Activity and Stability of Recombinant Bifunctional Rearranged and Monofunctional Domains of ATP-Sulfurylase and Adenosine 5’-Phosphosulfate Kinase

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Andrea T. Deyrup, Srinivasa Krishnan, Bhawani Singh, and Nancy B. Schwartz

From the Departments of Pediatrics and Biochemistry and Molecular Biology, The University of Chicago, Chicago, Illinois, 60637

Murine adenosine 3’-phosphate 5’-phosphosulfate (PAPS) synthetase consists of a COOH-terminal ATP-sulfurylase domain covalently linked through a nonhomologous intervening sequence to an NH2-terminal adenosine 5’-phosphosulfate (APS) kinase domain forming a bifunctional fused protein. Possible advantages of bifunctionality were probed by separating the domains on the cDNA level and expressing them as monofunctional proteins. Expressed protein generated from the ATP-sulfurylase domain alone was fully active in both the forward and reverse sulfurylase assays. APS kinase-only recombinants exhibited no kinase activity. However, extension of the kinase domain at the COOH terminus by inclusion of the 36 residue linker region restored kinase activity. An equimolar mixture of the two monofunctional enzymes catalyzed the overall reaction (synthesis of PAPS from ATP + SO4^-2) comparably to the fused bifunctional enzyme. The importance of the domain order and organization was demonstrated by generation of a series of rearranged recombinants in which the order of the two active domains was reversed or altered relative to the linker region. The critical role of the linker region was established by generation of recombinants that had the linker deleted or rearranged relative to the two active domains. The intrinsic stability of the various recombinants was also investigated by measuring enzyme deactivation as a function of time of incubation at 25 or 37 °C. The expressed monofunctional ATP-sulfurylase, which was initially fully active, was decaying with a t1/2 of 10 min at 37 °C. Progressive extension by addition of kinase sequence at the NH2-terminal side of the sulfurylase recombinant eventually stabilized sulfurylase activity. Sulfurylase activity was significantly destabilized in a time-dependent manner in the rearranged proteins as well. In contrast, no significant deactivation of any truncated kinase-containing recombinants or misordered kinase recombinants was observed at either temperature. It would therefore appear that fusion of the two enzymes enhances the intrinsic stability of the sulfurylase only.

The formation of adenosine 3’-phosphate 5’-phosphosulfate (PAPS) (1), the sole donor of activated sulfate in mammalian systems, requires the sequential actions of two enzymes: ATP-sulfurylase (ATP:sulfate adenylyltransferase, EC 2.7.7.4) and APS kinase (ATP:adenylylsulfate 3’-phosphotransferase, EC 2.7.1.25) (see Reaction 1).

\[
\text{ATP} + \text{SO}_4^{2-} \rightarrow \text{APS} + \text{PP} \\
\text{APS} + \text{ATP} \rightarrow \text{ADP} + \text{PAPS}
\]

**REACTION 1**

We have studied the PAPS-synthesizing enzymes in the context of a defect in the production of PAPS in the brachymorphic mutant mouse where a severe reduction in the kinase activity and a partial reduction in the sulfurylase activity was demonstrated (1–3). To understand this intriguing double enzyme defect, the relationship between the two activities was investigated by determining whether they represented two separate polypeptides or a single bifunctional polypeptide. The two sulfate-activating enzymes, ATP-sulfurylase and APS kinase, were purified from rat chondrosarcoma and shown to have nearly identical molecular properties and fractionation behavior (4). To distinguish whether a single polypeptide with multiple active sites or two tightly complexed polypeptides pertain, characterization of both kinetic mechanisms (5, 6), as well as affinity purification of the sulfate activation complex were accomplished (7). These studies identified the mammalian sulfurylase/kinase as a bifunctional enzyme (7) that uses a channeling mechanism to transfer the intermediate APS efficiently from the sulfurylase to the kinase active site (8, 9). Subsequently, the murine and human sulfurylase/kinase have been cloned, sequenced, and expressed as a fused bifunctional enzyme (10, 11). The finding of multiple functions on a single polypeptide suggests that this complex enzyme is a critical locus for regulation and a vulnerable site for mutations.

