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KINETIC PROPERTIES OF PHOSPHORIBOSYLADENOSINE TRIPHOSPHATE SYNTHETASE

INHIBITION BY AGGREGATION AT HIGH ENZYME CONCENTRATIONS

HÅKON KRYVI AND LEIV KLUNGSØYR
Department of Physiology, University of Bergen, 5000 Bergen (Norway)

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SUMMARY

1. The specific activity of phosphoribosyladenosine triphosphate: pyrophosphate phosphoribosyltransferase (phosphoribosyladenosine triphosphate synthetase) from *Escherichia coli* decreases with increasing concentration of the enzyme, indicating aggregation to less active forms.

2. The decrease in specific activity at high enzyme concentrations is more pronounced at 35° than at 15°, and for this reason a very low activation energy for the synthetase reaction is found at high enzyme concentrations.

3. 2.5 mM AMP inhibits the synthetase reaction under standard assay conditions and low enzyme concentrations while little inhibition is seen at high enzyme concentrations. Even at high enzyme levels, 2.5 mM AMP changes the response of the enzyme to histidine from a cooperative to a more hyperbolic type.

4. At high enzyme concentrations, inhibition by the product, phosphoribosyladenosine triphosphate, is temperature dependent and most pronounced at low temperatures.

5. The apparently sigmoidal response of synthetase activity to magnesium concentration may be explained by the binding of Mg^{2+} to ATP. After correction for this binding, half saturation of the enzyme with Mg^{2+} is observed at 0.13 mM.

INTRODUCTION

The enzyme phosphoribosyladenosine triphosphate synthetase of *Escherichia coli* is feedback inhibited by histidine, and its activity is also controlled by the energy charge parameter[1].

The histidine pathway is further controlled by synergistic inhibition of the synthetase by the first product and the pathway end product. These various control mechanisms were shown to be associated with an increased enzyme affinity for histidine caused by AMP and phosphoribosyladenosine triphosphate[2].

Studies of the sedimentation properties of phosphoribosyladenosine triphosphate synthetase in the analytical ultracentrifuge[3] showed that the enzyme may exist in many aggregate forms, one of which, the 8.0-S species, is stabilized by or

preferentially bound to AMP, histidine and phosphoribosyladenosine triphosphate. This species, however, is also seen with moderately high concentrations of ATP, which raises the question of whether the presumed hexamer really represents a specifically inhibited form.

We have studied this and other problems of kinetics at high enzyme concentrations, as they presented themselves from our knowledge about the molecular properties of the enzyme.

MATERIALS AND METHODS

Phosphoribosyladenosine triphosphate synthetase was prepared from our *E. coli* mutant strain X-1 as previously reported[2]. The enzyme was dissolved in 0.5 ml 10 mM imidazole buffer containing 0.5 ml 2-mercaptoethanol per l (Basal buffer). Further dilution of the enzyme was done in Basal buffer + 0.1 M NaCl.

The kinetic experiments were carried out in a Shimadzu MPS-50L multipurpose spectrophotometer by measuring the change in absorbance at 290 nm at a controlled temperature.

AMP, ATP, phosphoribosyl pyrophosphate and purified pyrophosphatase were obtained from Sigma Chemical Company, St. Louis.

Tris, imidazole, 2-mercaptoethanol and histidine were purchased from Merck AG, Darmstadt.

Protein content was determined as described by KLUNGSÖYR[4].

The rate in the kinetic experiments was determined by measuring the slope of the tangent to the curve immediately after substrate addition.

RESULTS

Enzyme concentration and activity

The specific activity of the enzyme at 30° depended upon protein concentration (Fig. 1). The activity increased with decreasing enzyme concentration and reached a maximum value beyond which it again decreased, probably due to enzyme denaturation.

In these experiments the assay mixture contained 5 mM ATP. We have earlier described the ability of ATP at high concentration to create the inhibited 8.9-S species of the enzyme, while other aggregate forms were seen with lower ATP concentrations or in the absence of ATP[3]. While protein concentration obviously will influence the aggregation of the enzyme, ATP might also be an important factor, both with regard to the extent of aggregation and the species of aggregate formed.

