DIRECT EVIDENCE FOR SEPARATE BINDING SITES FOR L-GLU AND AMINO ACID FEEDBACK INHIBITORS ON UNADENYLYLATED GLUTAMINE SYNTHETASE FROM E. COLI

S. G. Rhee, J. J. Villafranca*, P. B. Chock and E. R. Stadtman
Section on Enzymes, Laboratory of Biochemistry
NHLBI, NIH, Bethesda, Maryland 20014

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SUMMARY: Glutamine synthetase from E. coli is modulated by adenylylation of a tyrosine residue on each subunit of the dodecamer, as well as by feedback inhibition. With the stopped-flow fluorometric method, the binding constants for L-Glu, L-Ala, D-Val, and Gly to $E\overline{}^{1.0}Mg$, E**, in the absence or presence of ATP or ADP, and NH_3 were evaluated at pH 7.0, 15°. Strong synergistic effects between the amino acids and the nucleotide were observed. The fluorescence amplitude observed due to either simultaneous or sequential addition of 2 different amino acids to E or E·ATP indicate that L-Glu can bind to the enzyme simultaneously with L-Ala, Gly and D-Val; L-Ala can coexist with D-Val, Gly or D-Ala. NMR method also shows that L-Glu and L-Ala can bind simultaneously. Therefore, within our experimental conditions, the unadenylylated enzyme possesses allosteric site(s) for the amino acid inhibitors.

Woolfolk and Stadtman (1) reported that Escherichia coli glutamine synthetase (EC 6.3.1.2) is inhibited by eight end products of glutamine metabolism. Many of these compounds caused only partial inhibition when tested individually, but in combinations their effects were cumulative. From this observation and the fact that some of these feedback inhibitors, e.g. L-Ala, Gly, yielded mixed inhibition patterns, with respect to L-Glu, they proposed the existence of separate allosteric effector sites for the feedback inhibitors. In the meantime Dahlquist and Purich (2) have suggested that the eight feedback inhibitors simply bind to substrate binding sites of the enzyme. In an effort to distinguish between these two hypotheses the binding of feedback inhibitors to glutamine synthetase has been studied by means of fast reaction (3), NMR (4), equilibrium dialysis (5) and calorimetric techniques (6). We report here

*On leave from the Chemistry Department, Pennsylvania State University, University Park, PA 16802.

**The subscript indicates the average number of adenylylated subunits per dodecamer. In this paper, E and $E(Mn)_2$ represent $E\overline{}^{1.0}Mg$ and $E\overline{}^{1.0}(Mn)_2$, respectively.

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the results of binding studies using fluorescent and NMR methods. The data indicate that L-Ala, D-Val, and Gly do not bind to the L-Glu site, and L-Ala does not bind at D-Val or D-Ala site.

MATERIALS AND METHODS

Materials: Glutamine synthetase containing one equivalent of adenylyl groups per mole ($E_{1.0}$) was isolated from E. coli grown in a medium containing 35 mM L-Glu and 0.67 M glycerol. The Mg^{2+}-Zn^{2+} precipitation method (7) as modified by Stadtman was used for the isolation (8). The specific activity of the purified enzyme agrees well with the published values for purified enzyme (9). All amino acids and nucleotides were purchased from Sigma Chemical Co. The L-Glu and L-Ala was purified with Chelex 100 and recrystallization. 99.96% isotopic pure D_2O was obtained from Aldrich Chemical Co., Inc. HEPES, ultrol grade, was obtained from Calbiochem.

Methods: Fluorescence titration data was obtained with a home built stopped-flow fluorometer as described previously (9). The change in fluorescence intensity of the enzyme was followed by excitation with light at 300 nm and recording the emission at 336 nm. The emission observed can be ascribed solely to the three tryptophan residues in each enzyme subunit; interference by absorbance due to ATP or ADP is negligible. For the stopped-flow experiments, the same solution, which contained all the required reagents except enzyme and the particular ligand or inhibitor under investigation, was used to prepare the two pre-mixed solutions. The solutions were equilibrated at 15° in a constant-temperature bath followed by 10 more min. of incubation in the stopped-flow driving syringes. The buffer solution used contained 50 mM HEPES-KOH at pH 7.0, 20 mM $MgCl_2$ and 100 mM KCl. The NMR spectra were obtained at 18° using a Varian HR 220 spectrometer. T_1 data were obtained utilizing a 180-τ-90 pulse sequence.

