EVIDENCE FOR COOPERATIVE Mn-ATP BINDING WITH *BACILLUS SP.* GLUTAMINE SYNTHETASE*

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SUMMARY

The binding of Mn-ATP with *B. subtilis* glutamine synthetase, observed kinetically at 37°, pH 7.0, is cooperative (Hill $n = 2.3$, $S_{0.5} = 0.36$mM), a phenomenon overlooked in earlier studies. The Arrhenius plot is biphasic with a break at 26°C. Similar behavior is observed with the thermophilic *B. stearothermophilus* enzyme, but is absent with the enzymes from *E. coli*, plant, and mammaliam sources under optimal assay conditions. The temperature dependence of the intrinsic fluorescence of the protein is also non-linear, and the intersection point of 18° shifts to 30°upon binding of substrates. These results are interpreted as indicating that *Bacillus sp.* enzymes can assume multiple, functionally important conformational states related to Mn-ATP binding at 37°. They also emphasize further that critical differences in mechanism exist among glutamine synthetases from different sources.

Despite the dodecameric quaternary structure typically encountered for bacterial glutamine synthetases, cooperative effects among subunits have been observed so far but rarely. Several examples of inter-subunit effects observed with the very complex *E. coli* system include: the affinity for the first Mn (II) or Co (II) bound to the protein at the n_1 sites, non-linear responses to adenylylation of Mn^{2+}-, Mg^{2+}-, and Co^{2+}-stimulated activities, and cooperative L-glutamine binding to the Co^{2+} enzyme (1). Whether these effects come into play *in vivo* is quite uncertain. With the *B. subtilis* enzymes, previous data suggest the possibility of cooperative binding of Mn-ADP (2,3). Based on our recent investigations on the thermophilic *B. stearothermophilus* enzyme (4), the intriguing possibility

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exists that Mn-ATP binds cooperatively to the B. subtilis enzyme also.

EXPERIMENTAL

Bacillus subtilis (ATCC 6051) was grown on the defined MG media, with limiting ammonia according to Freese and Fortnagel (5). Glutamine synthetase was isolated and purified from 200g of cells according to Deuel et.al. (6). It was judged to be > 95% homogeneous by polyacrylamide gel electrophoresis pH 9.0, and exhibited a specific activity of 9.40 μ moles P_i released/min/mg in the Mn^{2+}- dependent assay at pH 7.0, 37°. The typical biosynthetic assay mixture contained (in μ moles/ml): 50 L-glutamate, 5 NH_4Cl, 7.5 Mn-ATP, and 50 imidazole-HCl at pH 7.0. Prior to addition of substrate mixture, diluted enzyme was pre-incubated at the temperature of interest, usually 37°. P_i release was measured colorimetrically (7).

Fluorescence measurements were carried out using a Turner Model 430 spectrofluorometer, with the cuvet block thermostated to ± 0.1°C with a Haake refrigerated circulator bath and the output attached to a Sargent SRLG recorder. Cuvet solution temperature was monitored with a YSI Model 42SC tele-thermometer.

RESULTS:

Kinetic observations. Although the kinetically-determined binding curves for substrates with B. subtilis glutamine synthetase at 25° published previously appear hyperbolic, the Mn-ATP curve was not well defined below half-saturation (2). Later studies (3) revealed non-hyperbolic effects associated with ADP binding in the γ-glutamyltransferase activity, strongly dependent on ionic strength. However, such salt effects are nearly negligible with the biosynthetic assay. Thus we have studied this activity at 25° and 37°: the result of varying Mn-ATP (1:1) concentration at 37° is seen in Figure 1A, and clearly produces a sigmoidal curve. The Hill n is calculated (inset) to be 2.3 and $S_{0.5}$ = 0.36 mM. Interestingly, the saturation curve at 25° (not shown) is more nearly hyperbolic (Hill n = 1.2) and Mn-ATP binds more tightly, $S_{0.5}$ = 0.10 mM. These data suggest that the enzyme can assume multiple conformational states, modulated by temperature.