In Fig. 2 results of experiments with varying ATP at two different levels of enzyme are presented. At high enzyme concentration, high concentrations of ATP caused considerable additional inhibition.

Effect of AMP

The effect of AMP on the activity of the synthetase is also shown in Fig. 1. At low concentrations of the enzyme, high concentrations of AMP had an inhibitory effect which is not seen at high enzyme concentrations. This may reflect a displacement of the aggregation–dissociation equilibria by AMP towards the 8.9-S species, analogous to our findings in the ultracentrifuge[3].

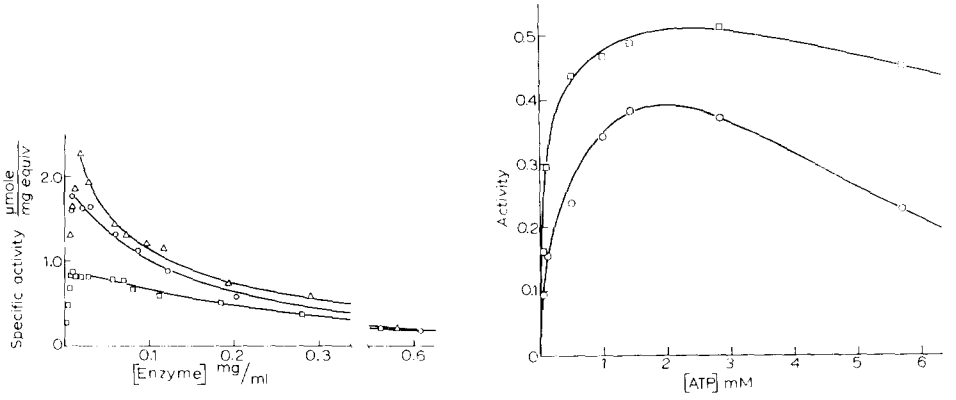

Fig. 1. Effect of the enzyme concentration and AMP on the specific activity of the enzyme. The reaction mixture contained: 5 mM $MgCl_2$, 5 mM ATP, 0.2 M Tris HCl (pH 8.5), 20 μl of the appropriate enzyme dilution in a final volume of 0.54 ml and AMP as specified below. The reaction was started by adding phosphoribosyl pyrophosphate to a concentration of 0.18 mM. The rate unit is μmole/min per mg enzyme. Curve identification: ○, No AMP; □, 50 μM AMP; △, 2.5 mM AMP.

Fig. 2. Synthetase reaction rate as a function of ATP concentration at two different enzyme concentrations. The temperature was 30°. Assay mixture: 0.1 M Tris, 0.15 M KCl, 6.0 mM $MgCl_2$. Curve identification: ○, 0.21 mg enzyme per ml; □, 0.014 mg enzyme per ml.

AMP and histidine sensitivity

Since AMP may act by inducing the hexameric structure in the synthetase, this ligand might be expected to influence the kinetics of the histidine inhibition of the enzyme. This has been shown to be true at low enzyme concentrations[2]. The results in Fig. 3 demonstrate that AMP affects histidine inhibition also at high en-