The expressed 624-amino acid murine ATP-sulfurylase/APS kinase has an amino-terminal region (residues 1–199) that is highly homologous to known APS kinases and a carboxyl-terminal region (residues 237–624) that is similar to known ATP-sulfurylases (10). These two domains are joined by a 37-amino acid intervening sequence (linker region) (10). This arrangement in which the kinase domain is positioned at the NH2 terminus of the bifunctional enzyme whereas the sulfurylase domain is toward the COOH terminus is maintained in the human (11), spoonworm (12), and Drosophila (13) sulfurylase/kinase, and is the reverse of the physiological reaction sequence, the gene order in the Escherichia coli operon (14), and

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† These authors contributed equally to this study.

‡ To whom correspondence should be addressed: University of Chicago, Dept. of Pediatrics, MC5058, 5841 S. Maryland Ave., Chicago, IL 60637. Tel.: 773-702-6426; Fax: 773-702-9234; E-mail: n-schwartz@uchicago.edu.

1 The abbreviations used are: PAPS, adenosine 3’-phosphate 5’-phosphosulfate; APS, adenosine 5’-phosphosulfate; IMAC, Tris-imidazole buffer; NTA, nitrilotriacetic acid.
the structural order of the protein product in *Penicillium chrysosogenum* where the sulfurylase has a partial kinase-like sequence fused to the COOH terminus (15).

The number of examples of single polypeptides that carry out multiple functions is increasing, and it has been suggested that such multifunctional enzymes have selective advantages over separate monofunctional counterparts. In some cases, benefits of bifunctionality such as improved regulation (16, 17) and enhanced stability (18) have been demonstrated. A fused sulfurylase/kinase that uses a kinetically channeled mechanism may offer advantages in overcoming obstacles inherent to the sulfate activation pathway. For instance, APS is both spontaneously labile, resulting in nonenzymatic degradation to AMP and free sulfate, and subject to breakdown by cytosolic sulfohydrolase/sulfatase activities if released into the medium (19). The unusually high equilibrium constant (\(>10^8\)) for the reverse sulfurylase reaction (toward ATP formation) implies that even in the presence of associated pyrophosphatases, APS must be phosphorylated to PAPS (which is not a substrate for ATP-sulfurylase), or it will be redirected in the reverse sulfurylase reaction. Thus a channeling bifunctional protein may shift the equilibrium of the reversible sulfurylase reaction in a favorable way or protect the APS intermediate from degradative effects of the solvent.

The availability of cloned and expressed sulfurylase/kinase allows us to address the question of the relationship of the bifunctional sulfurylase/kinase domains to monofunctional ATP-sulfurylases and ATP kinases and whether there is a distinct mechanistic or structural advantage to a fused protein.

To examine the ability of the kinase and sulfurylase domains to function independently (as occurs in plants, fungi, and bacteria), we have generated and expressed a series of constructs that encode independent monofunctional domains. The importance of the domain order and organization was examined through the construction of several mutants in which the order of the two active domains was reversed or altered in relation to each other or to the linker region. The critical role of the linker region was then established by construction of recombinants in which the linker region was deleted or rearranged in relation to the two active domains.

**EXPERIMENTAL PROCEDURES**

Restriction enzymes were obtained from New England Biolabs, unless otherwise indicated. T4 polynucleotide kinase was from Promega, AmpliTaq was from Perkin Elmer-Cetus, DNA polymerase and associated reagents for automated sequencing were from Applied Biosystems, nickel-nitrioltriacetic acid alkaline phosphatase conjugate used for Western blot analysis was from Qiagen. *Pfu* DNA polymerase and cloning vector plasmid were obtained from Stratagene. The *pcR 2.1* vector for direct ligation of polymerase chain reaction products was a component of the Invitrogen TA cloning kit. Thrombin protease for removal of the histidine tag, the *pet-15b* bacterial expression vector, and metal chelate resin for gravity purification of the expressed protein were purchased from Novagen. All enzymes were used with the buffers and metal chelate resin for gravity purification of the expressed protein were purchased from Novagen. All enzymes were used with the buffers recommended by the suppliers. Determination of protein concentration was done using the Bio-Rad protein assay and the Pierce BCA protein assay system.

