Conformational Changes in Glutamine Synthetase from *Escherichia coli*. I. The Binding of Mn^{2+} in Relation to Some Aspects of the Enzyme Structure and Activity*

M. D. Denton† and A. Ginsburg‡

ABSTRACT: The binding of $^{54}Mn^{2+}$ to glutamine synthetase from *Escherichia coli* has been studied because specific divalent cations are involved in the activation and structural stabilization of this enzyme. By equilibrium dialysis, three apparently independent sets of Mn^{2+} binding sites can be resolved. These include 12 apparently equivalent and independent very high-affinity sites, k_1, per molecule of glutamine synthetase of mol wt 600,000 (or 1/subunit), 12 sites of significantly lower apparent affinity, k_2', and approximately 48 relatively low-affinity sites, k_3'. The 12 sites of intermediate affinity, k_2', together with those of high affinity, k_1', are probably concerned in catalytic function, whereas some or all of the loose binding sites, k_3', appear to be involved in a gross stabilization of the enzyme structure. Mg^{2+} binding to the high-affinity sites of glutamine synthetase was studied indirectly by competition experiments with $^{54}Mn^{2+}$. The enzyme was found to have a nearly 400-fold lower apparent affinity for Mg^{2+} ($k_1' \simeq 4 \times 10^4$) than for Mn^{2+}, although Mg^{2+} has a uniquely specific catalytic function also. In contrast, the enzyme was found to have a very low affinity for the nonspecific Ba^{2+} cation ($k' \simeq 200$). Varying degrees of adenylylation of the glutamine synthetase molecule, as well as pH, influence the apparent intrinsic association constant at the high-affinity sites for Mn^{2+}, k_1', but do not affect the affinity of the other Mn^{2+} binding sites, k_2' or k_3', significantly. The binding of the first 12 Mn^{2+} to apparently equivalent sites appears to be associated with a relatively large over-all conformational change involved in the conversion of the catalytically inactive relaxed enzyme (divalent cation free) into the active taut or tightened form. Considered together with other physical and kinetic data, the results are consistent with the interpretation that the $\Delta F'$ of this confirmational change at pH 7 is a function of the state of adenylylation of the taut enzyme, rather than that of the relaxed enzyme form. As the pH is increased from pH 7 to 8, the increase in k_1' is consistent with previous observations that the quarternary structure of the relaxed enzyme is destabilized by increasing pH. The discharging of at least two ionizing groups per subunit appears to be involved in the pH-induced structural changes wihch indirectly affect k_1'. No cooperativity was observed in the binding of Mn^{2+} in the relaxed to taut enzyme conversion. Instead, a conformational change per subunit, which is modulated by the state of adenylylation of enzyme macromolecule, appears to be associated with the independent binding of each of 12 specific divalent cations.

Specific divalent cations (Mn^{2+} or Mg^{2+}) are directly involved in the catalytic function and the stabilization of the quarternary structure of the glutamine synthetase from *Escherichia coli*. Mn^{2+} or Mg^{2+} cations were shown by Woolfolk and Stadtman (1964) and Woolfolk *et al.* (1966) to be a necessary component of the *in vitro* biosynthesis of glutamine from glutamate, ammonia, and ATP. Co^{2+} or Ca^{2+} can substitute for Mn^{2+} in this reaction (Woolfolk *et al.*, 1966; Kingdon and Stadtman, 1967). Subsequently, it was shown that the enzyme as isolated (Woolfolk *et al.*, 1966) contained large amounts of bound Mn^{2+} (Shapiro and Stadtman, 1967). The removal of Mn^{2+} by excess EDTA and gel filtration treatments produced an enzyme form, called relaxed enzyme, which differed from the native taut enzyme in that the sulfhydryl groups were reactive with various sulfhydryl reagents (Shapiro and Stadtman, 1967). After the removal of Mn^{2+} ions, the enzyme can be dissociated into subunits by fairly mild treatments which include 1.0 M urea, or organic mercurials at pH 8, or sodium docedyl sulfate at pH 7 (Woolfolk and Stadtman, 1967; Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968). The electron micrographs of Valentine *et al.* (1968) show that glutamine synthetase is composed of 12 subunits arranged in two superimposed hexagons, and chemical analysis suggest that these subunits have the same primary amino acid structure (Woolfolk *et al.*, 1966; Shapiro and Stadtman, 1967).

In an investigation of the physical–chemical changes in the taut into relaxed enzyme conversion by the removal of Mn^{2+} or by the presence of excess EDTA, it was shown that at pH 7 at about 0.15 ionic strength the molecular weight remained unchanged (592,000), although some small increases in the hydrodynamic

* From the Laboratory of Biochemistry, Section on Enzymes, National Heart Institute, National Institutes of Health, Bethesda, Maryland. *Received December 16, 1968.*

† Present address: Thorndike Memorial Laboratory, Boston City Hospital, Boston, Mass.

‡ To whom requests for reprints should be addressed.

volume or frictional coefficient occurred (Shapiro and Ginsburg, 1968). Under these conditions, the removal of divalent cations causes a spectral perturbation consistent with the exposure to a more polar environment of 12–24 tyrosyl and tryptophanyl residues/mole of enzyme. The studies of Kingdon *et al.* (1968) show that the relaxed enzyme is catalytically inactive, but can be slowly reactivated at 25° by preincubation of the relaxed enzyme with Mn^{2+}, Mg^{2+}, or Ca^{2+}. The same kinetics and specificity were observed also in the readdition of divalent cations to relaxed enzyme (tightening process) in the difference spectra studies of Shapiro and Ginsburg (1968). However, the reactivation of relaxed enzyme in a Mg^{2+} assay mixture at 24° containing saturating levels of substrates is quite rapid and also is proportional to the concentration of nonadenylylated subunits (see below). Further, the experiments of Kingdon *et al.* (1968) suggest that the substrate glutamate may increase the affinity of the enzyme for specific divalent cations.

While these studies on the equilibrium binding of Mn^{2+} were in progress, it was discovered that, depending upon conditions of the growth of *E. coli*, preparations of glutamine synthetase differed dramatically in their response and specificity toward divalent cations (Kingdon and Stadtman, 1967). Further investigations showed that this property was a function of the number of adenylate groups covalently bound to the enzyme and that this modification could occur to the extent of 12 moles of AMP/600,000 g of enzyme or 1/subunit (Shapiro *et al.*, 1967; Kingdon *et al.*, 1967). These and other studies (Stadtman *et al.*, 1968) indicate that the adenylylation of glutamine synthetase by a specific ATP–glutamine synthetase adenylyltransferase of *E. coli* (Kingdon *et al.*, 1967; Wulff *et al.*, 1967) changes the divalent cation requirement in catalysis from Mg^{2+} to Mn^{2+} (Kingdon *et al.*, 1967).

The studies reported here are concerned with the direct measurement of Mn^{2+} binding to two preparations of glutamine synthetase which differ chemically only in the extent of adenylylation. Mn^{2+} binding as a function of pH was investigated also. The results are interpreted on the basis of the other studies outlined above on the role of divalent cations in the structure and function of the glutamine synthetase from *E. coli*.

Materials and Methods

Glutamine Synthetase was purified by the procedure of Woolfolk *et al.* (1966) from *E. coli* W cells, grown under the conditions described for the glutamine synthetase preparation II (Kingdon and Stadtman, 1967; Shapiro *et al.*, 1967). Two different enzyme preparations were used: $E_{\bar{9}}$ is preparation II described previously (Shapiro *et al.*, 1967) and contains an average of 9 equiv of covalently bound AMP/mole of enzyme (600,000 g); $E_{\overline{2.3}}$ was isolated also on a large scale and it was characterized as containing an average of 2.3 equiv of AMP by the procedures outlined by Shapiro *et al.* (1967). Amino acid analysis of $E_{\overline{2.3}}$ (hydrolyzed in sealed tubes at 107° for 16, 24, and 48 hr) were included and showed no significant deviations from the published amino acid composition of preparations I and II, $E_{\overline{1.2}}$ and $E_{\bar{9}}$, respectively (Shapiro *et al.*, 1967) with $E_{\bar{9}}$ hydrolysates analyzed as controls. The reason for the relatively low degree of adenylylation of the $E_{\overline{2.3}}$ preparation is unclear. Two possible reasons are: variations occurred in the time that the cells (8–10 batches yielding 12.5 kg) were in the stationary growth phase; the time was longer in handling the crude extract (~60 l.) when deadenylylating catalysis (Shapiro and Stadtman, 1968) could occur. The physical properties of these enzyme preparations as well as the spectrophotometric determination of protein concentrations have been described elsewhere (Shapiro and Ginsburg, 1968). Enzymatic activities were determined by either the phosphate assay (Woolfolk and Stadtman, 1964) or the spectrophotometric assay at 23–24° coupled with pyruvate kinase and lactic dehydrogenase to measure ADP formation (Kingdon *et al.*, 1968). pH determinations were made at 23–25° (and corrected if necessary to the temperature of the experiment using the predetermined temperature dependence of the buffer) with a Radiometer type PHM25 pH meter equipped with a scale expander and with the Leeds and Northrup microelectrodes (no. 124138) which have a low leakage of KCl (Ginsburg and Carroll, 1965).

