Probing the catalytic roles of n_2 -site glutamate residues in *Escherichia coli* glutamine synthetase by mutagenesis

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Abstract

The contribution of metal ion ligand type and charge to catalysis and regulation at the lower affinity metal ion site (n_2 site) of *Escherichia coli* glutamine synthetase (GS) was tested by mutagenesis and kinetic analysis. The 2 glutamate residues at the n_2 site, E129 and E357, were changed to E129D, E129H, E357H, E357Q, and E357D, representing conservative and nonconservative alterations. Unadenylylated and fully adenylylated enzyme forms were studied. The Mn^{2+} - K_D values, UV-vis, and fluorescence emission properties were similar for all mutants versus WTGS, except E129H. For kinetic determinations with both Mn^{2+} and Mg^{2+} , nonconservative mutants (E357H, E129H, E357Q) showed lower biosynthetic activities than conservative mutants (E129D, E357D). Relative to WTGS, all the unadenylylated Mn^{2+} -activated enzymes showed reduced k_{cat}/K_m values for ATP (>7-fold) and for glutamate (>10-fold). Of the unadenylylated Mg^{2+} -activated enzymes, only E129D showed kinetic parameters competitive with WTGS, and adenylylated E129D was a 20-fold better catalyst than WTGS. We propose the n_2 -site metal ion activates ADP for departure in the phosphorylation of glutamate by ATP to generate γ -glutamyl phosphate. Alteration of the charge density at this metal ion alters the transition-state energy for phosphoryl group transfer and may affect ATP binding and/or ADP release. Thus, the steady-state kinetic data suggest that modifying the charge density increases the transition-state energies for chemical steps. Importantly, the data demonstrate that each ligand position has a specialized spatial environment and the charge of the ligand modulates the catalytic steps occurring at the metal ion. The data are discussed in the context of the known X-ray structures of GS.

Keywords: glutamine synthetase; metalloenzyme; site-directed mutagenesis; structure–function studies

Glutamine synthetase (EC 6.3.1.2) is a central enzyme involved in nitrogen metabolism and catalyzes the reversible conversion of L-glutamate, ATP, and ammonia into L-glutamine, ADP, and inorganic phosphate. The reaction involves 2 distinct chemical steps and proceeds through ammonolysis of an enzyme-bound intermediate, γ -glutamyl phosphate (Midelfort & Rose, 1976). Glutamine serves as a biological ammonia equivalent and is vital for the biosynthesis of nucleic acids, amino sugars, and α -amino acids (Fig. 1).

In enteric bacteria, such as *Escherichia coli*, the enzyme is active as a dodecamer of identical subunits with a total mass

of 620 kDa (Stadtman & Ginsburg, 1974). The enzyme is the *gln A* gene product, comprised of 468 amino acids with a molecular mass of 51,628 Da. The enzyme from *E. coli* has been cloned (Backman et al., 1981) and the gene completely sequenced (Colombo & Villafranca, 1986). GS is a metalloenzyme that requires 2 divalent metal ions per monomer for catalytic activity. Magnesium is the biologically relevant metal, although manganese also supports biosynthetic activity. In addition, the enzyme is subject to multiple modes of regulation, including covalent modification of a specific tyrosine residue, Y397 (Rhee et al., 1988). Adenylyl transferase catalyzes the ATP-dependent adenylylation of 1 residue per monomer, Y397, which decreases magnesium-dependent biosynthetic activity by increasing K_m values and decreasing k_{cat}/K_m values of substrates (Abell & Villafranca, 1991). The steady-state kinetic parameters of the manganese-supported activity, however, are not as strongly affected by adenylylation.

The X-ray structure of the enzyme from *Salmonella typhimurium*, 98% identical to the *E. coli* enzyme, was determined with 2 manganese ions bound to the active site (see Kinemage 1; Almassy et al., 1986; Yamashita et al., 1989). The metal–metal distance obtained from the crystal structure is 5.8 Å. The do-

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Abbreviations: PIPES, piperazine- N,N' -bis-(2-ethanesulfonic acid); HEPES, N -(2-hydroxyethyl)piperazine- N' -(2-ethanesulfonic acid); GS, glutamine synthetase; WTGS, wild-type glutamine synthetase; BSA, bovine serum albumin; MSOX, methionine sulfoxime.

Fig. 1. The reactions catalyzed by glutamine synthetase.

decamer is composed of 2 face-to-face stacked symmetrical hexamers, and the active sites are located at the interface between 2 adjacent subunits in a hexamer, within a partial β -barrel structure. Both manganese ions are in octahedral environments and 6 amino acids were identified as metal-binding ligands, including 5 glutamates (E129, E131, E212, E220, E357) and 1 histidine (H269). Interestingly, 5 of these 6 residues are strictly conserved across 17 species (Pesole et al., 1991). Only 1 glutamate, residue E131 in *E. coli*, is not strictly conserved in all the examined species. The role of metal-binding ligands in the catalytically active enzyme is a central issue addressed in this work.

Several groups have characterized the substrate and metal ion requirements of GS. Two divalent metal ions per monomer were demonstrated to be necessary for catalytic activity and substrate binding, and several metal ions, including magnesium, manganese, cobalt, zinc, and calcium can support enzyme activity (Kingdon et al., 1968; Ginsburg, 1972; Hunt & Ginsburg, 1972; Segal & Stadtman, 1972a, 1972b; Stadtman & Ginsburg, 1974; Hunt et al., 1975; Rhee et al., 1976). Upon binding of the first metal ion to the higher-affinity or n_1 site, a conformational change in the enzyme was shown by both fluorescence and UV spectroscopic methods. In contrast, binding of a second equivalent caused no further observable change. The equilibrium binding constants for 2 manganese ions per monomer were measured by EPR and were shown to exhibit a >50-fold difference in affinity for the 2 sites (Villafranca et al., 1976a, 1976b). On the basis of these and other experiments, distinct roles have been proposed for the 2 metal ions in GS.

