Variations in the Response of Mouse Isozymes of Adenylosuccinate Synthetase to Inhibitors of Physiological Relevance*

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Vertebrates have acidic and basic isozymes of adenylosuccinate synthetase, which participate in the first committed step of de novo AMP biosynthesis and/or the purine nucleotide cycle. These isozymes differ in their kinetic properties and N-leader sequences, and their regulation may vary with tissue type. Recombinant acidic and basic synthetases from mouse, in the presence of active site ligands, behave in analytical ultracentrifugation as dimers. Active site ligands enhance thermal stability of both isozymes. Truncated forms of both isozymes retain the kinetic parameters and the oligomerization status of the full-length proteins. AMP potently inhibits the acidic isozyme competitively with respect to IMP. In contrast, AMP weakly inhibits the basic isozyme noncompetitively with respect to all substrates. IMP inhibition of the acidic isozyme is competitive, and that of the basic isozyme noncompetitive, with respect to GTP. Fructose 1,6-bisphosphate potently inhibits both isozymes competitively with respect to IMP but becomes noncompetitive at saturating substrate concentrations. The above, coupled with structural information, suggests antagonistic interactions between the active sites of the basic isozyme, whereas active sites of the acidic isozyme seem functionally independent. Fructose 1,6-bisphosphate and IMP together may be dynamic regulators of the basic isozyme in muscle, causing potent inhibition of the synthetase under conditions of high AMP deaminase activity.

Adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP-forming), EC 6.3.4.4) is present in almost all organisms, the only exceptions being some intracellular prokaryotic parasites (1). Adenylosuccinate synthetases are well conserved through evolution, exhibiting, for instance, ~40% sequence identity between eubacteria and mammals (2, 3). Eukaryotic synthetases differ from their prokaryotic counterparts by the presence of N-terminal leader sequences (~30 residues) and by truncations (~10 residues) at their C termini (2–4). The synthetase catalyzes the first committed step of the de novo biosynthesis of AMP from IMP and, in vertebrates, is also a component of the purine nucleotide cycle (PNC)1 (2, 5–7). Mammals have two different synthetase isozymes (2, 3, 7–11). The basic isozyme (hereafter AdSS1) has a higher $K_m$ value for IMP and a lower $K_m$ value for L-aspartate than the acidic form (AdSS2). AdSS2 is less susceptible to inhibition by fructose 1,6-bisphosphate (Fru-1,6-P$_2$) than AdSS1 but more strongly inhibited by nucleotides (7, 12). On the basis of these findings alone, investigators assigned AdSS2 to de novo biosynthesis of AMP and AdSS1 to the PNC (2, 6, 12). The PNC is active in muscle, brain, kidney, liver, and pancreatic islets (5, 13–17) and involves adenylosuccinate synthetase, adenylosuccinate lyase, and AMP deaminase in the following net reaction (Reaction 1):

$$\text{L-aspartate} + \text{GTP} + \text{H}_2\text{O} \rightarrow \text{fumarate} + \text{GDP} + \text{P} + \text{NH}_3$$

REACTION 1

The PNC may have multiple roles as follows: 1) shifting the adenylate kinase equilibrium in the direction of ATP formation by converting AMP into IMP; 2) liberating ammonia from amino acids by using L-aspartate as a donor; 3) regulating glycolysis (IMP activates glycogen phosphorylase and both AMP and ammonia activate phosphofructokinase); and 4) providing Krebs cycle intermediates (fumarate) in tissues that lack pyruvate carboxylase (2, 6).

The impact of the PNC on the metabolism of various tissues is unsettled (18–21), as is the assignment of the two isozymes to mutually exclusive metabolic roles (18). Moreover, in muscle, where exercise can increase IMP concentration up to 50-fold, the PNC may work asynchronously; AMP deaminase works only when AdSS1 and/or adenylosuccinate lyase is quiescent (3, 22–25). During recovery (restoration of basal IMP levels) AMP deaminase is inactive (24, 26). Indeed, interactions with myosin activate AMP deaminase, a process regulated by the decrease in the ATP concentration during exercise (24, 26). No study has demonstrated regulation of AdSS1 in the context of the PNC, although slight inhibition of the basic isozyme occurs at high concentrations of IMP (8, 13, 27).

