this site as the first step in a branched pathway that leads ultimately to the biosynthesis of a large number of compounds.

While the physiological relevance of these aggregration phenomena to regulatory control remains to be established, the evolutionary significance of isozymic regulatory control for several dehydrogenases is now well recognized (Markert et al., 1975).

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Glutamine Synthetase of *Bacillus stearothermophilus.* Regulation, Site Interactions, and Functional Information†

F. C. Wedler,* J. Carfi, and A. E. Ashour

ABSTRACT: The action of various feedback modifiers on *Bacillus stearothermophilus* glutamine synthetase has been investigated by initial velocity kinetics, using the Mn^{2+}-stimulated biosynthetic assay at 55 °C. The most potent inhibitors, used singly, are AMP, L-glutamine, and L-alanine. Other modifiers of significance include glycine, CTP, L-histidine, glucosamine 6-phosphate, and GDP. Marked synergism of action is observed for AMP in the presence of L-glutamine, L-histidine, ADP, or glucosamine 6-phosphate (glucosamine-6-P), and for CTP with ADP or GDP. Inhibition by saturating levels of many modifiers is either <100%, or is not overcome by elevated substrate levels, or both. This argues for modifier binding sites separate from substrate sites, notably in the cases of AMP, L-glutamine, glycine, L-alanine, glucosamine-6-P, and CTP. Glycine and L-alanine are V_{max} inhibitors, whereas L-glutamine, glucosamine-6-P, GDP, and CTP alter the binding of L-glutamate. ADP and L-histidine apparently can compete directly with MnATP, but AMP alters Mn-ATP binding from a separate site. The action of several modifiers requires or is enhanced by bound substrates. Considerable antagonistic interaction is observed in experiments with modifier pairs, but the most potent inhibitors show synergistic or cumulative (independent) interactions. One may interpret antagonistic effects as due to (a) overlapping modifier domains, or (b) separate but antagonistically interacting sites. Either interpretation leads to a scheme for modifier–substrate and modifier–modifier site interactions in which the thermophilic enzyme must maintain and stabilize a great deal of complex functional information under extreme environmental conditions.

The regulation of glutamine synthetase activity in microorganisms can occur primarily by end-product metabolite feedback inhibition, and in some cases by direct product inhibition (Shapiro and Stadtman, 1970). Specific mechanisms of regulation from one source to another are quite variable, however. Among mesophiles, for enzymes from gram-negative organisms, such as *Escherichia coli,* sensitivity to inhibitors is modulated by covalent adenylylation–deadenylylation, whereas for enzymes from gram-positive organisms, such as *Bacillus sp.,* inhibitors act synergistically and product inhibition by glutamine is a key feature (Hubbard and Stadtman, 1967a,b; Deuel and Prusiner, 1974).

† From the Chemistry Department, Cogswell Laboratory, Rensselaer Polytechnic Institute, Troy, New York 12181. *Received July 8, 1975.* This research was supported in part by grants from the American Chemical Society Petroleum Research Fund (2401-G1), the National Science Foundation (GB-34751), and the National Aeronautics and Space Administration (NSG-7065).

Earlier studies on the glutamine synthetase from the thermophile *Bacillus stearothermophilus* involved elucidation of its physical, chemical, and kinetic properties (Wedler and Hoffmann, 1974a,b). For those molecules showing significant inhibition, it was determined whether a given modifier was competitive or noncompetitive vs. each substrate. In the present communication, these preliminary results are supplemented by experiments designed to yield insights to the spatial relations and interactions among substrate and modifier sites. Taken overall, these studies indicate that modifier binding domains interact considerably and that modifier–substrate site interactions can be very complex. Synergy of modifier action is also a key observation. Such results have important consequences for investigations on macromolecular evolution and on the amount of complex functional information that can be maintained in key thermophilic enzymes.

Experimental Section

Materials. Glutamine synthetase was purified to homogeneity from *B. stearothermophilus,* strain 4S (8,12), according to the procedures of Wedler and Hoffmann (1974a). All biochemicals used were of the highest purity obtainable from Sigma Chemical Company. Inorganic salts and metal ions were of analytical grade from Fisher Scientific.

