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REGULATION OF ASPARTATE CARBAMOYLTRANSFERASE OF *ESCHERICHIA COLI* BY THE INTERRELATIONSHIP OF MAGNESIUM AND NUCLEOTIDES

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Summary

Purified aspartate carbamoyltransferase from *Escherichia coli* K12 (carbamoylphosphate: L-aspartate carbamyltransferase, EC 2.1.3.2) shows greater activity with nucleotide effectors as the magnesium nucleotide complex than with similar amounts of the sodium nucleotide. Regulation of aspartate carbamoyltransferase activity in vivo may occur by changes in the total concentration of regulatory nucleotides or, under conditions of magnesium-limited growth, by variation of the saturation of the nucleotides with magnesium.

The work of Kleppe and Spaeren [1] indicated that $Mg^{2+} \cdot$ CTP complexes have different regulatory effects on aspartate carbamoyltransferase (carbamoylphosphate: L-aspartate carbamyltransferase, EC 2.1.3.2), when compared with the Na^+ nucleotides. In *Escherichia coli* growing in a glucose-salts minimal medium, the free Mg^{2+} level has been found to be approximately 1 mM [2]. This suggests that nucleoside triphosphates in vivo will be largely present as their Mg^{2+} complexes, assuming that the stability constants of these complexes are similar to that for $Mg^{2+} \cdot$ ATP [3,4]. Previous kinetic studies of the effect of nucleotides on aspartate carbamoyltransferase [5—7] and studies on the equilibrium binding of CTP or ATP to the enzyme [8,9] have not used Mg^{2+} nucleotides and the substrates or substrate analogues were not at physical concentrations.

In *E. coli*, the steady state concentrations of the principal effector nucleotides (ATP, GTP, CTP) of the enzyme are proportional to the growth rate of the organism [10] and are also maintained in a fairly constant ratio (approximately, ATP : GTP : CTP = 10 : 5 : 3) despite fluctuations in cells undergoing adaptive responses to changes in growth conditions [11]. For *E. coli* K12 growing exponentially in a glucose-salts minimal medium the approximate

intracellular concentrations are ATP 3.5 mM, GTP 1.9 mM, and CTP 1.0 mM [11], while those for aspartate and carbamoyl phosphate are 310 μM * and 840 μM [13], respectively. Using these substrate concentrations and the above ratios of effector nucleotides as representing an approach towards physiological conditions, we have measured the response of aspartate carbamoyltransferase to changing concentrations of Mg^{2+} and Na^{+} nucleotides.

Materials and Methods

The concentration of uniformly labelled L-[^{14}C]aspartic acid (ICN Chemicals) was standardised in a Beckman Model 120B amino acid analyser. Solutions of dilithium carbamoyl phosphate were standardised by enzymatic conversion to [^{14}C]carbamoyl aspartate with an excess of [^{14}C]aspartate.

Mg^{2+} complexes of disodium ATP and CTP were prepared by neutralising the solutions with solid $MgCO_3$ to pH 7.0. Excess $MgCO_3$ was removed by centrifugation. The Mg^{2+} complex of trisodium GTP was prepared by adding one equivalent of magnesium acetate and then neutralising the solution to pH 7.0 with NaOH. Magnesium acetate solutions were standardised using an atomic absorption spectrophotometer. The concentrations of neutralised ATP, CTP and GTP solutions were determined spectrophotometrically. A modification of the method of Neuhard et al. [14] was used to check the purity of the nucleotides. Each nucleotide was chromatographed on a polyethyleneimine-cellulose thin-layer chromatogram with 1.0 M acetic acid/0.75 M LiCl as the developing solvent. The nucleotide samples moved as single spots when viewed under ultraviolet light except for the GTP sample which contained a trace of GDP.