The above interpretation assumes that the main effect observed is due to enzyme-Mn-ATP interaction. Alternative

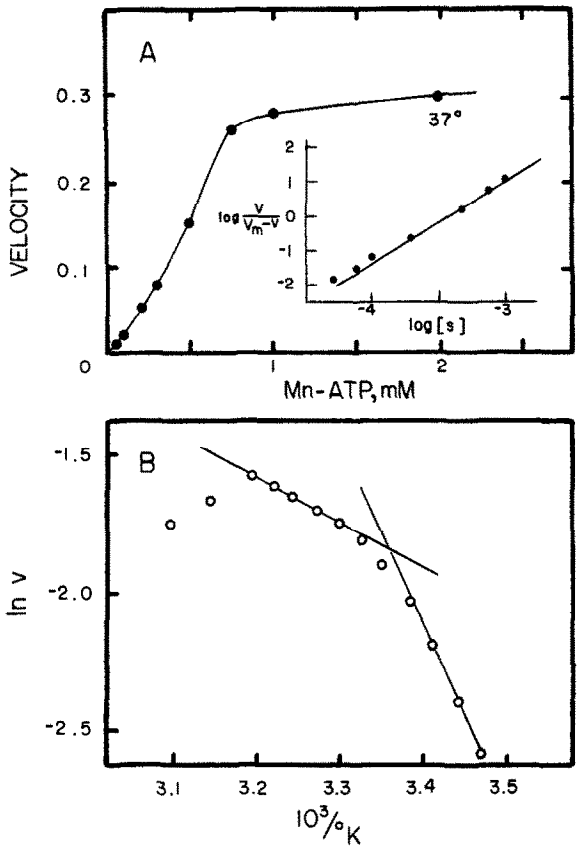

Figure 1: (A) Kinetic response of the biosynthetic assay of B. subtilis glutamine synthetase, pH 7.0, 37°, to variation in Mn-ATP concentration. See Experimental section for details of the assay. (Inset): Hill plot of the kinetic data; calculated Hill n = 2.30.
(B) Arrhenius plot of biosynthetic activity vs temperature, over the range of 15-50°C.

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explanations to consider are: (a) An ionic strength effect. This is unlikely, since μ changes < 5% and since the data of Deuel and Turner (3) indicated insensitivity of the biosynthetic assay to increasing salt. (b) Multi-order kinetics due to Mn^{2+} and ATP concentrations below the dissociation constants for Mn-ATP and E-Mn-ATP complex formation, to produce artifactual sigmoidal curves as calculated by London and Steck (8). This is ruled out by the lack of cooperativity at 25°. Also model calculations using Kd ≅ 2.7 x 10^{-5} M for the $MnHATP^-$ complex and 3.2 x 10^{-7} M for the $MnATP^=$ complex at 25° (9), show that the Mn-ATP complex is > 95% formed before < 2% E-Mn-ATP is formed. (C) Intra-site substrate syrergism between Mn-ATP and

L-glutamate or NH_3, as observed with other glutamine synthetases under dynamic conditions (10). This possibility is one which the present data do not eliminate, but one not supported by data on interdependence of substrate Km's (2,3). Thus, overall a homotropic effect induced by Mn-ATP seems the most likely explanation.

In support of this hypothesis, the Arrhenius plot shown in Figure 1B is strikingly biphasic, with calculated E_a values of 3.0 and 14.7 Cal/mole. The higher value is similar to those (4) reported for glutamine synthetases from E. coli and ovine brain, both monophasic plots, but differ rather markedly from those obtained with the B. stearothermophilus enzyme which also exhibits a biphasic plot. The intersection point occurs at 26°C. Above 40° substantial enzyme denaturation occurs within the 10 min. assay incubation.