The metal ion at the n_1 site was suggested to be involved in glutamate binding (Villafranca et al., 1975; Balakrishnan & Villafranca, 1978; Hunt & Ginsburg, 1980) and may further function as an electrophilic catalyst in polarizing the carbonyl group of the covalent intermediate, γ -glutamyl phosphate, for attack by ammonia. Additional support for the role of the n_1 metal ion in catalysis comes from EPR studies involving the transition-state analog methionine sulfoxime (Eads et al., 1988). In recent X-ray structures, the substrate glutamate, the product glutamine, and MSOX all bind to the n_1 metal ion, adding fundamental structural data to the proposed catalytic mechanism (Liaw & Eisenberg, 1994).

The lower-affinity, or n_2 -site, metal ion has been proposed to bind and activate ATP for attack by glutamate (Hunt et al., 1975) and may assist in departure of the leaving group, ADP. This site contains 2 glutamates, E129 (carboxyl oxygen atoms

at 1.54 and 3.37 Å from the metal ion) and E357 (carboxyl oxygen atoms at 1.93 and 3.33 Å), 1 histidine, H269 (δ nitrogen atom at 2.11 Å), all strictly conserved residues, 2 water molecules (in the absence of ATP), as well as another histidine, not close enough to be a ligand (ϵ nitrogen atom at 3.88 Å). The spatial relationships of the n_2 -site residues are demonstrated in Figure 2 and Kinemage 2. When nucleotide substrate is co-crystallized with the enzyme, the phosphoryl oxygen atoms of ADP bind to the metal ion (Liaw & Eisenberg, 1994).

We have used site-directed mutagenesis to alter the charge density at the metal ions and thus to probe the importance of the metal-binding residues in catalytic activity of GS. Other studies from our laboratory addressed the role of His-269 in catalysis (Abell et al., 1994). In this study, the n_2 -site glutamate residues were changed in conservative (i.e., retention of ligand charge) and nonconservative (i.e., change from negative charge to neutral ligand) fashion (see Kinemage 2). We have studied the effects of the ligand environment on the mutant enzymes' binding and catalytic parameters for comparison to wild-type enzyme. Several mutations were chosen for these studies: a conservative change with respect to charge (Glu to Asp: E357D, E129D); an isosteric, uncharged change (Glu to Gln: E357Q); and a nonconservative change (Glu to His: E357H, E129H). The importance of these amino acid residues in determining the metal ion binding environment and the functional role in catalysis at the n_2 site is described in this paper. A preliminary account of this work has appeared (Witmer et al., 1994), and, in this paper, additional steady-state kinetic data from the adenylylated proteins and steady-state fluorescence data are presented and the results discussed in more detail.

Results

Mutagenesis, protein expression, and physical characterization

The desired mutant *gln A* genes were constructed by in vitro site-directed mutagenesis using the phagemid vector pTZglnA. Following successful mutagenesis, the *gln A* gene was subcloned into the expression vector pglu35, and all mutants were completely sequenced. Transformation of the *E. coli* strains YMC21E and YMC21D guaranteed isolation of glutamine synthetase in an unadenylylated ($n = 0$, physiologically active) or fully adenylylated ($n = 12$, physiologically down-regulated) state. All the

Fig. 2. The spatial orientation of the n_2 -site ligands are shown, including the 3 metal-binding ligands E129, H269, and E357 and H271 (not a ligand), as identified in the manganese-enzyme complex. The metal ion is shown as a white sphere, with carboxyl oxygen atoms in red and histidine nitrogen atoms in blue.

proteins were expressed well by these strains and no significant differences in protein yields were observed. Quantitation of released acid-catalyzed inorganic phosphate from the phosphodiester group of residue Y397 confirmed all enzymes were unadenylylated or fully adenylylated.

Spectroscopic characterization of the enzymes

The UV-vis and fluorescence spectra exhibited by the purified mutants were similar to WTGS, indicating no major global conformational differences were caused by mutagenesis. Furthermore, UV-difference spectra of the mutants in the absence and presence of 5 mM Mg^{2+} or Mn^{2+} showed peaks at 283.5 nm and 290 nm (data not shown), a documented characteristic of wild-type enzyme (Shapiro & Ginsburg, 1968). This is attributed to a conformational change induced by metal ion binding at the n_1 site. The saturating concentration of metal ion (i.e., 5 mM) exceeded the determined K_D values for Mn^{2+} binding to all the enzymes and should also exceed the Mg^{2+} - K_D values, which are ~ 200 -fold weaker for WTGS (Denton & Ginsburg, 1970), ensuring 2 metal ions bound per subunit. The spectroscopic change demonstrated that mutagenesis at the n_2 site did not prevent metal binding or a similar conformational change as occurs with WTGS. That all unadenylylated mutants supported at least some biosynthetic activity with either metal ion furthermore is consistent with a similar environment at the n_1 site in spite of an altered n_2 binding site.

The calculated K_D values for WTGS and the mutant enzymes from fluorescence experiments with ATP and ADP are given in Table 1. The fluorescence emission spectrum of GS is sensitive to conformational changes, reflecting the environments of the tryptophans. GS has only 2 tryptophans, W57 and W158, and ATP binding causes an increase in emission intensity (nearly

100%) and a 2-nm blue shift (330 nm to 328 nm) attributed to movement of a loop containing W57, located 14 Å from the n_2 -site metal ion (Atkins et al., 1991). The nonconservative mutants, E357H and E357Q, bound ATP 300-fold and 60-fold less tightly, respectively, than WTGS, whereas the aspartate mutants maintained tight binding. E129D bound both nucleotides more tightly than WTGS, and E357D maintained tight binding of ATP but showed weaker binding of ADP. Fluorescence titrations of unadenylylated manganese-form enzymes with ATP and ADP showed the mutants behaved similarly to WTGS, except for E129H. This strongly suggests that the overall tertiary struc-

Table 1. Equilibrium nucleotide binding constants (K_D) from steady-state fluorescence titration experiments^a

Enzyme	Nucleotide	K_D (μ M)
WTGS	ATP	1.0 ± 0.1
WTGS	ADP	4.3 ± 0.2
E357H	ATP	320 ± 120
E357Q	ATP	65 ± 6
E357Q	ADP	20 ± 4
E357D ^b	ATP	4.7 ± 1.9
E357D	ADP	76 ± 8
E129D	ATP	$0.071 \pm .019$
E129D	ADP	2.8 ± 0.3
E129H	ATP/ADP	nd

^a Conditions used for experiments were 1.0 μ M enzyme subunit concentration (unadenylylated manganese-form enzymes) in 100 mM PIPES, 100 mM KCl, 8 mM $MnCl_2$, pH 6.5. nd, Not determined; failed to exhibit fluorescence change.