Mouse recombinant AdSS1 has a significantly lower $K_m$ for IMP than that reported for the basic isozyme isolated from either rat or rabbit (28). Reported $K_m$ values for adenylosuccinate synthetases vary considerably (2, 6, 28) due, in part, to variations in assay protocols and conditions of assay, as well as intrinsic differences in the synthetases themselves (2, 6). The low natural abundance of AdSS2 has been an impediment to its purification and rigorous evaluation (7, 9, 18). Preparations of AdSS2 from malignant cells, such as Novikoff ascites tumor cells (29) and Yoshida sarcoma tumor cells (30), have provided,
save in one instance (30), specific activities significantly lower than that of AdSS1 (2, 6). Human and mouse AdSS2 have been cloned (3, 4) and the latter overexpressed in COS (African green monkey kidney) cells, but no kinetic characterization was reported (3). Native states of oligomerization for each isozyme remain ambiguous, as reports of monomeric and dimeric AdSS1 and AdSS2 are in the literature (8, 10, 29–31). In contrast, the synthetase from Escherichia coli is active as a dimer (32, 33) and exists in a monomer-dimer equilibrium (34). Reported here are first instances of heterologous overproduction and kinetic characterization of mouse AdSS2. The $k_{cat}$ values for recombinant AdSS1 and AdSS2 are almost identical. AdSS2, relative to AdSS1, has a slightly lower $K_m$ for IMP and GTP and a significantly higher $K_m$ for L-aspartate. High (but physiologically relevant) concentrations of IMP inhibit AdSS1 but not AdSS2. Adenylosuccinate, GDP, and GMP are strong inhibitors of both isoforms, but AMP, which potently inhibits AdSS2, is a weak inhibitor of AdSS1. Furthermore, the kinetic mechanism of AMP inhibition differs for the two isoforms. Fru-1,6-P$_2$ might be a physiologically significant inhibitor of AdSS2 but not AdSS1. Truncated isozymes (N-terminal Fru-1,6-P$_2$ might be a physiologically significant inhibitor of the mechanism of AMP inhibition differs for the two isozymes. Recombinant $E.~coli$ adenylosuccinate synthetase was overproduced and purified as described elsewhere (34).

Enzyme Assay—Protein concentration was determined by the method of Bradford (36), using bovine serum albumin as a standard. Enzyme activity was determined at an absorbance of 280 nm and at 22 °C as described previously (37). A standard assay buffer for AdSS1 contained 40 mM HEPES, pH 6.7, 8 mM magnesium acetate, 150 μM GTP, 250 μM IMP, and 2 mM aspartate. For AdSS2, the assay buffer contained 40 mM HEPES, pH 6.7, 8 mM magnesium acetate, 150 μM GTP, 200 μM IMP, and 8 mM L-aspartate. The reaction was started by the addition of up to 1 μg/mL enzyme. Under these conditions the reaction was linear for 1 min. The Hill coefficient for Mg$^{2+}$ was determined by varying the concentration of magnesium acetate from 0.2 to 4 mM for AdSS1, 0.05 to 2 mM for AdSS2, and 0.05 to 2 mM for Fru-1,6-P$_2$, AMP, GDP, and GMP were determined by holding two substrates at saturating levels and varying the concentration of the third substrate over 1–8 $K_m$, at different fixed concentrations of inhibitors ranging over 0.5–2 $K_m$. In experiments to determine the $K_m$ of IMP inhibition, concentrations of GTP varied from 15 to 250 μM, those of IMP ranged from 40 to 4,000 μM, and concentrations of L-aspartate and Mg$^{2+}$ were 2 and 8 mM, respectively. Kinetic data were analyzed with the computer program GraFit (38).

