Recombinant Mouse Muscle Adenylosuccinate Synthetase

OVEREXPRESSION, KINETICS, AND CRYSTAL STRUCTURE*

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Vertebrates possess two isozymes of adenylosuccinate synthetase. The acidic isozyme is similar to the synthetase from bacteria and plants, being involved in the de novo biosynthesis of AMP, whereas the basic isozyme participates in the purine nucleotide cycle. Reported here is the first instance of overexpression and crystal structure determination of a basic isozyme of adenylosuccinate synthetase. The recombinant mouse muscle enzyme purified to homogeneity in milligram quantities exhibits a specific activity comparable with that of the rat muscle enzyme isolated from tissue and K_m parameters for GTP, IMP, and L-aspartate (12, 45, and 140 μM, respectively) similar to those of the enzyme from *Escherichia coli*. The mouse muscle and *E. coli* enzymes have similar polypeptide folds, differing primarily in the conformation of loops, involved in substrate recognition and stabilization of the transition state. Residues 65–68 of the muscle isozyme adopt a conformation not observed in any previous synthetase structure. In its new conformation, segment 65–68 forms intramolecular hydrogen bonds with residues essential for the recognition of IMP and, in fact, sterically excludes IMP from the active site. Observed differences in ligand recognition among adenylosuccinate synthetases may be due in part to conformational variations in the IMP pocket of the ligand-free enzymes.

Adenylosuccinate synthetase (IMP: L-aspartate ligase (GDP-forming), EC 6.3.4.4) catalyzes the first committed step in the de novo biosynthesis of AMP. In vertebrates the synthetase is also a component of the purine nucleotide cycle, which interconverts IMP and AMP (1–3). Adenylosuccinate synthetases from various sources exhibit significant sequence conservation (~40% sequence identity between eubacteria and mammals, for instance (4)). Nonetheless, synthetases from nearly every eukaryotic organism have ~30-residues added to their N-termini and truncations at their C-termini relative to the bacterial synthetases. Moreover, vertebrates express two distinct forms of adenylosuccinate synthetase (5). The basic isozyme (often called the muscle enzyme or AdSS1) is associated with the purine nucleotide cycle (1), whereas the acidic isozyme (often called the liver enzyme or AdSS2) is associated with de novo biosynthesis of AMP (5). The two isozymes differ not only in their isoelectric points but also in their kinetic properties, regulation, and tissue distribution (2, 3).

Adenylosuccinate synthetase from *Escherichia coli* is the most studied of all synthetases. Its kinetic mechanism is rapid equilibrium random (6). 6-Phosphoryl-IMP is an intermediate in a two-step process that begins with the transfer of the γ-phosphoryl group of GTP to the 6-keto group of IMP, followed by the displacement of P_i from the intermediate by L-aspartate to form adenylosuccinate (7–11). In the absence of ligands the synthetase from *E. coli* is an equilibrium mixture of monomers and dimers (7, 12). Active site ligands and/or high subunit concentrations favor dimer formation. Hence, crystallization experiments have thus far resulted only in dimer assemblies (9–11, 13–19). IMP alone may stabilize the synthetase dimer and by itself triggers conformational changes in the active site of the *E. coli* synthetase.1 However, recent structures of the synthetase from plants, *Arabidopsis thaliana* and *Triticum aestivum*, reveal organized active sites in the presence of GDP alone (19).

More than two decades ago, the basic isozymes from rabbit (20) and rat (21) were purified and characterized. Subsequent studies confirmed a random sequential kinetic mechanism for these muscle synthetases (6, 8). Several groups reported crystals of the basic isozyme (20, 21), as well as preliminary x-ray diffraction data (22), but no structure of the basic isozyme has appeared in the literature. Only the cDNA encoding the muscle muscle isozyme has been cloned (4). Reported here are first instances of heterologous overexpression and structure determination of the basic isozyme of adenylosuccinate synthetase. The enzyme is expressed in *E. coli* using an N-terminal, polyhistidyl tail to facilitate purification. The purified enzyme crystallizes readily in the absence of ligands. The resulting crystal structure of the ligand-free protein at 2.5 Å resolution reveals a polypeptide fold similar to those of synthetases from *E. coli* and plants. The active sites of the basic isozyme and the *E. coli* synthetase, however, differ significantly in the conformation of loops, which define, in part, the IMP and GTP binding sites. Indeed, the basic isozyme, as seen here, cannot bind IMP as does the *E. coli* synthetase without a conformational change involving residues that up to now have been conformationally invariant among all known synthetase structures.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes, DNA ligase, and Vent Polymerase came from New England Biolabs. Plasmid pET28b, *E. coli* strain BL21