Enzyme Assays. Enzyme activity was assayed during purification by the γ-glutamylhydroxamate synthetase assay, and with pure enzyme by the biosynthetic assay as described earlier (Wedler and Hoffmann, 1974a). The biosynthetic assay mixture with "saturating" substrate levels contained (in μmol/ml): 10 of NH_4Cl, 25 of L-glutamate, and 7.5 of MnATP. Those assays designated as "limiting" in one substrate contained either 2.5 μmol of NH_4Cl, 5 μmol of L-glutamate, or 1 μmol of MnATP, respectively, with all other substrate levels at "saturating" levels.

Inhibition Studies. Modifier levels were varied by the following technique: a single assay reaction mixture was prepared and divided into two parts, with the modifier added to one of these at its maximal concentration. The solutions were then mixed in different proportions to a fixed final volume (0.2 ml) to make a series of reactions.

In studies of synergistic effects in which modifier levels were varied, a constant level of the first modifier was added to each such reaction, and then the level of the second modifier was varied as above. The activity exhibited by the enzyme in the presence of the first modifier (but in the absence of the second one) was taken as, or was "normalized" to, 100%.

In all cases, i, the fractional inhibition, was defined as $(V_0 - V)/V_0$, where the terms V_0 and V are defined as the reaction velocities at zero and at the finite level of the inhibitor being varied, respectively. Equations relating i to inhibitor concentration, [I], have been derived and discussed extensively elsewhere (Webb, 1963; Wedler and Boyer, 1972), and it has been shown that one can plot $1/i$ vs. $1/[I]$, in analogy to a Lineweaver–Burk plot.

In such double-reciprocal plots, if $1/i_{max} = 1.0$, this implies 100% inhibition at saturating modifier, whereas partial inhibitions are indicated by a $1/i_{max}$ value >1.0. A lower K_i(app) value is indicated by a more shallow slope of the line.

Experiments involving modifier competition with substrates or inhibitions by pairs of modifiers were performed as described previously (Wedler and Hoffmann, 1974b). In the substrate-pairs experiments, each inhibitor was used at a concentration that gave i values in the range of 0.15–0.30. Under these conditions, one obtains significant differences between the calculated values for cumulative vs. additive models of inhibition, but minimal deviation from a linear dependence of i on total inhibitor concentration. To provide accurate kinetic data, each inhibition value reported (in Table I) was the result of five or more separate determinations, so that these values showed errors below 5% in all cases.

Results

Modifier–Modifier Site Interactions. The effects of virtually all possible combinations of modifiers, singly and then in pairs, were first considered (Table I). A variety of effects is observed, but additive inhibitions are virtually absent, which tends to exclude the involvement of isozymes in this system. The single additive effect of Gly + L-His may result in fact from mild synergistic interaction.

For the pairs Gly + ADP, Gly + GDP, L-Ala + ADP, CTP + AMP, L-Gln + ADP, and L-Gln + CTP, the effects are essentially cumulative, indicative of separate, independent sites for these moieties. Synergistic interactions are noted for AMP + L-Gln, AMP + ADP, AMP + L-His, AMP + glucosamine-6-P[1] (GA-6-P), CTP + ADP, and CTP + GDP; these effects were studied in greater depth in experiments reported in the next section. It is also apparent that many of the modifiers interact antagonistically, the interpretation of which is also discussed below.

Modifier–Substrate Site Interactions. The effect of varying modifier levels at fixed substrate levels, either saturating or limiting, was next observed. Plots of fractional inhibition, i, as a function of modifier concentration were made, and these data were replotted as $1/i$ vs. $1/[I]$. From these replots one can readily tell whether, at infinite modifier, activity is diminished to zero or to a finite value. If activity is diminished less than 100%, modifier binding at a separate (allosteric) site is indicated. Similar experiments with mesophilic enzymes have proved quite revealing (Woolfolk et al., 1966; Hubbard and Stadtman, 1967a,b; Deuel and Prusiner, 1974).

In Figure 1 are shown the effects of glycine, L-histidine, and L-glutamine in the absence and presence of added AMP. Glycine and L-glutamine show partial inhibitions with saturating substrates, whereas histidine shows biphasic binding but complete inhibition under all conditions. Apparently, the action of glycine is essentially independent of the level of L-glutamate or other substrates, so that it is most likely a V_{max} inhibitor, as proposed earlier. The principal effect of added AMP is to raise the i_{max} of glycine to 1.0, i.e., to increase the extent of maximal inhibition.

The action of histidine is weakened by limiting glutamate, but is strengthened by limiting ATP. The biphasic nature of the histidine binding is abolished and modifier binding is enhanced by added AMP. These results, considered with earlier kinetic studies (Wedler and Hoffmann, 1974b), strongly suggest that L-histidine is competitive with ATP. Added L-glutamine produces no synergistic effects with histidine (data not shown), but is antagonistic (cf. Table I).

