GLUTAMINE SYNTHETASE DEADENYLYLATION: A PHOSPHOROLYTIC REACTION YIELDING ADP AS NUCLEOTIDE PRODUCT

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SUMMARY- Previous studies showed that in Escherichia coli detachment of adenylyl groups from the adenylylated form of glutamine synthetase is catalyzed by a complex deadenylylation enzyme system composed of at least two protein components, and is activated by α-ketoglutarate, ATP and UTP. It is now found that cleavage of the energy-rich adenylyl-O-tyrosyl bond of adenylylated glutamine synthetase is coupled with the esterification of orthophosphate to form ADP as the deadenylylation product. No deadenylylation occurs in the absence of orthophosphate. When arsenate is substituted for orthophosphate, AMP is the only product of deadenylylation.

INTRODUCTION- The activity of Escherichia coli glutamine synthetase (GluSyn) is modulated by the covalent attachment of one 5'-adenylyl group to each subunit of the enzyme (1-3). The effects of adenylylation on the catalytic activity, and other physical properties of glutamine synthetase have been discussed elsewhere (4,5). The transfer of the adenylyl moiety of ATP to glutamine synthetase is catalyzed by a specific ATP: glutamine synthetase adenylyltransferase (ATase). Shapiro and Stadtman (6) showed that the 5'-adenylyl group is bound in phosphodiester linkage to the phenolic hydroxyl group of a specific tyrosyl residue on each of the twelve subunits of glutamine synthetase. In a recent report, Mantel and Holzer (7) demonstrated reversibility of the adenylylation reaction and established the energy-rich nature of the adenylyl-O-tyrosine bond. Nevertheless, under physiological conditions, removal of co-valently bound adenylyl moieties from glutamine synthetase is catalyzed by a complex deadenylylating enzyme

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system (DA system) (8-10) consisting of at least two protein components, denoted P_I and P_{II}, and is stimulated by α-ketoglutarate, ATP, and UTP (10). Moreover, ATase was recently shown to be a subunit of the P_I deadenylylation component (11,12).

In earlier studies, Shapiro (10) showed that orthophosphate (Pi) or arsenate stimulates the deadenylylation reaction as catalyzed by a relatively impure DA system and that both AMP and ADP accumulated as reaction products. However, since this DA system catalyzed interconversion of AMP and ADP the primary product of deadenylylation was uncertain. Subsequently, Heilmeyer et al (9) showed that AMP was the only product to accumulate during deadenylylation of glutamine synthetase by crude dialyzed enzyme preparations. Since then it has been generally assumed (5,13) that deadenylylation involves a hydrolytic cleavage of the adenylyl-O-tyrosyl linkage.

Meanwhile, as reported here, it was shown that activity of a highly purified DA system is almost completely dependent upon the presence of P_i; moreover, ADP is the major if not sole product of deadenylylation. Thus, deadenylylation involves phosphorolysis rather than hydrolysis of the phosphodiester linkage.

METHODS- The 5'-^{14}C-adenylylated glutamine synthetase (51 cpm/10 μμmole subunits of enzyme) used as substrate for the deadenylylation experiments contained 11.6 equivalents of AMP/mole of enzyme and was prepared as described elsewhere (11). The unlabeled, fully adenylylated glutamine synthetase preparation was prepared by adenylylation of an enzyme (containing 7.9 moles of AMP/mole of enzyme) with ATP in the presence of Mg^{2+}, glutamine, and adenylyltransferase as previously described (10,14).

The P_I and P_{II} deadenylylating components, initially described by Shapiro (10), were obtained from extracts of E. coli W cells that were grown on a glycerol-NH_3 medium and harvested after the onset of stationary growth induced by NH_4Cl depletion. The P_I and P_{II} preparations (purified

several hundred fold) were obtained by a multistep procedure which will be described in detail elsewhere. Both P_I and P_{II} were dialyzed extensively against 20 mM Tris-0.5 mM dithiothreitol-0.25 mM K_2 Mg EDTA buffer, pH 7.2, to remove phosphate present in the buffer systems used during purification.