[1] Z. Hou, H. J. Fromm, and R. B. Honzatko, unpublished data.
Harvested cells were disrupted by sonication in lysis buffer (50 mM
NaCl, 10 mM imidazole, pH 8). The column was washed sequentially
with two buffers (10 volumes of each), differing from that above only in
imidazole concentration of 300 mM, and the culture was maintained at 15
°C overnight. The cells were collected at 4000 x g for 10 min at 4 °C.
Harvested cells were disrupted by sonication in lysis buffer (50 mM
NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 10% glycerol, pH 8). After
centrifugation at 20,000 x g for 30 min, SDS-polyacrylamide gel elec-
trophoresis of samples from both the supernatant and pellet revealed
the protein (~52 kDa) accounted for ~25% of the total protein. Most of the protein, however, was in inclusion bodies.

The supernatant solution was loaded onto a nickel-nitriilotriacetic
column equilibrated with the dialysis buffer. After the cell culture reached
~50 mL of the precipitant solution. Equal dimensional
bands of protein. The recombinant protein can be frozen at
contaminating proteins bind to the resin, whereas the recombinant
protein can be frozen at

RESULTS AND DISCUSSION

Enzyme Characterization—Previous studies of the rat and
rabbit muscle isozymes isolated from tissue reported a high Km
value for IMP and a low Km for l-aspartate relative to the acidic
isozymes (5, 20, 21, 35). Largely because of these findings, the
basic and acidic isozymes were cast as integral components of
the purine nucleotide cycle and de novo AMP biosynthesis,
respectively (5, 35). The Km values for GTP, IMP, and l-aspar-
tate of the recombinant mouse muscle isozyme are 12 ± 2, 45 ±
7, and 140 ± 15 , respectively, values that are more similar to
those of the E. coli synthetase (20–50, 20–60, and 200–350
, respectively) (36–38), than values reported for other
muscle isozymes isolated from tissue (10–120, 107–700, and 250–
330 , respectively) (5, 20, 21, 35, 39). The relatively large
range in reported Km values for the synthetase is probably due
in part to technical difficulties in establishing linear progress
curves. GTP is a potent product inhibitor of all known adeny-
osuccinate synthetases (2) and greatly limits the linear range of
progress curves in the determination of Km for GTP. Furth-
more, the synthetase may exist as a mixture of monomers and
dimers governed by an equilibrium constant sensitive to sub-
strate concentration (12). Hence, even the oligomeric state of
the synthetase may change with substrate concentration at a
fixed enzyme concentration. The highest values for Km come
from single point, stopped assays (5, 21), which in addition to
the complications noted above, provide no information by which
to assess the linearity of the progress curve. Excluding deter-
minations based on single point assays, the Km values for
enzymes from rabbit and rat muscle are 10–24, 107–320, and
290–300, respectively (20, 35, 39, 40), for GTP, IMP, and l-
aspartate, values approximately 2-fold higher than those re-
ported here for the recombinant enzyme from mouse muscle.

The recombinant mouse muscle isozyme has almost the same
specific activity as the rat muscle enzyme (5.74 versus 6.24
µmol/min/mg) (21) and is approximately five times more active
than the protein isolated from rabbit muscle (1.0 µmol/min/mg)
(41). We observe no evidence of substrate inhibition for IMP up
to a maximum concentration of 500 µM. Furthermore, thrombin
cleavage of the His-tagged synthetase and subsequent purifi-
cation do not alter the kinetic parameters of the recombinant
enzyme. In fact, the thrombin-cleaved protein forms crystals
identical to those described below, with a structure identi-
cal to within experimental uncertainty.

