The Mechanism of the Adenylosuccinate Synthetase Reaction as Studied by Positional Isotope Exchange*

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In an attempt to gain insight into the mechanism of the rat muscle adenylosuccinate synthetase reaction, experiments using the technique of positional isotope exchange (isotope scrambling) were undertaken. [γ-18O]GTP was prepared and incubated with Mg2+ and the synthetase in the presence of various ligands. Positional isotope exchange occurred, as measured by nuclear magnetic resonance spectroscopy, when IMP was present. In the absence of IMP, with or without aspartate or succinate, the [γ-18O]GTP did not exhibit scrambling. These results suggest that the adenylosuccinate synthetase reaction involves the participation of 6-phosphoryl-IMP as an obligatory intermediate. On the basis of experiments carried out in our laboratory as well as in others, we believe the GDP remains bound to the enzyme until the product, adenylosuccinate, is formed. All products may then dissociate randomly from the enzyme. The positional isotope exchange experiments, along with initial-rate experiments carried out in our laboratory, serve to explain the lack of partial exchange reactions associated with the synthetase (Fromm, H. J. (1958) Biochim. Biophys. Acta 29, 255-262), as well as the net inversion of configuration when chiral thio-GTP is converted to thiophosphate (Webb, M. R., Reed, G. H., Cooper, B. F., and Rudolph, F. B. (1984) J. Biol. Chem. 259, 3044-3046).

Adenylosuccinate synthetase (IMP-L-aspartate ligase (GDP-forming), EC 6.3.4.4) catalyzes the reaction:

\[ \text{GTP} + \text{IMP} + \text{L-aspartate} \rightleftharpoons \text{GDP} + \text{P} + \text{adenylosuccinate} \]

Because this reaction is the first step in the biosynthesis of adenine nucleotides from IMP, the enzyme plays an important role in the regulation of purine nucleotide interconversion. For an extensive review of adenylosuccinate synthetase, see Stayton et al. (1983).

To date, there have been three mechanisms proposed for the adenylosuccinate synthetase reaction. The earliest was suggested by Lieberman (1956) and involves a 6-phosphoryl-IMP intermediate. The second, proposed by Miller and Buchanan (1962), involves a concerted reaction in which all three substrates participate simultaneously. The third mechanism, proposed by Markham and Reed (1978), has aspartate attacking the C-6 of IMP in the first step of the reaction.

Recently, Webb et al. (1984) have followed the stereochemical course of the adenylosuccinate synthetase reaction using chiral [16O,18O,18O]thiophosphate in the γ position of GTP. They found that the reaction proceeds with net inversion of configuration. These results are consistent with all three mechanisms if one proposes direct phosphoryl transfer from the γ position of GTP to the O-6 of IMP, with subsequent cleavage of the carbon-oxygen bond. To postulate a phosphorylated enzyme intermediate, one would have to propose three phosphoryl transfers; however, it seems unlikely that there are two phosphoryl-enzyme intermediates.

Positional isotope exchange is technical in which one can investigate the existence of phosphate intermediates in a reaction mechanism (see Rose, 1979). By labeling the γ-phosphate of GTP with 18O, one can observe the exchange of the label from the β-γ bridge position to the β nonbridge of GTP, if exchange occurs. If exchange does occur, it can be observed by two methods: mass spectroscopy and 31P NMR spectroscopy. With the method of 31P NMR spectroscopy, 18O-labeled in the β-γ bridge position can be differentiated from 18O-labeled in the β nonbridge position. The difference between these two resonances was found to be 0.012 ppm for ATP (Cohn and Rao, 1979). If an exchange is observed, one can hypothesize a phosphoryl-enzyme or a phosphoryl-substrate as a reaction intermediate.

Positional isotope exchange experiments were performed with adenylosuccinate synthetase to determine the reaction mechanism. We report that an exchange reaction occurs when [γ-18O]GTP is incubated with IMP. No exchange reaction was observed when IMP was absent. These findings support a reaction mechanism that involves a 6-phosphoryl-IMP intermediate.

EXPERIMENTAL PROCEDURES

Adenylosuccinate synthetase was purified from rat muscle by the method of Baugher (1980) and was dialyzed against a buffer containing 100 mM HEPES (pH 7.0) and 5 mM dithiothreitol. 18O2 (97 atom % purity) was obtained from MSO Isotopes (Merck and Co.). CDTA, dithiothreitol, GDP, GTP, and HEPES were purchased from Sigma. Solvents used in the synthesis of [γ-18O]GTP were dried, 1

1 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CDTA, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid; EnNH·HCO3, a buffer of triethylammonium bicarbonate at pH 8.0; GTPyS, guanosine 5'-O-(3-thiotri-phosphate).
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RESULTS

Fig. 1. A and B show the purity of the isotopic enrichment of the [γ-31O]GTP alone (Fig. 1A) and with approximately equimolar unlabeled GTP added (Fig. 1B). The total shift between major peaks of the γ-31P spectrum was 0.105 ppm. The value reported for ATP is 0.085 ppm (Cohn and Rao, 1979). In the β region of the spectrum, the difference was 0.018 ppm, compared with a literature value of 0.016 ppm for ATP. The minor resonance seen in the γ spectrum was found to have a shift of 0.027 ppm. This compares quite well with the expected shift for a nonlabeled oxygen atom in the γ nonbridge position. Using the integrated areas of the peaks, we found the isotopic enrichment of the γ-phosphate to be 91.2%.