The standard assay procedure for deadenylylating activity (DA activity) was as described by Shapiro (10), except that the complete reaction mixture contained 50 mM 2-methyl imidazole, 1.25 mM dithiothreitol, 15 mM α-ketoglutarate, 18 mM potassium phosphate, 1.0 mM UTP, 20 mM ATP, 1.25 mM $MnCl_2$, 80 μg of ^{14}C-adenylylated glutamine synthetase, and the highly purified deadenylylating components (7.5 μg of P_I and 2.5 μg of P_{II}) in a final reaction volume of 0.2 ml (pH 7.2).

RESULTS: Figure 1 illustrates dependence of the deadenylylation reaction

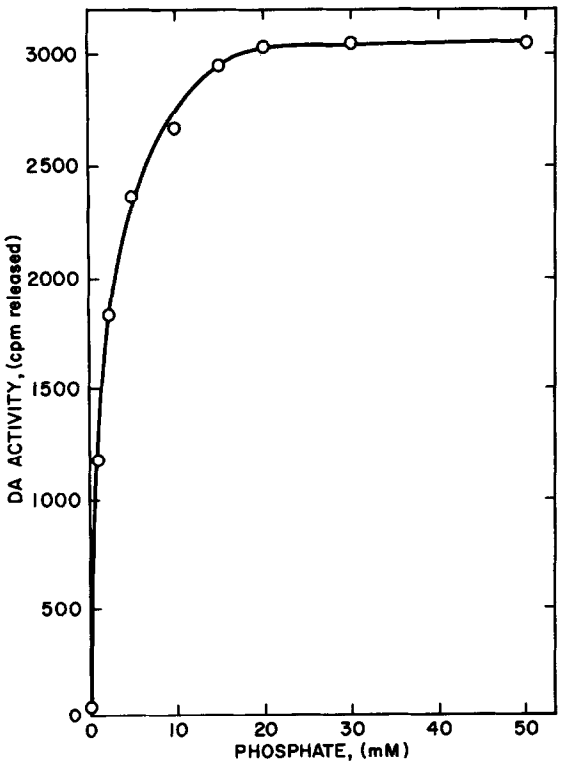

Figure 1. Phosphate requirement of the deadenylylation reaction. Activity was determined as described in Methods, except that the concentration of phosphate was varied in the reaction mixture as indicated. DA activity is reported as counts per minute released per 30 minutes.

upon the potassium phosphate concentration. Activity increases progressively with elevation of phosphate concentration up to 20 mM. Half maximal activity is obtained with 1.5 mM phosphate. Potassium arsenate replaces phosphate, but potassium sulfate does not support activity. In other studies methyl-phosphate was about 10% as effective as phosphate under comparable conditions. The slight activity noted in the absence of added phosphate may be due to traces of phosphate left in the P_I and P_{II} preparations after dialysis.

As shown in Table I, when ^{14}C-adenylylated glutamine synthetase is incubated with the DA system in the presence of orthophosphate, nearly all of the ^{14}C-adenylyl groups removed are recovered in ADP; little or no AMP or ATP is produced. This suggests that deadenylylation involves a phosphorolytic cleavage of the phosphodiester bond. This is supported further by the fact that deadenylylation of unlabeled adenylylated glutamine synthetase in the presence of $^{32}P_i$ results in the production of ^{32}P-ADP. In contrast,

Table 1

Product of the Deadenylylation Reaction

Experiment	Adenylylated Substrate	Adenylyl Acceptor	Isotope in Product: AMP	ADP	ATP
			cpm	cpm	cpm
(A)	GlnSyn-(^{14}C-AMP)$_{12}$	K-PO_4	101	3670	86
(B)	GlnSyn-(^{12}C-AMP)$_{12}$	K-$^{32}PO_4$	2630	3448	-
(C)	GlnSyn-(^{14}C-AMP)$_{12}$	K-AsO_4	3068	126	-