Reagents. $^{54}MnCl_2$ (carrier-free) was purchased from the New England Nuclear Corp. Stock solutions of imidazole (Eastman Organic Chemicals) were treated lightly with charcoal and filtered to remove any yellow color; 2-methylimidazole (Aldrich Chemical Co.) was recrystallized three times from deionized water and then stock solutions of 2-methylimidazole were further treated with Chelex resin (California Corp. for Biochemical Research) to remove traces of metal ions. Inorganic salts used were reagent grade. The deionized water and the materials used for the enzyme assays are described in an accompanying paper (Ginsburg, 1969).

Equilibrium Dialysis. Materials and techniques for equilibrium dialysis were obtained, prepared, and used as noted by Ginsburg and Mehler (1966) unless otherwise noted. Equilibration with manganous ions ($^{54}Mn^{2+}$) at low free concentrations was carried out in dialysis bags and flasks rather than in equilibrium dialysis cells. The dialysate buffers were either 0.1 M KCl and 0.02 M imidazole chloride (pH $\leq$ 7.6) or 0.1 M KCl and 0.02 M 2-methylimidazole chloride (pH $\geq$ 7.7) in the $^{54}Mn^{2+}$ binding experiments.

Enzyme solutions were predialyzed at pH 7.4 (4°) against the above imidazole buffer containing also 5 mM $MnCl_2$. The enzyme solutions at pH 8 were equilibrated with 0.1 M KCl, 0.02 M 2-methylimidazole chloride, and 0.1 mM $MnCl_2$ (pH 8) by gel filtration at 25° through G-25 Sephadex. $^{54}Mn^{2+}$ was added then to the equilibrated protein solutions (~11 mg/ml), and equilibration against a Mn^{2+}-free buffer (with an otherwise identical salt composition) was continued (~3 days) in a 5-ml dialysis cell. Then, the specific activity of the $^{54}Mn^{2+}$ was determined by measuring the radioactivity and the manganous ion concentration on the solvent side of the cell by the method of Cook

(1941) or by atomic absorption spectroscopy (using the Perkin-Elmer instrument made available by Dr. Gerald Auerbach). (In the studies with $E_{\bar{9}}$ and $E_{\overline{2,3}}$, the specific activities of the $^{54}Mn^{2+}$ buffers were approximately 3×10^5 and 1×10^6 cpm per μmole, respectively.) Radioactivity was determined in 20 ml of the solution of Bray (1960) in a Packard Model 314 liquid scintillation spectrometer or in a Packard series 410A Auto-Gamma spectrometer. Quenching was detected by the channel ratio or internal standard techniques. The efficiency of counting varied with the volume of buffer counted, but there was no detectable quenching due to the presence of protein. Therefore all samples and standards were adjusted to the same volume with additional buffer, and no quenching correction was required. The free concentration of manganese in the stock enzyme solution was lowered to the desired concentration by dialysis against varying volumes of solvent with or without diluting the protein solution at the desired pH. After equilibration, the total and free concentrations of manganese were determined by counting aliquots from the protein and solvent sides of the dialysis membrane, respectively. Protein concentration (0.8–11 mg/ml), enzymic activity, and solvent pH (variation = ± 0.05 pH unit within a study at a fixed pH) were determined at this time also.

The procedure outlined above eliminated difficulties that could arise from prolonged dialysis in the absence of Mn^{2+}, under which conditions the *relaxed* enzyme is of questionable stability, or from addition of Mn^{2+} to relaxed enzyme giving rise to *tightened* enzyme, which possibly could differ from the native taut form. That is, the taut state of the enzyme was maintained until the final equilibration partially removed the protein-bound $^{54}Mn^{2+}$ to different extents.

In the competition experiments, large volumes (20 ml or greater) of 0.01 M $MgCl_2$ or 0.01 M $BaCl_2$ in the $^{54}Mn^{2+}$ buffers described above were equilibrated with the protein–$^{54}Mn^{2+}$ mixture. In this way, the concentration of the competing divalent cation for $^{54}Mn^{2+}$ sites was held constant. The decrease in the protein-bound $^{54}Mn^{2+}$ was measured as described above.

Analysis of the Data. Electrostatic corrections to the Mn^{2+} binding data (pH 6.5–8.5) were not made. However, electrostatic effects will be minimized by the high ionic strengths used in these studies (Klotz, 1953; Saroff, 1957a,b; Saroff and Lewis, 1963) even though glutamine synthetase has an acid isoelectric point, as determined electrophoretically. The pH dependence of the binding data will reflect changes in the net charge of the protein, Z, which unfortunately cannot be calculated from the amino acid composition of glutamine synthetase (Shapiro *et al.*, 1967) because the amide nitrogen value is not known. However, comparisons in the binding data for the same or different enzyme preparations are made between measurements made at the same high ionic strength. Further, the magnitude and the internal consistency of the binding constants observed indicate that these may be interpreted directly, with electrostatic factors included in the constants. Then, a form of the mass law binding expression (see Klotz, 1953) for nonequivalent independent binding sites (Ginsburg and Mehler, 1966) was directly applicable

$$\bar{\nu}_{Mn} = \sum_{n'=i} \frac{n_i' k_i' c_{Mn}}{1 + k_i' c_{Mn}} \tag{1}$$

where $\bar{\nu}_{Mn}$ is the average number of Mn^{2+} cations bound to one molecule of protein, k_i' is the apparent intrinsic association constant for the binding of Mn^{2+} to the ith site(s) at the concentration c_{Mn} of the free Mn^{2+} cations, and n_i' is the apparent maximum number of equivalent ith binding sites on each protein molecule. Activity coefficients of the cations are assumed to be unity and free concentrations, c_{Mn}, are expressed as molar concentrations. A molecular weight of 600,000 for glutamine synthetase was used for all calculations of $\bar{\nu}_{Mn}$ (Shapiro and Ginsburg, 1968).

For equivalent, noninteracting binding sites with k' a constant, binding data can be treated in the manner suggested by Scatchard (1949) in which

$$\bar{\nu}_{Mn} = -\left(\frac{1}{k'}\right)\frac{\bar{\nu}_{Mn}}{c_{Mn}} + n' \tag{2a}$$

where $1/k'$ is the apparent dissociation constant. A plot of $\bar{\nu}$ *vs.* $\bar{\nu}/c$ will have a slope and an intercept on the $\bar{\nu}$ axis equal to $1/k'$ and n', respectively. This treatment of the data obtained for the binding of Mn^{2+} cations to glutamine synthetase gave more complex results, but the data could be fit with three straight lines giving different k' and n' values (see Results and Figure 1a, for example). Since k_i' values were sufficiently different for rather large numbers of sites within each set of apparently equivalent binding sites, it can be shown that this treatment gives approximations of k_1', k_2', and k_3' ($k_1' \gg k_2' \gg k_3'$) with intercepts on the $\bar{\nu}$ axis of n_1', $n_1' + n_2'$, and $n_1' + n_2' + n_3'$, respectively. For example, an expanded form of eq 2a from eq 1 which is of special interest in the case of $^{54}Mn^{2+}$ binding ($k_1' \simeq 10\ k_2'$) for two terms with $k_1' \gg k_2'$ (two groups of binding sites, n_1' and n_2' of apparent affinity k_1' and k_2', respectively) yields

$$\frac{\bar{\nu}}{c}[1 + c(k_1' + k_2' + k_1'k_2'c)] = n_1'k_1' + n_2'k_2' + (n_1' + n_2')k_1'k_2'c \tag{2b}$$