The action of L-glutamine is weakened with limiting ammonia, in terms of both tightness of binding and i_{max}, but is

[1] Abbreviation used: GA-6-P or glucosamine-6-P, glucosamine 6-phosphate.

Table I: Inhibition of *Bacillus stearothermophilus* Glutamine Synthetase by Pairs of Modifiers.[a]

Modifier: A	Obsd (% *i*)	B	Obsd (% *i*)	A + B (% *i*)	Calcd A + B (Cuml. % *i*)[b]	Conclusion[c]
Gly	(24)	L-Ala	(15)	34	35	Ant.
	(24)	L-His	(23)	46	42	Add.
	(24)	L-Gln	(22)	35	41	Ant.
	(24)	AMP	(23)	18	42	Ant.
	(24)	ADP	(21)	41	40	Cuml.
	(24)	GDP	(7)	28	29	Cuml.
	(24)	CTP	(20)	16	39	Ant.
	(24)	GA-6-P	(11)	26	32	Ant.
L-Ala	(15)	L-His	(23)	25	35	Ant.
	(15)	L-Gln	(22)	17	34	Ant.
	(15)	AMP	(23)	33	35	Ant.
	(15)	ADP	(21)	31	33	Cuml.
	(15)	GDP	(7)	6	21	Ant.
	(15)	CTP	(20)	8	32	Ant.
	(15)	GA-6-P	(11)	4	24	Ant.
L-His	(23)	L-Gln	(22)	27	40	Ant.
	(23)	AMP	(23)	47	41	Syn.
	(23)	ADP	(21)	26	39	Ant.
	(23)	GDP	(7)	15	28	Ant.
	(23)	CTP	(20)	11	38	Ant.
	(23)	GA-6-P	(11)	18	31	Ant.
L-Gln	(22)	AMP	(23)	49	40	Syn.
	(22)	ADP	(21)	39	38	Cuml.
	(22)	GDP	(7)	10	27	Ant.
	(22)	CTP	(20)	40	38	Cuml.
	(22)	GA-6-P	(11)	11	31	Ant.
AMP	(23)	ADP	(21)	46	39	Syn.
	(23)	GDP	(7)	20	28	Ant.
	(23)	CTP	(20)	40	38	Cuml.
	(23)	GA-6-P	(11)	43	31	Syn.
ADP	(21)	GDP	(7)	16	26	Ant.
	(21)	CTP	(20)	44	37	Syn.
	(21)	GA-6-P	(11)	26	30	Ant.
GDP	(7)	CTP	(20)	40	26	Syn.
	(7)	GA-6-P	(11)	5	17	Ant.
CTP	(20)	GA-6-P	(11)	8	29	Ant.

[a] Biosynthetic assay, pH 6.0, 55 °C, using the saturated substrate assay mixture (see Experimental Section). The concentrations of modifiers used were [in mM]: Gly [3.0], L-Ala [0.5], L-His [6.0], L-Gln [0.5], AMP [0.3], ADP [1.0], GDP [7.5], CTP [10.0], and glucosamine-6-P (GA-6-P) [20]. Observed percent inhibitions are shown in parentheses beside each moiety. [b] Calculated as described previously (Wedler and Hoffmann, 1974b). [c] Add. = additive, Cuml. = cumulative, Ant. = antagonistic, Syn. = synergistic.

strengthened in both parameters by limiting L-glutamate or added AMP. The observation of incomplete inhibition (i_{max} <1.0) suggests strongly that, although glutamine alters glutamate binding, the modifier binds at a separate site, which in turn interacts with bound AMP or other substrates. This hypothesis was tested in terms of the ability of each of the three substrates to overcome the effect of bound L-glutamine (Figure 2). Clearly, L-glutamine alters not only the K_m for glutamate, as seen previously, but also V_{max}; that is, high glutamate cannot abolish the inhibiting effect of bound glutamine, nor can high levels of ammonia or MnATP. Although (Figure 1C) glutamate levels can somewhat moderate the extent of the inhibition (i_{max}) by glutamine, these data argue strongly for a site for glutamine separate from substrate sites.