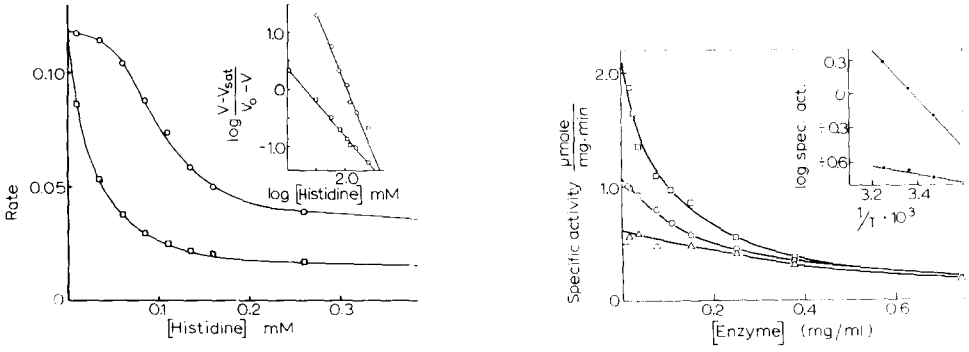

Fig. 3. Effect of AMP on the inhibition by histidine of phosphoribosyladenosine triphosphate synthetase. The reaction mixture was as given in Fig. 1. Data from two different series of experiments at 30° have been combined. The enzyme concentrations were approx. 0.2 mg/ml. The Hill coefficients were calculated by the least-squares method. Rate units are arbitrary. Curve identification: ○, 50 μM AMP; □, 2.50 mM AMP.

Fig. 4. Effect of temperature and enzyme concentration on synthetase activity. Conditions as given in Fig. 1 with no AMP. The rate unit is μmole/min per mg enzyme. The points are average values of 3–5 determinations. Curve identification: ○, 15°; △, 25°; □, 35°. Inset, protein concentrations: upper line, 0.01 mg/ml; lower line, 0.37 mg/ml.

zyme concentrations, when AMP alone has little effect, and the enzyme is already inhibited by aggregation. Homotropic histidine interaction is apparent at the lowest AMP concentration, and the Hill plot of the histidine effect had a slope of 2.3. At 2.5 mM AMP hyperbolic kinetics of the histidine inhibition is approached with a Hill plot slope of 1.3.

Temperature

For a better understanding of the association–dissociation phenomena, the effect of the temperature was studied. Fig. 4 illustrates the specific activity as a function of enzyme concentration at three different temperatures. At low enzyme concentration the rate of reaction increased with the temperature. However, as the protein concentration increased the specific activity decreased much more at 35° than at 15°, and at the highest concentrations tested, nearly similar rates were measured at the three temperatures. An Arrhenius plot of the rates at high and low concentrations of the enzyme is included in Fig. 4. For the dilute enzyme the activation energy was estimated to 9.12 kcal, whereas at high enzyme concentrations 1.6 kcal was found.

Product inhibition

In the synthetase rate assay the traces recording the increase in absorbance at 290 nm with time had different shapes depending upon enzyme concentration and temperature. In Fig. 5 this is illustrated with copies of recorder tracings from experiments with high enzyme concentration at 15°, 25° and 35°. The inhibition caused by product formation at 15° could be duplicated by addition of corresponding concentrations of the product from the start of the assay. The initial phase of rapid decrease in rate was then abolished. Addition of product at high enzyme concen-

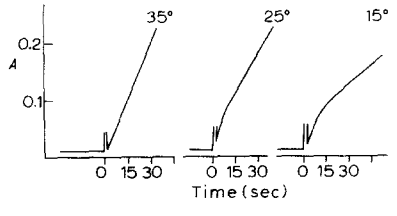

Fig. 5. Product inhibition. Copies of recorder tracings of phosphoribosyladenosine triphosphate synthetase assays at different temperatures. The enzyme concentration was 0.37 mg/ml. Conditions as in Fig. 1 (no AMP).

trations at 35° had no appreciable effect on the rate. At 15° the accumulation of phosphoribosyladenosine triphosphate seemed to participate in the process of activity decrease which at 35° was already accomplished initially. Probably at this temperature and concentration the enzyme was aggregated and inhibited, while stabilization of the inhibited 8.9-S species by phosphoribosyladenosine triphosphate was seen at the lower temperature. Similar temperature effects on the histidine affinity to the enzyme in the presence of phosphoribosyladenosine triphosphate have been reported previously[2].

Magnesium

It has been shown that Mg^{2+} is necessary for the catalytic activity of the enzyme[5]. With high ATP/Mg^{2+} ratios the enzyme had little activity independent of the absolute concentration of ATP. The magnesium/ATP complex is relatively stable ($\log k = 4.3$)[6]. An apparently sigmoidal dependence of rate on Mg^{2+} was observed (Fig. 6), but when the Mg^{2+} concentrations were corrected for ATP binding, hyperbolic kinetics were observed, and a K_m value of 0.13 mM was estimated.