The deadenylylation reaction mixture was as described in Methods in a final volume of 0.3 ml, but with the following experimental variations: (A) Contains 17 mM K-phosphate, 0.5 mg of 5'-^{14}C-adenylylated glutamine synthetase, 15 µg of P_I and 5 µg of P_{II}. (B) Contains 20 mM ^{32}-P-K-phosphate (5-10 X 10^5 cpm/µmole), 0.5 mg of unlabeled, adenylylated glutamine synthetase, 8 µg of P_I and 5 µg of P_{II}. (C) Contains 25 mM K-arsenate, 0.5 mg of 5'-^{14}C-adenylylated glutamine synthetase, 15 µg of P_I and 5 µg of P_{II}. After 5 hrs at 37°, 3% perchloric acid and 1 µmole each of unlabeled adenosine, AMP, ADP, and ATP were added. The supernatant was adjusted to pH 5.0 with 2N KOH, cooled to 0°, and the $KClO_4$ precipitate was removed by centrifugation. Adsorption of the labeled nucleotide on H_3PO_4 washed charcoal, elution with 40% acetone containing 0.3% NH_3, and thin-layer chromatography were performed as previously described (10).

when the deadenylylation reaction is carried out in the presence of arsenate, AMP is the only significant product; this is presumed to arise by arsenolysis of that phosphodiester bond, followed by spontaneous hydrolysis of the AMP-arsenate intermediate. In each of the experiments, the amount of radioactivity recovered in nucleotides other than the principle product is probably due to imperfect separation of the nucleotides by the chromatographic procedure used in their isolation.

DISCUSSION- The discovery that ADP is the product of deadenylylation is understandable in view of the demonstration by Mantel and Holzer (7) that the AMP-O-tyrosyl-linkage is energy-rich. These results demonstrate that the energy of the adenylyl-O-tyrosyl bond is not lost by hydrolytic cleavage but is preserved by phosphorolysis and the formation of ADP.

Since ATase is a subunit of the P_I-protein component of the deadenylylation enzyme system (11,12), it appears significant that orthophosphate will not replace pyrophosphate in reversal of the adenylylation reaction (7). Moreover, we find that pyrophosphate will not replace orthophosphate in the complex deadenylylation system. Since P_I-protein participates in both adenylylation and deadenylylation reactions it appears possible that an adenylyl-P_I intermediate is common to both processes. Then, the acceptor specificity of the adenylyl-P_I intermediate could be dictated by the P_{II}-protein and the allosteric effects of glutamine, α-ketoglutarate, ATP and UTP; the adenylylation reaction could be represented by two half-reactions (Eqs. 1 and 2), and deadenylylation may proceed by equations 3 and 4.

$$ATP + P_I \underset{}{\overset{\text{glutamine}}{\rightleftharpoons}} P_I\text{-AMP} + PP_i \qquad (1)$$

$$P_I\text{-AMP} + \text{GluSyn} \overset{\text{glutamine}}{\rightleftharpoons} \text{GlnSyn-AMP} + P_I \qquad (2)$$

$$\text{GlnSyn-AMP} + P_I \xrightarrow[\text{ATP, Kg, UTP}]{P_{II}} \text{GlnSyn} + P_I\text{-AMP} \qquad (3)$$

$$P_I\text{-AMP} + P_i \xrightarrow[\alpha\text{KG, UTP, ATP}]{P_{II}} \text{GlnSyn} + \text{ADP} \qquad (4)$$

These possibilities and the alternative possibility that P_{II} has a catalytic rather than a regulatory role, are under investigation.

Conceivably, the adenylyl moiety of the P_I-AMP intermediate could be transferred to acceptors other than P_i or PP_i. The slight activity of methylphosphate in the deadenylylation reaction supports this possibility. However, several other potential acceptors, including FMN and NMN, did not support activity under the conditions used for phosphorolytic deadenylylation.

The earlier finding of Heilmeyer et al (9) that AMP is the only nucleotide to accumulate when deadenylylation of glutamine synthetase is catalyzed by dialyzed crude extracts is most likely due to the presence of side reactions that led to dephosphorylation of the primary product, ADP. Nevertheless, the possibility that in crude extract regulatory effects of endogeneous metabolites or other factors favor a hydrolytic cleavage of the adenylyl-O-tyrosyl bond has not been excluded.

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