Purified aspartate carbamoyltransferase from *E. coli* K12 was prepared by the method of Gerhart and Holoubek [15]. The specific activity of the purified enzyme assayed under the conditions of Gerhart and Pardee [5] using the radioisotopic assay system previously described [13] was 7070 units per mg of protein. A unit of enzyme is defined as the amount catalysing the formation of 1 μmol carbamoyl aspartate per h. The enzyme preparation was shown to be homogeneous by polyacrylamide gel electrophoresis.

Incubation mixtures of 50 μl contained 29.9 ng of purified aspartate carbamoyltransferase, 2.5 μg of crystalline bovine serum albumin, 310 μM [^{14}C]-aspartate (0.03 μCi/μmol), 840 μM carbamoyl phosphate and 201 mM imidazole acetate pH 6.8. Na^{+} or Mg^{2+} nucleotides or mixtures were added as required and after equilibration of the assay mixtures to 28°C, the reaction was initiated by addition of the carbamoyl phosphate. Three samples were taken from each assay mixture at appropriate times (usually 12, 24 and 36 min) and applied to polyethyleneimine-cellulose chromatograms. The [^{14}C]carbamoyl aspartate product was isolated and quantitated as previously described [13]. The aspartate carbamoyltransferase activity for each sample was taken from the gradient of the line of best fit through the three points on a plot of carbamoyl phosphate formation versus time. Values determined in this way had a standard

* Based on an aspartate content of 1.04 μmol/g dry weight [12] and an intracellular volume of 3.4 ml/g dry weight.

error of ±7%. They were expressed as units/mg or as a percentage of the activity of a control incubation containing no nucleotide or Mg^{2+}.

Results and Discussion

Using the physiological substrate concentrations of 840 μM carbamoyl phosphate and 310 μM aspartate, we investigated the response of aspartate carbamoyltransferase to varying concentrations of effector nucleotides as their Mg^{2+} complexes or in the Na^+ form.

Fig. 1 shows that the enzyme is activated more rapidly with increase in concentration of Mg^{2+} · ATP than of Na^+ · ATP and that the maximal activation is about twice as great with Mg^{2+} · ATP as with Na^+ · ATP. Fig. 1 also shows that Na^+ · CTP is more inhibitory than Mg^{2+} · CTP at concentrations below 2 mM. At the lower concentrations Na^+ · GTP is also more inhibitory than Mg^{2+} · GTP. The inhibition for either nucleotide above 5 mM is the same for Na^+ and Mg^{2+} forms. The activity curves with Na^+ · ATP and Na^+ · GTP are almost identical with those presented by Gerhart in Fig. 4 of ref. 7, despite the difference in conditions in relation to the buffers and the concentrations of aspartate and carbamoyl phosphate used in the assays. With CTP however, our curve shows a maximal inhibition at 200 μM Na^+ · CTP whereas in Gerhart's data [7] the same maximum of approximately 80% inhibition is only reached with inhibitor at 5 mM. Either the difference in buffer or in aspartate concentration used might be a cause of such difference in the observations. Comparison of the effects of Na^+ and Mg^{2+} nucleotides suggests that the cations modify the affinity of the nucleotides for the enzyme with Mg^{2+} increasing the affinity of ATP and decreasing the affinity of CTP.

Fig. 2 shows the effect of Mg^{2+} concentration on aspartate carbamoyltransferase at various total concentrations of the nucleotide mixture ATP : GTP: CTP (10 : 5 : 3). Without nucleotides, increase in Mg^{2+} gives increasing inhibi-

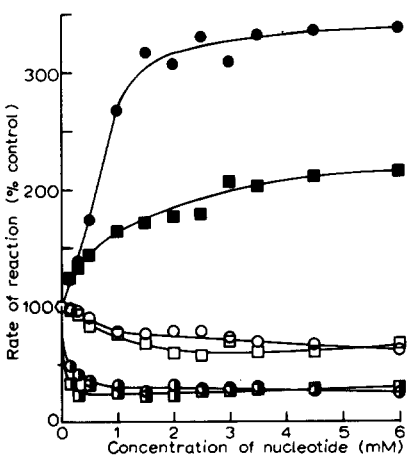

Fig. 1. The effects of regulatory Na^+ ribonucleoside triphosphates or their Mg^{2+} complexes on the activity of aspartate carbamoyltransferase. The conditions of assay are described in Materials and Methods. Added nucleotide: ●, Na_2MgATP; ■, Na_4ATP; ◑, Na_2MgGTP; □, Na_4GTP; ◐, Na_2MgCTP; ◧, Na_4CTP.