Synergism of inhibition, induced by AMP, clearly occurs with glycine, L-histidine, and L-glutamine. The inhibitory effect of AMP plus glutamine is greater than the sum of the effects obtained with either alone. In Figure 3A this concept was explored further in terms of the inhibition by AMP. At saturating levels of modifier, with either limiting or saturating levels of ATP, inhibition is incomplete (partial), although AMP clearly binds more tightly if ATP is limiting, and vice-versa (Wedler and Hoffmann, 1974b). Thus, it is unlikely that AMP competes directly for the ATP site, but rather possesses a separate (allosteric) site. This is supported by the lack of any cooperativity in AMP binding, in contrast to the markedly cooperative binding of ATP observed previously with this and with *Bacillus subtilis* glutamine synthetases (Wedler and Hoffmann, 1974a,b; Wedler, 1974).

Second, the action of AMP is altered by added amino acids: it is synergistically enhanced by bound L-histidine or L-glutamine. Bound glycine and L-alanine are antagonistic, however, in terms of their effect on the extent of inhibition and tightness of binding for AMP.

Figure 3B shows that ADP binds to the enzyme in a noncooperative manner when ATP is limiting but interacts cooperatively when substrate levels were saturating. ADP binds much less tightly than does AMP under similar conditions, but i_{max} with ADP is 1.0 in all cases. The tightness of ADP binding is strongly altered by ATP levels and, thus,

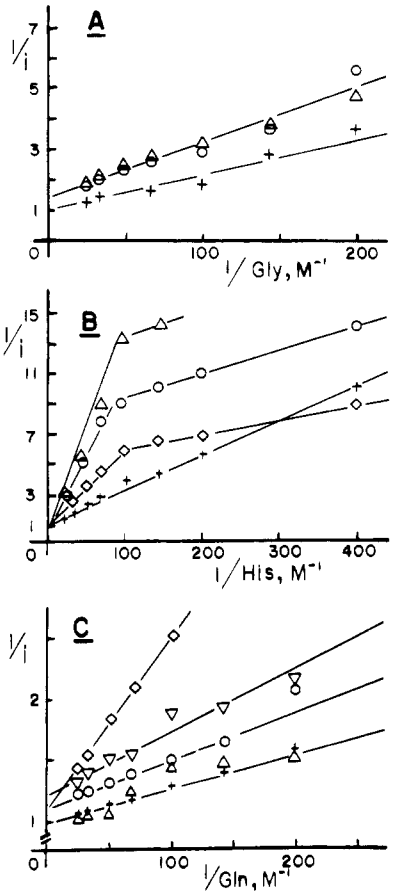

FIGURE 1: Inhibition of *B. stearothermophilus* glutamine synthetase by (A) glycine, (B) L-histidine, and (C) L-glutamine, as measured by the Mn^{2+}-stimulated biosynthetic assay, pH 6.0, 55 °C. The fractional inhibition, i, is plotted as $(i)^{-1}$ vs. [modifier]$^{-1}$. Symbols: saturating substrates (○), limiting L-glutamate (△), limiting ammonia (▽), limiting MnATP (◇), and saturating substrate plus 5 mM MnAMP (+). Actual substrate levels used for each type of assay are given in the Experimental Section.

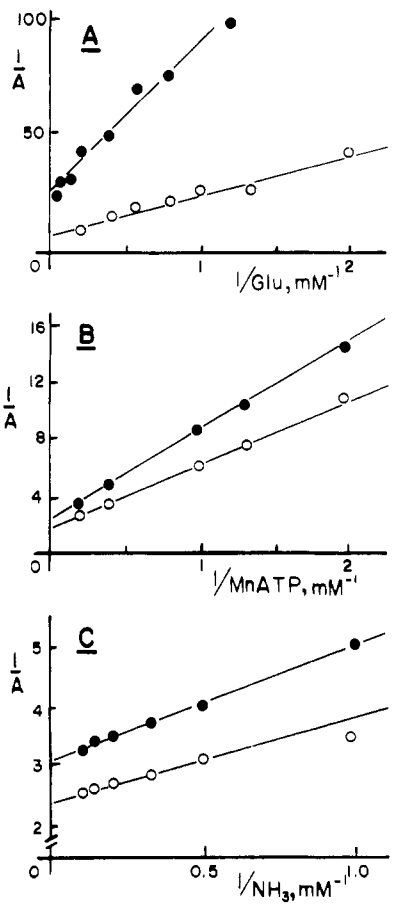

FIGURE 2: Competition of L-glutamine vs. substrates of *B. stearothermophilus* glutamine synthetase, pH 6.0, 55 °C, as double-reciprocal plots of A_{660} as a function of substrate concentration. Symbols: (○) no added L-glutamine; (●) 5 mM L-glutamine added.