Analytical Ultracentrifugation—Sedimentation equilibrium experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge using an An-60 Ti rotor, rotor speeds of 9,000 and 15,000 rpm, and a temperature of 4 °C. AdSS1 and AdSS2, loaded onto a DEAE-Sepharose column equilibrated with the same buffer. AdSS2 was eluted by a linear gradient (0–200 mM NaCl). The column retained the acidic but not the basic isozyme. 1M ammonium sulfate was used to elute the two isozymes. Mouse isozymes exhibit a monomer-dimer equilibrium, and both GTP and IMP stabilize the dimer. Differences in mouse synthetases reported here do not support mutually exclusive metabolic roles for the two isoforms. Moreover, our findings support the asynchronous operation of the PNC in muscle.

EXPERIMENTAL PROCEDURES

Materials—E. coli strain BL21 (DE3), plasmid pET28b, nickel-nitritrotetrazolium acid-agarose, and the thrombin cleavage capture kit were from Novagen, Inc. Restriction enzymes, DNA ligase, and Vent Polymerase were from New England Biolabs. All other reagents were from Sigma unless noted otherwise.

Construction of Full-length and Truncated Synthetases—The cloning of full-length AdSS1 into the expression plasmid pET28b was described previously (28). cDNA for mouse acidic adenylosuccinate synthetase (AdSS2) was kindly provided by Dr. F. B. Rudolph (Department of Biochemistry and Cell Biology, Rice University, Houston, TX) as a pSPORT1clone (11). A fragment of 1,371 bp was amplified using the following primers: forward, 5'-CCCTTGCTATATCGTCCCTGAGGACGGCAGC-3' (Ndel restriction site underlined), and reverse, 5'-CCGCTCGAGTGAGAAGAAGGCGATCGATGACC-3' (Xho1 restriction site underlined). Insertion of the amplified fragment into corresponding sites of the pET28b expression vector resulted in the plasmid pAdSS2a. An Ndel restriction site located into the AdSS2 open reading frame was removed by a silent mutation. Truncated AdSS1 (AdSS1-Tr) and AdSS2 (AdSS2-Tr) were generated using the forward primers 5'-CCCTTGTGCTATATCGTCCCTGAGGACGGCAGC-3' and 5'-CCCTTTGCTATATCGTCCCTGAGGACGGCAGC-3', respectively (Ndel restriction sites underlined). All constructs were checked by sequencing (Iowa State University DNA sequencing facility).

Sequence Alignments—DNA and protein sequences were aligned using Multalin (35). Published sequences of mouse AdSS2 (GenBank™ gi, 404056 and 6671520) (11) were compared with other full-length sequences (GenBank™ gi, 128889641, 12901898, 13048093, 14817011, and 14676160). The published sequence of human AdSS2 (GenBank™ gi, 415848) (4) was compared with other (redundant) sequences (GenBank™ gi, 10438053 and 15214462). The sequence for human AdSS1 came from available full-length clones and the latter overexpressed in COS (African green monkey kidney) cells, but no kinetic characterization was reported (3). Native states of oligomerization for each isozyme remain ambiguous, as reports of monomeric and dimeric AdSS1 and AdSS2 are in the literature (8, 10, 29–31). In contrast, the synthetase from Escherichia coli is active as a dimer (32, 33) and exists in a monomer-dimer equilibrium (34).
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et al.  

quences of mouse AdSS2, reported here and by Guicherit

source (mouse or human) is

Sequence identity between AdSS1 and AdSS2 from the same

human AdSS2 then each have 26 amino acid residues (Fig. 1).

after position 24. The N-terminal leader sequence in mouse and

should be arginine and an additional residue (proline) comes

appear in inclusion bodies. In contrast, yields of recombinant

Opti-4CN kit (Bio-Rad).

0.05% SDS. Detection of antigen-antibody complexation employed the

RESULTS

Sequence Comparison and Validation—Nucleotide se-

quences of mouse AdSS2, reported here and by Guicherit et al.

(11), differ at positions 499 (C in Ref. 11 is now G) and 595 (A

becomes G). As a consequence, Arg167 and Thr199 become gly-

cine and alanine, respectively, identical to corresponding resi-

dues in human AdSS2 (4). Moreover, Gly186 is invariant among

synthetases from 43 organisms, representing 30 major phylo-

genetic lineages. The published sequence of human AdSS2 (4) (purA) synthetases.