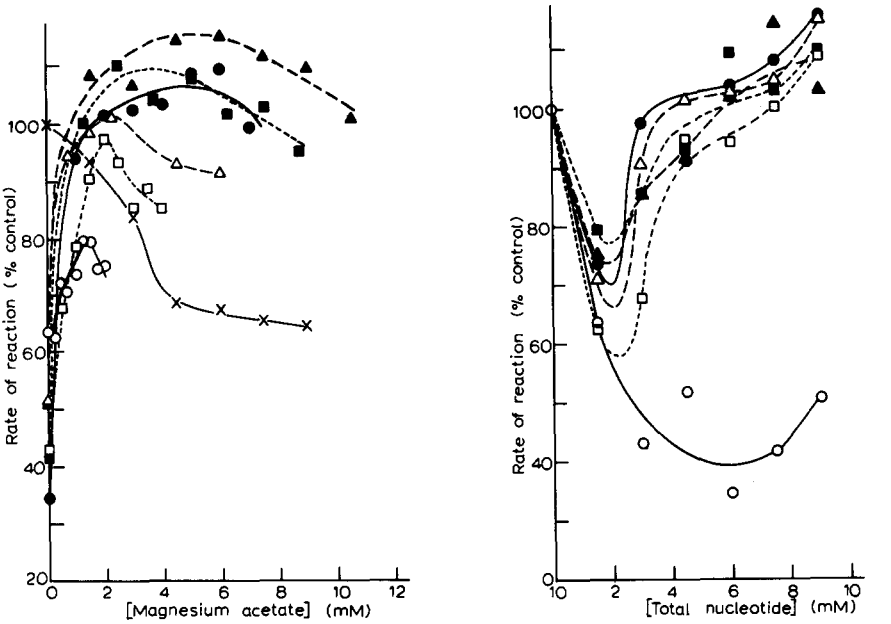

Fig. 2. The effect of Mg^{2+} concentration on the activity of aspartate carbamoyltransferase at various concentrations of regulatory nucleotide mixture: X, no nucleotide; ○, 1.5 mM; □, 3.0 mM; △, 4.5 mM; ●, 6 mM; ■, 7.5 mM and ▲, 9.0 mM. The conditions of assay are identical to those of Fig. 1.

Fig. 3. The effect of the concentration of regulatory nucleotide mixture on the activity of aspartate carbamoyltransferase at various fractions of Mg^{2+}-saturation of the regulatory nucleotides: ○, no Mg^{2+}; □, $\frac{1}{6}$; △, $\frac{1}{2}$; ●, $\frac{2}{3}$; ■, equimolar Mg^{2+} nucleotide; ▲, $\frac{4}{3}$. The conditions of assay are identical to those of Fig. 1.

tion, that is, there is maximal activity at zero Mg^{2+} concentration. As the total concentration of the nucleotide mixture increases the concentration of Mg^{2+} for maximal activity also increases. With the nucleotide mixture present, increases in Mg^{2+} up to the concentration giving maximal activity presumably act by decreasing the inhibitory effects of CTP and GTP and increasing the activating effect of ATP (Fig. 1). The inhibition caused with higher concentrations of Mg^{2+} may be similar in the incubations with nucleotide mixture and in the control, that is, resulting from effects of Mg^{2+} unrelated to chelation to nucleotides.