ADP probably competes directly for the ATP site, but may also bind to the site for AMP or another nucleotide modifier.

The action of GDP (Figure 3C) is highly dependent on substrate levels, especially in terms of its tightness of binding, i.e. (slope of the double-reciprocal plots)$^{-1}$. Bound ammonia and ATP are required for effective inhibition, especially the latter, whereas diminished levels of L-glutamate allow tighter GDP binding. That GDP does not alter the binding of ATP was found by competitive inhibition studies (not shown).

The effects of L-alanine, CTP, and glucosamine-6-P, shown in Figure 4, indicate that bound substrates also influence the effects of these modifiers. The effect of alanine apparently is not overcome by substrates, but requires bound ammonia and glutamate for effective inhibition. With limiting glutamate, alanine binding is biphasic, possibly indicating multiple sites of interaction. CTP, however, inhibits only 50% with saturating substrates present. Limiting glutamate markedly raises the extent (i_{max}) of the effect of CTP to 100%. CTP raises K_m (Glu), but not that of ATP, and has no effect on V_{max}; this was determined in separate experiments (not shown).

Glucosamine-6-P shows weakened binding with limiting ATP, but i_{max} is drastically decreased with limiting ammonia. With decreased glutamate, i_{max} is increased to 1.0 but modifier binding affinity decreases.

Other Studies. Other amino acid modifiers of potential significance were also studied more extensively, but were not found to have clearcut effects. D- or L-serine (40 mM) and L-tryptophan (10 mM), in the presence of limiting or saturating substrates or in the presence of other modifiers, gave less than 10% inhibition. Hence, these modifiers were considered of negligible importance and were not studied further. Carbamoyl phosphate was too labile at 55 °C to test as an inhibitor by those techniques.

The inhibitory patterns seen with the Mg^{2+}-stimulated activity in single-level inhibitor experiments (Wedler and Hoffmann, 1974b) also were pursued somewhat further. Basically, these studies revealed that all inhibition effects were markedly weaker with Mg^{2+} than with Mn^{2+} bound, with the exceptions of glucosamine-6-P, CTP, and GDP. The relationship of Mn^{2+} and Mg^{2+} to the growth and sporulation phases of *Bacillus sp.* organisms, and how this may relate to glutamine synthetase control, has been discussed by Deuel and Prusiner (1974).

Discussion

The most important observations made in this present research are that: (1) AMP interacts in a synergistic manner with several other modifiers, especially L-glutamine, but also L-histidine, glucosamine-6-P, and ADP; (2) because inhibitions by many of the modifiers are either incomplete or not overcome by saturating substrate levels (Figures 1–4),

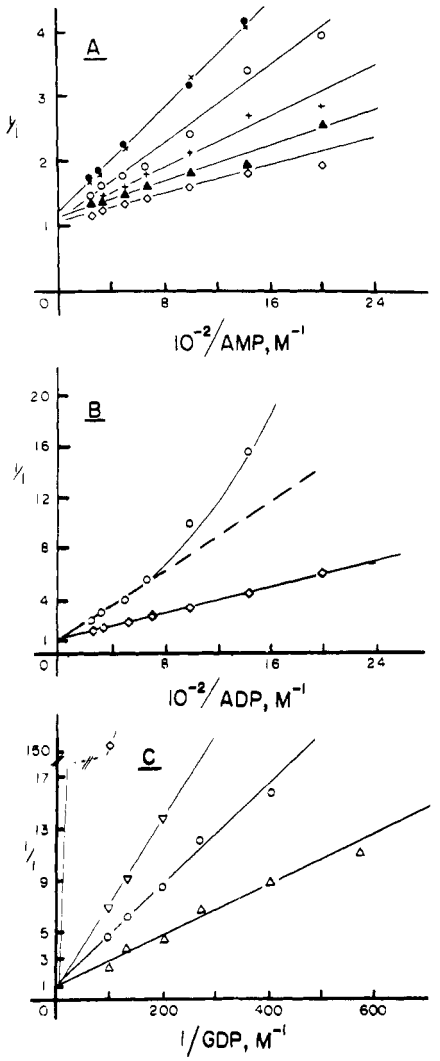

FIGURE 3: Inhibition of *B. stearothermophilus* glutamine synthetase by (A) MnAMP, (B) MnADP, and (C) MnGDP, with $[Mn^{2+}]$ = [total nucleotide]. Reaction conditions and symbols were as in Figure 1 and, in addition, those for 5 mM of added amino acids in (A) were: (●) Gly, (X) L-Ala, (+) L-His, and (▲) L-Gln.