FIG. 1. Alignment of amino acid sequences of mouse (M), hu-

man (H), and E. coli (purA) synthetases. Conserved amino acids

among mouse and human AdSS1 and AdSS2 are boxed. Sequence

corrections are in boldface italics. Vertical arrows indicate sites of

N-terminal truncation of the mouse isozymes. Active site residues are
designated by .

FIG. 2. Thermal stability of mouse and E. coli synthetases.

Samples of 20 µl of protein solution at a concentration of 0.5 mg/ml

protein were incubated for 1 min at different temperatures. Plotted are

activities (determined as described under “Experimental Procedures”) relative to fully active systems at 40 °C of mouse AdSS1 (○ and ●), mouse AdSS2 (□ and ■), and the E. coli synthetase (▲ and ▼) in the absence and presence of IMP/GTP (open versus filled symbols, respectively).

AdSS2 and AdSS2-Tr were ∼25 mg/liter of cell culture, ∼20%
of the total soluble protein. SDS-PAGE of samples revealed a

single band of ∼50 kDa. Full-length and truncated AdSS2 are

stable for several days at 4 °C. AdSS2 is stable with respect to freeze/thaw cycles in buffers supplemented with 30% glycerol. Full-length AdSS1, with or without their N-terminal polyhisti-
dyl tags, have identical kinetic parameters and crystallize under

the same conditions (28). Similarly, truncation of the N-terminal polyhistidyl tag from AdSS2 does not change its kinetic properties (data not shown).

Thermal Stability—In the absence of ligands, the E. coli synthetase is in a monomer–dimer equilibrium (Kd ∼10 µM) (34). The presence of active site ligands, such as IMP and GTP, significantly increases thermal stability of E. coli synthetase. Moreover, the thermal stability of the E. coli synthetase increases with protein concentration.2 In the absence of ligands, AdSS1 and AdSS2 are more stable than the E. coli synthetase, but IMP and GTP do not greatly enhance the thermal stability of the mammalian isozymes (Fig. 2). AdSS1-Tr and AdSS2-Tr have the same thermal stability as their full-length counterparts, ruling out an effect due to the N-terminal leader sequence.

Native Molecular Weight of AdSS1 and AdSS2—Equilibrium sedimentation ultracentrifugation indicates single species of molecular mass 86.0 ± 3.9 and 90.7 ± 2.5 kDa for AdSS1 and AdSS2, respectively. Predicted masses are 101.1 and 100.7 kDa, derived from amino acid sequences of recombinant AdSS1 and AdSS2, respectively. Equilibrium ultracentrifugation of samples in the presence of IMP, GTP, Mg(ace-
tate)2, and hadacdin (N-hydroxy-N-formylglycine, a potent competitive in-
hibitor with respect to l-aspartate) showed increased molecular masses of 103.6 ± 4.2 and 101.5 ± 4.3 kDa for AdSS1 and AdSS2, respectively. The behavior of AdSS1-Tr and AdSS2-Tr was similar to their full-length counterparts. In the absence of active site ligands, molecular masses were 86.1 ± 5.9 and 84.8 ± 2.7 kDa for AdSS1-Tr and AdSS2-Tr, respectively; and in the presence of substrate/substrate analogues, their molecular masses were 101.1 ± 7.2 and 96.4 ± 0.8 kDa, respectively. Molecular masses of the recombinant mouse isozymes did not change over a protein concentration from 0.1 to 0.5 mg/ml, whereas the molecular mass of the E. coli synthetase varied from 47.8 ± 2.1 to 56.9 ± 2.0 kDa (experiments at 15,000 rpm). Evidently, over the range of protein concentration accessible to analytical centrifugation, AdSS1 and AdSS2 are predomi-