If $k_1' \gg k_2'$, $c \ll 1/k_2'$, and $n_2' \leq n_1'$, eq 2b approaches eq 2a for the estimates of k_1' and n_1'. Rearrangement of eq 2b in the form

$$\left(\frac{1}{k_1'k_2'c}\right)\left(\frac{\bar{\nu}}{c}\right) = n_1' + n_2' - \bar{\nu} + \left(\frac{1}{c}\right)\left(\frac{n_2' - \bar{\nu}}{k_1'} + \frac{n_1' - \bar{\nu}}{k_2'}\right) \tag{2c}$$

shows that in the concentration range of $c \simeq 1/k_2'$ ($k_1' \gg k_2'$), eq 2c approaches the form

$$\left(\frac{1}{k_2'}\right)\left(\frac{\bar{\nu}}{c}\right) = n_1' + n_2' - \bar{\nu} + \frac{n_1'}{k_2'c} \tag{2d}$$

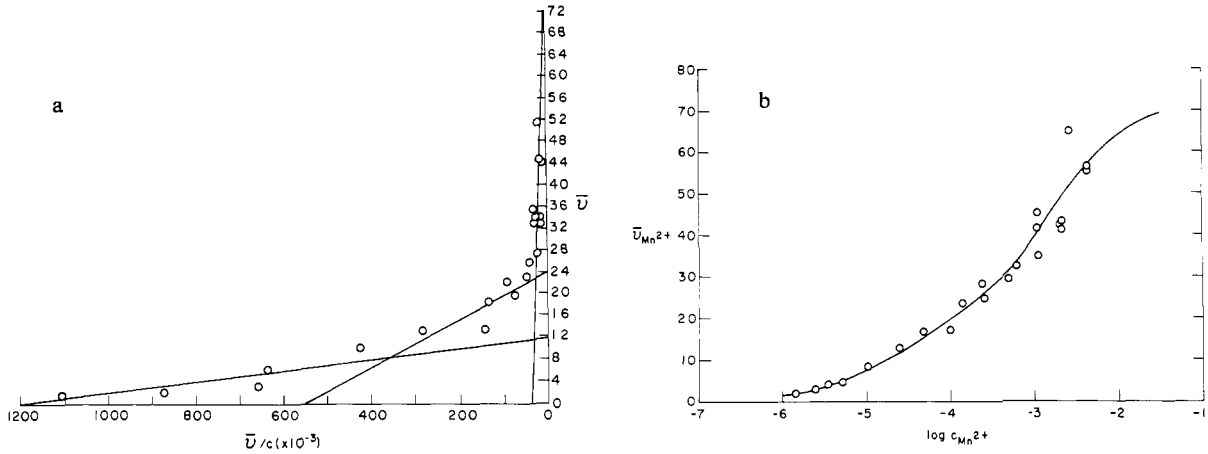

FIGURE 1: Experimental data. (a) Scatchard plot of the experimental data for the binding of manganous ions to $E_{\bar{9}}$ in 0.02 M imidazole and 0.1 M KCl at pH 7 and 4° (see Methods). (b) Alternate representation of the experimental data of a with a theoretical curve constructed with eq 1, for $n_1' = 12$, $k_1' = 10^5$; $n_2' = 12$, $k_2' = 10^4$; and $n_3' = 48$, $k_3' = 500$.

so that a Scatchard plot (eq 2a) gives approximately a slope of $-(1/k_2')$, where $c \simeq 1/k_2'$ and an intercept of $\bar{\nu} = (n_1' + n_2')$ as $c \to \infty$.

More accurate approximations of all of the parameters were obtained by curve fitting the data with eq 1 in a plot of $\bar{\nu}$ *vs.* log c (see Results and Figure 1b, for example), where the values for k_2', k_3', and n_3' were allowed to fluctuate about the values obtained as a first approximation from the Scatchard plot (eq 2a) in the concentration ranges of $1/k_1'$, $1/k_2'$, and $1/k_3'$.

The competition of other divalent cations for identical equivalent $^{54}Mn^{2+}$ binding sites in the concentration range $c_{Mn} \simeq 1/k_1'$ (eq 3) is described by (Lewis

$$\bar{\nu}_{Mn} = \frac{n'k_1'c_{Mn}}{1 + k_1'c_{Mn} + k_c''c_c} \qquad (3)$$

and Saroff, 1957), where $\bar{\nu}_{Mn}$ is the average number of $^{54}Mn^{2+}$ bound per molecule of enzyme, n_1' is the apparent maximum number of binding sites for $^{54}Mn^{2+}$ with the apparent intrinsic association constant, k_1', observed in the absence of added divalent cation ($n_1' = 12$), c_{Mn} and c_c are the free concentrations of $^{54}Mn^{2+}$ and other divalent cation (0.01 M, see above), respectively, and k_c'' is the apparent intrinsic association constant for the added divalent cation. The parameter k_c'' could be directly computed from eq 3.

For equilibrium dialysis, the ionic strength of the medium is increased so that the Donnan effect is negligible. The cations (K^+ or imidazole$^+$) added for this purpose may be considered as possible competing ions but these monovalent species will not alter significantly the constants k_1', k_2', n_1', and n_2' for $^{54}Mn^{2+}$ binding, or k_1' and n_1' for Mg^{2+} binding, although k_3' and n_3' for Mn^{2+} and k_1' for Ba^{2+} may be somewhat influenced (Ginsburg and Mehler, 1966). That is, the corrections for monovalent cation competition with the divalent species will not be significant as long as there is a sufficient difference in the magnitude of the association constants (k' and k'' in eq 3). Imidazole itself will bind Mn^{2+}, but this constant must be small also in comparison with the constants measured here for Mn^{2+} binding to the enzyme.

Results

The binding of manganous ions at pH 7 to a glutamine synthetase preparation which is adenylylated to the extent of 9 equiv of AMP ($E_{\bar{9}}$) is shown in Figure 1. In Figure 1a the apparent maximum total, n', of the Scatchard plot approaches 72 Mn^{2+} binding sites/enzyme molecule; 24 of these sites have a much greater affinity for Mn^{2+} than do the other 48 sites, and these 24 sites are partially resolved further into approximately equal numbers of high- and intermediate-affinity sites. The resolution of these two types of high-affinity sites, with k_1' and k_2' apparent intrinsic association constants, is insufficient to allow unequivocal estimates of the apparent n_1' and n_2' values for these sites. However, the enzyme is composed of 12 subunits (Valentine *et al.*, 1968), each of which appears to have the same amino acid composition (Woolfolk *et al.*, 1966; Shapiro and Stadtman, 1967). On a structural basis it is reasonable then to classify the tight sites into two groups of equal number, where $n_1' = 12$ and $n_2' = 12$ per enzyme molecule. With the Mn^{2+} binding sites of different affinity (k_1', k_2', or k_3') equally distributed throughout the protein macromolecule, the results of Figure 1a can be interpreted as indicating that each subunit of the enzymes has one high-, one intermediate-, and four relatively low-affinity sites for Mn^{2+}.

The best fit of the Mn^{2+} binding data for $E_{\bar{9}}$ at pH 7 is shown in Figure 1b by the theoretical curve constructed from the indicated apparent intrinsic association constants (k_1', k_2', and k_3') and n' values with the use of eq 1.