^b Titration performed in the presence of 100 mM L-glutamate.

tures were not significantly altered relative to WTGS for these mutants. Only E129H, which failed to show an increase in fluorescence upon titration regardless of the order of substrate addition, is suggested to have an altered structure relative to WTGS. This may account for why the mutant supported reduced Mn^{2+} -dependent biosynthetic activity but only Mg^{2+} -dependent ATPase activity.

EPR titration experiments

Manganese titration experiments characterized the effects of mutagenesis on metal ion binding. Unadenylylated enzyme samples, titrated over a range of Mn^{2+} concentrations that encompassed the expected K_D values, exhibited saturation upon binding 2.0 ± 0.2 equivalents of metal ion per monomer. The dissociation constants are given in Table 2, and an upper limit on K_D^1 was estimated at $0.1 \mu M$ for all enzymes.

All the enzymes examined bound 2 Mn^{2+} ions per subunit and exhibited >50-fold difference in affinity for the 2 sites. The tighter binding site for all the enzymes was estimated at $K_D \leq 0.1 \mu M$, below the detection limit of the technique. This value, determined for completely unadenylylated enzyme, is less than the previously determined value of $K_D = 0.5 \mu M$ for WTGS in low adenylation state ($n = 1-2$) (Villafranca et al., 1976a). In addition, the second binding constant, $K_D = 23 \pm 14 \mu M$, is also lower than was previously determined. The tighter K_D values for WTGS can be attributed to the difference between 0 adenylyl ($n = 0$) and 1-2 groups ($n = 1-2$) per dodecamer. These data suggest that the presence of a few adenylylated subunits in a dodecamer contributes to an overall global conformational change, giving an average binding constant that is higher than that determined for 0 adenylylated enzyme.

The binding constants for the n_2 sites in mutant enzymes ranged from 18 to $63 \mu M$, representing small variations in these values relative to WTGS. No trend is apparent from the data, and E129D, 1 of the 2 most active mutants, showed the weakest n_2 -site binding. This indicates mutagenesis does not drastically affect the ability of the enzymes to bind metal and the variations may reflect altered geometry of ligands and/or substrates at this site. As discussed below, Mn^{2+} , but not Mg^{2+} , binds well to both nitrogen and oxygen ligands, and the mutations at E357 and E129 apparently preserve the coordination

environment for manganese. The number of ligands to the manganese, however, is not known, nor is the geometry or identity of the ligands, and the mutants may have recruited an additional ligand or may substitute with an adjacent ligand, such as His-271.

Steady-state kinetic characterization

To quantitatively evaluate the effects of mutagenesis on catalysis, steady-state kinetic experiments were performed. GS catalyzes the ATP- and ammonia-dependent conversion of L-glutamate to L-glutamine and, in the absence of ammonia, supports very low ATPase activity. The glutamate- and ammonia-independent ATPase activities of unadenylylated enzymes with manganese and magnesium were shown to be very low for all mutants, as well as for WTGS, except for magnesium-form E129H, which supported minimal ATPase activity only.

The biosynthetic activities with manganese were determined at pH 6.5 and are given in Table 3 for unadenylylated and fully adenylylated enzymes and include comparative k_{cat}/K_m values. The comparative $\log(k_{cat}/K_m)$ is shown in Figures 3 and 4. The analogous data for magnesium-form enzymes are listed in Table 4 and shown in Figures 5 and 6.

The kinetic mechanism of unadenylylated manganese-form WTGS fits an ordered mechanism better than either a ping-pong or rapid equilibrium mechanism. The kinetic mechanism, therefore, is assumed to follow the same order as the magnesium-form enzyme, which is the preferred order of ATP followed by L-glutamate and ammonia (Meek & Villafranca, 1980). The mutant unadenylylated manganese-form enzymes E357D and E129D also fit best to an ordered mechanism, as did E357Q GS assayed at pH 7.85. Additionally, unadenylylated magnesium-form E129D also fit best to an ordered mechanism.

The steady-state kinetic parameters for magnesium-form, unadenylylated enzymes illustrates mutagenesis of glutamate to uncharged isosteric glutamine reduced the activity (k_{cat}) by >60,000-fold, and change to histidine reduced activity by >14,000-fold (E357H), and generated a very poor ATPase (E129H). The effects are less severe on K_m values than on k_{cat} values, suggesting that with magnesium, catalysis may be more strongly affected than substrate binding steps. The specificity constants for glutamate for the mutants relative to WTGS show the expected severe decreases, except for E129D, which shows only a 1.2-fold reduction, indicating that E129D is an excellent catalyst. Similarly, the specificity constants for ATP show significant reductions except for E129D, reduced only 3.7-fold. Other mutants made at the n_2 -site ligands that fail to support magnesium-dependent activity include H269D, H269E, and H269Q (Abell et al., 1994), and 4 double mutants, E129H/H269E, E129H/H269Q, E129H/H269D, and E129H/H269N (Witmer et al., 1994). All of the double mutants are catalytically inactive with manganese except for E129H/H269E, which supported very low biosynthetic activity.