2 T. Borza and H. J. Fromm, unpublished results.
Table I
Kinetic parameters for full-length and truncated mouse isozymes

| Enzyme    | $k_{cat}$ | $K_m$ GTP | $K_m$ IMP | $K_m$ Asp |
|-----------|-----------|-----------|-----------|-----------|
| AdSS1     | 5.4 ± 0.4 | 12 ± 2    | 45 ± 7    | 140 ± 20  |
| AdSS1-Tr  | 3.9 ± 0.3 | 9 ± 2     | 43 ± 5    | 150 ± 15  |
| AdSS2     | 4.2 ± 0.4 | 15 ± 2    | 12 ± 2    | 950 ± 90  |
| AdSS2-Tr  | 4.0 ± 0.5 | 13 ± 2    | 9 ± 1     | 1030 ± 80 |

nantly dimers, but a small shift from monomer to dimer occurs in the presence of ligands. The $K_m$ value for the mammalian isozymes then, in the absence of ligands, is significantly lower than that of E. coli synthetase (34). The molecular weight (78 kDa) of a yeast synthetase, determined by gel filtration, lies between that of a monomer and dimer (40), whereas plant synthetases are either monomers or dimers, depending on the methodology of mass determination (41).

Values of $K_m$, $k_{cat}$, pH, and Buffer Effects—$K_m$ values for AdSS1-Tr are comparable with those of AdSS1 (28), but $k_{cat}$ is slightly lower, perhaps due to a small component of misfolded AdSS1-Tr. Relative to AdSS1, AdSS2 has a lower $K_m$ for IMP, a similar $K_m$ for GTP, and significantly higher $K_m$ for l-aspartate (Table I). AdSS2-Tr has a slightly higher $K_m$ value for l-aspartate than that of AdSS2. $K_m$ values for IMP and l-aspartate of tissue-derived AdSS1 and AdSS2 were similar to those of the recombinant isozymes (data not shown).

Metal Requirement—AdSS2 and the E. coli synthetase reach maximum activities in 2 mM Mg(acetate)$_2$, but AdSS1 requires 8 mM Mg(acetate)$_2$. Concentrations above 10 mM Mg(acetate)$_2$ are inhibitory for all systems. Inhibition is more pronounced when Cl$^-$ is a counterion to Mg$^{2+}$ instead of acetate. Hill coefficients for Mg$^{2+}$ are 1.1 ± 0.1 for AdSS1 and 1.0 ± 0.2 for AdSS2. The Hill coefficient for Mg$^{2+}$ is 2 for the E. coli synthetase (43). Crystal structures of AdSS1 (44, 45), AdSS2 (3), and the E. coli synthetase (46, 47), however, reveal only one Mg$^{2+}$ per subunit. Evidently, studies of AdSS1 and AdSS2 will not clarify the role of the "second" Mg$^{2+}$ inferred by the kinetics of the E. coli synthetase.

DISCUSSION

E. coli and mouse synthetases exhibit a monomer-dimer equilibrium, in which GTP and IMP stabilize the dimer. Mouse isozymes in the absence of ligands, however, are more stable than the E. coli synthetase on the basis of thermal stability and analytical ultracentrifugation. The monomer-dimer equilibrium may be a property common to all adenylosuccinate synthetases. The dimer is the active form of the E. coli synthetase, and evidently each of its subunits independently achieves maximum velocity in the presence of saturating substrates (32). Indeed, an arginyl side chain (Arg$^{143}$ in the E. coli synthetase) critical to the recognition of IMP (34) comes to the active site from a symmetry-related subunit of the dimer and is present in all known sequences of synthetase. Hence, regulatory mechanisms that impair subunit dimerization are conceptually possible but have not been demonstrated for any synthetase in vivo. The increased stability of the mouse dimers relative to E. coli dimer could be exploited, however, in the design of drugs (antibiotics) that target the subunit interface.

N-terminal truncations of the mouse isozymes do not alter dimer stability or kinetics. Therefore, the functional differences exhibited by the two isozymes arise from their core sequences, which are ~75% identical. Nevertheless, the N- leader sequences of AdSS1 and AdSS2 diverge significantly, and yet each is conserved across mammalian species. The latter observation suggests possible differences in regulation, protein-pro-
Table II

| Ligand   | AdSS1          | AdSS2          | Type of inhibition relative to substrate |
|----------|----------------|----------------|------------------------------------------|
| Adenylosuccinate | $K_i$ (μM) | $K_i$ | Competitive/IMP |
| GDP      | 19 ± 2        | 30 ± 2        | Competitive/GTP |
| GMP      | 12 ± 1        | 14 ± 1        | Competitive/GTP |
| AMP      | 700 ± 20      | 59 ± 6        | Non-competitive/IMP in AdSS1; competitive/IMP in AdSS2 |
| Fru-1,6-P$_2$ | 16 ± 3$^a$ | 19 ± 4$^a$ | Noncompetitive/IMP |
|          | 46 ± 6$^b$   | 128 ± 3$^b$  | Noncompetitive/GTP |
|          | 63 ± 7       | 400 ± 30     | Noncompetitive/Asp |
|          | 81 ± 6       | 670 ± 20     | Noncompetitive/Asp |