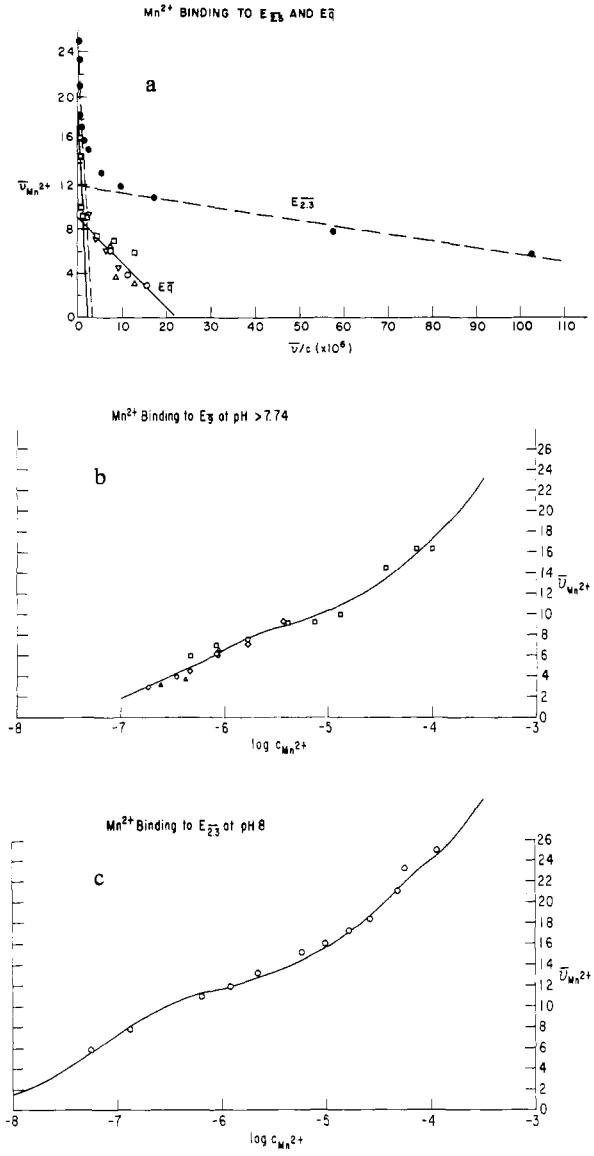

FIGURE 2: The binding of Mn^{2+} to $E_{\overline{2.3}}$ and $E_{\overline{9}}$ at 24° and alkaline pH in 0.02 M 2-methylimidazole and 0.1 M KCl. $E_{\overline{2.3}}$ at pH 8 (●), $E_{\overline{9}}$ at pH 7.74 (○), 7.80 (□), 8.15 (△), and 8.63 (▽, ◇). (a) Scatchard plots of the data (see Methods). (b) The experimental data for $E_{\overline{9}}$ at pH $\geq$ 7.74 with a theoretical curve constructed from eq 1 and $n_1' = 9$, $k_1' = 2.5 \times 10^6$; $n_2' = 9$, $k_2' = 2 \times 10^4$; $n_3 = 48$, $k_3' = 500$. (c) The experimental data for $E_{\overline{2.3}}$ at pH 8 with a theoretical curve constructed from eq 1 for $n_1' = 12$, $k_1' = 1.5 \times 10^7$; $n_2' = 12$, $k_2' = 4 \times 10^4$; $n_3' = 48$, $k_3' = 500$.

Preparations of glutamine synthetase that are adenylylated to varying extents have quite different biosynthetic specific activities in assays with either Mg^{2+} or Mn^{2+} and saturating substrate concentrations. This has been correlated to an absolute specificity of nonadenylylated and adenylylated subunits either for Mg^{2+} or Mn^{2+}, respectively (Kingdon *et al.*, 1967; Stadtman *et al.*, 1968). For this reason, studies were made on the binding of Mn^{2+} to another enzyme preparation, containing an average of 2.3 AMP groups/molecule ($E_{\overline{2.3}}$) and therefore differing from $E_{\overline{9}}$ in the magnitude of its catalytic responses to Mn^{2+} and Mg^{2+} (Figure 2a,c). Since other studies with $E_{\overline{9}}$ showed that greater resolution of the high-affinity sites for Mn^{2+} were obtained at higher pH values (compare Figures 1 and 2a,b) and that no further changes in k_1' occur at pH $\geq$ 7.7 (Figure 3), Mn^{2+} binding to $E_{\overline{2.3}}$ was measured at pH 8. Data for the binding of Mn^{2+} to the $E_{\overline{9}}$ and $E_{\overline{2.3}}$ enzyme preparations at pH 8 are shown in Figure 2a–c.

The Scatchard plot for $E_{\overline{2.3}}$ at pH 8 (Figure 2a) shows that there is a sharp break in the data which extrapolates to $n_1' = 12$ for the high-affinity sites, again suggesting that there is one tight site per subunit. Also, there appears to be 12 intermediate affinity sites for $E_{\overline{2.3}}$.

The data for E_9 at pH $\geq$ 7.7 and the lowest Mn^{2+} concentrations (Figure 2a) extrapolate to $n_1' \simeq 9$ instead of 12. The loss in high-affinity sites for $E_{\overline{9}}$ as the pH is increased from pH 7 (Figure 1a) to 8 is attributed to the occurrence of a small amount of dissociation of the partially relaxed $E_{\overline{9}}$ at the high pH. It has been observed in sedimentation studies[1] that a relaxed preparation of $E_{\overline{9}}$ (divalent cation free) at pH 8 and ~0.15 ionic strength is ~15% disaggregated. Further, destabilization of the quarternary structure of relaxed enzyme forms produced by increasing the pH above pH 7.7 is well documented (Woolfolk and Stadtman, 1967; Shapiro and Ginsburg, 1968). Although the Mn^{2+} was never completely removed from the enzyme preparations in these studies, they did undergo prolonged dialysis at 24° (see Methods). Intermediate states of relaxation and therefore partial instability can be inferred from studies on the reactivity of sulfhydryl groups (Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968). It is of interest that about the same loss in Mn^{2+} sites of intermediate affinity of the $E_{\overline{9}}$ preparation occurred at pH 7.7 ($n_2' \simeq 9$). This observation suggests that the n_1' and n_2' sites are distributed evenly, with each subunit having one of each type of binding site only when the subunit is part of the native dodecameric aggregate. The slopes for $E_{\overline{9}}$ in the Scatchard plot of Figure 2a will give estimates of k_1' and k_2' (see Methods), despite the partial loss of n_1' and n_2' binding sites if it is assumed that the remaining Mn^{2+} binding sites are left intact as part of the native enzyme structure. The less adenylylated enzyme ($E_{\overline{2.3}}$) appears to be a more stable aggregate in a partially relaxed form at pH 8 under the same conditions, although less Mn^{2+} was removed from the enzyme in this case due to the very high affinity of $E_{\overline{2.3}}$ for Mn^{2+} (Figure 2a). Lower free concentrations of $^{54}Mn^{2+}$ than those shown in Figure 2c could not be measured accurately.

Figure 2b,c illustrates the theoretical curves constructed from eq 1 to fit the data for $E_{\overline{9}}$ and $E_{\overline{2.3}}$ at pH $\geq$ 7.7, respectively. The apparent intrinsic association constants for the sites of intermediate and low affinity (k_2' and k_3') estimated by this procedure are approximately the same for both $E_{\overline{2.3}}$ and $E_{\overline{9}}$. However, the k_1' values are markedly different for the two

[1] B. M. Shapiro and A. Ginsburg, unpublished observations.

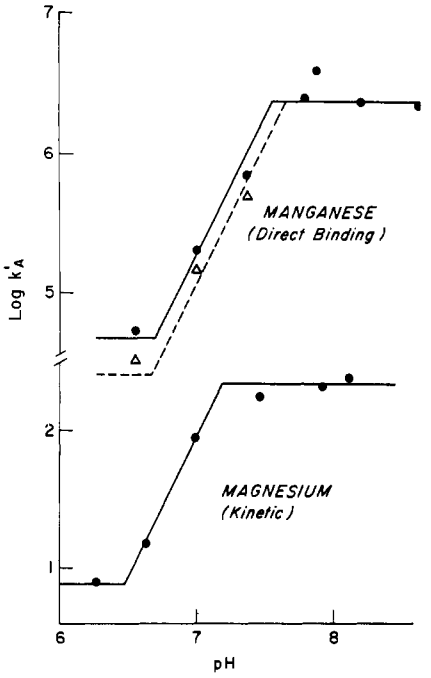

FIGURE 3: The influence of pH on the apparent intrinsic association constant, k_A', of the high-affinity sites of $E_{\overline{9}}$. The association constants for manganese were determined from Scatchard plots of direct binding studies; k_A' values calculated for $n_1' = 9$ (●) and $n_2' = 12$ (△) are shown. The k_A' values for magnesium were obtained kinetically, $1/K_m'$, using the phosphate assay at 25° (see Methods); the assay contained 100 mM glutamate, 50 mM NH_4Cl, 5 mM ATP, and 50 mM Tris-maleic buffer (pH value as shown determined at 25°).

enzyme preparations as seen most simply by comparing the slopes in Figure 2a. The k_1' of $E_{\overline{2.3}}$ is about sixfold greater than k_1' for $E_{\overline{9}}$ at the same pH. It is important to realize that a good fit of the data in Figure 2b,c is obtained by the use of eq 1 which assumes nonequivalent independent Mn^{2+} binding sites. However, this is not absolute proof that this assumption is correct.