The biosynthetic activities of the mutants with manganese are less affected, especially for the conservative aspartate mutants. A conservative change can maintain a suitable coordination environment for binding and catalysis, allowing that some conformational differences may be necessary to accommodate the shorter aspartate side chain. Mutagenesis of the n_2 -site glutamates, consequently, indicates the metal ion is sensitive to orientation and spatial arrangement of these ligands in the active

Table 2. Equilibrium Mn^{2+} binding constants (K_D) from EPR titration experiments^a

Enzyme	K_D^1 (μM)	K_D^2 (μM)
WTGS	≤ 0.1	23 ± 14
E357H	≤ 0.1	40 ± 14
E357Q	≤ 0.1	31 ± 10
E357D	≤ 0.1	18 ± 6
E129D	≤ 0.1	63 ± 11
E129H	nd	nd

^a Using $25 \mu M$ protein subunit concentrations (unadenylylated apo-enzymes, pH 7.2), and $5-600 \mu M$ Mn^{2+} concentrations. The data were fit to a 2-state model using a nonlinear least-squares fitting program. K_D^1 and K_D^2 are the binding constants to the n_1 and n_2 metal ion sites, respectively. nd, Not determined.

Table 3. Steady-state kinetic parameters for manganese-WTGS and mutant enzymes, in unadenylylated and fully adenylylated forms^a

Enzyme	<i>n</i> ^b	K_m^{Glu} (mM)	K_m^{ATP} (μM)	k_{cat} (s ⁻¹)	$k_{\text{cat}}/K_m^{\text{Glu}}$ ^c	$(k_{\text{cat}}/K_m)^{\text{ATP}}$ ^c
WTGS	0	0.050 ± 0.003	1.1 ± 0.1	1.83 ± 0.18	36,600	1,830,000
WTGS	12	3.23 ± 0.18	49 ± 4	8.03 ± 0.54	2,490	164,000
E357H	0	3.60 ± 0.20	460 ± 100	0.0029 ± 0.0008	0.8	6
E357H	12	2.22 ± 0.24	398 ± 52	0.0038 ± 0.0002	1.7	10
E357Q	0	3.23 ± 0.10	68 ± 20	0.0079 ± 0.0007	2.5	116
E357Q	12	4.74 ± 0.40	80 ± 6	0.0075 ± 0.0002	1.6	94
E357D	0	1.11 ± 0.19	33 ± 2	5.60 ± 0.25	5,050	170,000
E357D	12	1.22 ± 0.20	40 ± 2	0.082 ± 0.005	6.7	2,050
E129D	0	1.52 ± 0.30	2.6 ± 0.4	0.13 ± 0.01	86	50,000
E129D	12	0.85 ± 0.14	1.6 ± 0.2	0.844 ± 0.001	993	528,000
E129H	0	4.74 ± 0.33	11.4 ± 0.5	0.138 ± 0.010	29	12,100
E129H	12	nd	nd	nd	nd	nd

^a All data were determined at 25 °C using the coupled spectrophotometric assay described in the Materials and methods section. nd, Not determined.

^b *n* is adenylation state, as adenylyl groups per dodecamer.

^c k_{cat}/K_m values are M⁻¹ s⁻¹.

site and supports the notion of inequivalency of the 2 glutamate residues.

The kinetic parameters for the fully adenylylated mutant enzymes differ relative to WTGS, with both metals. Whereas both the K_m and k_{cat} values increase upon adenylation, and k_{cat}/K_m values decrease for WTGS, each mutant behaves differently upon adenylation. For magnesium-form enzymes, only adenylylated E129D GS maintains significant biosynthetic activity and, interestingly, is a better catalyst than adenylylated WTGS, considering k_{cat}/K_m values. Adenylation reduces specificity constants for WTGS by 50-fold and 125-fold for glu-

tamate and ATP, respectively, whereas for E129D the specificity constants are reduced only by 1.6-fold and 1.8-fold. The specificity constants for adenylylated E129D are increased relative to WTGS, by 27-fold for glutamate and 19-fold for ATP. Comparison of $\log(k_{\text{cat}}/K_m)$ values for ATP and glutamate for Mg²⁺-activated WTGS and E129D are given in Figures 7 and 8, respectively, illustrating the high catalytic efficiency of the adenylylated mutant.

Adenylylated manganese-form mutant enzymes exhibit different effects. E357H and E357Q show similar K_m , k_{cat} , or k_{cat}/K_m values versus respective unadenylylated forms. In con-

Fig. 3. The compiled specificity constants for ATP expressed as $\log(k_{\text{cat}}/K_m)$ with unadenylylated and adenylylated manganese-form enzymes, at pH 6.5 and 25 °C. The data were determined as described in the Materials and methods.

Fig. 4. The compiled specificity constants for L-glutamate expressed as $\log(k_{cat}/K_m)$ with unadenylylated and adenylylated manganese-form enzymes, at pH 6.5 and 25 °C.

trast, the k_{cat} values of the aspartate mutants are strongly affected, but the K_m values are relatively unaffected. Although adenylylation increases the turnover number for WTGS, the specificity constants decrease by 15-fold for glutamate and 11-fold for ATP. For E357D, adenylylation reduces both k_{cat} and k_{cat}/K_m values and, in contrast, E129D exhibits increases in both k_{cat} and k_{cat}/K_m values. On the basis of specificity constants, E129D is nearly as effective at catalyzing glutamine

formation as is WTGS, and thus adenylylation does not down-regulate this mutant with either manganese or magnesium.

Discussion

We have addressed the catalytic importance of metal-binding residues of GS that were identified from the static X-ray structure (Kinemages 1, 2). Mutagenesis of the n_2 -site ligands, which

Table 4. Steady-state kinetic parameters for magnesium-WTGS and mutant enzymes, unadenylylated, and fully adenylylated forms^a

Enzyme	<i>n</i> ^b	K_m^{Glu} (mM)	K_m^{ATP} (μM)	k_{cat} (s ⁻¹)	k_{cat}/K_m^{Glu} ^c	$(k_{cat}/K_m)^{\text{ATP}}$ ^c
WTGS	0	7.1 ± 0.8	289 ± 17	50 ± 5	7,040	173,000
WTGS	12	6.6 ± 1.0	657 ± 53	0.9 ± 0.3	136	1,370
E357H ^d	0	6.2 ± 1.8	184 ± 32	0.0035 ± 0.0006	0.6	19
E357H ^e	12	nd	nd	≤0.00001	nd	nd
E357Q ^f	0	4.2 ± 0.4	470 ± 105	0.00081 ± 0.00005	0.2	1.7
E357Q ^e	12	nd	nd	≤0.00003	nd	nd
E357D	0	5.79 ± 0.81	359 ± 19	0.0166 ± 0.0005	2.9	46
E357D	12	1.76 ± 0.62	419 ± 78	0.0042 ± 0.0004	2.4	10
E129D	0	2.13 ± 0.16	265 ± 14	12.3 ± 0.6	5,780	46,400
E129D	12	1.95 ± 0.20	278 ± 40	7.13 ± 0.13	3,670	25,800

^a All data were determined at 25 °C using the coupled spectrophotometric assay described in the Materials and methods section. nd, Not determined.