RESULTS

Effects of ATP and NH_3 on amino acid binding: Data in Tables I and II show that the binding of either L-Ala, L-Glu or D-Val to E causes a quenching of intrinsic tryptophan fluorescence. In contrast, the binding of these ligands to E-ATP results in enhancement of the fluorescence. From titrations of the ligand induced fluorescence changes it was determined that the dissociation constants (K_d's) of complexes between E and L-Glu, L-Ala and D-Val are 17 ± 4 > 60 and > 50 mM, respectively; whereas the corresponding K_d's for the E·ATP-amino acid complexes are 2, 29 and 9 mM, respectively. Thus ATP increases the affinity of the enzyme for all of these amino acids. The K_d values for L-Glu determined from these fluorescence measurements are in good agreement with previously reported values of 20 mM for L-Glu (10) and of 24 mM for L-Glu in the presence of ADP plus Pi (11), and with the reported K_i of 30 mM for L-Glu as an inhibitor of the E catalyzed γ-glutamyl transferase reaction (11).

TABLE I. Effects of ADP, ATP and NH_3 on the Dissociation Constants of Amino Acid-E Complexes Determined by Stopped-Flow Fluorescence Measurements[a]

	K_d, mM	Polarity of Δ Fluorescence
E + L-Glu	17 ± 4	-
E + L-Ala	> 60	-
E + D-Val	> 50	-
E·ADP + L-Glu	4 ± 2[b]	+
E·ATP + L-Glu	2.0[c]	+
E·ATP + L-Ala	29	+
E·ATP + D-Val	9	+
E·ATP + Gly	25	+
E·ATP·NH_3 + L-Ala	20	+
E·ATP·NH_3 + D-Val	9	+

[a]All data were obtained at 15", pH 7.0 in 50 mM HEPES-KOH, 20 mM $MgCl_2$ and 100 mM KCl. Concentrations of ATP, ADP and NH_3 used were 4 mM, 1 mM and 20 mM, respectively. - and + indicate decrease and increase in fluorescent intensity.

[b]Due to an additional L-Glu binding site at high [L-Glu], a biphasic titration curve is observed. This constant is calculated for the tight binding site. Shrake and Ginsburg reported a K_d of 1.7 mM at 30° (Ref. 6). A value of 24 mM cited in Ref. (11) for L-Glu dissociation constant from E·ADP·L-Glu is in fact from E·ADP·Pi·L-Glu.

[c]Calculated with L-Glu K_d from E·L-Glu and k_2, k_{-2}, k_3 and k_{-3} given in Ref. (8).

Other data in Table I and in Fig. 1 show that NH_3 increases both affinity of E·ATP for L-Ala and L-Ala induced fluorescent change but not of D-Val. The synergistic effect of NH_3 on L-Ala binding is consistent with the demonstration that NH_3 augments the ability of L-Ala to inhibit the γ-glutamyl transferase activity of E·ADP (12).

Nonexclusive binding of L-Ala, L-Glu and D-Val: From the fluorescence changes elicited by each amino acid separately (Exp. I-III, VII-X in Table II), and the K_d values in Table I, one can calculate fluorescence change that would occur in the presence of any two of these ligands if they compete with one another for the same binding site on the enzyme. For example, with K_d of 17 and 60 mM for the binding of L-Glu and L-Ala to E and the fluorescence change elicited by 100 mM L-Glu and L-Ala independently (Exp. I & II, Table II), one expects a 35 mV decrease in fluorescence intensity when 100 mM of L-Ala is

TABLE II. Relative Fluorescence Intensity Changes due to Amino Acid Binding to the Unadenylylated Glutamine Synthetase