Figure 3 shows the influence of pH on the affinity of the $E_{\overline{9}}$ enzyme preparation toward Mn^{2+} and Mg^{2+} cations. The apparent association constant, k_1', for Mn^{2+} was determined at each pH from Scatchard plots of Mn^{2+} binding data as illustrated in Figures 1a and 2a. No significant differences in k_1' for the high-affinity Mn^{2+} sites was observed pH 7.7–8.5. Below pH 7.7 values for n_1' of 9 and 12 are shown because the loss in n_1' sites discussed above was observed at pH 7.7. Below this pH, there is some uncertainty in the n_1' value due to the relatively poor resolution of high and intermediate sites. However, an analysis of the Mn^{2+} binding data for $E_{\overline{9}}$ at pH $\leq$ 7.4 by the graphical method of Wyman (1964), as illustrated below (Figure 4), indicates that both $n_1' = 12$ and $n_2' = 12$ since other values for these variables give unsymmetrical Hill plots.

The apparent affinity constant for Mg^{2+} (Figure 3) was estimated kinetically by the phosphate assay so that this is really the reciprocal of the Michaelis kinetic constant, $1/K_m'$. The enzyme requires free Mg^{2+} in excess of ATP present for activity.[2] The free concentration of Mg^{2+} ions was calculated by assuming a 1:1 ATP–Mg^{2+} complex (Perrin and Sharma, 1966) and subtracting the amount of ATP from the total concentration of Mg^{2+}. The high affinity of ATP^{2-} for Mg^{2+} (O'Sullivan and Perrin, 1964) makes the error involved in assuming the 1:1 ATP–Mg^{2+} complex negligible relative to the excess free Mg^{2+} present. The double-reciprocal plots of velocity *vs.* the calculated free Mg^{2+} concentration were linear, and the intercept of the abcissa was used to determine $1/K_m'$.

The lines shown in Figure 3 have slopes of 0 or 2, and these were drawn according to the procedure outlined by Dixon and Webb (1964). The zero slopes above pH 7.5 and below pH 6.8 or 6.5 in the Mn^{2+} or Mg^{2+} cases, respectively, indicate that in these pH ranges the k_1' (k_A' in Figure 3) is not a function of pH. The slope of two in the pH range where k_A' is a function of pH suggest that there are two ionizing groups involved in this effect of pH on divalent cation binding. Since the binding of 12 Mn^{2+} cations is associated with k_A', the results of Figure 3 are normalized to one-twelfth; that is, the pH dependence of k_A' is intimately associated with the discharging of 2 ionizing groups/subunit or a total of 24 amino acid residues/molecule. Groups that could have pK's in this range include α-amino, imidazolium, or abnormal sulfhydryl or ϵ-amino groups (Cohn and Edsall, 1943). The pK's of the groups associated with the Mg^{2+} binding (Figure 3) appear to be lower than those involved in the very tight Mn^{2+} binding sites. This may be due to the presence of other substrates (ATP, NH_3, or glutamate) in the kinetic determinations, or it may reflect an intrinsic difference in the binding of the two cations (see below). It is apparent also that there is about 3.5–4 orders of magnitude difference between the observed k_A' for Mg^{2+} and Mn^{2+} at most pH values in Figure 3.

The influence of pH on the high-affinity sites for Mn^{2+} or Mg^{2+} (k_1', $n_1' = 12$) may be direct or indirect. Since the magnitude of the apparent intrinsic association contant, k_1', most probably reflects conformational changes of the protein (see Discussion), this effect will be involved indirectly also in the magnitude of the pH dependence of k_A' for Mn^{2+} and Mg^{2+}. If the contribution of k_A' of a conformational change is not fairly constant over the pH range in question, the slopes of Figure 3 will be estimates only of the minimum number of ionizing groups involved. The estimate of two or more ionizing groups per subunit from the slopes of Figure 3, then would pertain indirectly to structural changes induced in the protein by the pH increase. A direct effect would involve the discharging of groups participating directly in the tight binding sites for divalent cation, but in this case an increase in the apparent total number of sites would be expected to occur (Saroff, 1957a,b). There

[2] Unpublished data

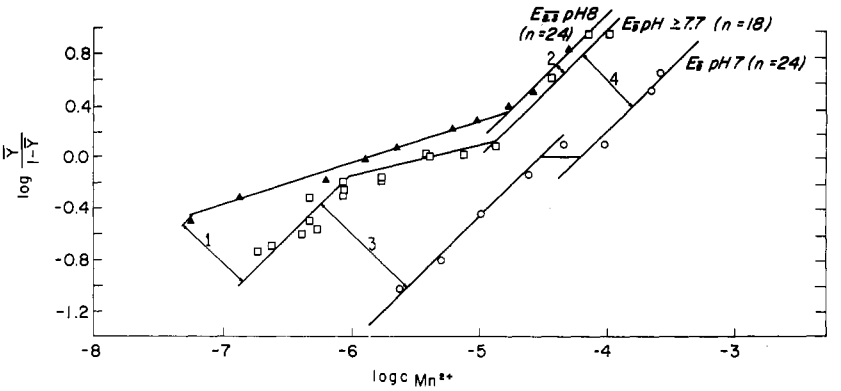

FIGURE 4: Hill plots of the manganese binding data (see Wyman, 1964). $\bar{Y}/(1 - \bar{Y})$ is the fractional saturation of the two high-affinity sites, where $\bar{Y}$ is the moles of Mn^{2+} bound per mole of enzyme divided by $n_1' + n_2'$ ($\bar{Y} = \bar{\nu}/(n_1' + n_2')$). The lines at the extremes of each set of data (missing in the case of $E_{\overline{2.3}}$ at the very low Mn^{2+} concentrations) are slopes of one and are connected by a line with a slope less than one. Shown are (▲) $E_{\overline{2.3}}$ at pH 8, $n_1' + n_2' = 24$; (□) $E_{\bar{9}}$ at pH $\geq$ 7.7 $n_1' + n_2' = 18$; (0) $E_{\bar{9}}$ at pH 7, $n_1' + n_2' = 24$.

TABLE I: Magnesium Competition with [^{54}Mn]Manganous Cations in Binding to $E_{\overline{2.3}}$ at pH 8.

Assume $k_{Mn}' = 1.5 \times 10^7$, $n' = 12$		$C_{Mg} = 0.01$ M	
C_{Mn} (M $\times 10^6$)	$\bar{\nu}_{Mn}$ (no Mg^{2+})	$\bar{\nu}_{Mg}$ (obsd)	k_{Mg}'' (calcd $\times 10^4$)
0.92	11.9	0.45	3.5
3.39	14.1	1.22	4.5
3.45	14.2	1.43	3.8
6.56	15.8	2.31	4.1
12.61	17.5	4.11	3.6
			av 3.9×10^4

was no evidence of a gain in n_1' sites of the enzyme with increasing pH in the pH range studied. The n_1' binding sites of $E_{\bar{9}}$ at pH 7 and of $E_{\overline{2.3}}$ at pH 8 $\pm$ Mg^2 (Figure 1ab, Table I) were the same. The only observed change in n_1' was a loss of Mn^{2+} binding sites in the case of $E_{\bar{9}}$ at pH $\geq$ 7.7, and then a loss of n_2' sites was observed too. The n_1' and n_2' Mn^{2+} binding sites might be expected to involve similar charged groups, but the n_2' sites of intermediate affinity, k_2', are not influenced significantly by the same pH change (Figures 1b and 2b). For these reasons, the pH dependence of k_1' shown in Figure 3 appears to be an indirect consequence of a pH-induced structural change.