**Polymerase Chain Reaction and Sequencing**—Polymerase chain reaction actions were performed in a Perkin Elmer GeneAmp 2400 thermal cycler using either *Taq* polymerase from Perkin Elmer or *Pfu* polymerase from Stratagene. Standard cycling parameters included 1–5 min of preincubation at 94 °C followed by twenty cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. Following the cycling step, a 10-min extension step at 72 °C allowed completion of all unfinished transcripts. All oligonucleotides were purchased from the University of Chicago oligonucleotide core facility. Automated DNA sequencing was performed on an ABI PRISM 377 DNA sequencer.

**Expression and Purification of Separate Enzyme Domains**—Three constructs corresponding to the APS kinase region were designed: APS kinase alone (1MAPSK) (amino acid residues 1–199); APS kinase plus linker (APSKL) (residues 1–236); and finally, APS kinase plus linker and an extension into the ATP-sulfurylase region (APSK456) (residues 1–456). The latter primer (N9) was intended to introduce a TAA stop codon following Gly-456 of the sulfurylase region, but in fact altered the reading frame so that translation ran on into the downstream sequence, adding a tryptophan residue. The following sequence (NSRILLKTKPERIKLSWLLPLPSNNN) to the C terminus of the APSKL vector sequence, adding a 24-residue heterologous peptide sequence (residues 237–624): ATP-sulfurylase plus linker (LATPS) (residues 200–624); and ATP-sulfurylase with an NH2-terminal extension into the kinase region (3MSK) (residues 70–624).

Plasmid pET-15bSK1 containing the coding region for the sulfurylase/kinase was used as a polymerase chain reaction template for generation of inserts. Primers were synthesized that contained restriction enzyme cutting sites to facilitate post-amplification ligation of products into the pET-15b expression vector. The sites chosen, *NdeI* and *XhoI*, do not occur in the sulfurylase/kinase coding sequence. The amino acids Gly-Ser-His were added to the amino terminus of all constructs by vector sequences, and an additional artificial Met was added at the amino end of all constructs except 1MAPSK, APSKL, APSK456, and 3MSK.

The Novagen pET-15b vector was used for bacterial expression of the separate enzyme domains. This plasmid provides elements needed for translation in *E. coli* as well as His-Tag and thrombin digestion site coding sequences to facilitate purification of the expressed proteins. Amplified fragments were doubly digested with *NdeI* and *XhoI* and ligated into the likewise digested vector. Recovered cloned inserts were sequenced in their entirety before transformation into JM109 DE3 cells by the CaCl2 method.

Protein expression and purification were accomplished as described previously (16). Briefly, isopropyl-1-thio-β-D-galactopyranosidase was added to overnight bacterial cultures and incubation was continued for 3–4 h at 37 °C with shaking. Cells were pelleted and sonicated in IMAC 5 buffer (5 mM imidazole, 50 mM Tris, pH 7.9). Following removal of cellular debris by ultracentrifugation, the soluble protein fraction was diluted 1:2 in IMAC 5 sonication buffer and loaded onto a His-bind column from Novagen according to the supplier’s protocol. Following incubation at 4 °C for 20 min with intermittent mixing, the flowthrough was collected, and the column was washed with 30 mM imidazole in 50 mM Tris, pH 7.9, to remove the majority of nonspecifically bound bacterial proteins, after which the expressed protein was eluted with 400 mM imidazole in 50 mM Tris, pH 7.9. The purified protein was dialyzed into phosphate buffer (25 mM NaHPO4/K2HPO4, pH 7.8, 1 mM dithiothreitol, 1 mM EDTA) overnight in preparation for enzymatic assays.

