromatography plate and resolved in 750 mM KH2PO4, pH 3.5. The 2–5(A) formed was then quantitated by PhosphorImager analysis and expressed as arbitrary units.

Bacterial Expression and Purification of Recombinant 2–5(A) Synthetase—Hexahistidine-tagged synthetase was expressed in bacteria and purified as described previously (17).

Electrophoretic Mobility Shift Assay—The binding reaction contained approximately 100 ng of purified 2–5(A) synthetase, 100 μg/ml poly(A), 1 μg/ml bovine serum albumin, 20 μg Tris-HCl, pH 7.5, 20 mM magnesium acetate, and 50,000 cpm of an 85-bp labeled dsRNA (19). The reaction mixture was incubated on ice for 20 min followed by the addition of glycerol dye and underwent electrophoresis onto a 4% polyacrylamide gel at 150 V in 50 mM Tris/glycine buffer. The gel was imaged on a GelStar (FMC) gel and stored at -20 °C. The bands of interest were excised and subjected to gel electrophoresis. The 2–5(A) synthetase was purified and used for binding experiments.

ATP-agarose Chromatography—[32P]Methionine-labeled hexahistidine-tagged WT and P7Q mutant proteins were synthesized in vivo using a coupled T7 transcription translation system (Promega) and the cognate PET15b constructs. The proteins were bound to nickel-Sepharose 6 Fast Flow (Amersham) and eluted with a buffer containing 100 mM EDTA, 400 mM NaCl, and 20 mM Tris-Cl, pH 7.5. The eluted samples were analyzed against a buffer containing 20 mM Tris-Cl, pH 7.5, and 10% glycerol and concentrated to a smaller volume using microcon concentrators. The purified radiolabeled proteins were bound to ATP-agarose or agarose in the binding buffer (20 mM Tris-Cl, pH 7.5, 5 mM magnesium acetate, and 2.5 mM dithiothreitol) for 1 h at 4 °C in the presence of 50 μM poly(I)-poly(C) (21). Before binding, the resins were presoaked and washed three times in 20 mM Tris-Cl, pH 7.5, incubated with reticulocyte lysate at 4 °C for 30 min in the same buffer, washed with a buffer containing 20 mM Tris-Cl, pH 7.5, and 500 mM NaCl, and equilibrated against the binding buffer. This protocol of resin pretreatment reduces nonspecific binding of proteins (21). After binding the radiolabeled proteins to ATP-agarose, the resin was washed three times with 20 mM Tris-Cl, pH 7.5. Finally, the proteins bound to the resin were eluted by boiling in SDS-PAGE sample buffer, electrophoresed, and quantitated by autoradiography and PhosphorImager analysis.

Poly[I]-poly(C) Chromatography—This assay was done as previously described (12).

Construction of 9–2 FLAG cDNA—For adding the FLAG epitope (DYKDDDDK) to the carboxyl terminus of the 9–2 protein, the TGA stop codon in 9–2 was mutated to AGA, and a TCT codon appeared after the deletion mutations were achieved by PCR using the appropriate primers. Since the newly created AGATCT sequence is a BglII site, the 9–2 cDNA can be cut open there and joined to a synthetic DNA containing a BglII sticky end at the 5' followed by codons to encode the FLAG epitope, DYKDDDDK, and a stop codon. The final protein contained two residues, Arg and Ser (encoded by the BglII site) in between the last residue of 9–2 (Ser) and the first residue of FLAG (Asp). For carrying out this construction, part of 9–2 cDNA was copied by PCR using a sense primer from nucleotide 630 to 653 and an antisense primer containing the BglII site for mutating the termination codon. The blunt-ended PCR product was cloned into the EcoRV site of pBluescript. The clone was digested with BglII (cuts at the end of 9–2) and SmalI (cuts in the vector polylinker), and the FLAG double-stranded oligonucleotide (BglII overhang at the 5' end and blunt at the 3' end) was ligated to the plasmid. The synthetic oligonucleotide was not phosphorylated to prevent its oligomerization. Finally, the 9–2 FLAG sequence from this clone was released by BamHI (cuts in the 9–2 body) and KpnI (cuts in the plasmid) digestion and swapped in the place of the same fragment generated from NS9–2 in pGEM4 (12). The 9–2 FLAG mRNA can be transcribed from the final plasmid, pG1 in pGEM4, using SP6 RNA polymerase.