Table I shows data from competition experiments in which Mg^{2+} was added to glutamine synthetase with $^{54}Mn^{2+}$. The Mg^{2+} competition with $^{54}Mn^{2+}$ for high-affinity $E_{\overline{2.3}}$ binding sites at pH 8 is shown. Incidentally, at this pH the affinity of $E_{\overline{2.3}}$ for Mn^{2+} is so high that data at lower $\bar{\nu}$ than shown in Figure 2c could only be obtained by adding sufficient amounts of a competing divalent cation (see above). Mg^{2+} at 0.01 M concentration lowered the fractional saturation of the enzyme with $^{54}Mn^{2+}$ at the free concentrations of Mn^{2+} shown in Table I. Further, the displacement of $^{54}Mn^{2+}$ from the high-affinity sites, k_1' and n_1', by Mg^{2+} could be expressed by eq 3 which assumes a direct competition between divalent cations at the same n_1' sites. The calculated apparent intrinsic association constants, $k''_{Mg^{2+}}$, for Mg^{2+} from eq 3 at the observed $\bar{\nu}_{Mn} \leq 12$ were in good agreement, and indicate that $k''_{Mg} \simeq 4 \times 10^4$. The constant k''_{Mg} will reflect any conformational change of the protein involved in the binding of the first 12 Mg^{2+} ions (see Discussion), as in the binding of the first 12 Mn^{2+} ions.

There is a discrepancy between the relative affinities of Mn^{2+} and Mg^{2+} in the competition and kinetic measurements of Table I and Figure 3, respectively. At pH 8, the apparent affinity of $E_{\overline{2.3}}$ is ~400-fold lower for Mg^{2+} than for Mn^{2+}. In the comparison of the kinetic Mg^{2+} constant to k_1' for Mn^{2+} in Figure 3, $E_{\bar{9}}$ appeared to have $\sim 10^4$ lower affinity for Mg^{2+}. The differences observed in the affinity for Mg^{2+} (Figure 3 and Table I) could represent a difference between the enzyme preparations ($E_{\overline{2.3}}$ and $E_{\bar{9}}$), and/or probably more important, between the kinetic affinity constant measured with substrates present and the binding measurements of Table I.

Table II shows Ba^{2+} competition with $^{54}Mn^{2+}$ for binding to the Mn^{2+} high-affinity sites of $E_{\bar{9}}$ at pH 7, k_1' and n_1'. The estimated apparent intrinsic association constant for Ba^{2+}, k''_{Ba}, from eq 3 was considerably lower than that observed for Mg^{2+} by the same procedure. It is of interest that the apparently low specificity of the Ba^{2+} binding ($k'' \simeq 200$)

TABLE II: Barium Competition with [^{54}Mn]Manganous Cations in Binding to $E_{\bar{9}}$ at pH 7.

Assume $k'_{Mn} = 1 \times 10^5$; $n = 12$		$C_{Ba} = 0.01$ M	
C_{Mn} (M $\times 10^6$)	$\bar{\nu}_{Mn}$ (no Ba^{2+})	$\bar{\nu}_{Mn}$ (obsd)	k_{Ba}'' (calcd)
1.75	2.00	0.69	185
9.47	7.20	3.01	183
23.99	11.50	6.53	101
55.70	16.25	7.99	180
			av 160

TABLE III:

Scheme of Manganese Binding to Glutamine Synthetase

A[a] $[E_{\bar{n}}{}^r]_{12} \rightleftharpoons [E_{\bar{n}}{}^t]_{12}$

B $[E_{\bar{n}}{}^t]_{12} + 12Mn^{2+} \overset{k_1}{\rightleftharpoons} [E_{\bar{n}}{}^t]_{12}\,Mn_{12}{}^{2+}$

C $[E_{\bar{n}}{}^t]_{12}\,Mn_{12}{}^{2+} + 12Mn^{2+} \overset{k_2}{\rightleftharpoons} [E_{\bar{n}}{}^t]_{12}\,Mn_{24}{}^{2+}$

D $[E_{\bar{n}}{}^t]_{12}\,Mn_{24}{}^{2+} + 48Mn^{2+} \overset{k_3}{\rightleftharpoons} [E_{\bar{n}}{}^t]_{12}\,Mn_{72}$

	pH	Temp (°C)	k_1'	k_2'	k_3'	ΔF_A[c] (cal)
			Data Summary for Two Enzyme Preparations[b]			
$E_{\overline{2.3}}$	8	24	1.5×10^7	4×10^4	500	−3500
$E_{\bar{9}}$	≥7.7	24	2.5×10^6	2×10^4	500	−2850
	7	4	1×10^5	1×10^4	500	−1250

[a] $[E_{\bar{n}}{}^r]_{12}$ represents relaxed enzyme (12 subunits) with $n = 0, 1, 2, 3, \ldots 12$ AMP, where $\bar{n}$ is the average extent of adenylylation per 600,000 g of protein; $[E_{\bar{n}}{}^t]_{12}$ represents taut enzyme which may exist in different conformations as a function of the state of adenylylation; equilibria A + B are functions of both $\bar{n}$ and pH since structural changes in $[E_{\bar{n}}{}^r]_{12}$ are induced also by a pH increase. [b] Dialysis buffers contained $^{54}Mn^{2+}$, 0.1 M KCl, and 0.02 M imidazole (pH 7) or 2-methylimidazole (pH > 7.6). [c] $\Delta F_A' \simeq -RT \ln k_1' + RT \ln k_2'$, if $\Delta F_B' \simeq \Delta F_C'$.

corresponds to the nonspecificity of this divalent cation by other criteria. Ba^{2+} is not an activating divalent cation (Kingdon *et al.*, 1968) and is not effective in reversing the pertubation of aromatic amino acids produced by the removal of specific divalent cations from the active taut form (Shapiro and Ginsburg, 1968), whereas the specific Mn^{2+}, Mg^{2+}, or Ca^{2+} ions are effective tightening agents by these criteria.

Figure 4 shows Hill plots of the Mn^{2+} binding data. The data are plotted according to the method of Wyman (1964) for comparative purposes. The saturation, $\bar{Y}$, was obtained by dividing the moles of Mn^{2+} bound per mole of enzyme, $\bar{\nu}$, at a free concentration of Mn^{2+}, c_{Mn}, by $n_1' + n_2'$ obtained from the Scatchard plots of Figures 1a and 2a. The total n' was 24 with the exception of $E_{\bar{9}}$ at alkaline pH which had $n_1' + n_2' = 18$ (see above). Each set of binding data shows symmetry about half-saturation and a right-hand displacement with the final asymptote of unit slope lying below the initial one. This type of Hill plot will result from nonidentical independent binding sites where the intrinsic association constant $k_1{}^0 \neq k_2{}^0$ or from identical sites with a negative interaction where the apparent $k_1' \neq k_2'$ (Wyman, 1967). The Mn^{2+} binding data can be interpreted most simply then as fitting the case where the observed apparent $k_1' \neq k_2'$, making no distinction between these two cases. The perpendicular distance between the asymptotes of unit slope, as shown by the arrows in Figure 4, is a function of the differences in Mn^{2+} affinity between the $E_{\overline{2.3}}$ and $E_{\bar{9}}$ enzyme preparations or between $E_{\bar{9}}$ at pH 7 and ≥7.7. Adenylylation of the glutamine synthetase decreases the apparent affinity of the high-affinity sites (arrow 1 in the saturation range of n_1' sites), whereas there is little or no effect of adenylylation on k_2' of the intermediate-affinity sites (arrow 2 in the saturation range of n_2' sites). The effect of increasing pH is considerably greater on the k_1' sites than on the k_2' sites as seen by comparing the lengths of arrows 3 and 4 in the n_1' and n_2' saturation range, respectively. These differential effects allowed the greater resolution of the n_1' and n_2' sites at the higher pH and/or with enzyme having the lower degree of adenylylation.

Discussion

The scheme presented in Table III summarizes the principal results from the equilibrium dialysis studies

with $^{54}Mn^{2+}$ at relatively high ionic strength and two preparations of glutamine synthetase ($E_{\overline{2.3}}$ and $E_{\overline{9}}$), and our interpretation of these results. There are three sets of resolvable and apparently independent Mn^{2+} binding sites (represented as equilibria B–D). These include 12 very high-affinity sites (B)/mole of enzyme (1/subunit), 12 sites of slightly lower affinity (C), and up to 48 relatively low-affinity sites (D). Varying degrees of adenylylation of the enzyme influence the apparent intrinsic association constant at the high-affinity site, k_1', but do not affect the affinity of the other sites for Mn^{2+} (k_2' or k_3'). Also, a change in pH effects k_1', without influencing significantly the binding parameters of the other sites.

