Vol. 117, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1983 Pages 238-244

INACTIVATION OF CARBAMOYL PHOSPHATE SYNTHETASE (AMMONIA) BY ELASTASE AS A PROBE TO INVESTIGATE BINDING OF THE SUBSTRATES

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Received September 6, 1983

SUMMARY Rat liver carbamoyl phosphate synthetase I is inactivated by elastase. Addition of ATP, Mg^{2+}, K^+ and N-acetyl-L-glutamate (the physiological allosteric activator) protects entirely, whereas acetylglutamate alone speeds inactivation. We have exploited these properties to investigate binding of these ligands. Acetylglutamate binds with low affinity (K_D 0.25 mM) in the absence of other ligands, and with higher affinity (K_D<<0.1 mM) when ATP, Mg^{2+} and K^+ are present. The apparent K_D for ATP in the presence of acetylglutamate is intermediate between the K_D values for the two ATP binding sites present in the enzyme; thus, binding of ATP to both sites is involved in protecting the synthetase. The data also indicate binding of MgATP and Mg^{2+} in the absence of acetylglutamate. The results provide further evidence for conformational changes associated with allosteric activation of the enzyme.

Carbamoyl phosphate synthetase I from mammalian liver mitochondria has a nearly absolute requirement for N-acetyl-L-glutamate, an allosteric activator (1). To ascertain the mechanism of activation by this agent we studied its binding to the rat enzyme using rate of dialysis and ultracentifugation techniques (2); we showed binding of one molecule of acetylglutamate in the presence of ATP, K^+ and Mg^{2+}. In the absence of these ligands we failed to observe binding, possibly due to low sensitivity of the binding assays, which would only detect binding with a K_D<0.2-0.3 mM.

It was noted earlier in our laboratory (3) that substrates alter the proteolytic susceptibility of carbamoyl phosphate synthetase. More recently an abstract has appeared (4) reporting protection by the substrates against inactivation by elastase of hamster's carbamoyl phosphate synthetase. By using similar experiments of protection and inactivation of the rat enzyme we have extended our binding data. The results, presented here, demonstrate binding of acetylglutamate in the absence of other ligands; they also show binding of

0006-291X/83 $1.50

ATP and of Mg^{2+} to the enzyme, and provide evidence for conformational transitions associated with binding of these ligands.

MATERIALS AND METHODS Elastase (from porcine pancreas, 70 units/mg), elastatinal (an inhibitor of elastase) and phenyl methyl sulphonyl fluoride (PMSF) were from Sigma. Carbamoyl phosphate synthetase from rat (specific activity 29-32 µmol carbamoyl phosphate/15 min/mg) and hamster (25-27 µmol/15 min/mg) were prepared as described for the rat enzyme (5). The frog liver synthetase (29 µmol/15 min/mg) was purified as described (6). Enzyme activity was assayed using a NADH-coupled assay (6). Except where indicated, carbamoyl phosphate synthetase (2.5 mg/ml) was treated with elastase (0.5 % of the synthetase in terms of protein) at 37°C in 30-100 µl of a solution of Tris-HCl 30 mM pH 7.2, KCl 20 mM, dithioerythritol 1.5 mM, glycerol 8 % (v/v) and the concentrations specified of other ligands. At the indicated times, 10 µl samples were diluted in 100 µl of K phosphate buffer 10 mM pH 7.4 at 0°C containing 1 mM dithioerythritol and 5 µM elastatinal, and the activity was assayed immediately. It remained constant when elastase was omitted or when 1 mM PMSF was included in the incubation. None of the ligands of carbamoyl phosphate synthetase had an effect on elastase activity (assayed with azoalbumin). For other details, see (5)

RESULTS Table I confirms (4) that hamster liver carbamoyl phosphate synthetase is inactivated by elastase and that addition of a mixture of ATP, acetylglutamate, Mg^{2+} and NH_4^+ prevents inactivation. The Table also shows nearly identical behavior of the hamster and rat liver enzymes on incubation with elastase with or without substrates. In contrast, the more stable frog liver enzyme was not inactivated by elastase, whether or not there were substrates.

TABLE I. Effect of elastase on hamster, rat and frog liver carbamoyl phosphate synthetases. Where used, 10 mM ATP, 10 mM acetylglutamate, 20 mM $MgSO_4$ and 7 mM $(NH_4)_2SO_4$ were added. Less than 10 % of the ATP was consumed during the two hours' incubation. When the frog enzyme was used, it was at a concentration of 1.2 mg/ml and elastase represented 1 % of the protein. For other details, see Materials and Methods.