Experiment	Amino Acid Added (mM)	Observed ΔF (mV)		Expected ΔF for Competitive Binding (mV)	
I	E + L-Glu (100)	410	-		
II	E + L-Ala (100)	360	-		
III	E + D-Val (100)	320	-		
IV	E·L-Glu(100) + L-Ala(100)	80	+	35[b], 47[c]	-
V	E·L-Glu(100) + D-Val(100)	260	+	16[b], 38[c]	-
VI	E·L-Ala(100) + D-Val(100)	380	+	50[b],148[c]	-
VII	E·ATP + L-Ala(150)	470	+		
VIII	E·ATP + D-Ala(100)	810	+		
IX	E·ATP + Gly(100)	650	+		
X	E·ATP + D-Val(100)	800	+		
XI	E·ATP·L-Ala(150) + Gly(100)	300	+	135	+
XII	E·ATP·L-Ala(150) + D-Ala(100)	400	+		
XIII	E·ATP·L-Ala(150) + D-Val(100)	450	+	257[d]	+
XIV	E·ATP·L-Ala(150) + L-Ala(100)	60	+	31[d]	+
XV	E·ATP + L-Ala(100), Gly(100)	800	+	615	+
XVI	E·ATP + L-Ala(100), D-Ala(100)	920	+		
XVII	E·ATP + L-Ala(100), D-Val(100)	920	+	747[e]	+
XVIII	E·ATP·D-Val(100) + D-Ala(100)	30	+	40[e]	+
XIX	E·ATP·Gly(170) + L-Ala(170)	120	+	60	-
XX	E·ATP·Gly(170) + Gly(170)	0			

[a]Data obtained from stopped-flow fluorometer under the conditions described in Table I. The uncertainty for ΔF is ± 30 mV for all experiments. The notation used is same as given in Table I.

[b]Calculated with the K_d value of 60 mM and 50 mM for L-Ala and D-Val, respectively.

[c]Calculated with the K_d value of 300 mM and 150 mM for L-Ala and D-Val, respectively.

[d]For one site binding.

[e]Calculated with the K_d value of 9 mM for D-Ala.

added to E·L-Glu (100 mM), if their binding is mutually exclusive. However, as noted in Table II, exp. IV, addition of L-Ala causes an 80 mV increase in fluorescence intensity; there is an even greater discrepancy between observed and calculated values if a K_d value of 300 mM for L-Ala (as was reported previously (2)) is used for the calculation. Similarly, a comparison of the calculated and observed fluorescence changes for other pairs of ligands, either in the presence or absence of ATP, shows that for the ligand pairs L-Glu - D-Val, L-Ala - D-Val, L-Ala - Gly, L-Ala - D-Ala and L-Ala - L-Glu, the observed fluorescence changes are inconsistent with mutually exclusive binding

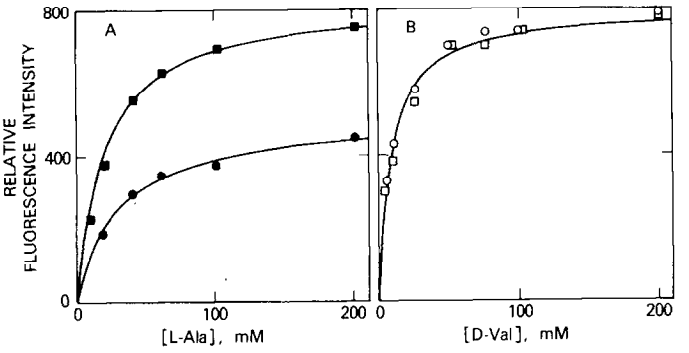

Figure 1: Computer simulated titration curves for (A) L-Ala binding to E•ATP (●) with K_d = 29 mM; to E•ATP•NH_3 (■) with K_d = 20 mM; (B) D-Val binding to E•ATP (o) and to E•ATP•NH_3 (□) with K_d = 9 mM for both cases. For experimental conditions see Table I.

mechanisms. Exp. XVIII in Table II shows that the binding of D-Val and D-Ala is mutually exclusive. These results indicate that L-Glu, L-Ala, D-Ala and Gly bind to separate sites on the enzyme; however, D-Ala and D-Val bind to the same site. Other titration and kinetic data (13) indicate that Gly can bind to both the L-Ala and the D-Ala sites.