**Western Blot Analysis**—A nickel nitrioltriacetic acid (Ni-NTA) alkaline phosphatase conjugate was used to detect the His-tagged expressed protein on Western blots. Briefly, the purified protein was electrophoresed in a 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked in 3% bovine serum albumin in TBS (150 mM NaCl, 10 mM Tris·HCl, pH 7.4) buffer overnight and then washed three times in TBST (500 mM NaCl, 0.05% Tween 20, 20 mM Tris·HCl, pH 7.5) buffer. The blot was then incubated with the Ni-NTA alkaline phosphatase conjugate at a 1:500 dilution in TBST buffer for 2 h and washed as before. The protein band was visualized using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate in a Tris buffer (100 mM NaCl, 5 mM MgCl2, 100 mM Tris·HCl, pH 9.5) followed by a 10% trichloroacetic acid wash and storage in H2O.

**Generation of Rearranged Mutant Proteins**—Both the ATPS/APS and ATPS/APS/L constructs were created from the natural S/R cDNA clone using the methodology of Ali and Steinkasserer (21). For the former, the APS kinase region was amplified with a 5’ primer (SW1), which had no restriction site, and a 3’ primer (N6), which included a *XhoI* site, whereas the ATP-sulfurylase region was amplified with a 5’ primer with an *NdeI* site (N6) but a 3’ primer without a restriction site using Vent DNA polymerase. Following gel purification of the two bands, the blunt-ended DNA fragments were phosphorylated for 30 min at 37 °C and then ligated for 15 min at room temperature in a 10-μl reaction. 1 μl of the ligation reaction was then re-amplified using the two internal primers (N5 and N6). An additional step was required in the construction of the ATPS/LPSK/L mutant. The ATPS/LPSK construct was generated through polymerase chain reaction incorporation of restriction sites 5’ and 3’ to each domain (APSK, ATPS, and linker) followed by digestion and ligation of the respective half-sites to give the desired order. An EcoRI site was inserted between the sulfurylase and linker sequence, causing insertion of the dipeptide.
Expression and Analysis of Individual Sulfurylase and Kinase Domains—Expression constructs were created, and expressed protein was purified as described under “Experimental Procedures.” In addition to the entire bifunctional enzyme (1MSK), seven constructs, each encoding one of the catalytic domains plus various amounts of additional peptide sequence, were designed (Fig. 1A). Four constructs corresponding to the APS kinase region were made: APS kinase only, (1MAPSK) (residues 1–199); APS-kinase plus linker (APSKL) (residues 1–236); and APS-kinase plus linker with two extensions into the sulfurylase domain (APSK257) (residues 1–257); and APSK456 (residues 1–456). The ATP-sulfurylase domain was encoded in three constructs: sulfurylase only, ATPS (residues 237–624); sulfurylase plus linker, LATPS (residues 200–624); and sulfurylase with NH2-terminal extension into the kinase region, 3MSK (residues 70–624).

Detection of three of these expressed protein constructs is illustrated in Fig. 2. Following purification of the soluble fractions from the bacterial lysate on the His-bind histidine affinity resin, expressed proteins of sizes corresponding to the expected molecular weights were visible on SDS-polyacrylamide gel electrophoresis (Fig. 2A). To ascertain that these proteins were expressed from our constructs and not bacterial contaminants, a Western blot using a nickel-NTA alkaline phosphatase conjugate visualized a major band for each recombinant protein preparation corresponding to the expected size (Fig. 2B). The other expression products were characterized in the same manner.

To assess the level of expression and the activity of each recombinant protein, three assays were routinely used, the reverse sulfurylase assay, the kinase assay, and the overall reaction assay; the forward sulfurylase assay was used when appropriate. Homogenates were assayed to verify that overexpression of the recombinant protein was accompanied by an increase in enzyme activity. Extracts obtained from vector-free DE3 cells alone and cells transfected with empty vector or the
normal 1MSK construct were all assayed to verify that there was no activity in the absence of a construct. Expressed recombinant proteins were purified as described under “Experimental Procedures” for all subsequent assays.