$^a$ Represents the dissociation of Fru-1,6-P$_2$ from the enzyme-GTP-(L-aspartate)-Fru-1,6-P$_2$ complex.

$^b$ Represents the dissociation of Fru-1,6-P$_2$ from the enzyme-GTP-(L-aspartate)-IMP-Fru-1,6-P$_2$ complex.

FIG. 3. Variation in the relief of IMP inhibition of mouse isozymes by GTP. IMP inhibition of AdSS1 at 10 μM GTP (□) and 150 μM GTP (■) exhibits little change, whereas that of AdSS2 differs significantly at 10 μM GTP (△) and 150 μM GTP (▲). Curves are smoothed fits through data points and do not correspond to a kinetic model.

FIG. 4. Comparison of observed and predicted initial velocities for IMP inhibition of mouse AdSS1. GTP and IMP concentrations vary over 10–200 and 40–4000 μM, respectively. The velocities were determined at 40 (○), 70 (●), 100 (□), 200 (■), 800 (△), 1600 (▲), 2400 (○), 3200 (■), and 4000 (☉) μM IMP. Curves represent theoretical lines obtained from fitted kinetic parameters and Equation 1.

FIG. 5. View down the molecular 2-fold axis of AdSS1. Conformational mobility of helix 5 specific to AdSS1, but not observed in structures of AdSS2, may communicate conformational changes in the IMP loop of one active site to that of the other. The IMP pockets and flexible IMP loops (last turn of the C-terminal end of helix 3 and the 10 residues that follow) of the synthetase dimer are adjacent to, but make only weak interactions with the helix 5 pair. Adenylosuccinate (labeled SAMP) occupies the IMP pocket in this particular structure from Ref. 45. This illustration was drawn by MOLSCRIPT (61)

Adenylosuccinate Synthetase

Previous studies (8, 13, 27) have documented IMP inhibition of AdSS1, but have disagreed as to whether GTP antagonizes or reinforces the phenomenon (8, 13). Nonetheless, IMP does bind to the GTP pocket of AdSS1 as evidenced by the crystal structure of an IMP complex (44). Furthermore, all residues of the GTP pocket are identical in AdSS1 and AdSS2. The EA$_2$ complex in Scheme I then may well represent IMP molecules bound to the IMP and GTP pockets within the same subunit of a dimer. High levels of GTP would relieve such inhibition, but AdSS1 remains sensitive to IMP even in the presence of saturating GTP. Hence, AdSS1 has an alternative mechanism (competitive non-competitive) that allows IMP to inhibit in the presence of GTP (EA$_2$ complex of Scheme I). As will be discussed below, the alternative inhibitory site for IMP in AdSS1 may be the symmetry-related IMP pocket of the dimer.

