

SYNERGISTIC INHIBITION OF ADENYLOSUCCINATE SYNTHETASE BY NITRATE AND GDP

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1. Introduction

Adenylosuccinate synthetase (IMP: L-aspartate ligase (GDP), EC 6.3.4.4) occupies a central position in the biosynthesis of AMP and in purine nucleotide cycling [1]. Because of the enzyme's unique function in adenine nucleotide synthesis, regulation and inhibition of this enzyme are of potential significance in control of cellular proliferation.

Recent studies have shown that the planar nitrate anion may mimic the hypothetical metaphosphate intermediate in enzyme catalyzed phosphoryl transfer reactions [2–5]. In several cases there is a strong interaction between nitrate and abortive enzyme-substrate complexes which lack only the γ -phosphoryl group of the nucleotide moiety to be catalytically functional. This type of nitrate interaction was observed for the ligase enzyme, formyltetrahydrofolate synthetase, where nitrate enhanced the binding affinities of MnADP and of tetrahydrofolate ~ five fold in the abortive complex, enzyme-MnADP- H_4 folate-formate [5]. This observation suggested the possibility for nitrate inhibition of other enzymes of the ligase class. The present paper reports the synergistic inhibition of adenylosuccinate synthetase by nitrate and GDP. The influence of nitrate and GDP on the inhibitory action of the antineoplastic agent, 6-mercaptopurine ribotide has also been investigated.

2. Experimental

HEPES*, phosphoenolpyruvate, dithiothreitol, IMP,

*Abbreviations used: HEPES, *N*-2 hydroxyethyl-piperazine-*N'*-2 ethanesulfonic acid; DTT, dithiothreitol, AMPS, adenylosuccinate; H_4 folate, tetrahydrofolate.

GDP, L-aspartate and GTP (type I) were purchased from Sigma. 6-Mercaptopurine ribotide was purchased from P and L Biochemicals. DEAE 52 cellulose was a product of Whatman Biochemicals Ltd. *E. coli* B were purchased from Grain Processing Co. Pyruvate kinase was prepared from rabbit muscle by the method of Tietz and Ochoa [6]. All other materials were of the highest quality commercially available.

Adenylosuccinate synthetase was prepared by the method of Liebermann [7] with the following modifications: 1) The ammoniumsulfate-fractionated enzyme was dialyzed against one change of 100 vol of 25 mM P_i , pH 6.5, 0.2 mM DTT. 2) The dialysate was loaded onto a column of DEAE cellulose pre-equilibrated with 25 mM P_i , pH 6.5, 50 mM KCl, and the protein eluted with a linear KCl gradient (50–350 mM) in 25 mM P_i , 0.2 mM DTT buffer. Adenylosuccinate synthetase activity eluted near the end of the gradient, and the active fractions were pooled. This preparation was free of contaminating GTPase and AMPS lyase activities. The enzyme was dialyzed into 50 mM HEPES/KOH, pH 7.7, 0.2 mM DTT before use in kinetic experiments.

Kinetic assays were performed on a Cary 14 spectrophotometer at 280 nm where there is a $\Delta\epsilon$ of $11.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ on the conversion of IMP to AMPS [8]. All reaction mixtures were buffered with 50 mM HEPES/KOH, pH 7.7, 1 mM DTT, and the temperature was regulated at 23°C. Sufficient concentrations of all substrates were added to saturate the enzyme in the absence of added inhibitor (0.2 mM GTP, 0.2 mM IMP, 5.0 mM L-aspartate and 1.2 mM Mg (acetate) $_2$). In some assays phosphoenolpyruvate (1 mM) and pyruvate kinase (10 $\mu\text{g/ml}$) were present

to convert the GDP formed during the reaction to GTP. In GDP inhibition experiments, sufficient Mg (acetate)₂ was added to maintain the free Mg(II) level at approx. 1 mM.

3. Results and discussion

A Dixon plot of nitrate inhibition of adenylosuccinate synthetase in the presence of a GTP regenerating system is shown in fig.1, curve A. While substantial inhibition occurs at 10 mM nitrate, the rate of reaction is insensitive to 100 mM acetate (fig.1, curve B), indicating a specific effect of the nitrate anion. Other anions which give measureable inhibition are SO_3^{2-} , SCN^- , HCO_2^- , NO_2^- and Cl^- , in order to decreasing effectiveness. SCN^- gives linear competitive inhibition with respect to GTP ($K_i = 16$ mM). In the presence of nitrate, substrate inhibition by GTP seems to occur. However, a plot of $1/v$ versus nitrate concentration in the presence and absence of SCN^- gives parallel lines, which indicates that both anions compete for the same binding site [9]. Fig.1, curve C, shows that nitrate inhibition is markedly enhanced by the pre-