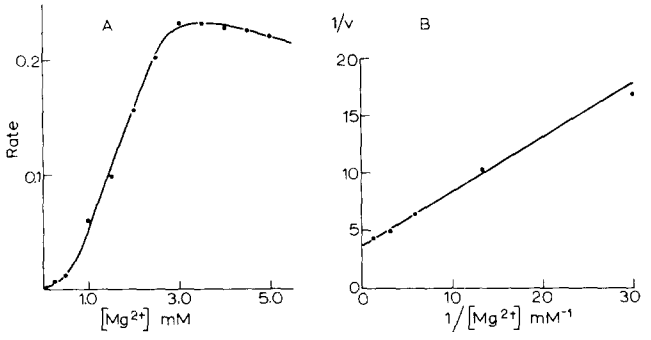

Fig. 6. A. The effect of magnesium on the catalytic activity of the synthetase. The reaction mixture contained: 2.4 mM ATP, 0.05 mM AMP, 0.2 M Tris·HCl (pH 8.5). The temperature was 30°. The rate units are arbitrary. B. Double-reciprocal plot of the data in A after correction for binding of Mg^{2+} by ATP.

We were, however, unable to demonstrate any effects of Mg^{2+} on the histidine inhibition of the enzyme, except possibly a decreased histidine sensitivity at high Mg^{2+} concentrations. If Mg^{2+} was necessary for the histidine effect as indicated by the sedimentation data[3], this function was saturated before or simultaneously with the catalytic function of the metal ion.

DISCUSSION

In a previous paper[3] we attempted to summarize our knowledge of *E. coli* phosphoribosyladenosine triphosphate synthetase in the form of a model. This model aided in the formulation of several questions, some of which could be attacked with kinetic methods.

First: Are all inhibited forms of the enzyme to be found among the 8.9-S species? This question may now be answered in the negative. Even at low concentrations of ATP, inhibition was observed at high enzyme concentrations. Under these conditions little 8.9-S material was seen in the ultracentrifuge. However, increased ATP concentration gave even more inhibition, and therefore the conditions of maximum inhibition were those that created the 8.9-S species.

Second: Since AMP is such efficient stabilizer of the 8.9-S species, why is it not a more efficient inhibitor of enzyme action? This seems partly to be a matter of concentrations. At low enzyme concentrations, relatively high concentrations of AMP did inhibit the enzyme. At high enzyme concentration, enzyme aggregation caused inhibition by itself and AMP gave little additional inhibition. Also, the

AMP and ATP stabilized 8.9-S forms were only partially inhibited, since histidine caused considerably additional inhibition.

Third: Are the 8.9-S species stabilized by AMP and ATP identical? This seemed unlikely from the ultracentrifuge observations, where the histidine effect was much facilitated by AMP and not by ATP. The results from the kinetic experiments at high enzyme concentration confirm that different species must be involved. With high AMP hyperbolic kinetics of histidine, inhibition was seen, while with low AMP the histidine effect was clearly cooperative. Therefore, the ATP-created 8.9-S species must in some way be reorganized before the histidine-stabilized 8.9-S form appears, while this is not necessary for the AMP form.

Of great interest are the observations on the temperature effect on enzyme activity decrease, probably caused by aggregation. The greater tendency to aggregate at 35° than at 15° indicates that hydrophobic bonds may be involved either in the aggregation process itself or in the creation of "sticky" protomers.

The data may also be used for the construction of crude Arrhenius plots and as such display an interesting difference from the data of HUANG AND GRAVES[7] on muscle phosphorylase *a*. These authors observed a doubling of the activation energy when the enzyme concentration was increased, whereas we observed a decrease towards zero. Obviously, these energies merely represent reactions concerned with aggregation and dissociation of the enzyme, not the catalyzed reaction. Is activity in the aggregated enzyme perhaps determined by the rate of dissociation of the aggregate, making possible the release of product which was hindered in the aggregate?

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