Interaction Assay—The 9–2 FLAG protein (48.8 kDa) and the wild type or mutant 9–2 protein (47.5 kDa) were co-translated using the TNT translation system and [35S]cysteine according to the manufacturer’s instructions. The translated products were separated by SDS-PAGE, and the amount of each protein synthesized was quantitated by PhosphorImager analysis of the gel. For measuring potential interaction between the co-translated proteins, the 9–2 FLAG protein was immunoprecipitated with anti-FLAG M2 antibody-Sepharose, and coprecipitation of the 9–2 protein without a FLAG was monitored. The immunoprecipitation was done in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris-Cl, pH 8.0). 30 μl of the antibody-Sepharose (2.38 mg/ml) was suspended in 500 μl of RIPA buffer and washed twice. It was then incubated in 500 μl of buffer containing 1.5 μg/ml bovine serum albumin and 3 μl/ml reticulocyte lysate (21). For resolution of complexes, the FLAG immunoprecipitated 9–2 protein was centrifuged down and resuspended in 500 μl of buffer containing the sample of 5 μl of translation products in reticulocyte lysate. The suspension was incubated at 25 °C for 1 h with continuous mixing. The continuous mixing during presoaking and sample binding was achieved by tapping the Eppendorf tube containing the suspension horizontally to the platform of an orbital shaker and shaking it at 160 rpm. After sample binding, the Sepharose beads were centrifuged down and washed four times with RIPA buffer. The beads were suspended in SDS-PAGE sample buffer and boiled. The eluted proteins were analyzed by gel electrophoresis.

Velocity Gradient Sedimentation—11.4 ml of glycerol gradients (5–20% v/v) were prepared in the gradient buffer containing 400 mM NaCl, 50 mM KCl, 5 mM MgCl2, 5 mM β-mercaptoethanol, and 50 mM Tris-Cl, pH 7.5, and equilibrated to 4 °C. 10 μl of in vitro translated proteins were diluted with 100 μl of gradient buffer containing 0.2% Triton X-100 and layered on the top of the glycerol gradient. The gradients were centrifuged in a Beckman SW 41 ultracentrifuge rotor at 4 °C for 30 h at 180,000 rpm. After centrifugation, the gradients were fractionated by using a peristaltic pump and a capillary tube inserted from the top and reaching to the bottom of the tube. 300-μl fractions were collected. Proteins present in 200 μl of alternate fractions were precipitated with 1.0 ml of cold acetone in the presence of 50 μg of bovine serum albumin. The protein pellets were centrifuged out, dissolved in SDS-PAGE sample buffer, and subjected to gel electrophoresis. The 9–2 band in each fraction was quantified by PhosphorImager analysis.
active (bind dsRNA. In the current study, we inquired if deletions from ever, destroys the protein’s ability to polymerize ATP but not to required for 2–5(A) synthetase activity. Further deletion, how- present at the carboxyl terminus of the 9–2 isozyme, are not Activity— Nine residues at the amino terminus of 9–2 were totally con- served in the mouse 3–9 and the human E-16 and E-18 69K 1–9 synthetases. Residues Leu, Pro, and Ala at positions 3, 7, and 8 were also conserved in the L-3 isozyme at positions 5, 9, and 10, respectively. The 69K medium isozyme is thought to be a composite of two halves, each of which resembles the small synthetase isozyme. We found that the Leu and Pro residues are also conserved in both halves of the medium isozyme, whereas the Ala residue is conserved in the amino-terminal half but replaced by Gly in the carboxyl-terminal half. Thus, it appears that the LXXXPA motif is present near the amino termini of all known 2–5(A) synthetase isozymes.

To examine the functional significance of the conserved Leu, Pro, and Ala residues at positions 3, 7, and 8, we mutated them individually in the context of the 9–2 cDNA sequence. A double mutant at positions 7 and 8 was also produced. Each of these mutant proteins was generated by in vitro translation and assayed for enzyme activity. They were all translated equally well (Fig. 2A and data not shown) and bound dsRNA as efficiently as the wild type protein (data not shown). Their enzymatic activities, however, were quite different. The L3H mutant was 30% as active as the wild type protein, whereas the A8D mutant had about 18% activity. In contrast, the activities of the P7Q and the P7Q/A8D mutants were not any higher than the background activity present in the reticulocyte lysate. Since this background activity varied from lot to lot of the commercial lysates used, these experiments were repeated 10 times with different lots of cDNAs and reticulocyte lysates (Fig. 2B). Given the small range of variability from experiment to experiment, it was reasonable to conclude that the P7Q and the double mutant were totally inactive, whereas the L3H and A8D mutants were partially active.