When the APS kinase-containing recombinants were assayed, 1MAPSK expressed protein exhibited neither kinase nor sulfurylase activity. Recombinant proteins expressed from the series of extended constructs were then assayed to determine the minimal amount of additional sequence necessary to restore kinase activity (Table I). APSKL, APSK257, and APSK456 were all found to have kinase activity with comparable specific activities (~0.2 nmol/min/mg). Of the three, APSKL was the shortest active recombinant, extending the kinase-like domain by inclusion of the 37-residue linker region only. None of these recombinants exhibited sulfurylase activity.

In contrast to the requirement for additional sequence at the COOH terminus to generate an active APS kinase, the expressed protein from the sulfurylase-only (ATPS) construct was active in both the forward and reverse sulfurylase reactions; the specific activity of ATPS in the reverse sulfurylase assay was 2.77 μmol/min/mg compared with 2.44 μmol/min/mg for 1MSK (Table I). Addition of the linker region at the NH₂-terminal end of the sulfurylase sequence did not increase the specific activity of this construct (2.64 μmol/min/mg) and further extension to the third methionine (Met-70) in the kinase sequence (3MSK) caused a moderate decrease in specific activity (1.62 μmol/min/mg).

To examine the possibility that combining the separately expressed monofunctional domains in solution might enhance the individual activities or facilitate a coupled reaction, a series of mixing experiments was conducted. Initial experiments demonstrated that mixtures of either ATPS or LATPS proteins at a molar ratio of 1:1 with the 1MAPSK protein did not yield kinase or overall activity. However, when ATPS or LATPS was combined with APSKL protein, overall PAPS production was comparable to that produced by the intact enzyme (Fig. 3).

Gel filtration experiments on Sephadex G-200 indicated that the wild type enzyme and the two individual domains, ATPS and APSKL, are present as monomers at concentrations of 6.5 or 65 nM in the absence of substrate (data not shown). Similarly an equimolar mixture of ATPS and APSKL did not show the presence of heterodimers.

To examine the importance of the linker region and domain order on enzyme activity, a recombinant without the 37-residue linker region (APSK/ATPS) as well as a series of switched-order mutants in which the domain organization was rearranged so that the sulfurylase domain preceded the kinase domain: 1) without the linker region (ATPS/APSK), 2) with the linker region between the sulfurylase and the kinase domains (ATPS/L/APSK); and 3) with the linker region at the extreme carboxyl terminus, i.e. following the kinase domain (ATPS/APSK/L) were constructed (Fig. 1B).

When these structural mutants were assayed for sulfurylase, kinase, and overall activities (Table II), neither ATPS/APSK, ATPS/L/APSK, nor APSK/ATPS exhibited sulfurylase activity, and hence did not synthesize PAPS. Additionally ATPS/APSK and ATPS/L/APSK lacked kinase activity (~10% of the wild type 1MSK) (Table II). The APSK/ATPS recombinant, which lacked the linker region, exhibited partial kinase activity (~25% of wild type) (Table II). However, the ATPS/APSK/L recombinant exhibited normal sulfurylase, kinase, and overall activity (2.42 μmol/min/mg, 210 pmol/min/mg, and 10.5 nmol/min/mg, respectively).

Stability Experiments—To determine whether fusion of the sulfurylase and kinase domains has a stabilizing or destabilizing effect on the component activities, inactivation of the purified bifunctional and monofunctional recombinants was compared using prolonged incubation at different temperatures. The bifunctional enzyme (1MSK) maintained sulfurylase activity for 2 h as monitored at both 25 and 37 °C (Fig. 5; Table III). In contrast when the monofunctional proteins containing the

### Table I

| Construct | Sulfurylase activity | Kinase activity |
|-----------|----------------------|----------------|
| 1MSK      | 2.44                 | 160.8          |
| 1MAPSK    | ND                  | ND             |
| APSKL     | ND                  | 200            |
| APSK257   | ND                  | 220            |
| APSK456   | ND                  | 200            |
| ATPS      | 2.77                | ND             |
| LATPS     | 2.64                | ND             |
| 3MSK      | 1.62                | ND             |