Structural Features of the Muscle Isozyme—Crystals of li-
gand-free, mouse muscle enzyme diffract to a resolution (2.5 Å)
comparable with crystalline synthetases from other sources.
The space group and unit cell dimensions are virtually identi-
k to those reported for the rat muscle isozyme (22). Statistics of
data collection and refinement are in Table I. The stereo-
chemistry of the refined model, as analyzed by the program
PROCHECK (32), exceeds that typically observed for a struc-
ture of 2.5 Å resolution. No residues lie in disallowed regions of
the Ramachandran plot, and 87% of all the residues are in the
most favored regions. The average uncertainty in coordinates is
~0.3 Å. Thermal parameters vary from 16 to 100 Å², the latter
being an upper limit imposed by the software.
We observe reliable electron density beginning with residue 26. Hence, the first 25 residues plus the His tag construct are disordered and not included as part of the model. The His tag/leader element (~50 residues) is presumably intact, given the successful isolation of the recombinant protein from nickel-nitritotriacetic acid-agarose and the lack of any evidence for heterogeneity (by gel electrophoresis) in the purified protein. The successful isolation of the recombinant protein from nickel-nitritotriacetic acid-column chromatography is presumably intact, given the low level of sequence identity (Fig. 2) and the absence of disordered structures.

As expected from the high level of sequence identity (Fig. 2), the overall fold of the mouse muscle synthetase is almost identical to that of other synthetases (Fig. 1). The structure consists of a β-sheet core made up of nine parallel strands (B14, B10, B7, B1, B6, B2, B3, B4, and B5) and one antiparallel strand (B15). The core β-sheet is flanked by subdomains that contain 11 α-helices, seven antiparallel β-strands, and five 3_α helices.

Secondary structures of the mouse muscle, E. coli, and plant synthetases are in agreement, with only a few differences. Residues 393–421 of the ligand-free, mouse muscle synthetase correspond to Phe264, Val236, Phe264, and Val353, but Met206 and Met352 of the mouse muscle enzyme correspond to polar residues in the synthetase. Catalytic function of adenylosuccinate synthetase from E. coli is sensitive to the integrity of the subunit interface. Mutations of Lys342 and Asp351, which correspond to Lys174 and Asp234, respectively, of the mouse muscle enzyme, inactivate the synthetase (42) or elevate the Km for IMP and GTP by 20- and 20-fold, respectively (38). At subunit concentrations below 10 μM, and in the absence of active site ligands, the E. coli synthetase is largely monomeric (12) and presumably inactive, although the latter has yet to be demonstrated. Synthetases from plants reportedly behave as monomers in gel filtration, but light scattering data are consistent with a dimer (19). The basic isoform is reportedly a stable dimer (2, 3, 21, 39). Conformational variations among synthetases in regions corresponding to residues 184–193, 204–213, and 385–395 of mouse muscle enzyme (Figs. 1 and 3) are at or close to the subunit interface of the dimer. Known crystal structures of synthetases, however, reveal no certain basis for differences in dimer stability, assuming that such differences exist.

The present structure provides no insight as to the role (if any) of the 30-amino acid extension to the N terminus of the mouse muscle enzyme (hereafter called the leader sequence). Leader sequences among members of the synthetase family are dissimilar. The fusion of lacZ to the N terminus of the acidic human isoform, however, results evidently in an inactive construct (43). As significant evidence stands against the direct participation of the leader sequence in catalysis (the E. coli synthetase, for instance, has no leader sequence), the fusion protein may be incapable of forming an active dimer. No evidence suggests, however, any change in catalytic properties or structure because of the presence of the N-terminal His tag.