NMR Data for L-Glu and L-Ala: The 220 MHz proton NMR spectrum of L-Glu shows well resolved multiplets due to the 3 sets of protons (α, β and γ protons). In the presence of 90 μM (subunit) of $\overline{E_{1.0}}$ and Mg^{2+} (20 mM), the $1/T_1$ values for the 3 sets of protons at 18° (pH 7.0) are 0.46, 0.87 and 1.16 sec^{-1} for α-CH, γ-CH_2 and β-CH_2, respectively. The Mg^{2+} on the tight site was replaced by Mn^{2+} when 90 μm of Mn^{2+} was added to the enzyme solution. The paramagnetic effect on the relaxation rate of these protons is shown by the large increase of the $1/T_1$ values. When the enzyme is saturated with 2 Mn^{2+} per subunit (0.18 mM Mn^{2+}, 0.25 mM ADP) (14), the values of $1/T_{1m}$ (the relaxation rate of the enzyme bound ligand) for the proton of L-Glu bound to $E(Mn)_2$•ADP were evaluated at 220 MHz to be 200, 320 and 450 sec^{-1} for α-CH, β-CH_2 and γ-CH_2, respectively. Similarly, the $1/T_{1m}$ values for the proton of L-Ala when bound to $E(Mn)_2$•ADP are 92 and 119 sec^{-1} for β-CH_3 and α-CH, respectively.

If L-Glu and L-Ala are mutually exclusive on the enzyme surface, the observed $1/T_{1m}$ for the protons of L-Glu would decrease and approach zero when

L-Ala was titrated into $E(Mn)_2$•ADP•L-Glu solution. To test this hypothesis, experiments were carried out with $E(Mn)_2$•ADP in the presence of 25 mM or 40 mM L-Glu to which various amounts of L-Ala (10-110 mM) were added. No significant change was obtained for the value of $1/T_{1m}$ for the proton of L-Glu when compared to the values obtained in the absence of L-Ala. Likewise, there is no significant change in $1/T_{1m}$ values for the protons of L-Glu or L-Ala when L-Ala was maintained at 40 or 75 mM while L-Glu was varied from 10 to 90 mM.

DISCUSSION

The dissociation constants given in Table I show that there is strong synergism in the binding of ATP or ADP and one of the amino acids, L-Glu, L-Ala, Gly and D-Val to E at pH 7.0. In addition, in the presence of ADP, Pi antagonizes the binding of L-Glu, but not of L-Ala (6). Similarly, with respect to γ-glutamyl transferase activity, Pi increases the apparent K_i for L-Glu but does not affect the K_i for L-Ala (15). This is likely due to some degree of overlapping at the oxygen binding site between the extra oxygen atom in the ADP-Pi system (compared with the ATP system) and one of the γ-carboxyl oxygen atoms of L-Glu. The absence of a γ-carboxyl group in L-Ala precludes such overlapping. These appear to be consistent with the common-site hypothesis. However, based on the fluorescence and proton NMR measurements reported here, it is unlikely that L-Glu and L-Ala compete with one another at a common binding site.

Table II shows that the fluorescence amplitude observed upon either simultaneous or sequential addition of two different amino acids is invariably higher than the value calculated for competitive binding mechanisms. In some cases (Exp. IV, V, VI and XIX), an opposite polarity is observed. These data indicate that L-Glu, L-Ala and D-Val occupy separate sites on the enzyme subunit. Because the fluorescence change observed for any given pair of amino acids does not equal the sum of the changes when measured separately, some interaction between binding sites is evident; i.e., the binding of one amino acid to the enzyme induces a protein conformational change which affects the fluorescence amplitude that is associated with the binding of another amino

acid. The discrepancy between the observed and calculated fluorescence changes shown in Table II cannot be accounted for by the subunit interaction between neighboring L-Glu and L-Ala-bound subunits. This is supported by the fact that normal fluorescence saturation curves were obtained when a given amino acid was titrated into E·ATP in the presence of another amino acid at saturating level (13).

The NMR data show that no significant change was observed in $1/T_{1m}$ values for the protons of L-Glu or L-Ala when L-Ala or L-Glu was titrated into $E(Mn)_2$·ADP·L-Glu or $E(Mn)_2$·ADP·L-Ala, respectively. This indicates that both L-Ala and L-Glu can coexist on the enzyme surface. The separate site hypothesis is also consistent with the report that L-Ala is a non-competitive (mixed) inhibitor with respect to L-Glu in the biosynthetic reaction (1), and the results obtained from calorimetric measurements for L-Ala binding to E·ADP or E·ADP·Pi in the presence or absence of L-Glu (6).

In conclusion, under the experimental conditions described, the unadenylylated glutamine synthetase from E. coli possesses allosteric site(s) for feedback amino acid inhibitors. The data reported here are inconsistent with the hypothesis (2) that amino acid inhibitors bind at the L-Glu site.

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