Enzyme	Additions	Activity remaining after 2 hours
		%
Hamster	—	35
	ATP, acetylglutamate, Mg^{2+}, NH_4^+	102
Rat	—	30
	ATP, acetylglutamate, Mg^{2+}, NH_4^+	100
Frog	—	100
	ATP, acetylglutamate, Mg^{2+}, NH_4^+	100

Since our binding studies have used the rat synthetase, further experiments were done with the enzyme from this source.

Carbamoyl phosphate synthetase is an unstable enzyme and ligands have been documented to influence its thermal stability under a number of conditions (7). However under the present conditions the ligands used did not affect carbamoyl phosphate synthetase activity if elastase was omitted or inactive. Fig 1 shows that in the absence of ATP and Mg^{2+} 10 mM acetylglutamate speeds considerably inactivation by elastase. In these experiments 60 mM K^{+} (an activator of the synthetase) was present; when this cation was omitted (acids were neutralized with Tris) acetylglutamate also speeded inactivation (not shown). Therefore, acetylglutamate binds in the absence of other ligands and induces changes in the synthetase rendering it more susceptible to inactivation by elastase.

Fig 1 also shows that addition of ATP or Mg^{2+}, in the presence of acetylglutamate, has no effect on the time-course of inactivation, compared with acetylglutamate alone. In contrast, addition of ATP and Mg^{2+} together, in the presence of acetylglutamate, completely prevents inactivation. This dramatic

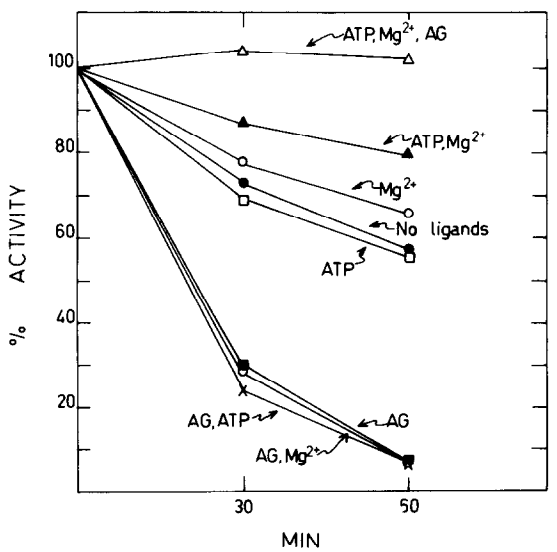

Fig 1. Effects of ATP, Mg^{2+} and acetylglutamate on the inactivation of the rat enzyme by elastase. Where used, the concentrations of the ligands were: ATP, 10 mM, Mg^{2+}, 20 mM, acetylglutamate (AG), 10 mM. The concentrations of K^{+} and Na^{+} were kept constant at 60 and 20 mM respectively by addition of the corresponding chlorides.

effect must result from changes in the enzyme associated with binding of MgATP. Addition of NH_4^+ had no further effect (not shown).

In the absence of acetylglutamate (Fig 1) ATP alone does not protect (as expected; MgATP is the substrate) and Mg^{2+} alone protects somewhat; the latter may be related to the requirement by the enzyme of free Mg^{2+}. Addition of ATP and Mg^{2+} together protects more, albeit not completely. This confirms that MgATP binds in the absence of acetylglutamate (1).

Fig 2 illustrates the effect of various concentrations of acetylglutamate on elastase inactivation of the enzyme. In the presence of Mg^{2+} and ATP complete protection is already attained at 0.1 mM acetylglutamate, indicating that under these conditions acetylglutamate binds with a K_D<<0.1 mM. In contrast, comparatively high concentrations of acetylglutamate are required to speed inactivation in the absence of Mg^{2+} and ATP. The curve has been fitted assuming hyperbolic binding with a K_D for acetylglutamate of 0.25 mM.

Fig 3 shows that in the presence of acetylglutamate ATP protects more at a concentration of 10 mM than at 1 mM, and therefore the enzyme is not saturated at the latter concentration. Since there are two distinct binding sites for ATP in the enzyme (5) the curve needs not be hyperbolic. However, if a hyperbola is aproximated to the points, an apparent K_D of ca. 50 μM is

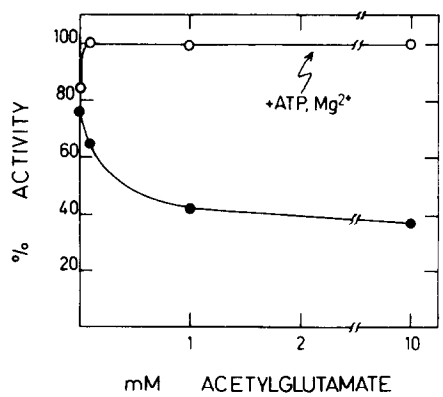

Fig 2. Effect of acetylglutamate concentration on carbamoyl phosphate synthetase inactivation by elastase. When used, ATP and $MgSO_4$ were present at concentrations of 10 and 20 mM respectively. The concentrations of K^+ and Na^+ were kept constant at 60 and 20 mM respectively by addition of the corresponding chlorides. Incubation time was 30 min in the absence of ATP and Mg^{2+} and 60 min when they were present. For other details see Methods.