* ND, none detected.
sulfurylase domain, i.e. ATPS, LATPS and 3MSK were similarly treated, ATPS, which contains the sulfurylase portion only, lost activity within 50 min at 37 °C with a half-life close to 10 min. At 25 °C the loss was much slower, i.e. a 40% activity loss in 2 h. When the length of the protein was increased at the amino-terminal end of the ATPS domain by including the 37-residue linker region to the COOH-terminal end, the sulfurylase remained completely active at 25 °C and showed improved stability at 37 °C, with an approximate 2-fold increase in the half-life and 20% residual activity after 2 h. When the length of the protein was increased further on the amino-terminal end to include sequence to the third methionine (Met-70) in the kinase sequence, but not including the P-loop (3MSK) (20), there was a dramatic increase in the stability of the sulfurylase activity; i.e. complete activity for the 2-h incubation period at both 25 and 37 °C.

In contrast to the increased lability of the most truncated constructs bearing the sulfurylase domain, no significant deactivation of APS kinase activity was observed for either the intact bifunctional protein (1MSK) or the recombinant APSKL at 25 or 37 °C (Table III). These results suggest that the individual kinase portion of the enzyme is as stable as the native bifunctional enzyme. To determine whether the separate active kinase protein might stabilize the sulfurylase protein, equimolar amounts of recombinant APSKL and ATPS were mixed, and the mixture was subjected to incubation at the different temperatures. No difference in the inactivation of the individual sulfurylase domain versus the mixture of ATPS and APSKL was observed at either 25 or 37 °C (Fig. 5; Table III), indicating either that the domains do not interact, or the complex formed does not stabilize the sulfurylase activity. Alternatively, it may be that the global geometry of the protein in addition to complex formation is important in stabilizing the enzyme activity.

Finally, the significance of the domain order in the bifunctional enzyme was addressed with respect to the stability of the sulfurylase domain by analyzing the rearranged recombinant, ATPS/APSK/L, which exhibits all three activities, sulfurylase, kinase, and overall PAPS synthetase. Monitoring the sulfurylase and kinase activities as a function of incubation time at 25 and 37 °C showed that the sulfurylase activity was susceptible to destabilization, losing activity with a half-life of 15 min and exhibiting a residual activity of only 2% after incubation for 2 h at 37 °C (Fig. 5; Table III). Although it is relatively more stable at 25 °C, the recombinant still lost about 20% of the activity in 2 h, compared with no loss of activity by the native enzyme. As before, the kinase activity remained resistant to inactivation at both 25 and 37 °C, similar to the native enzyme (Table III).

**DISCUSSION**

The ability of individual domains of some multifunctional enzymes to function independently has recently received increasing attention (18, 22). In the case of murine ATP-sulfurylase/APS kinase we have shown that the native bifunctional enzyme, which possesses both sulfurylase and kinase activities, efficiently converts all APS synthesized into PAPS (7, 10). The native bifunctional enzyme has an NH₂-terminal region homologous to known APS kinases and a COOH-terminal region highly homologous to known ATP-sulfurylases (10). To understand the selective advantages of the bifunctional enzyme over expressed monofunctional domains, we engineered segments of sulfurylase/kinase to produce the two individual enzyme activities on separately expressed polypeptides.

The ability of the ATP-sulfurylase domain alone to catalyze the sulfurylase reaction in both the forward and reverse directions was the first evidence that the bifunctional enzyme can be divided into active fragments containing each enzyme activity. Although a construct encoding the APS kinase domain-only (1MAPSK) was inactive for kinase activity, addition of the 37-residue linker region to the COOH-terminal end of the kinase polypeptide (APSKL) restored kinase activity. However, the importance of this sequence to kinase activity cannot be attributed a priori to a catalytic or structural role, because it is possible that the APSK polypeptide is incapable of proper folding and is therefore inactive. These results do however indicate that fusion of the two activities does not preclude their ability to function independently. Furthermore, isolation of the two enzyme activities on individual polypeptides and the ability of each domain to function separately implies that each domain
must have the independent ability to bind ATP and APS.