Considered together with various kinetic data and the previously reported relaxation phenomena (Kingdon *et al.*, 1968; Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968), the results of these studies are consistent with the interpretation that the binding of the first 12 Mn^{2+} is associated with a relatively large conformational change of the protein. Kingdon *et al.* (1968) found that there was a time-dependent reactivation of inactive relaxed enzyme produced by the addition of specific divalent cations (Mn^{2+}, Mg^{2+}, or Ca^{2+}). The half-maximal amount of total Mn^{2+} required for the reactivation was found by these workers to be 2.5×10^{-5} for 9×10^{-7} M enzyme ($E_{\overline{9}}$). Also, the reactivation in the presence of near-saturating ATP, NH_3, and glutamate concentrations with Mg^{2+} is first order with respect to the concentration of non-adenylylated subunits (Shapiro and Ginsburg, 1968). This suggests a conformational change of nonadenylylated or Mg^{2+}-specific subunits, acting independently in catalysis (see Stadtman *et al.*, 1968). It has been established that at pH 7 and about 0.15 ionic strength the molecular weights of the relaxed $[E^r]_{12}$ and taut $[E^t]_{12}$ forms are the same (592,000) and that differences between the forms can be detected by hydrodynamic and spectral measurements (Shapiro and Ginsburg, 1968). Differences in the spectrum between relaxed and taut forms could be eliminated by adding certain divalent cations (Mn^{2+}, Mg^{2+}, or Ca^{2+}) to relaxed enzyme. The cation specificity and the kinetics of the changes in the spectrum were the same as were found for reactivation of relaxed enzyme (Kingdon *et al.*, 1968). The constant estimated for $^1/_2(\Delta\epsilon_{290.5\,m\mu})_{max}$ was 1.5×10^{-5} M Mn^{2+} for 3.33×10^{-6} M enzyme ($E_{\overline{9}}$). The k_1' value for $E_{\overline{9}}$ at pH 7 (Figure 1b) can be used to calculate the total concentration of Mn^{2+}, C_T, at half-saturation, $\bar{\nu} = 6$, if the protein concentration is known, where $C_T = C_F + C_B$ with C_F the free concentration and C_B protein-bound concentration of Mn^{2+}. Values at $\bar{\nu} = 6$ of $C_T = 1.5 \times 10^{-5}$ M Mn^{2+} and $C_T = 3.0 \times 10^{-5}$ M Mn^{2+} were calculated for the enzyme concentrations used in the reactivation and spectral studies, respectively. These values are sufficiently close to the observed values that the first 12 Mn^{2+} bound can be associated with the attendent conformational changes. Although the shapes of the saturation curves are changed by using total rather than the free concentration of Mn^{2+}, the midpoint shift is less significant.

It is of interest too that K'_m values for Mn^{2+} estimated by E. R. Stadtman[3] in the γ-glutamyl transfer assay with almost fully unadenylylated and fully adenylylated gultamine synthetase were similar in magnitude and to the $1/k'_1$ values determined here for $E_{\overline{2.3}}$ and $E_{\overline{9}}$, respectively. It seems probable therefore that the first 12 Mn^{2+} bound to the enzyme are directly involved in the relaxed to taut (or tightened) enzyme conversion and also play a direct catalytic role. The function of the second set of 12 Mn^{2+} ions with the intermediate affinity (equilibrium C in Table III) is more difficult of assess. However, excess Mn^{2+} in the biosynthetic assay is inhibitory[2] and perhaps these sites are involved in this secondary reaction. Alternatively, these sites of intermediate affinity may be directly concerned with the binding of ATP to form an enzyme–metal–ATP complex that is active in the catalytic reaction. This interpretation is consistent with the observation that the K'_m for ATP–Mn^{2+} is in the same range as $1/k'_2$. We believe then, that the Mn^{2+} binding sites of high affinity, and possibly also of the intermediate affinity, can be assigned a catalytic function. The relatively low-affinity sites for Mn^{2+} (equilibrium D of Table III) are probably involved in a gross stabilization of the enzyme structure since prolonged dialysis at 4°, ~pH 7.4, and low ionic strength against buffers with <1 mM Mn^{2+} or <1 mM Mg^{2+} results in some irreversible activity loss (Shapiro and Ginsburg, 1968).

The studies reported here suggest that each subunit has several independent, nonequivalent binding sites for Mn^{2+}. Further, each subunit appears to act independently of the others in the macromolecule in the uptake of Mn^{2+} cations. This is shown in Table III by the subscript 12 for the number of subunits per molecule of enzyme (Valentine *et al.*, 1968) and by multiples of 12 being associated with each B, C, or D. Changes in the state of adenylylation or in pH appear to involve alteration of the conformation of the entire enzyme molecule, which must be mediated by subunit interaction in such a way that all subunits are affected about equally. Thus, variations in pH or in the state of adenylylation modulate the conformation of relaxed and taut forms of the enzyme illustrated as equilibrium A (Table III) and thereby specifically affect the affinity of each subunit for Mn^{2+} at the first binding site (sum of equilibria A and B of Table III). The independent nature of the binding of Mn^{2+} ions implies that there are conformers intermediate between relaxed and taut states. These and previous studies on the reactivity of the sulfhydryl groups of glutamine synthetase at low levels of Mn^{2+} suggest that partially relaxed enzyme forms do exist (Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968).

As shown in Table III, the values of k'_1, and k'_2 approach each other as the pH is lowered from 8 to 7. This suggests that similar or identical functional groups may be involved in the binding of Mn^{2+} at both the n_1' and n_2' sites, illustrated by B and C in

[3] E. R. Stadtman, unpublished observations, which Dr. Stadtman kindly allows us to quote here.

Table III. If so, both kind of sites would be expected to have the same intrinsic binding constants and the marked differences in these constants observed at high pH would reflect changes in the conformation of the protein that could differentially affect the local environment at the separate sites. Since k_2' varies only slightly with pH whereas k_1 increases by at least one order of magnitude in going from pH 7 to 8, it is evident that the assumed alteration in conformation must specifically affect the binding of Mn^{2+} at the n_1' site, involved in B (Table III). Adenylylation also differentially influences the apparent affinity of the n_1' sites for Mn^{2+}, without any evidence that the site of adenylylation is involved directly in the binding mechanism. That is, the enzyme preparations with varying equivalents of AMP bind Mn^{2+} to the same extent. The $E_{\overline{2.3}}$ and $E_{\overline{9}}$ enzyme preparations have the same amino acid composition (see Methods) and presumably the same primary amino acid sequence. Adenylylation of the enzyme then must indirectly alter the configuration at the n_1' sites so that, as in the case of pH variations, the local environment of the n_1' and n_2' sites are differentially changed. If the intrinsic association constants for the binding of Mn^{2+} to the n_1' and n_2' sites are the same, the apparent intrinsic association constants, k_1' and k_2', will differ if these sites are sufficiently close together for interaction to occur (see Klotz, 1953). However, this cannot account for the observed differences in k_1' and k_2' entirely since both pH and adenylylation differentially influence k_1'.

As was discussed above, the binding of Mn^{2+} to the n_1' site appears to be associated with the conversion of the relaxed into the taut form of glutamine synthetase. It is therefore possible that the conformational changes responsible for the differences in k_1' and k_2' at high pH are the same as those involved in the interconversion of the relaxed and taut forms of the enzyme as illustrated by A in Table III. From the thermodynamic point of view and without regard as to the mechanisms involved, equilibrium B (Table III) might be considered to represent the over-all equilibrium obtained by the coupling of A with the binding of Mn^{2+} to the intrinsic n_1' binding site (*i.e.*, binding in the absence of pH-dependent conformational change). The apparent free energy of the Mn^{2+} binding can be calculated for each condition shown in Table III. As discussed above, the intrinsic binding constants for the n_1' and n_2' sites can be considered to be nearly the same, and then the relationship follows: $B \simeq A + C$. The change in free energy associated with A can therefore be calculated from k_1' and k_2' as follows, $\Delta F_A' = \Delta F_B' - \Delta F_C' = -RT \ln k_1' + RT \ln k_2'$. It should be stressed that the calculated values for $\Delta F_A'$ represent contributions in free-energy change only effected through the binding of the first 12 Mn^{2+} ions. Previously observed differences between relaxed and taut enzyme forms may not necessarily involve the same regions of the protein structure. That is, the exposure of sulfhydryl, tyrosyl, and tryptophanyl residues in the conversion of taut into relaxed enzyme (Shapiro and Stadtman, 1967; Shapiro and Ginsburg, 1968) do not necessarily involve the same free-energy change as represented by $\Delta F_A'$.

