REGULATION OF MAMMALIAN ASPARAGINE SYNTHETASE BY ADENINE NUCLEOTIDES

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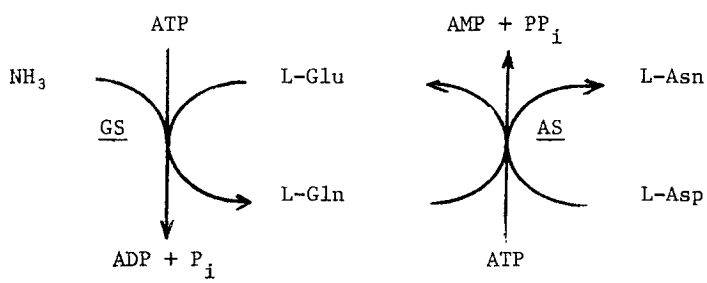
Received February 4,1976

SUMMARY: Initial velocity kinetic data indicate that ADP and AMP are inhibitors of mammalian liver asparagine synthetase. The non-product nucleotide ADP is a much more potent inhibitor than AMP, although both apparently compete for the same site. This modifier site, however, does not overlap spatially with the substrate site for ATP. Both ADP and AMP are V_{max} inhibitors, but ADP also raises the K_m for ATP. Adenylate energy charge, calculated at various levels of ATP and ADP show typical correlations with activity, but with AMP these correlations are weak and atypical.

Asparagine synthetase (L-aspartate: ammonia ligase (AMP), EC (6.3.1.1), AS, is coupled to the glutamine synthetase reaction, GS, in the sense that L-Gln serves as the nitrogen donor for the formation of L-Asn, as shown by:



The GS-catalyzed reaction involves β-γ cleavage in ATP whereas the AS-catalyzed reaction involves α-β cleavage, yielding ADP + P_i and AMP + PP_i, respectively. Since adenylate energy charge has been seen to regulate glutamine synthetase activity [1], one may properly inquire whether AS is similarly controlled. In the present study, we have used the mammalian enzyme [2] to probe the extent of inhibition by AMP and ADP, and how these modifiers may interact with substrate

sites or each other, or alter the catalytic process. Potentially, these results may have significance for the known relationship of aspartate and asparagine metabolism to growth of certain tumors [3,4].

EXPERIMENTAL

Materials: Asparagine synthetase was partially purified from guinea pig liver by the procedures of Holcenberg [2], and was stored in Tris-HCl buffer 0.05 M, pH 8.0, with 20% sucrose added, at -20°C. All biochemicals were of highest purity obtainable from Sigma Chemical Company. Inorganic salts were analytical reagent grade. Water was double-distilled from glass. [^{14}C] L-Aspartate was a New England Nuclear product.

Methods: Enzyme activity was assayed by formation of [^{14}C] L-asparagine. The standard assay mixture contained (in mM): 200 $(NH_4)_2SO_4$, 1.5 L-Asp, 1.0 ATP, 10.0 $MgCl_2$, and 50 Tris-HCl at a pH of 8.5, in a volume of 0.4 ml, with [^{14}C]-labeled L-Asp at 2.8 x 10^5 dpm/reaction. It was found that the substitution of 20 mM L-Gln for the $(NH_4)_2SO_4$ did not alter the kinetic results. Standard incubations were at 37° for 2 hr.

Because of the long incubation times for the reactions, checks were made as to the linearity of the kinetics over two hours. Also the enzyme was investigated at several stages of purification with regard to regulatory properties and interference of such activities as adenylate kinase, ATPase, and aspartokinase. It was found that the state of purity (after removal of nucleic acids) did not affect the nature of the enzyme's responses to ADP or AMP, comparing enzyme in a crude extract to that purified 200 X. Nor did any of the activities cited appreciably interfere with the assay, it was found.

In those experiments in which modifier levels were varied, two reaction mixtures were prepared: one contained modifier at the maximum level desired, the other none. These two mixtures were then mixed in varying proportions to the same constant volume (0.4 ml). In those experiments in which ATP was varied in the presence or absence of ADP or AMP, two mixtures were again prepared, in this case with all components at double the levels cited above. These were similarly mixed to constant volume of 0.2 ml in variable ratio. The remaining 0.2 ml volume of the reaction mixture consisted of added modifier, water, and enzyme. At the end of the incubation period, enzyme was removed by a 5 min. heating in a 100° water bath, chilling, and centrifugation. This technique of stopping was found to be far

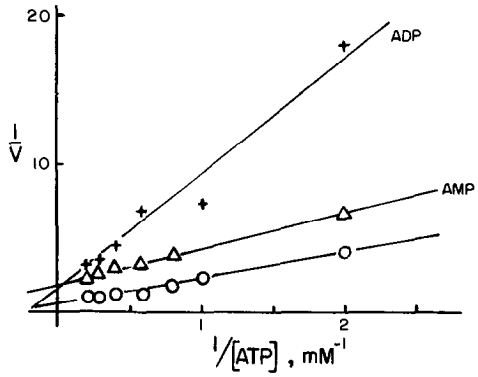


Fig. 1: Inhibition of asparagine synthetase by ADP and AMP, in competition with ATP, presented as double reciprocal plots. See Experimental section and text for reaction conditions. Symbols: (0) no modifier, (Δ) 12.5 mM AMP, (+) 12.5 mM ADP.