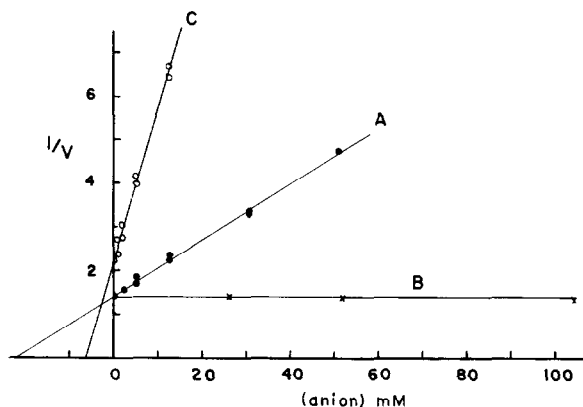


Fig.1. Dixon plot of the inhibition of AMPS synthetase by anions. Curves A and B display the effects of KNO_3 and K-acetate, respectively, on the initial velocity in the absence of GDP. Curve C shows the inhibition by KNO_3 in the presence of 29 μM GDP. Solutions were buffered with 50 mM HEPES/KOH pH 7.7, 1 mM DTT. Substrate concentrations were 0.20 mM IMP, 4.9 mM L-aspartate, 1.2 mM $\text{Mg}(\text{acetate})_2$, 0.20 mM GTP. In A and B, 1.0 mM phosphoenolpyruvate and 10 $\mu\text{g/ml}$ pyruvate kinase were present. Temperature = 23°C. The same amount of enzyme was used in all experiments, and velocity is expressed as $\Delta A_{280}/\text{min}$.

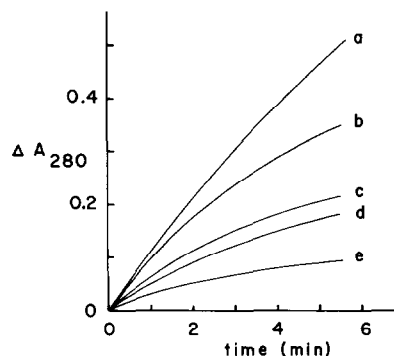


Fig.2. Progress curves of the AMPS synthetase reaction under various conditions. Curves a and b show the course of the reaction in the presence and absence of 10 $\mu\text{g/ml}$ pyruvate kinase, respectively. Curves c and d show the inhibitory effects of 11 μM 6-mercaptopurine ribotide and of 5.2 mM KNO_3 , respectively, in the absence of pyruvate kinase. Curve e shows the progress curve when both 11 μM 6-mercaptopurine ribotide and 5.2 mM KNO_3 are added in the absence of pyruvate kinase. Reaction conditions and substrate concentrations are the same as in fig.1 except $[\text{IMP}] = 0.42$ mM. An identical amount of enzyme was used in all experiments.

sence of 29 μM GDP. Intersection of curves A and C above the abscissa shows the presence of synergism in the inhibition by GDP and nitrate [9]. The point of intersection of curves A and C gives an interaction coefficient of 0.1 corresponding to a ten-fold synergism in binding of the two inhibitors [9]. An interaction coefficient of 0.3 is obtained for GDP and SCN^- from similar experiments.

The combined effects of two or more inhibitors are readily visualized in progress curves of the adenylosuccinate synthetase reaction, as is shown in fig.2. Curves a and b illustrate the effect of GDP product inhibition in the absence of other inhibitors. Curves c, d and e show the influence of 11 μM 6-mercaptopurine ribotide, 5 mM nitrate and the combination of 11 μM 6-mercaptopurine ribotide and 5 mM nitrate, respectively, on the progress of the reaction. The results of curve e suggest that the effects of the three inhibitors are at least cumulative since the sensitivity of the rate towards GDP inhibition is enhanced by the presence of the other two inhibitors.

Potentiation of nitrate inhibition by GDP is analogous to observations with enzymes such as creatine kinase [2], arginine kinase [4], and formyl-

tetrahydrofolate synthetase [5] where a strong interaction of nitrate is expressed in the presence of abortive complexes which include the nucleoside diphosphate. The results for adenylosuccinate synthetase are compatible with the suggestion [2] that nitrate mimics the structure of the trigonal-bipyramidal phosphoryl group in a complex which may resemble the structure of the transition state of the reaction.

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