The magnitude of the association constants, k_1', k_2', or k_3', measured for Mn^{2+} with glutamine synthetase implicate a clustered arrangement of charged amino acids at the binding sites as proposed by Velick (1949) and Saroff and Lewis (Saroff, 1957a,b; Saroff and Lewis, 1963). Protons, as well as other cations, would be expected to compete with Mn^{2+} at these binding sites (Lewis and Saroff, 1957). The protonation of amino acid residues involved in these binding sites would effect n' and k' of the binding parameters (Saroff, 1957a; Saroff and Lewis, 1963). In the pH range studied, the apparent number of different affinity sites for Mn^{2+} did not decrease with decreasing pH and even the apparent association constants of the intermediate-and low-affinity sites remained unchanged. (It is of interest that from pH 7 to 8, the binding of Ca^{2+} ions to isoionic serum albumin was found by Saroff and Lewis (1963) to only increase from $\sim$1 to 2.6, the large increases in Ca^{2+} binding occurring well above pH 8.) For these reasons, it seems unlikely that the observed increase in k_1' is due to the discharging of amino acid residues directly participating in the n_1' binding sites.

The effect of increasing pH on k_1' is considered therefore to be an indirect effect which influences the magnitude of $\Delta F_A'$ ($\sim$1600-cal change/subunit from pH 7 to $\geq$7.7 in the case of $E_{\overline{9}}$; Table III). It seems probable that the pH effect is related to previous observations that a destabilization of the quarternary structure of the relaxed enzyme $[E^r]_{12}$ occurs as the pH is increased from pH 7.2 to >8 (Woolfolk and Stadtman, 1967; Shapiro and Ginsburg, 1968). It has been shown further that disaggregation of the enzyme is enhanced by 1 M urea at pH 8, whereas high ionic strength protects the relaxed enzyme against dissociating agents. Then, in these studies, the effect on k_1' of increasing pH at moderate ionic strength can be attributed to a loosening of the quarternary structure of the partially relaxed enzyme. (Mn^{2+} ions are never completely removed from the enzyme in the experiments reported here; see Methods.) The pH-induced structural change is associated with the discharging of at least two amino acid residues per subunit. Nitrogenous basis with pK's in the pH range of 7.2–8, or other groups with abnormal pK's, such as SH groups, could be implicated (Cohn and Edsall, 1943).

No significant physical–chemical differences were observed at pH 7 among relaxed preparations of glutamine synthetase of varying degrees of adenylylation (Shapiro and Ginsburg, 1968). Instead, the various taut enzyme forms appeared to exhibit subtle differences in hydrodynamic behavior. It should be recalled also that it is the taut active enzyme forms which exhibit dramatic differences in catalytic behavior (see Stadtman *et al.*, 1968). It is logical then to attribute the effect of adenylylation at pH 7 on $\Delta F_A'$ of Table III to different conformational states of the taut enzyme forms (assuming the k_1' of $E_{\overline{2.3}}$ to have the same pH dependence as the $E_{\overline{9}}$ preparation). However, these differences in the conformations of the taut enzyme forms

cannot have significant effect on the subsequent binding of Mn^{2+} (C and D of Table III) since k_2' and k_3' were not functions of the adenylylation state of the enzyme. From the data of Table III, it is readily apparent that the $\Delta F_A'$ change as a function of adenylylation is rather small, or only ~100 cal/equiv of covalently bound AMP as manifested by each subunit. This might be represented better as a 1200-cal change/AMP group for the dodecameric aggregate. $\Delta F_A'$, as estimated by the difference between k_1' and k_2', is potentially an index of rather subtle structural changes influencing the microenvironment of the Mn^{2+} binding sites. The hydrodynamic measurements only detect gross structural changes which may or may not directly involve the regions of charged clusters in the protein binding sites. However, the loss in n_1' and n_2' sites of $E_{\bar{9}}$ at pH $\geq$ 7.7 does suggest indirectly that the integrity of these sites requires the maintenance of the quarternary enzyme structure. This could be simply a decrease in k_1' and k_2', reflecting an inability of dissociated subunits to undergo conformational changes of the same magnitude as the native structure.

The competition experiments between Mg^{2+} and Mn^{2+} reported here for the $E_{\overline{2.3}}$ enzyme preparation indicated that the k_1' value for Mg^{2+} (A + B in Table III) is ~400-fold lower than that for Mn^{2+}. The electrostatic factors involved in Mn^{2+} and Mg^{2+} binding should be similar. The lower k_1' value for Mg^{2+} could be due to the intrinsic difference between the Mg^{2+} and Mn^{2+} ions themselves. Also, Mg^{2+} may induce less of a conformational change as defined by $\Delta F_A'$ in the relaxed to taut enzyme conversion. There is no way to evaluate the Mg^{2+} k_1' value further since the corresponding k_2' value is unknown.

The metal ion binding data should be considered together with the divalent cation specificity of glutamine synthetase. The Ba^{2+} ion which is not an activating divalent cation (Kingdon *et al.*, 1968) was found to have about the same k_1' values as k_3' (D in Table III) for Mn^{2+}. This suggests that Mn^{2+} or Mg^{2+}, which are activating cations, induce structural changes which are reflected in both k_1' and k_2'. The considerably less specific binding of Ba^{2+} ions is compatible too with the inability of this ion to dispel the spectral changes associated with relaxation (Shapiro and Ginsburg, 1968). It has been shown by Kingdon *et al.* (1967) that adenylylation of glutamine synthetase changes the specificity of the enzyme toward divalent cations. With subunits acting apparently independently in the biosynthetic assay, only adenylylated subunits are active with Mn^{2+} and, conversely, nonadenylylated subunits require Mg^{2+} (Stadtman *et al.*, 1968). It is tempting to speculate that the nonadenylylated form of the enzyme is "over tightened" to an inactive form with Mn^{2+}, since k_1' increases with decreasing adenylylation. Also, the lower affinity of $E_{\overline{2.3}}$ for Mg^{2+} could mean that the Mg^{2+} enzyme form is closer to the relaxed state than the Mn^{2+} conformation in that the Mg^{2+} ion is unable to induce the same structural changes as the Mn^{2+} divalent cation. The apparent affinity of $E_{\bar{9}}$ for Mg^{2+} in an assay system was even about two orders of magnitude lower than that estimated for $E_{\overline{2.3}}$ by binding measurements. The significance of that result cannot be evaluated as comparable data for the $E_{\overline{2.3}}$ preparation was not obtained. It is obvious that the subunits binding either Mn^{2+} or Mg^{2+} cannot be equivalent in the presence of the substrates, ATP, NH_3, and glutamate. However, both adenylylated and nonadenylylated subunits are catalytically active with Mn^{2+} in the γ-glutamyl transfer assay (Stadtman *et al.*, 1968). A variable response to substrate is observed with different extents of adenylylation and these effects are related to the specific divalent cation present.

The mechanism of the high-affinity Mn^{2+} binding to glutamine synthetase could involve a type of induced fit such as proposed by Koshland (1958), or the independent stabilization of relaxed and taut conformations which fluctuate per subunit to a degree dictated by the adenylylation state of the macromolecule. In either case, multiple conformational states are involved. No cooperativity was observed in the binding of the first 12 Mn^{2+} ions. That is, all 12 of these cations appear to be associated in a linear fashion with the over-all conversion of inactive relaxed into active taut enzyme. Then, the simple two-state allosteric model of Monod *et al.* (1965) or the more extended models of Rubin and Changeux (1966) and Koshland *et al.* (1966) do not adequately describe the activation of glutamine synthetase by divalent cations.

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