preferrable to the $HClO_4$-K^+acetate procedures described in the literature [2], thus avoiding high salt levels, found to interfere with chromatographic separation of labeled L-Asn from L-Asp. This separation was accomplished with a 0.7 x 14 cm column of Whatman DE-52 (DEAE-cellulose, Cl^--form). Once applied to this column, the L-Asn (2.5 μmoles carrier L-Asn added) from each reaction was eluted by 20 ml of distilled water, with complete retention of [^{14}C] L-Asp. The reaction velocity was expressed as μmoles L-Asn produced/hr., or as dpm. The fractional inhibition of activity, i, could be plotted versus modifier level, [M], then replotted and analyzed as 1/i versus 1/[M] as with a Lineweaver-Burke plot, according to previously derived relationships [5,6]. Adenylate energy charge [7] was calculated from the equation:

$$E.C. = \frac{ATP + 1/2\ ADP}{ATP + ADP + AMP}$$

Each assay was performed in triplicate with controls in which ATP was omitted.

RESULTS AND DISCUSSION

The kinetically determined saturation curves for ATP (as 1/v vs 1/[S]) in the presence and absence of added ADP or AMP are presented in Fig. 1. It is apparent that both modifiers alter V_{max} rather dramatically, and that K_m(ATP) is also increased. These data suggest that neither inhibitor competes directly for the ATP substrate binding site.

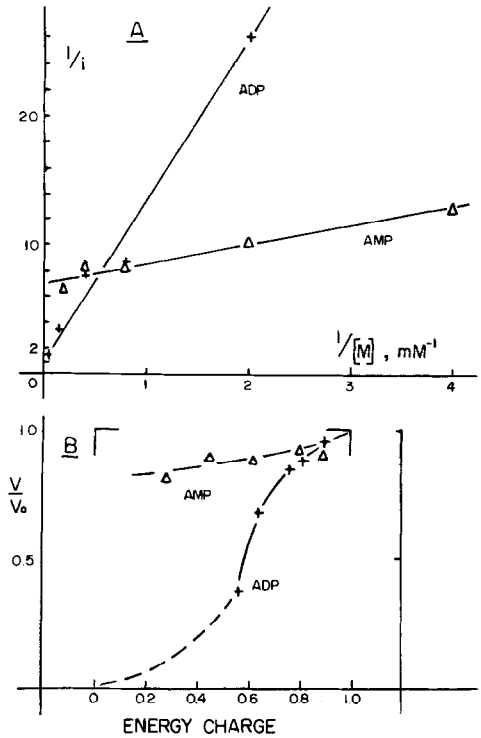


Fig. 2: Effect of the variation of ADP and AMP levels on the asparagine synthetase activity, using fixed levels of substrates. See Experimental section for procedures and conditions. Symbols as in Fig. 1. (A) Double reciprocal plot of fractional inhibition vs modifier level, (B) Replot of modifier inhibition data as relative velocity vs adenylate energy charge.

The extent and manner of inhibition by a modifier can reveal its mode of interaction on the enzyme surface with other ligand sites. For example, a modifier that competes directly with a substrate site must give 100% inhibition at infinite modifier. Modifiers binding at non-substrate sites may give 100% inhibition, or less, as discussed at length elsewhere [5]. Although the data of Fig. 1 show that ADP and AMP inhibit V_{max}, AMP binding is seen in Fig. 2A to give tight binding, but incomplete inhibition ($i_{max} < 1$). In contrast, infinite levels of ADP appear to give nearly 100% inhibition, even though the K_i(app) for ADP is lower than that for AMP (in these plots, the slope of the line is related to K_i directly). These observations suggest that ADP is the more potent modifier of the enzyme.

TABLE I:

Antagonistic Action of ADP and AMP with Mammalian Asn Syn

Each inhibitor was used at 12.5 mM in the standard assay with substrate levels as described for Figure 1., i represents the fractional inhibition (100% inh. = 1.0).

	i			
Modifier(s)	observed	calculated [a]		
		add.	cuml.	antag.
AMP	0.20	-	-	-
ADP	0.63	-	-	-
AMP + ADP	0.59	0.83	0.70	<0.63

[a] Additive, cumulative, or antagonistic inhibitions were calculated as described previously [8].

By using both AMP and ADP together, and comparing this inhibition with their effects alone, one may determine whether their binding domains overlap antagonistically or whether they are separate and interact cumulatively or synergistically. Similar experiments have been used successfully with glutamine synthetase enzymes, where multiple feedback modifiers interact with the enzyme [1,8]. Table I presents the results of such experiments with asparagine synthetase. The effect of ADP + AMP is less than the effect of ADP alone, so the type of interaction is clearly antagonistic. The simplest explanation for this is that ADP and AMP compete for the same site. Alternatively, antagonism could occur by interactions transmitted between separate sites.

The data of Fig. 2A were used to calculate the relationship of the relative velocity, V/V_o, to the adenylate energy charge [7], as shown in Fig. 2B. AMP shows little effect, but the ADP data follow the sort of response curve expected for an ATP-utilizing system. Interestingly, the curve shows its midpoint at E.C. $\simeq$ 0.6 rather than in the range of 0.7 - 0.9 as seen in many other cases. This midpoint is, however, dependent on ATP levels, probably for reasons similar to those discussed by Purich and Fromm [9,10], e.g., pH, total adenylate

pool size, and other factors. Thus, complete understanding of the regulatory properties of this enzyme must await more extensive studies of the effects of all these parameters.

Acknowledgements: This research was supported in part by NSF grant GB-34751.

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