^b *n* is adenylylation state, as adenylyl groups per dodecamer.

^c k_{cat}/K_m values are M⁻¹ s⁻¹.

^d Determined at pH 7.85.

^e Estimated at pH 8.0.

^f Determined at pH 7.90.

Fig. 5. Comparative specificity constants for L-glutamate, expressed as $\log(k_{cat}/K_m)$ for magnesium-form unadenylylated enzymes, at optimal pH values and 25 °C. This includes all but E129H, which supports only low magnesium- and glutamate-independent ATPase activity.

Fig. 6. Comparative specificity constants for ATP, expressed as $\log(k_{cat}/K_m)$ for magnesium-form unadenylylated enzymes, at optimal pH values and 25 °C. Again, E129H is not included.

Fig. 7. A comparison of the specificity constants for ATP, expressed as $\log(k_{cat}/K_m)$ for WTGS and E129D in unadenylylated and adenylylated magnesium-form enzymes at pH 7.5 and 25 °C. Note the specificity constant for adenylylated E129D is greater than that for adenylylated WTGS.

Fig. 8. A comparison of the specificity constants for L-glutamate, expressed as $\log(k_{cat}/K_m)$ for WTGS and E129D in unadenylylated and adenylylated magnesium-form enzymes.

comprise the ATP-binding site, allowed experimental evaluation of the effects of altering the charge density around the metal ion site on binding and catalytic steps. The mutant enzymes are similar to WTGS structurally, as demonstrated by UV-vis and fluorescence spectroscopic methods and by Mn^{2+} EPR titration.

Metal ion binding selectivity

Selectivity in metal ion binding is determined by the nature, number, and orientation of ligands and by the size of the metal ion and its Lewis acidity, as addressed in a review on metalloenzymes (Glusker, 1991). Overall, the metal ions Mg^{2+} and Mn^{2+} are quite similar in many respects, and Mn^{2+} with unpaired d electrons is frequently used as a spectroscopic probe for enzymes that contain Mg^{2+} . Both ions prefer hexacoordinate, octahedral environments, especially Mg^{2+} . Although Mg^{2+} has a smaller ionic radius (0.65 Å) than Mn^{2+} (0.80 Å), the average Lewis acidity of Mg^{2+} is slightly less than that of Mn^{2+} , 0.334 versus 0.344, respectively (Brown, 1988). Magnesium shows a high selectivity for binding to aspartates and glutamates, whereas manganese will bind more tightly to nitrogen ligands that are disfavored by magnesium. An analysis of favored metal-carboxylate orientations in protein structures indicates Mg^{2+} preferentially binds to *anti* lone pair electrons, whereas Mn^{2+} binds equally to *anti* and the more basic *syn* lone pairs (Carrell et al., 1988). In addition, Mn^{2+} forms tighter complexes with similar ligands than Mg^{2+} (Mellor & Maley, 1947), judged on the respective critical stability constants (e.g., for Mg-EDTA, $\log K_{cs} = 8.79$ versus Mn-EDTA, $\log K_{cs} = 13.87$; Martell & Smith, 1974). Enzymes use both metal ions for catalysis involving chemistry of relatively weak bases (i.e., polyphosphates), with magnesium commonly required for phosphoryl group transfer reactions.

The metal-binding sites of GS are consistent with these general trends. The reaction involves phosphoryl group transfer from ATP to L-glutamate, which requires electrophilic catalysis, and both metal ions are in octahedral sites containing 5 glutamate side chains. Glutamine synthetase binds Mg^{2+} ~200-fold less tightly than Mn^{2+} (Denton & Ginsburg, 1970), and the internal equilibrium for phosphoryl group transfer from ATP to L-glutamate was shown to be closer to the ideal value (i.e., $k_{int} \sim 1$) for magnesium ($k_{int} \sim 2$) than for manganese ($k_{int} \sim 5$), reflecting a looser interaction between magnesium enzyme and γ -glutamyl phosphate (Abell & Villafranca, 1991).

Steady-state kinetic parameters

Mutagenesis at the n_2 site was expected to disrupt catalytic properties of the magnesium-form enzymes but not to disrupt the manganese-form enzymes as severely. Specifically, E357H, E129H, and E357Q were predicted to maintain favorable coordination environments for manganese but not for magnesium, in contrast to E357D and E129D, which should maintain favorable environments for both metal ions.

These predictions are consistent with the observed catalytic activities with manganese and magnesium, especially for the nonconservative mutants E129H, E357H, and E357Q. The observed kinetic parameters, especially k_{cat}/K_m values, for the magnesium-form enzymes support the hypothesis that the ligand environment for magnesium-dependent activity is more critical than for manganese and indicates the important role of E357 and E129 in metal ion binding under catalytic conditions.

The combined kinetic data indicate that mutagenesis affects both substrate binding and chemical steps, and demonstrates the importance of the n_2 -site glutamates for biosynthetic activity. Mutagenesis of the n_2 -site glutamates also clearly disrupts communication between the active site and the rest of the protein, specifically the adenylation site, Y397, which is ~15 Å from the n_2 metal ion. This reflects the complex nature of interactions, mediated by covalent modification from the protein surface and translated to the active site. The details of the conformational change that occurs upon adenylation of Y397 are not yet elucidated, although both steady-state and pre-steady-state kinetic parameters indicate changes in substrate activation upon adenylation (Abell & Villafranca, 1991). Adenylation also weakens manganese binding affinities, increasing $K_D^1 \geq 10$ -fold and $K_D^2 \sim 2$ -fold (Denton & Ginsburg, 1969).