**Active Site**—Loops 38–53, 120–131, 298–304, and 417–421 of the E. coli synthetase undergo significant conformational...
change in response to ligation of the active site (9, 38). The corresponding loops in the mouse muscle enzyme (residues 68–83, 151–165, 330–336, and 448–452, respectively) are probably sensitive to active site ligands as well. For the most part, residues in direct contact with active site ligands in the E. coli synthetase are identical to corresponding residues of the mouse muscle synthetase. By analogy to the E. coli synthetase, loops 151–165, 330–336, and 448–452 are part of the IMP-, the L-aspartate-, and the GTP-binding pockets, respectively, and specific residues of loop 68–83 probably interact with IMP, GTP, and Mg$^{2+}$.

Because the aforementioned loops seem important to the function of all known synthetases and yet have different sequence numbers, we suggest a generalized nomenclature. Hereafter, we suggest that the names Switch loop, IMP loop, Asp loop, and GTP loop represent segments corresponding to residues 38–53, 120–131, 298–304, and 417–421, respectively, of the E. coli synthetase (Fig. 1).

As noted above, the IMP loop of the mouse muscle enzyme is disordered. In structures of the E. coli and plant synthetases without IMP, the corresponding residues are also disordered. In the presence of IMP the conserved threonines at positions 162 and 163 of the mouse muscle enzyme should hydrogen bond with the 5'-phosphoryl group of IMP. The IMP loop of the mouse muscle enzyme has three more residues than those from E. coli and plant synthetases (Fig. 2). Glu$^{118}$ of E. coli synthetase, however, hydrogen bonds with the IMP loop of the ligated enzyme (9). If Glu$^{149}$ (the corresponding residue of the mouse muscle synthetase) plays a similar role, then the additional residues may influence the conformation of the mouse muscle

IMP loop only as it emerges from helix H3. Conformational differences in this area of the IMP loop, however, could still influence interactions between subunits of a synthetase dimer (Fig. 3).

In contrast to the disordered Asp loop of the E. coli synthetase, the corresponding loop of the mouse muscle enzyme adopts a well defined conformation, probably because of the stabilizing effect of lattice contacts (see above). The conformation of the Asp loop in the basic isozyme is similar to that of the E. coli synthetase in its complex with hadacidin (an analog of L-aspartate) (9). On the basis of model building and energy minimization, the Asp loop of the mouse muscle synthetase will retain its observed conformation but will move 5 Å toward the active site to hydrogen bond with hadacidin.

Superposition of the mouse muscle and E. coli synthetases reveals a 1.6 Å root mean square difference between their respective GTP loops. The observed difference may be related to a much larger conformational difference in the Switch loop, described below. Residues of the GTP loop define in part the binding pocket for the base of the guanine nucleotide. In the E. coli synthetase, Ser$^{414}$, the side chain of which hydrogen bonds with the O-6 atom of the guanine nucleotide, and Pro$^{417}$, which stacks against the base of the guanine nucleotide, are Gly$^{420}$ and Lys$^{423}$, respectively, in the mouse muscle isozyme. Neither changes in residue type nor conformation, however, greatly influence the $K_m$ for GTP (see above). In the plant enzymes Ser$^{414}$ is also replaced by glycine. Evidently, the backbone amide of the glycine recognizes the O-6 atom of the guanine nucleotide as well as the OG atom of Ser$^{414}$.

The Switch loop of the E. coli synthetase adopts a ligand-free
conformation or a ligated conformation, both of which differ significantly from that of the mouse muscle synthetase (Fig. 1). The root mean square difference between corresponding /H9251 -carbons of the mouse muscle loop relative to the ligand-free and ligated E. coli loops is 3.0 and 2.7 Å, respectively. Hydrogen bonds involving Asn 116, Lys119, and Gly 120 with Asp81, Glu79, and Tyr80, respectively, stabilize the observed conformation of mouse muscle Switch loop. The above residues, with the exception of Gly120, are different in E. coli and plant synthetases (Fig. 2). The ligand-free position of the corresponding loop in plant synthetases is unknown. Variations in the sequence of the plant synthetases relative to E. coli and mouse muscle synthetases may give rise to yet another loop conformation in the absence of ligands.