**RESULTS**

**Deletions from the Amino Terminus Cause a Loss of Enzyme Activity**—We have previously shown that residues 345–414, present at the carboxyl terminus of the 9–2 isozyme, are not required for 2–5(A) synthetase activity. Further deletion, however, destroys the protein’s ability to polymerize ATP but not to bind dsRNA. In the current study, we inquired if deletions from the amino terminus can be tolerated for enzyme activity. Two deletion mutants missing residues 1–23 (Δ23) and 1–9 (Δ9) were synthesized by in vitro translation using appropriate cDNAs. These proteins bound to poly(I)-poly(C)-agarose (data not shown) but were enzymatically inactive (Fig. 1). Although there was a low level of activity contributed by the endogenous 2–5(A) synthetase present in reticulocyte lysate, the in vitro translation products of Δ9 (lane 2) and Δ23 (lane 3) cDNAs were more active than the negative control (lane 5). In contrast, the in vitro translated wild type protein was highly active (lane 4). The way the Δ9 and the Δ23 mutants were constructed at their amino termini, there were two additional residues including a proline residue in addition to the initiator methionine. Since proline is known to profoundly influence local protein structure, we wondered whether the lack of activity of the mutants was due to an altered conformation near the amino terminus. This does not appear to be the case since another mutant, +2, carrying methionine, glycine, and proline at the amino terminus of full-length 9–2, was highly active (lane 1).

**The LXXXPA Motif Is Important for Enzyme Activity**—To find out if the residues present near the amino terminus of the 9–2 protein are conserved among other isozymes of 2–5(A) synthetase, we did an appropriate homology search (Table I). Nine residues at the amino terminus of 9–2 were totally conserved in the mouse 3–9 and the human E-16 and E-18 isozymes. Residues Leu, Pro, and Ala at positions 3, 7, and 8 were also conserved in the L-3 isozyme at positions 5, 9, and 10, respectively. The 69K medium isozyme is thought to be a composite of two halves, each of which resembles the small synthetase isozyme. We found that the Leu and Pro residues are also conserved in both halves of the medium isozyme, whereas the Ala residue is conserved in the amino-terminal half but replaced by Gly in the carboxyl-terminal half. Thus, it appears that the LXXXPA motif is present near the amino termini of all known 2–5(A) synthetase isozymes.

**Different Substitutions at Position 7 Have Different Effects**—Since the P7Q mutant was totally inactive, we wondered whether the Pro residue is absolutely necessary for the enzyme to be active. To examine this possibility, Pro was replaced by several other amino acids by site-directed mutagenesis. The corresponding mutant proteins were translated in vitro, and their enzyme activities were measured. All mutations caused partial losses of enzyme activity, but the degrees of inactivation were different for different substitutions (Fig. 9). Between the two aromatic residues, Phe and Tyr, substitution by Phe was more detrimental. Substitution of Pro by charged residues Glu or Lys also inactivated the protein partially. Substitution by Thr, a polar residue, had a degree of effect very similar to that of Tyr, another polar residue. Leu, a hydrophobic residue, had strong effects like Phe, which was the only other hydrophobic residue tested. These results demonstrated that the presence of Pro at position 7 was not absolutely required for enzyme activity. However, compared with all of the mutations tested, Pro was the best residue to be at that position, since no substitution increased the activity. The degree of reduction in the activity could not be readily correlated with any simple property of the substituting residue.

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**TABLE I**

Sequence homology near the amino termini of 2–5(A) synthetase isozymes

| Synthetase isozyme | Residue number | Amino acid sequence |
|--------------------|----------------|---------------------|
| 9–2                | 1–9            | MDLRTTPAK           |
| 3–9                | 1–9            | MDLRTTPAK           |
| L–3                | 3–11           | HGLRSITPPAW         |
| E–16               | 1–9            | MDLRTTPAK           |
| E–18               | 1–9            | MDLRTTPAK           |
| 69K I              | 6–14           | SQLSSTVPPAQ         |
| 69K II             | 338–346        | ALPTTPG           |
Bacterially Produced Mutants Are Inactive—To further test the properties of proteins carrying a mutation at position 7, the P7Q and the P7Q/A8D mutants were expressed in E. coli as hexahistidine-tagged proteins and purified by nickel-agarose chromatography. The mutant proteins were devoid of any enzymatic activity, whereas the wild type protein was highly active (Fig. 4). Over a large range of protein concentrations, the activity of the WT protein increased linearly, but even at the highest concentration tested, the mutant proteins were enzymatically inactive.