For these n_2 -site mutants, however, this communication is altered relative to WTGS, consistent with a changed active site geometry upon adenylation. The adenylylated mutant enzymes may each have an active-site geometry that is subtly different from adenylylated WTGS. In addition, the extensive network of active-site interactions, including distances and orientations between metal ion side-chain atoms and hydrogen bond partners, may be slightly altered by adenylation for each mutant enzyme relative to its unadenylylated form, and the consequences of this would be weakened substrate binding or especially weakened interactions in the transition states, manifest kinetically by decreased specificity constants. Removal of a single hydrogen bond between the enzyme and a transition state could result in an increase of $\Delta(\Delta G) \sim +4$ kcal/mol, which decreases specificity by 1,000-fold (Fersht et al., 1985). Comparing k_{cat}/K_m values (i.e., $\Delta(\Delta G) = -RT \ln[(k_{cat}/K_m)_{n=12}/(k_{cat}/K_m)_{n=0}]$) for ATP and L-glutamate between unadenylylated and adenylylated WTGS with both metal ions indicates the differences in transition states between the enzyme forms, reflected by increased $\Delta(\Delta G)$ values (i.e., between +1.4–2.9 kcal/mol) in the transition states with adenylylated enzyme. For magnesium-form adenylylated WTGS, E357D and E129D, increased $\Delta(\Delta G)$ values relative to WTGS are seen for E357D of +2.4 kcal/mol (L-Glu) and +2.9 kcal/mol (ATP), but decreased $\Delta(\Delta G)$ values for E129D of –2.0 kcal/mol (L-Glu) and –1.7 kcal/mol (ATP). Similar trends are observed for manganese-form adenylylated enzymes, which show $\Delta(\Delta G)$ increases in the transition states up to +5.8 kcal/mol (E357H with ATP), and the smallest changes for E129D. This indicates that interactions between substrate(s) and residue E357, which are preserved in E129D but not in E357 mutants, are important in the chemical steps. This is discussed in more detail below. The magnitude of some of these $\Delta(\Delta G)$ differences suggests that the hydrogen bonding network may be altered in these transition states, but the degree of electrophilic catalysis afforded by the metal ions may also be attenuated by adenylation.

Mutagenesis of other metalloenzymes

Metalloenzymes are popular targets for mutagenesis because modifications in metal binding can probe structure–function relationships as well as mechanism. The design of tailored metal-binding proteins has been facilitated by dissecting factors involved in metal ion binding, and recent examples include trypsin (Higaki et al., 1990; McGrath et al., 1993), thioredoxin (Helling & Richards, 1991), somatotropin (Suh et al., 1991), model

4-helix bundle proteins (Regan & Clarke, 1990; Handel et al., 1993), and antibodies (Wade et al., 1993a, 1993b). Other recent examples have addressed metal-binding residues in the copper proteins azurin (Karlsson et al., 1991; Mizoguchi et al., 1992) and myoglobin (Van Dyke et al., 1992), and the zinc proteins leukotriene A₄ (LTA₄; Medina et al., 1991) and neutral endopeptidase (Le Moual et al., 1991). The endopeptidase was shown by mutagenesis to contain 3 ligands to zinc, 2 histidine and 1 glutamate, and replacement of aspartate for the glutamate ligand (E646D), preserved binding of metal ion, the K_m value for substrate was unchanged, but k_{cat} was reduced to 3% that of wild-type enzyme.

Mutagenesis of calcium-binding ligands in EF-hand proteins reduced Ca²⁺-binding properties of calbindins (Forsen et al., 1989) and affected cooperativity of Ca²⁺ binding in calmodulin (Waltersson et al., 1993). Mutagenesis studies of Ca²⁺-binding ligands D21 and D40 in staphylococcal nuclease indicated loss of 1 carboxyl group resulted in ~10-fold reduction in affinity for Ca²⁺ (Mildvan & Serpersu, 1989). A similarity to our results was the demonstrated inequivalency of Ca²⁺-binding aspartate ligands with the mutants D21E and D40E, which showed large differences in k_{cat} values.

The metal ion binding environments of GS closely resemble that of D-xylose isomerase from *Streptomyces olivochromogenes* (Farber et al., 1987, 1988) and other bacterial species (Rey et al., 1988; Henrick et al., 1989). The crystal structure from *S. olivochromogenes* indicates an α/β -barrel protein, quite dissimilar to GS, except for the presence of 2 divalent metal ions per monomer, which can be either Mg²⁺ or Mn²⁺. The amino acids within binding distance to the metal ions comprise a nearly ideal octahedral environment at one site with the oxygen atoms of 2 glutamates (E181, E217), 2 aspartates (D245, D287), and 2 water molecules, whereas the other site displays a distorted octahedral environment with 1 glutamate (E217), 2 aspartates (D255, D257), 1 histidine (H220), and 1 water molecule. The 2 metal ions are bridged by another glutamate and the metal-metal distance of 4.9 Å is only 0.9 Å shorter than in GS. A mutagenesis study has been reported recently for this enzyme (Allen et al., 1994) that represents an interesting comparison for GS with respect to the chemical and structural roles supported by the metal ions.

The proposed roles of metal ions in GS

The roles of the metal ions in GS appear to be complex and may include subunit-subunit stabilization in addition to substrate binding and electrophilic catalysis. Residues E129 and E357 are proposed to serve as metal ion binding ligands, and not to serve as bases in the mechanism. The third metal-binding ligand at the site, His-269, may play a more complex role, but is important in metal binding (Abell et al., 1994). Oxidation of His-269 is known to "mark" GS for degradation by a proteolytic pathway, and studies of the thermodynamic stability of this modified enzyme (Fisher & Stadtman, 1992) support earlier indications of the importance of metal ion binding sites in the overall subunit-subunit stability (Woolfolk & Stadtman, 1967; Hunt & Ginsburg, 1972; Ciardi et al., 1973; Nakamura & Stadtman, 1984).