Because we could construct monofunctional units that express each activity, it was of interest to determine whether they could act in a coupled reaction to synthesize PAPS, when mixed in solution. Assays of such mixtures showed that although 1MAPSK and ATPS together had no appreciable kinase or overall activity, a mixture of APSKL and ATPS displayed kinase activity and overall synthesis of PAPS comparable to those of the intact enzyme (Fig. 3). In these mixing experiments, the amount of PAPS synthesized increased when the amount of one enzyme (APSKL or ATPS) was held constant, and the other (ATPS or APSKL) was increased. Thus it appears that the APS intermediate is efficiently transferred between the two separate polypeptides. Future experiments using isotopic dilution and enrichment will examine the ability of such mixtures to exhibit either efficient coupling like the separate fungal enzymes or channeling as demonstrated by the bifunctional rat chondrosarcoma enzyme (8).

Preliminary kinetic data suggest that there are modest differences in binding of the substrates, ATP and free sulfate, and the product, pyrophosphate, to the sulfurylase portion of the enzyme in 1MSK and the ATPS mutant, whereas the binding of APS is significantly different. Tighter binding of APS in the native enzyme, 1MSK, may be a reflection of the native enzyme to not release the APS intermediate into the medium and instead transfer it directly to the active site of the kinase portion of the enzyme. But because APS is the product that is released from the individual ATPS enzyme, its binding efficiency is lower. There is also a significant difference in the binding of the APS intermediate to the kinase portion of 1MSK compared with the monofunctional APSKL. These data would suggest that a covalent linkage between the two domains leads to a significant perturbation in apparent binding affinities without much effect on catalytic efficiency. Detailed kinetic analyses are underway to investigate these mechanistic aspects.

The results obtained from the rearranged structural mutants, ATPS/L/APS, ATPS/APS, APSK/ATPS, and ATPS/APSKL are particularly interesting. For instance, the inability of the first three of these constructs to catalyze the sulfurylase reaction is difficult to interpret, especially considering the fact that the individual sulfurylase domain is capable of functioning independently, and that the fourth rearranged mutant, ATPS/APSKL, exhibited normal sulfurylase activity comparable to that of the wild type. These results will become more comprehensible when the results of the stability experiments are considered.

The inability of the APS kinase domain to function without a linker region at its COOH terminus correlates with the inability of the ATPS/APS and ATPS/L/APS mutants to form PAPS (<10% of the wild type enzyme). These results imply either that the linker region is needed for kinase activity in addition to its positional specificity, i.e. the linker must be present at the COOH-terminal end of the kinase domain, or that the alteration of the sequence disrupts the kinase activity caused by improper folding. The later argument can be discounted based on the result of the third construct, APSK/ATPS. This construct, although not demonstrating normal kinase activity, did show about 25% of the kinase activity of the wild type. The partial preservation of kinase activity in this recombinant may be caused by the extension of the COOH-terminal region of the kinase domain by additional sulfurylase amino acid sequence. Interestingly when the linker sequence is aligned with the ATPS sequence (Fig. 4) there is a region toward the NH2 terminus of the ATPS sequence (residues 290–318 in the ATPS sequence) that is similar to the linker

FIG. 4. Sequence comparison. The similarity between the linker sequence (residues 200–236 of wild type protein) and the amino terminus of ATPS sequence (residues 268–328 of wild type protein) was found by FASTA sequence comparison (Wisconsin Package).
tivity of monofunctional domains were found to be less stable when compared with the bifunctional enzyme. To overcome this instability, microorganisms often produce higher amounts of the monofunctional enzymes compared with the bifunctional counterpart in mammalian cells (18).

The stability of the fused bifunctional sulfurylase/kinase versus the truncated or rearranged recombinant was thus examined. In our studies, stability is defined as a time-dependent inactivation process. It is fully recognized that loss of activity may be because of a number of factors, some of which are currently under investigation. In the murine sulfurylase/kinase system the wild type enzyme (1MSK), which has the domain arrangement APSKL/ATPS, is fully active in terms of both the kinase and sulfurylase activities for the entire incubation period at either 25 or 37 °C. However, the two separately expressed domains bearing a single activity (ATPS and APSKL) show significantly different stability characteristics. The shortest recombinant that exhibits normal kinase activity, APSKL, retained its kinase activity for the full incubation period at both 25 and 37 °C, which is similar to wild type. In contrast, ATPS, the shortest recombinant initially exhibiting normal sulfurylase activity is unstable at both temperatures as shown in Fig. 5 and Table III.