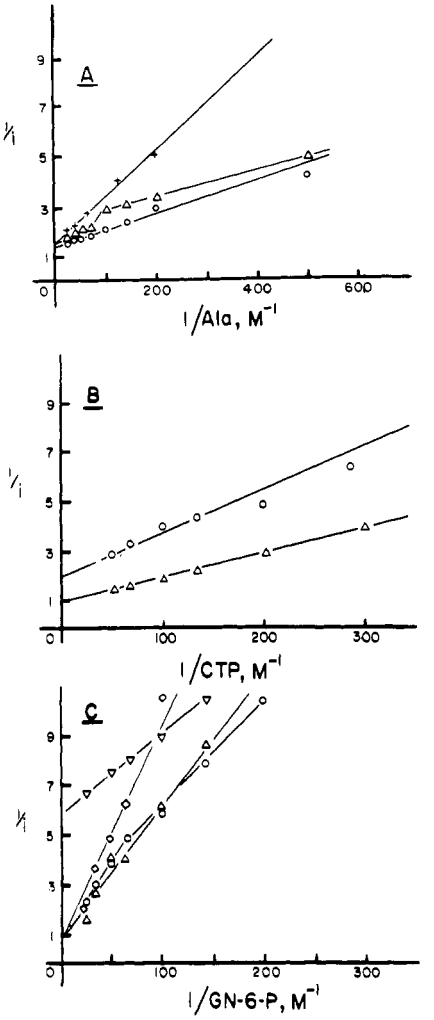

FIGURE 4: Inhibition of *B. stearothermophilus* glutamine synthetase by (A) L-alanine, (B) MnCTP, and (C) glucosamine 6-phosphate, with $[Mn^{2+}]$ = [total nucleotide]. Reaction conditions and symbols were as in Figure 1.

the number of modifier sites, separate from substrate binding sites, is much larger than previously suspected; (3) the action of the many modifiers depends on the presence of one bound substrate or another.

To draw together the various complex interrelated observations made with the modifiers of the *B. stearothermophilus* enzyme, the most salient features of the action of each modifier are summarized in Table II. A priori, such a summary might be considered incomplete without a comparison of calculated K_i values. However, the use of such quantitative terms has been avoided because the observed K_i value, as well as i_{max}, for many modifiers is highly dependent on the levels of substrates or of other modifiers. Partial and mixed inhibitions also are observed. In light of such complex interactions and dependencies, a given K_i value is of limited quantitative value. One may nonetheless qualitatively judge the relative potency of a modifier in terms of i_{max} and the modifier concentration required to give $\frac{1}{2}(i_{max})$, for example.

By these latter criteria, it can be seen, for example, that L-glutamine and AMP *alone* are much more potent inhibitors with the *B. stearothermophilus* system than with the enzyme from *B. subtilis* (Deuel and Prusiner, 1974). Basically the most potent end product feedback inhibitors are AMP, L-alanine, and CTP, with glutamine and ADP providing additional control as direct product inhibitors. It may be pointed out that these most potent modifiers do not interact antagonistically but act independently or in synergy, i.e., bind to regulatory sites separate from the substrate sites.

The strong synergism of action observed between AMP and other feedback modifiers raises the interesting possibility that bound AMP "potentiates" the (gram positive) *Bacillus* glutamine synthetase for inhibition in the same way as does covalent adenylylation of the (gram negative) *E. coli* enzyme.

Overall, a similar spectrum of inhibitors, with some additions or deletions, has been observed to interact with the enzymes from mammalian liver, brain, and kidney (Tate and Meister, 1973; Deuel et al., 1973) and from peas (Kingdon, 1974). Also, L-alanine, glycine, and L-glutamine are antagonistic, as with the *B. licheniformis* enzyme (Hubbard and Stadtman, 1967b). The inhibition pattern also agrees with that reported by Hachimori et al. (1974) for the enzyme from strain NCA 2184 of *B. stearothermophilus*. However, this organism apparently produces an enzyme which differs appreciably from that of strain 4S (8,12) in its

Table II: Summary of Modifier Effects and Interactions with *Bacillus stearothermophilus* Glutamine Synthetase.[a]