Mutant Protein Can Bind dsRNA and ATP—2–5(A) synthetases use ATP as the substrate and dsRNA as the activator, and hence, they have to be able to bind these two molecules for being enzymatically active. These binding properties of the P7Q mutant were examined in the next experiments. We have previously described a poly(I)-poly(C)-agarose binding assay for mapping the dsRNA-binding domain of 2–5(A) synthetase (12). The same assay was used for measuring the dsRNA binding activity of the P7Q mutant (Fig. 5). It could bind dsRNA as well as the WT protein. A different assay was used to measure the dsRNA binding ability of bacterially produced 2–5(A) synthetase proteins. A radiolabeled 85-bp dsRNA probe was used to perform electrophoretic mobility shift assays. No shifted complex was observed when bovine serum albumin was added to the probe (Fig. 6A, lane 2), but as reported previously (19) two shifted complexes were produced in the presence of the dsRNA-binding domain of PKR (Fig. 6A, lane 3). The wild type synthetase (Fig. 6B, lane 1) and the mutant (Fig. 6B, lane 4) produced one shifted complex that was not supershifted by a non-immune serum (Fig. 6B, lanes 2 and 5), but a synthetase-specific antipeptide antiserum (20) supershifted and stabilized the complexes (Fig. 6B, lanes 3 and 6). These results demonstrated that the synthetase protein could form a specific complex with dsRNA, and this property was not affected by the P7Q mutation.

In the next experiment, we examined the effect of the mutation on the protein’s ability to bind its substrate, ATP. ATP-agarose chromatography has been used for this purpose in the...
An 85-bp-labeled dsRNA was poly(I) to its inability to bind ATP. Thus, the inactivity of the mutant could not be attributed and 54% of the WT proteins bound to ATP-agarose, whereas ATP equally well (Fig. 7). Of the total input, 48% of the mutant demonstrate that the mutant and the WT synthetase could bind case of protein kinases (21). Using that method, we could dem-
strate that the mutant protein kinases could bind to wild type synthetase; for standardizing this assay, a FLAG epitope-

tag was monitored. The FLAG-tagged and untagged proteins

in vitro synthesized WT and P7Q proteins were subjected to poly(I)poly(C)-agarose chromatography (12). The amount of total and bound proteins was quantified by PhosphorImager analyses of the gel. About 20% of both proteins bound to the resin under our experimental conditions.

When an unrelated protein, luciferase, was co-translated, it did not co-purify with 9–2 FLAG (Fig. 8, lanes 1 and 2), but co-
translating 9–2 FLAG and 9–2 WT caused the untagged protein to co-purify with 9–2 FLAG (lanes 5 and 9). The same was true for P7Q 9–2 (lanes 6 and 10). When untagged 9–2 was translated by itself (lane 3), no labeled protein was purified (lane 7), thus validating the method. As expected, all of trans-
lated 9–2 FLAG protein was purified by the affinity resin (lanes 4 and 8). For co-purification, the two proteins required co-translating; mixing after separate translation did not yield the oligomeric complex (data not show). These results demonstrated that the 9–2 protein could bind to itself, and the P7Q mutation did not affect this interaction.

The oligomerization property of the mutant protein was investigated by another method in the experiment shown in Fig. 9. Velocity gradient sedimentation was used to measure the apparent size of the 2–5(A) synthetase proteins synthesized in vitro. Appropriately sized markers were provided by centrifugation of standard proteins of known molecular weights. The WT and P7Q proteins produced similar profiles (Fig. 9). The majority of the in vitro translated proteins was present as a monomer, but a substantial portion of both proteins formed a tetrameric complex. The mutant protein was as efficient as the WT protein in formation of the tetramer. The results shown in Figs. 8 and 9 clearly demonstrated that the P7Q protein was capable of participation in both homomeric and heteromeric interactions.