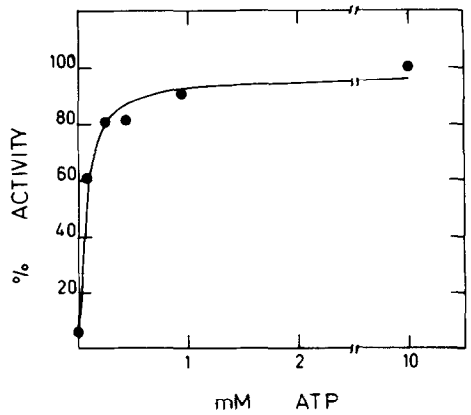

Fig 3. Effect of ATP concentration on carbamoyl phosphate synthetase inactivation by elastase. $MgSO_4$ was 10 mM in excess of the concentration of ATP, K^+, Na^+ and acetylglutamate where at concentrations of 60, 20 and 10 mM respectively, and elastase accounted for 5 % of the protein. The incubation was terminated after 5 min by dilution in cold buffer containing 50 µM elastatinal. For other details, see Methods. More than 80 % of the ATP remained at the end of the incubation, except when 0.1 mM ATP was used, where only 50 % remained. The concentrations specified are the average between these at the start and at the end of the incubation. The curve is a hyperbola for a K_D of 55 µM.

obtained. In the absence of acetylglutamate similar protection was attained with 10 mM ATP and 1 mM ATP (not shown).

DISCUSSION Carbamoyl phosphate synthetase is an allosteric enzyme. Transition from the inactive (T) to the active (R) form is greatly favored by acetylglutamate (2). We showed earlier binding of acetylglutamate after ATP_A (the molecule of ATP that yields P_i) (2). However, indirect kinetic evidence suggested that acetylglutamate may also bind first (1,8). We now demonstrate that this is indeed the case, by showing acetylglutamate binding in the absence of other ligands. The high K_D for this binding (0.25 mM) probably prevented detection in rate of dialysis and ultracentrifugation binding assays (2). In these previous experiments we observed binding of acetylglutamate with high affinity (K_D of ca. 10 µM) when K^+, Mg^{2+} and ATP were present. This is in agreement with the findings in this paper in the presence of these ligands, which are indicative of a K_D<<100 µM for acetylglutamate.

Carbamoyl phosphate synthetase has distinct binding sites for the two molecules of ATP used in the reaction. ATP_B (the molecule of ATP that yields carbamoyl phosphate) binds with high affinity (K_D of ca. 20 µM) whether or

not there is acetylglutamate whereas ATP_A binds with low affinity (K_D 0.2-0.7 mM) when there is acetylglutamate and with much lower affinity in the absence of the activator (1). Since in the presence of acetylglutamate the enzyme is not fully protected by 1 mM ATP (Fig 3), binding of ATP_A is involved in protecting the synthetase. The apparent K_D for ATP of 50 μM (Fig 3) is lower than the K_D for ATP_A and, therefore, it appears that binding of ATP_B also plays a role in pretecting the enzyme. This is also evident in experiments in the absence of acetylglutamate in which 1 mM ATP protects to the same extent than 10 mM ATP. Since in the absence of acetylglutamate K_D for ATP_A is 10 mM (1), binding of ATP_B has to be responsible for this protection.

Acetylglutamate may accelerate inactivation by elastase by exposing susceptible bonds in the synthetase, and thus, as expected for an allosteric activator, acetylglutamate induces conformational changes in the enzyme. Although binding of ATP_A (and possibly of ATP_B) appears to be involved in the allosteric changes (1), our present results indicate that some of these changes are induced by acetylglutamate even when ATP is absent.

Earlier evidence from pulse-chase experiments (5,9) was indicative of conformational changes associated with binding of ATP_A and ATP_B. This paper supports this view by showing that both molecules of ATP are involved in protecting the enzyme against inactivation by elastase. However, protection by ATP might also result from direct shielding by the nucleotide of bonds susceptible to elastase at the ATP binding sites. This appears unlikely, at least for ATP_B, for the binding site for ATP_B is normally unexposed to the environment (9).

ACKNOWLEDGEMENTS We thank C. Navarro for helping with the manuscript. Part of the work was supported by Grant n°3472/79 from the Comisión Asesora de Investigación Científica y Técnica of the Spanish Ministry of Science.

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