Because the sulfurylase activity of intact 1MSK is completely stable over the 2-h incubation period, we sought to determine whether additional sequence would stabilize the sulfurylase domain or if the entire kinase and linker sequence is necessary for complete stability. The sulfurylase portion with the linker at the NH₂ terminus did exhibit somewhat improved stability compared with ATPS, with the half-life increasing from 10 to 25 min at 37 °C. Thus the linker region does enhance sulfurylase stability, but linker alone is not sufficient to completely stabilize sulfurylase.

Interestingly, the sulfurylase component of the 3MSK protein that includes a truncated portion of the kinase domain, excluding the P-loop and thus not exhibiting any kinase activity (20), demonstrated complete stability comparable to the wild type enzyme. This result indicates that neither the entire kinase portion of the enzyme nor active kinase is required for stabilization of the sulfurylase activity and further that the P-loop plays a role predominantly in catalysis (20) rather than in stabilization of the enzyme structure. Last, a mixture of APSKL and ATPS in equimolar ratio, did not protect the sulfurylase activity from inactivation, indicating that noncovalent association of active kinase (APSKL) with active sulfurylase (ATPS) does not stabilize the sulfurylase region, although there is efficient coupling of the reactions by simple mixing of the monofunctional components.

Most interesting was the ATPS/APS KL recombinant, which exhibited activity similar to the wild type for sulfurylase, kinase, and overall, suggesting the order of domains has little effect on catalytic activity. As with all the other truncated and rearranged recombinants, the kinase activity is as stable as the wild type protein. However the sulfurylase activity was not even as stable in this rearranged recombinant as in LATPS, but rather was nearly identical to ATPS, exhibiting a half-life close to 15 min and complete loss of sulfurylase activity in 2 h, although at a slightly slower rate. These findings suggest that stabilization of sulfurylase activity has an absolute requirement for additional sequence at the NH₂ terminus only.

Overall, the stability ranking of the recombinants that possess sulfurylase activity, 1MSK = 3MSK > LATPS > ATPS/APS KL = ATPS, suggests that the requirements for initial activity and maintenance of that activity are different. Stabilization of the sulfurylase domain appears to require some sequence preceding its NH₂ terminus. Second, the linker region appears to also be required and positioned in such a way that it is able to interact with the sulfurylase portion. The lack of sulfurylase activity in the ATPS/APS KL and APS KL/ATPS recombinants, which lack the linker region, and the ATPS/APS KL recombinant, which contains the linker positioned between the COOH terminus of the sulfurylase and NH₂ terminus of the kinase, are commensurate with these being highly unstable recombinants that do not even exhibit initial activity.

In light of these findings, the origin and relevance of the COOH-terminal extension of the P. chrysogenum sulfurylase (15) is especially puzzling.

In conclusion, the bifunctional murine ATP-sulfurylase/APS kinase can be separated into active monofunctional domains. Because the linker region is required for kinase activity and the short kinase + linker recombinant (APSKL) is fully stable, it can be suggested that the linker is an integral part of the kinase region. In contrast, the sulfurylase domain by itself (ATPS) is fully active but unstable. The positional requirements of the three regions, kinase, linker, and sulfurylase, appear to be arrangement-specific and contribute significantly to the stability of sulfurylase activity in the bifunctional enzyme, rather than for catalytic activity per se. Hence the wild type protein, APSKL/ATPS, may have been selected for over the ATPS/APS KL arrangement, which also exhibits all the activities but has lower intrinsic stability. Although these studies demonstrate there is both arrangement- and sequence-specific requirements for maintenance of sulfurylase activity, it was not determined how the time-dependent loss of activity is related to structural perturbations in the protein, i.e. folding/ unfolding or aggregation. Studies to discriminate these possibilities are in progress.

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