Kleppe and Spaeren [1] proposed that inhibition of aspartate carbamoyltransferase by Mg^{2+} was due to the progressive lowering of the aspartate concentration by formation of a Mg^{2+} aspartate complex which could not serve as a substrate, whereas Bigler and Atkinson [16] concluded that such inhibition may result from direct interaction of cation with enzyme. Examination of the sigmoidal curve for rate versus aspartate concentration presented by Gerhart in Fig. 3 of ref. 7 shows a point of inflexion at an aspartate concentration of approximately 5 mM. Below this point a given percentage change in aspartate concentration produces a greater percentage change in the rate of reaction, at the point the percentage change in concentration and rate are equal, while above it there is a lesser percentage change in the rate of reaction. Kleppe and Spaeren's data [1] with 6 mM aspartate show a decrease in rate of reaction that closely follows the calculated decrease (calculated from K_s = 0.2 mM^{-1} [17]

for the reaction Mg^{2+} + Asp ⇌ Mg^{2+} (Asp)) in concentration of free aspartate with increase in Mg^{2+} concentration. That is, the percentage change in rate is approximately equal to the calculated percentage change in aspartate concentration. This result is not incompatible with Gerhart's data on the relationship between rate and aspartate concentration [3] and the hypothesis that Mg^{2+} acts by lowering the aspartate concentration, since the characteristics of the sigmoidal curve are not greatly different at 6 mM than at 5 mM aspartate.

In our experiments with a Mg^{2+} concentration well below 5 mM, any percentage change in aspartate concentration should give a greater percentage change in the rate of reaction. However, calculated percentage decreases in aspartate concentration by chelation with Mg^{2+} are much greater than the observed percentage decreases in rates of reaction produced by Mg^{+} at the respective concentration. For example, at 9 mM Mg^{2+} calculations how only 36.2% of the 0.31 mM aspartate to be unchelated whereas 64.5% of the enzymatic activity remains (Fig. 2). Thus our data are incompatible with the hypothesis [1] that Mg^{2+} inhibits the enzyme by decreasing the effective aspartate concentration through chelation. They are compatible with a direct effect of Mg^{2+} on aspartate carbamoyltransferase [16].

Variation of the concentration of the regulatory nucleotide mixture (Fig. 3) shows that activity of aspartate transcarbamoylase decreases initially and then increases in a way which is dependent upon the degree of Mg^{2+}-saturation of the regulatory nucleotides. The initial decrease in activity as the total nucleotide concentration is increased (Fig. 3) reflects the sensitivity of the enzyme to inhibition by low concentrations of CTP (Fig. 1). The increase in activity as the nucleotide concentration is increased would depend upon greater binding of ATP to the enzyme. The earlier occurrence of this increase at higher Mg^{2+} concentrations is consistent with greater ability of Mg^{2+} · ATP to compete with CTP for the regulatory sites.

The activity of aspartate carbamoyltransferase in vivo depends upon competition between ATP, CTP and GTP for binding at the regulatory sites of the enzyme. Changeux et al. [18] have shown in vitro that the binding of Na^{+} · CTP, at least to the high affinity CTP sites of the native enzyme, is diminished in the presence of Na^{+} · ATP in a manner suggesting simple competition between Na^{+} · ATP and Na^{+} · CTP for binding to a common nucleotide site. Conclusions from a comparison of Fig. 1 with binding data [8,9] suggest that the extent of binding of Na^{+} · ATP and Na^{+} · CTP can be correlated with enzymatic activity. However binding studies using Mg^{2+} nucleotides will be more relevant to an understanding of the mechanisms of control of the enzyme in vivo.

Increases in growth rate due to growth on different carbon sources are associated with increase in the concentration of the regulatory nucleotide mixture [10]. From the results presented here, this would be expected to stimulate the activity of aspartate carbamoyltransferase in keeping with the increased demand for pyrimidine nucleotides at increased growth rate. Under conditions of growth with Mg^{2+}-limitation, inhibition of the enzyme may be increased by lower saturation of the nucleotides with Mg^{2+}. Under conditions of low energy charge [16], increase in the concentrations of free Mg^{2+} through release of cation normally bound to nucleoside triphosphates would contribute to increased inhibition of the enzyme.

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