Regardless of the different ligand-free conformations for the above loop, the hydrogen bond between His71 and Asp51 (His41 and Asp21, respectively, in the E. coli synthetase) is present in each of the ligand-free synthetases, for which crystal structures are available. Furthermore, His41 of the E. coli synthetase is an essential catalytic residue (9, 36), and Asp 21 represents one-half of an important switching mechanism, which defines the ligated and ligand-free conformations of the E. coli synthetase (7, 9, 38). In the proposed switching mechanism (7), hydrogen bond formation between Asn38 and the 5'-phosphoryl group of IMP leverages the Switch loop into its ligated conformation, which is stabilized further by the salt link between Asp21 and Arg419 (of the GTP loop). Because these residues are invariant over the known synthetase family, the histidine-aspartate and arginine-aspartate links, defined above, are probably hallmark marks of the ligand-free and ligated conformations, respectively, of all adenylosuccinate synthetases.

Conformational differences in the aforementioned loops are not entirely surprising, because these structures are flexible. The gain or loss of a single hydrogen bond can result in a significant conformational difference. Residues 35–38 of the E. coli synthetase, however, are well ordered with low thermal parameters. Hence, the significant difference in conformation for residues 65–68 of the mouse muscle enzyme relative to residues 35–38 of the E. coli enzyme is unanticipated (Fig. 4). In fact, not only is Asn 68 of the mouse muscle synthetase removed from the 5'-phosphoryl pocket of IMP, segment 65–68 sterically excludes IMP from binding (Fig. 4). Furthermore, a hydrogen bond between Asn256 and backbone amide 67 may stabilize the observed conformation of residues 65–68. Asn256 may itself be an important catalytic residue. Gln224 of the E.
coli synthetase, which corresponds to Asn<sup>256</sup> of the mouse muscle enzyme, hydrogen bonds with the atoms N-7 and O-6 of IMP and putatively stabilizes the 6-oxyanion form of IMP (9, 37). Finally, residues 65–68 have elevated thermal parameters that suggest conformational mobility and the possibility of a ligand-induced conformational change. The conformation of this loop has been verified by the collection of a data set from a second crystal, by the use of omit electron density maps, and by the refinement of an E. coli-like model, the latter resulting in an inferior fit to the electron density. The altered conformation of residues 65–68 may be a distinct feature of the ligand-free, ligand-induced conformational change in residues 65–68 and associated omit electron density. The altered conformation of segment 65–68 of the mouse muscle synthetase (bold lines) and corresponding residues of the ligand-free (middle panel) and ligated (bottom panel) E. coli enzyme. The dashed lines represent donor-acceptor interactions.

Any functional significance attributed to the altered conformation of segment 65–68 is merely speculative, but the muscle isoform is inhibited at least 10-fold more potently by fructose 1,6-bisphosphate than the E. coli synthetase.<sup>2</sup> Others have suggested a connection between glycolysis and the purine nucleotide cycle on the basis of the in vitro inhibition of the muscle isoform by fructose 1,6-bisphosphate (2, 5), but whether the in vivo concentrations of fructose 1,6-bisphosphate in muscle ever rise to levels, which could cause appreciable inhibition of the synthetase, is unknown. Spector and Miller (40) report allopurinol ribonucleotide as a weak inhibitor of the synthetase from rabbit muscle, yet this IMP analog is a substrate for the synthetase from the protozoan Leishmania donovani (44). Variations in substrate specificity between muscle and protozoan synthetases may arise from conformational differences involving residues 65–68. Atom N-7 of IMP may need to hydrogen bond with Asn<sup>256</sup> of the mouse muscle enzyme to trigger a conformational change in residues 65–68. If so, atom N-7 of IMP would compete with backbone amide 67 for the side chain of Asn<sup>256</sup> (Fig. 4). The absence of a proton acceptor at position 7 of allopurinol ribonucleotide would give backbone amide 67 a clear advantage in stabilizing a conformation, which is antagonistic toward the IMP analog. The existence of an active site conformation that excludes IMP also allows for alternative non-competitive toward the IMP analog. The existence of an active site conformation that excludes IMP also allows for alternative

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