Spectrofluorometric observations. In these experiments the effect of temperature and bound substrates on intrinsic fluorescence emission of the B. subtilis enzyme was observed. First, each substrate (5mM NH_3, 50mM L-glutamate, or 7.5mM Mn-ATP,) was added separately. The major change occurred with Mn-ATP, which diminished the intensity of emission by ca. 28% whereas the others caused < 5% change. Addition of substrates sequentially in the order Mn-ATP, then glutamine, then NH_3, also showed that only Mn-ATP caused a major change. This is very different from the enhanced fluoresence observed upon addition of Mn-ATP and L-glutamine reported with the E. coli enzyme (11), interpreted as indicating an "activated E-ATP-Glu complex", the nature of which was not specified.

Next, the emission intensity (I) of native enzyme was observed as a function of temperature in the presence and absence of all three substrates (concentrations as above). If an equilibrium, $C_1 \overset{K}{\rightleftharpoons} C_2$ exists, where C_1 and C_2 are different protein conformations with different fluorescent properties, a plot of log I vs 1/T is analogous to a van't Hoff plot. Clearly, the data of Figure 2 indicate a non-linear response in both cases. The slopes of the lines are quite similar, but the intersection point is shifted from 18° to 30°C upon addition of substrates.

Finally, it was observed that upon continuous variation of temperature in the experiments of Figure 2 that the enzyme

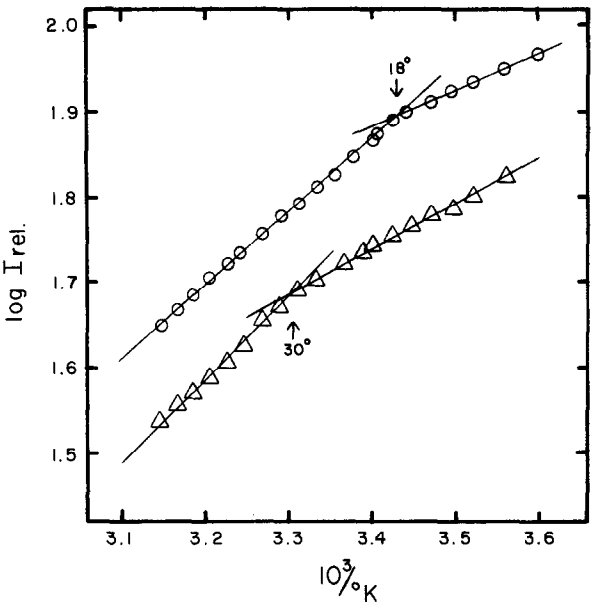

Figure 2: Intrinsic fluorescence emission of B. subtilis glutamine synthetase as a function of temperature (10°-45°) in the absence (0) and presence (Δ) of saturating levels of substrates. Excitation was at 290 nm and emission at 355 nm, using ca. 1 mg/ml protein at pH 7.0 in 0.05M phosphate buffer. Temperature was varied continuously upward at a rate not exceeding 0.4° per min.

with bound substrates was much more labile to denaturation and precipitation than the free enzyme. This effect is opposite to that observed with the B. stearothermophilus enzyme, where substrate protection occurs, particularly with L-glutamate (4).

DISCUSSION:

Taken together, the above data support the hypothesis that functionally important conformational changes occur in B. subtilis glutamine synthetase and that these appear to be related to the binding of Mn-ATP. The observed fluorescence decrease upon binding of Mn-ATP suggest that nucleotide binding may open up or loosen the protein structure, perhaps to facilitate binding of other substrates or the approach to transition states in catalytic steps. Different degrees of cooperativity at 37° and 25° further suggest that temperature can control conformational flexibility in this enzyme. Previous investigators may have overlooked these effects because all assays were conducted at 25° although bacteria were grown at 37° (2).

The observed non-linear Arrhenius plot and the hyperbolic Mn-ATP binding curve have been observed so far with Bacillus enzymes (4), not with others studied in this manner, including adenylylated and unadenylylated forms of E. coli enzyme, and these from ovine brain and pea seeds (10). The B. licheniformis enzyme has not been studied by probes for such effects (12).

These results also support the concept that considerable important differences in "mechanism" (at several levels of interpreation) appear to exist among the glutamine synthetase enzymes from different sources (10).

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