As shown in Figures 5 and 6, Mg²⁺-activated E129D supports high biosynthetic activity, in contrast to E357D. The X-ray crystal structure of the Mn²⁺-form enzyme illustrates the inequivalency of these 2 glutamate residues, illustrated in Figure 9 and Kinemage 2. E129 is 2–3 Å closer to the n₁ metal ion

(within 6 Å) than is E357, and closer to the 3 glutamates at the n₁ site, whereas E357 is >10 Å distant from the n₁ metal and is 2–3 Å closer to several arginines than is E129 (i.e., E357 carboxyl oxygen atoms are within 7 Å of guanidinium nitrogen atoms of residues R339, R344, R355, R359). These arginines are H-bond donors that appear to be involved in nucleotide binding contacts (Liaw & Eisenberg, 1994). Steady-state kinetic parameters of the mutant enzymes and fluorescence titration experiments indicate that the n₂-site glutamate residues are important for substrate binding under catalytic conditions and suggest that alteration in the charge density at this metal ion strongly affects the chemistry. Replacement with a similar charge preserves a favorable charge density for metal ion binding, substrate binding, and catalysis. It also argues against these residues being involved in any acid/base chemistry at the active site because mutagenesis would be expected to severely affect catalysis and have little effect on binding, although the network of interactions between enzyme and substrate involved in binding and catalysis may be altered differently by mutagenesis. In contrast to our results, mutagenesis of an active-site glutamate to aspartate in staphylococcal nuclease (Chaikan & Sanchez, 1972; Hibler & Gerlt, 1986), bacterial triosephosphate isomerase (Raines et al., 1986), and *Agrobacterium* β -glucosidase (Withers et al., 1992) all yielded enzymes with severely reduced catalytic activities.

Hershlag and Jencks suggested roles for electrophiles in dissociative and associative transition states leading to phosphoryl group transfer in model systems (Hershlag & Jencks, 1987, 1989, 1990). On the basis of our experiments, we propose that the n₂-site metal ion activates ADP for departure in the phosphorylation of glutamate by ATP to generate γ -glutamyl phosphate. Alteration of the charge density at this metal ion is expected to affect the transition-state energy for phosphoryl group transfer but may also affect ATP binding and/or ADP release. Our steady-state kinetic data, including k_{cat}/K_m values for ATP and glutamate, indicate that altering the charge density increases the transition-state energies for chemical steps as discussed above.

Several X-ray crystal structures of GS from *S. typhimurium* containing Mn²⁺ and the substrate glutamate, AMPPNP (an ATP analog), the products glutamine and ADP, and the transition-state analog MSOX are now available (Liaw & Eisenberg, 1994). These structures confirm our kinetic results and indicate that, upon substrate binding, the n₂-site metal ion ligands identified in the uncomplexed form remain intact. Furthermore, the n₂-site metal ion is involved in ATP and ADP binding. Residues E129 and E357 retain unique microenvironments and each has an additional interaction with a nearby residue, not seen in the uncomplexed structure.

The carboxyl oxygen atoms of E357 contact the guanidinium nitrogen atoms of R344 (each at 2.9 Å), consistent with a salt bridge. Because R344 is within 2.5 Å of the β -phosphate non-bridging oxygen of ATP, a potentially critical H-bond interaction for ADP departure, this suggests that E357 is important for metal ion binding and for substrate binding and activation. Removal of this conjugate base by mutagenesis (i.e., E357H or E357Q) should both affect the charge density around the metal ion and disrupt the interaction with R344 and thereby diminish ATP binding and activation. Conservative mutagenesis (i.e., E357D) may maintain these contacts and this hypothesis is supported by our steady-state kinetic and fluorescence data.

On the other hand, 1 carboxyl oxygen atom of E129 is within 2.6 Å of the ϵ nitrogen atom of H271, which is 2.5 Å from the

Fig. 9. A view of the active site including both metal ions, metal-binding ligands, and 4 arginines, illustrating the unique micro-environments of the n_2 -site glutamate residues, as taken from the manganese enzyme crystal structure. The n_1 -site metal is to the left, coordinated to 3 glutamates, E131, E212, and E220, and the n_2 -site metal (at 5.8 Å from the n_1 -site metal ion) is to the right coordinated by E129, H269, and E357. The guanidinium nitrogen atoms of the 4 arginines (R339, R344, R355, and R359) are all 2–3 Å closer to the carboxyl oxygens of E357 than to E129, and the orientation and distance of 2.9 Å between the respective atoms of E357 and R344 is consistent with a salt bridge. The arginines and histidine nitrogen atoms are shown in blue, the carboxyl oxygen atoms are shown in red, and the 2 metal ions are shown as white spheres.

α -phosphate moiety of ATP. This H-bond interaction is not expected to be as important in ADP departure as is the interaction between the β -phosphate group and R344 and may explain why E129D generally supports higher biosynthetic activities than E357D. Additionally, adenylated E129D shows the highest specificity constants, competitive with or higher than WTGS, suggesting that adenylation may disrupt the hydrogen bonding network surrounding the nucleotide binding site, least affected in mutant E129D, which contains the wild-type glutamate at position 357.

This is further reflected in the fluorescence titration experiments with unadenylated manganese-form enzymes and ATP and ADP. The large difference (~ 15 -fold) between E357D K_D values for ADP (76 μ M) and ATP (5 μ M) and versus WTGS for ADP (4 μ M) reflects weakened interactions between the enzyme and ADP originating from this metal-binding ligand (i.e., E357). In contrast, E129D shows K_D values more in line with WTGS. The inactive double mutants (Witmer et al., 1994) can be rationalized from these structures, which clearly indicates a complex 3-dimensional network of active-site interactions. Structural information on the magnesium form of GS, however, is not yet delineated, so mutagenesis can play a powerful role in clarifying important residues.