DISCUSSION

Continuing our investigation of the structure-function relationship of the mouse 9–2 isozyme of 2–5(A) synthetase, we created deletion mutants missing the first 9 or the first 22 residues from the amino terminus. These proteins were expressed using the translation initiation vectors that we designed previously for doing a similar analysis of PKR (5). Neither of the amino-terminal deletion mutants of the 9–2 protein had enzyme activity, although they were translated as efficiently as the wild type protein (data not shown). Another protein carrying two additional residues at the amino terminus of the full-length 9–2 protein was highly active. These results suggested that some alterations, but not others, near the amino terminus of the protein are tolerated. That the addition of extra residues to this end of the protein does not change its confor-
FIG. 8. Protein-protein interaction assay for the 9–2 protein. WT 9–2, P7Q 9–2, WT 9–2 FLAG, and luciferase (Luc.) were translated in vitro either singly or in combinations. A portion of the translation product (2 µl) was analyzed directly by gel electrophoresis, and another portion (5 µl) was subjected to immunoprecipitation as described under “Materials and Methods” before gel electrophoresis. Lane 1, total products from translation of 9–2 FLAG and luciferase; lane 2, the same as lane 1 after anti-FLAG immunoprecipitation. Lanes 3–6 are total translation products, and lanes 7–10 are products after immunoprecipitation. Lanes 3 and 7, 9–2; lanes 4 and 8, 9–2 FLAG; lanes 5 and 9, WT 9–2 plus 9–2 FLAG; lanes 6 and 10, P7Q 9–2 plus 9–2 FLAG.

FIG. 9. Velocity gradient sedimentation analysis. In vitro translated WT and P7Q proteins were analyzed as described under “Materials and Methods.” The direction of centrifugation was from the right to left. The positions of the molecular mass markers are shown on top. The data are presented as percent of radioactivity in the 9–2 band of each fraction with total radioactivity being 100.

mation to an inactive form was also demonstrated by the production of active hexahistidine-tagged 9–2 protein. We, therefore, concluded that specific residues present near the amino terminus are essential for the activity of this enzyme.

Sequence comparison revealed that the first nine residues are completely conserved among the murine 9–2 and 3–9 isozymes and the human E-16 and E-18 isozymes (Table I). Remarkably, when the sequence of the other murine small synthetase L-3 was compared, we noted the presence of a motif XXXPA in all isozymes. The leucine residue is at position 3 of the 9–2 protein, but it is at position 5 of the L-3 protein. Yet the spacing of the invariant residues is the same, and no other residue in this region is conserved between the two proteins. The 69K medium synthetase consists of two halves, which have strong sequence homology. We searched for the LXXXP motif near the amino termini of the two halves and found that the leucine and the proline residues are conserved there as well. The alanine residue after proline is also conserved in the amino half, but it is conservatively replaced by a glycine residue in the carboxyl half. The spacing of the conserved residues was again identical. The above observations strongly suggested that conservation of the LXXXP motif has a functional significance.

Site-directed mutagenesis of the invariant residues at positions 3, 7, and 8 clearly established that these residues are important for the enzymatic activity of the 9–2 protein. Replacement of the hydrophobic leucine residue at position 3 with histidine, a polar residue, reduced the activity by about 70%, whereas replacement of alanine at position 8 with a charged aspartic acid residue reduced the activity by more than 80%. Thus, specific defects in the enzymatic properties of these partially active mutants remains to be delineated. Similarly, more mutational studies will be required to determine which substitutions are tolerable at positions 3 and 8. Replacement of proline at position 7 with glutamine destroyed the activity of the protein completely. Proline residues are known to create discrete structural changes in protein conformations. Hence, it is quite likely that the substitution of proline at position 7 resulted in a crucial conformational change of the protein, resulting in a loss of activity. This putative conformational change was affected differently by different substitutions at this position. However, the degree of effect of a given substitution could not be easily correlated with any property of the replacing amino acid.

In addition to the totally inactive point mutation P7Q described here two other inactive point mutants, K199R and K199H, have been described recently (22). The latter mutations apparently affect the enzyme’s active center and its ATP binding. In contrast, the P7Q mutant could bind ATP as well as the wild type protein. Similarly, the dsRNA binding property and the oligomerization property of the protein were also unaffected by this mutation. For testing the oligomerization properties of the protein, we developed two new assays. The glycerol gradient centrifugation assay has the advantage of providing the molecular weight of the native protein and hence the estimate of its number of subunits. It is, however, a time-consuming assay, and it cannot distinguish between a heteromeric and a homomeric interaction. To circumvent these problems, we developed the FLAG-antibody interaction assay and established its validity. The observation that co-translation was required for the formation of heteromeric complexes suggests the complexes are quite stable, and they do not exchange subunits at an appreciable rate under our experimental conditions. Such information will be valuable in the future for expressing enzymatically inactive mutants in whole cells as potential trans-dominant inhibitors of the endogenous wild type enzyme.

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