AMP inhibits AdSS2 ~12-fold more strongly than AdSS1, and by a different kinetic mechanism (competitive versus non-competitive with respect to IMP). Furthermore, in crystalline complexes of the E. coli synthetase (52), AdSS1 (45), and AdSS2 AMP binds only to the IMP pocket. AMP ligation of the IMP pocket accounts for competitive inhibition of AdSS2, but noncompetitive inhibition of AdSS1 suggests one of two alternative mechanisms. (i) AMP promotes the dissociation of an active AdSS1 dimer into inactive monomers. (ii) AMP inhibits one subunit of the AdSS1 dimer by binding to the IMP pocket of the other subunit. The former mechanism is unlikely, given the stabilizing effect of IMP on the dimer (34), but the latter is
plausible because of the proximity of IMP pockets in the dimer (Fig. 5). Helix 5 and the IMP loop (the latter binds the 5'-phosphoryl group of AMP) are conformationally dynamic elements in AdSS1 and could interact across the subunit interface. In contrast, helix 5 in AdSS2 is immobilized by hydrogen bond interactions and hence is less likely to transmit conformational changes between active sites. IMP and AMP inhibition in AdSS1 then could stem from a common mechanism that involves the interaction of active sites of the dimer. AdSS2 on the other hand seems more like the E. coli synthetase in its properties of IMP and AMP inhibition. Kang et al. (32) have demonstrated functionally independent (non-interacting) subunits in the dimer of the E. coli synthetase. Irrespective of whether subunit interactions in the dimer are the basis for noncompetitive AMP inhibition of AdSS1, an unresolved issue still remains. How does AdSS1 exclude AMP from its IMP pocket and still retain high affinity for IMP? The conformation of the pre-Switch loop in AdSS1 in its ligand-free state differs from that of other synthetases (28), including AdSS2. The pre-Switch loop in AdSS1 blocks the 5'-phosphoryl pocket and holds the side chain of Asn256 in an intramolecular hydrogen bond. Hence, IMP and AMP must sacrifice binding energy to overcome the antagonistic conformation of the pre-Switch loop in AdSS1. The 4–5-fold increase in the $K_m$ of IMP and the 11–12-fold increase in $K_c$ of AMP are consistent with less favorable interactions of each nucleotide with the IMP pocket of AdSS1 relative to AdSS2. Unlike AMP, however, IMP can in principle recover free energy lost in its binding interaction by forming 6-phosphoryl-IMP; atoms N-7 and N-6 of AMP cannot both hydrogen bond with the side chain of Asn256 whereas atoms N-7 and O-6 of 6-phosphoryl-IMP can do hydrogen-bond with the side chain of Asn256. As IMP and AMP levels in most tissues are ~60 and 200 μM, respectively (53), fluctuations in the relative concentrations of AMP and IMP would influence the activity of AdSS2, whereas variations in the concentration of IMP alone would influence the activity of AdSS1.

The assignment of AdSS1 and AdSS2 to separate metabolic roles by Nakagawa and co-workers (7, 12) rests largely on different susceptibilities to Fru-1,6-P2 inhibition (7, 12); however, the reported $K_i$ values (0.6 and 1.6 mM for AdSS1 and AdSS2, respectively) are 30–80-fold higher than physiological concentrations of Fru-1,6-P2. Nonetheless, all kinetic parameters reported by Nakagawa and co-workers (7, 10, 12, 30) are equal for AdSS1 and AdSS2. On the other hand, Fru-1,6-P2 inhibits AdSS2.3 The pre-Switch loop in AdSS1 blocks the 5'-phosphoryl group of AMP) are conformationally dynamic elements in vivo and in vitro. However, the concomitant increase in levels of IMP and Fru-1,6-P2 inhibit AdSS1. Cessation of vigorous exercise leads to the restoration of ATP and Fru-1,6-P2 levels, the inactivation of AMP deaminase, and the relief of Fru-1,6-P2 inhibition of AdSS1. As the IMP concentration diminishes, the activity of AdSS1 actually accelerates until IMP is no longer saturating.

Continuous operation of the PNC in muscle is difficult to reconcile with the accumulation of IMP in vivo and the requirement for GTP to drive the AdSS1 reaction. The re-conversion of GDP to GTP is at best unfavorable in the face of diminished concentrations of ATP (24). Hence, the PNC is an unlikely source for the ammonia produced during muscle contraction and probably cannot provide fumarate to the Krebs cycle as originally proposed (5, 13). Indeed, partial and complete deficiency in muscle AMP deaminase does not influence Krebs cycle anaplerosis, phosphocreatine hydrolysis, adenine nucleotide ratios, or exercise performance (21). Moreover, the rate of IMP accumulation during contraction varies with muscle type (56–59); IMP primarily goes to AMP via the PNC, but some is released as inosine and hypoxanthine. These last nucleotides must be re-synthesized de novo in order to restore adenine nucleotide pools (60). Hence, AdSS1 activity related to de novo purine biosynthesis and the PNC may be inseparable in muscle tissue.

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