Modifier	Inhibits V_{max}	Inhibits K_m	i_{max}	Notable Features
Gly	X		0.6	Independent of [substrate]
L-Ala	X		0.6	Biphasic inh with lim Glu
L-His		(ATP)	1.0	Competes with ATP directly, synergistic with AMP, K_i depends on [Glu], biphasic inh plots
L-Gln		(Glu)	<1.0	i_{max} depends on [Glu], NC vs. all substrates, synergistic with AMP (i_{max} incr), slightly synergistic with AMP
GA-6-P		(ATP)	<1.0	Requires bound ATP, synergistic with AMP
AMP		(ATP)	<1.0	Hyperbolic inh curves, synergistic with His, Gln, GA-6-P, ADP
ADP		(ATP)	1.0	Competes with ATP, cooperative inh with lim ATP, slightly synergistic with CTP
CTP		(Glu)	<1.0	i_{max} depends on [Glu], slightly synergistic with Gln, ADP, GDP
GDP	X	(Glu)	1.0	Requires bound ATP, NH_3

[a] With the Mn^{2+}-stimulated biosynthetic assay. 55 °C, pH 6.0. Abbreviations include: NC = noncompetitive, GA-6-P = glucosamine-6-P, lim = limiting, incr = increases, inh = inhibition.

activation by metal ions (Mg^{2+} vs. Mn^{2+}) and its amino acid composition.

Several unusual effects are not readily understood from experiments performed so far. First, the biphasic inhibition curves for L-alanine and L-histidine might be simply interpreted in terms of multiple sets of binding sites with distinctly different affinities and inhibitory effects on the enzyme, but the significance of these regulatory effects in vivo is obscure at present. Second, AMP and glycine appear to interact antagonistically in one case (Table I and Figure 3A) but synergistically in another (Figure 1A). This apparent discrepancy may be rationalized in part by noting that (a) AMP increases i_{max} for Gly, but Gly decreases the affinity of the enzyme for AMP. Certainly, changes in i_{max} and modifier binding may occur via separate mechanisms, and the data of Table I do not distinguish between changes in binding or i_{max}. (b) The data in the figures for mixed modifier experiments are normalized to $i = 0$ in the presence of the modifier held at a fixed level, as detailed in the Experimental Section. Thus, the i values for Table I and the Figures 1a and 3A are not readily compared. Therefore, although the Table I data indicate antagonism between AMP and glycine at fixed levels of each, other effects may be observed in modifier variation studies (Figures 1–4) by the application of this normalization technique. Similar experimental approaches have revealed a variety of interesting phenomena with the *B. subtilis* enzyme (Deuel and Prusiner, 1974).

Finally, the natures of the interactions of AMP, ADP, and L-histidine with each other and with the ATP site are not completely understood yet. The effects of all three inhibitors can be overcome by high ATP levels, indicating competitive (exclusive) interaction. Because ADP and L-His can give $i_{max} = 1.0$, one need not propose separate sites for them, although such sites may exist. ADP may be capable of interacting with several nucleotide sites, depending on the concentration of ATP or other modifiers. The antagonism of L-His and ADP need not indicate competition for the same binding domain, as discussed below. AMP, on the other hand, probably has a site separate from those for ADP, ATP, or L-His since it is synergistic with the two modifiers and gives $i_{max} < 1.0$.

At least two different explanations are possible for the various antagonistic modifier-modifier interactions noted in Table I. One is direct overlap or competition between modifiers for a common binding domain. An alternative explanation involves spatially separate sites that interact antagonistically. For any given pair of modifiers, one must consider these two models equally likely, as present data do not serve to distinguish between them. To do so will require additional experiments by other techniques, e.g., microcalorimetric measurements of binding with each pair of Table I, as done with the *E. coli* enzyme by Ross and Ginsburg (1969) for L-tryptophan and AMP.

If one assumes that the direct overlap model predominates for the spatial relations among modifier sites, one can then visualize a very compact arrangement of the sites. In an enzyme stable at 55 °C, this could provide distinct survival advantages for the organism as it would allow the inclusion of a large amount of complex functional information while minimizing the surface area of protein involved. Alternatively, if one assumes the separate site model as being true in the majority of cases, the logical consequence is that the thermophilic enzyme maintains roughly as many binding sites for modifiers as its mesophilic counterparts. Either line of reasoning leads one to conclude that this enzyme greatly resembles those from mesophilic *Bacilli* in terms of the complexity of functional information maintained in the protein structure. Therefore, our present research includes characterization of an enzyme from an extreme thermophile, *Bacillus caldolyticus*, grown at 70–75 °C. Preliminary evidence (Wedler et al., 1976) indicates that rather marked departures from the behavior of the 37 and 55 °C organisms occur.