Materials and methods

General

All reagents, buffers, and salts were of reagent-grade quality or better. ATP and ADP were obtained from Sigma as the diso-

dium salts and were titrated to pH 7 and stored at -20°C . Concentrations were determined by measuring the absorbance at 259 nm ($E = 15,400 \text{ M}^{-1} \text{ cm}^{-1}$). Protein samples were analyzed by SDS-PAGE using pre-poured gels from Bio-Rad (4–15% gradient), run at 200 V, and proteins stained using Coomassie blue. UV-vis spectroscopy was performed on the purified enzymes using a Gilford Response spectrophotometer with 1-cm quartz cells. The enzymes were diluted in 20 mM imidazole buffer, pH 7.2, containing 100 mM KCl and 10 mM MgCl_2 . Protein concentrations were determined by the bicinchoninic acid (BCA) assay (Pierce), with BSA as a standard. Protein samples were diluted in buffer containing 20 mM imidazole, 100 mM KCl, and 10 mM MgCl_2 , pH 7.2, omitting Mn^{2+} , which interfered with the assay. The average adenylation states of the purified enzymes were determined using the molybdc acid–malachite green assay for inorganic phosphate (Lanzetta et al., 1979) and a standard phosphate solution from Sigma. Enzyme samples (250 or 500 μ g) were treated with HCl (4 M) at 100°C for 30 min to liberate free inorganic phosphate. Complete details for all procedures are given on the Diskette Appendix (SUPLEMNT directory, file Witmer.txt).

Site-directed mutagenesis

The phosphorothioate-based in vitro oligonucleotide-directed mutagenesis kit (Amersham) was used to construct the mutant *glnA* genes. Complete details for construction of all mutant enzymes at the DNA level are given on the Diskette Appendix and elsewhere (Witmer et al., 1994).

Isolation and purification of wild-type and mutant GS

Wild-type and mutant enzymes were obtained from the *E. coli* strains YMC21E ($n = 0$) and YMC21D ($n = 12$) transformed with the *glnA* gene in the vector pgl35 (Backman et al., 1981; Chen et al., 1982). A total of 8 L of cells was grown on minimal media containing metal salts, glutamine as the sole nitrogen source, and glucose as the sole carbon source. Cells were grown at 30 °C until $OD_{550} = 0.8$ – 1.0 au, then harvested by centrifugation (8,000 rpm $\times$ 20 min, 4 °C). Purification of GS was achieved by the published Zn^{2+} precipitation method with modifications (Miller et al., 1974). Acetone precipitation at pH 5.7 gave a pellet that was resuspended in a Mn^{2+} buffer (10 mM imidazole, 100 mM KCl, 10 mM Mn^{2+} , pH 7.2). The final step involved ammonium sulfate precipitation at pH 4.40 (to 10% saturation, on ice; Rhee et al., 1985). The final pellet was redissolved in storage buffer (10 mM imidazole, 100 mM KCl, 1 mM $MnCl_2$, pH 7.2) and shown to be nearly homogeneous ($\geq 98\%$) by SDS-PAGE. Typically 180–250 mg enzyme were obtained from 35 to 40 g of wet cell paste.

EPR titration experiments

Apoenzyme samples were prepared as described on the Diskette Appendix and shown to be manganese-free by atomic absorbance. The peak-to-trough amplitude of the central signal of the Mn^{2+} sextet was measured and a standard curve determined by linear regression. The data were then fit to the following equation, which describes a 2-site binding model, using a nonlinear least-squares program (Villafranca & Mildvan, 1971). In this equation, K_D^1 and K_D^2 are the dissociation constants for the 2 sites, $[L]_{free}$ is the free ligand (Mn^{2+}), and v is $[L]_{bound}/[Enzyme]$.

$$v = (K_D^1[L]_{free}) / (1 + K_D^1[L]_{free}) + K_D^2[L]_{free} / (1 + K_D^2[L]_{free}). \quad (1)$$

Fluorescence titrations

A PTI model 3300 fluorimeter was used to record the fluorescence emission spectra of the enzymes. Enzyme samples were diluted to 1 μ M subunit concentration in 100 mM PIPES, 100 mM KCl, and 8 mM $MnCl_2$, pH 6.5. The emission intensity at 330 nm, F , was fitted to the following equation, using a nonlinear least-squares fit, where F_i is the initial fluorescence, ΔF is the observed difference from the initial state, $[E_0]$ is the enzyme subunit concentration, $[S]$ is the added substrate concentration, and K_D is the desired dissociation value.

$$F = F_i + (\Delta F/[E_0]) \{ (K_D + [E_0] + [S]) - \{ (K_D + [E_0] + [S])^2 - 4[E_0][S] \}^{1/2} \}. \quad (2)$$

Biosynthetic assays

The spectrophotometric assay couples GS activity to the oxidation of NADH by pyruvate kinase and lactic dehydrogenase. Standard conditions included 200 mM buffer (PIPES for pH 6–8; HEPES for pH 7–9), 100 mM KCl, 50 mM NH_4Cl , 2 mM PEP, 2 mM NADH, 33 μ g/mL lactic dehydrogenase, and 33 μ g/mL pyruvate kinase. Optimal metal concentrations were determined first under saturating substrate conditions, then a constant

metal:ATP ratio was maintained (Morrison, 1979). The substrate being examined was varied keeping all other substrate levels at $>10 K_m$. Each data set was comprised of at least 10 concentration values and all were run in duplicate. Data analysis was performed using a software package for enzyme kinetics (KinetAsystII) on a Macintosh II computer, fitting to the Michaelis–Menton equation. Further details are given on the Diskette Appendix.

Biosynthetic activities of fully adenylylated magnesium-form enzymes

Standard conditions were used to determine kinetic parameters for WTGS, E129D, and E357D. For E357H and E357Q, 200 mM HEPES assay buffer was titrated to pH 7.0, 7.5, 8.0, and 8.5, and several samples were prepared using high substrate concentrations shown to be saturating for the other adenylylated enzymes. Blank samples were run at each pH value for comparison. The mutants exhibited minimal rates of ADP production even in the presence of high concentrations of enzyme (500 μ g/mL or 10 μ M subunit concentration). These rates were used to estimate upper limits for k_{cat} values.

ATPase activity

The enzymes were added to the standard assay mixtures omitting glutamate or ammonia or both, and the enzymatic rate followed for 30 min. After several minutes, the observed rates decreased dramatically as ambient ammonia was consumed. The observed ATPase rates were $\leq 5\%$ that of biosynthetic activity. After 30 min, saturating glutamate and ammonium chloride were added to demonstrate that full catalytic activity was maintained.

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