Fig. 2 depicts the spectra of the β peak of labeled GTP alone (A), after incubation with adenylosuccinate synthetase (B), after incubation with 1.5 mM IMP (C), and after incubation with 1.5 mM IMP and 10 mM succinate (D).

Fig. 3 illustrates the γ resonances for the same set of experiments. Succinate, a competitive inhibitor for aspartate (Rudolph and Fromm, 1969), decreased the extent of exchange as compared with incubation with IMP alone. Table I summarizes the differences in the peak positions for all the experiments conducted. From these data, it can be seen that there is no exchange in the absence of IMP or in the presence of ligands that are known to bind to the enzyme in the absence of IMP (Rudolph and Fromm, 1969). When IMP is present, there was an exchange of isotope between the β-γ bridge and the β nonbridge positions of GTP. This exchange was decreased when succinate was added to the incubation medium.

In the experiment in which all the substrates were present...
of GTP, generating the 6-phosphoryl-IMP intermediate. To date, this intermediate has eluded chemical synthesis. In the next step, the α-nitrogen of aspartate makes a nucleophilic attack on the C-6 of IMP, leading to a tetrahedral transition state. This breaks down to yield adenylosuccinate and orthophosphate. Lieberman based this mechanism on the finding that all the label in [6-18O]IMP ended up as [18O]P, and because there was no GTP = [32P]P exchange when aspartate was missing from the reaction mixture. Fromm (1958) generated results from isotope exchange at equilibrium experiments that also support the hypothesis of a 6-phosphoryl-IMP intermediate. Lieberman's mechanism is depicted in 1a of Scheme 1.

In 1962, Miller and Buchanan suggested a concerted mechanism for adenylosuccinate synthetase. In this mechanism, the α-nitrogen of aspartate makes a nucleophilic attack on the C-6 of IMP at the same time that the O-6 of IMP makes its attack on the γ-phosphorus of GTP, thus leading to the tetrahedral transition state. This mechanism was proposed to account for the finding that arsenolysis or phosphorylation of adenylosuccinate does not occur when GDP is missing from the reaction mixture. It also is consistent with the lack of partial exchange reactions for adenylosuccinate synthetase.

This mechanism is shown in part 7b of Scheme 1.

A third mechanism was proposed by Markham and Reed in 1978. The first step of this mechanism has the α-nitrogen of aspartate attacking the C-6 of IMP creating an "oxy-anion" intermediate. The activated oxygen then makes a nucleophilic attack on the γ-phosphorus of GTP leading to the tetrahedral transition state. This study used GTPyS, which lowers the turnover number of the enzyme by 12.5-fold. At this lower rate, Markham and Reed (1978) were able to observe a transient in the UV spectrum. They attributed this transient to the oxy-anion intermediate, reasoning that phosphorylation of the N-6 of AMP leads to a spectral red shift that is in the opposite direction of the transient that was observed with GTPγS. The Markham and Reed mechanism is outlined in part 1c of Scheme 1.

The data from the experiments reported in this paper show that positional isotope exchange did take place between the β-γ bridge and the β-nitrogen positions of GTP in the presence of adenylosuccinate synthetase. The finding that the exchange took place only in the presence of IMP is indicative of a 6-phosphoryl-IMP intermediate. This would support the mechanism originally proposed by Lieberman (1958). The other two mechanisms can be eliminated since they require aspartate be present before the GDPo-PO bond is broken.

Data used to support other models can be interpreted to fit mechanism 1a of Scheme 1 as follows. The finding that arsenolysis does not occur without GDP being present (Miller and Buchanan, 1962) can be interpreted as a need by the enzyme for the guanosine nucleotide for catalytic activity. In the absence of GDP, the enzyme cannot enter into a catalytic conformation. This is supported by the finding that GTP and IMP bind synergistically to the enzyme (Markham and Reed, 1978).

The sequential kinetics reported for adenylosuccinate synthetase from a variety of sources (Rudolph and Fromm, 1969; Nagy et al., 1973; Van Der Weyden and Kelly, 1974; Clark et al., 1977; Baugher, 1980) are consistent with the participation of 6-phosphoryl-IMP as an intermediate, if it is assumed that all substrates and products remain bound to the enzyme until adenylosuccinate is formed (Fromm, 1975). This proposal also would account for the lack of partial exchange reactions.

The results of this report on positional isotope exchange, along with the findings of Webb et al. (1984), which demon-
strate the inversion of configuration when GTP is cleaved, are consistent with 6-phosphoryl-IMP being an intermediate in the adenylosuccinate synthetase reaction. Other proposals advanced to explain the mechanism of the reaction seem not in harmony with the results of the present investigation.

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