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Mechanistic Studies of Glutamine Synthetase from *Escherichia coli:* Kinetics of ADP and Orthophosphate Binding to the Unadenylylated Enzyme†

S. G. Rhee and P. B. Chock*

ABSTRACT: The kinetics of protein fluorescence change exhibited by ADP or orthophosphate addition to the Mg^{2+}- or Mn^{2+}-activated unadenylylated glutamine synthetase from *Escherichia coli* were studied. The kinetic patterns of these reactions are incompatible with a simple bimolecular binding process and a mechanism which required protein isomerization prior to substrate binding. They are consistent with a mechanism in which direct substrate binding is followed by a substrate-induced conformational change step, $ES \rightleftharpoons ES^*$. At pH 7.0 and 15 °C, the association constants for the direct binding (K_1) of ADP to $MnE_{1.0}$ and of P_i to $MnE_{1.0}ADP$ are 3.9×10^4 and 2.28×10^2 M^{-1}, respectively. The association constant for the direct binding of ADP to $MnE_{1.0}P_i$ is 2.3×10^4 M^{-1} at pH 7.0 and 19 °C. The $\Delta G°$ for the substrate-induced conformational step are -3.5 and -1.3 kcal mol^{-1} due to ADP binding to $MnE_{1.0}P_i$ and $MnE_{1.0}$, respectively, and -1.4 kcal mol^{-1} due to P_i binding to $MnE_{1.0}ADP$. Rate constants, k_2, and k_{-2}, for the isomerization step are: 90 and 9.5 s^{-1} for ADP binding to $MnE_{1.0}$, 440 and 0.36 s^{-1} for ADP binding to $MnE_{1.0}P_i$, and 216 and 1.8 s^{-1} for P_i binding to $MnE_{1.0}ADP$. Due to low substrate affinity, the association constant for direct P_i binding to $MnE_{1.0}$ was roughly estimated to be 230 M^{-1} and $k_2 = 750$ s^{-1}, $k_{-2} = 250$ s^{-1}. At 9 °C and pH 7.0, the estimated association constants for the direct ADP binding to $MgE_{1.0}$ and $MgE_{1.0}P_i$ are 1.8×10^4 and 1.6×10^4 M^{-1}, respectively; and the rate constants for the isomerization step associated with the corresponding reaction are $k_2 = 550$ s^{-1}, $k_{-2} = 500$ s^{-1}, and $k_2 = 210$ s^{-1}, $k_{-2} = 100$ s^{-1}. From the kinetic analysis it is evident that the inability of Mn^{2+} to support biosynthetic activity of the unadenylylated enzyme is due to the slow rate of ADP release from the $MnE_{1.0}P_iADP$ complex. In contrast the large k_{-2} obtained for ADP release from the $MgE_{1.0}ADP$ or $MgE_{1.0}P_iADP$ complex indicates that this step is not rate limiting in the biosynthesis of glutamine since the k catalysis obtained under the same conditions is 7.2 s^{-1}.

Glutamine synthetase, a key enzyme in nitrogen metabolism (Stadtman, 1973), has been extensively studied in recent years (Rhee et al., 1976; Stadtman and Ginsburg, 1974; Meister, 1974). The enzyme from *E. coli* is composed of 12 identical subunits (Woolfolk et al., 1966) and is regulated primarily by cascade control of the adenylylation and deadenylylation of a tyrosine residue in each subunit (Adler et al., 1974; Holzer, 1969; Shapiro et al., 1967). This enzyme is known to catalyze several reactions involving the conversion of glutamate to glutamine, or vice versa, glutamate or glutamine to γ-glutamyl hydroxamate and glutamate to pyrrolidonecarboxylate (Rhee et al., 1976; Stadtman and Ginsburg, 1974). Of these reactions, the biosynthetic and transferase reactions (reaction 1 and 2, respectively) are the most widely studied

† From the Laboratory of Biochemistry, Section on Enzymes, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received September 18, 1975.* This paper is Part III of a series on mechanistic studies of this enzyme. For Part II, see Rhee, S. G., Chock, P. B., and Stadtman, E. R. (1976), *Biochimie* (in press).