Some Kinetics of the Interaction of Divalent Cations with Glutamine Synthetase from *Escherichia coli*. Metal Ion Induced Conformational Changes†

J. B. Hunt‡ and A. Ginsburg*

ABSTRACT: The kinetics of the interaction of glutamine synthetase from *Escherichia coli* with activating and stabilizing divalent cations have been investigated. The time course of proton release during the binding of Mn^{2+}, Mg^{2+}, Co^{2+}, or Zn^{2+} to glutamine synthetase was followed by ΔpH measurements, while metal ion induced conformational changes in the enzyme structure were monitored by ultraviolet difference spectral measurements. Four kinetically distinct reactions were observed in ΔpH measurements at pH ≃7.2 and 37°. (I) The binding of 1 molar equiv of Mn^{2+} ($K_A' \simeq 2 \times 10^6$ M^{-1}) or of Mg^{2+} ($K_A' \simeq 2 \times 10^4$ M^{-1}) per subunit of the dodecameric enzyme releases 2 proton equiv: one too fast to measure here and the other in a slow first-order process with a half-time of 31 sec. (The observation agrees qualitatively with the calorimetric results of Hunt, J. B., Ross, P. D., and Ginsburg, A., *Biochemistry 11*, 3716 (1972).) The rate of the slow proton release corresponds to the rate of the Mn^{2+}- or Mg^{2+}-induced ultraviolet spectral perturbation of glutamine synthetase at saturating concentrations of these divalent cations, with $t_{1/2}$ = 29 sec at 37° for the latter process. An Arrhenius activation energy of 21 kcal (mole of enzyme subunit)$^{-1}$ for the metal ion induced absorbancy change at 290.3 nm was obtained. (II) After 12 molar equiv of Mn^{2+} is bound to the enzyme, the binding of a second Mn^{2+} to a lower affinity site of the subunit ($K_A' \simeq 2 \times 10^4$ M^{-1}) causes a fast release of a single proton, without an accompanying ultraviolet spectral perturbation. (III) Zn^{2+} appears to bind with high affinity ($K_A' \geq 10^6$ M^{-1}) to sites other than the high-affinity Mn^{2+} sites and causes the release of only one proton per enzyme subunit, with $t_{1/2} \simeq 5$ sec at 37° for ΔpH. (IV) When Co^{2+} binds to the enzyme ($K_A' = 5 \times 10^4$ M^{-1}), two protons are displaced instantaneously per enzyme subunit. Mn^{2+} competitively displaces Co^{2+} from the enzyme in a rapid reaction, without proton release or uptake, suggesting that Co^{2+} binds to the same high affinity sites that bind Mn^{2+}. There is a small rapid decrease, followed by a slow increase, in the absorbancy at 290 nm upon the addition of Co^{2+} to the divalent cation-free enzyme. The subsequent addition of Mn^{2+} to the enzyme in the presence of Co^{2+} results in a slow increase in the ultraviolet difference spectrum until that of the enzyme plus Mn^{2+} alone is achieved. Thus, the binding of divalent cations to glutamine synthetase in these cases clearly precedes metal ion induced conformational changes. Despite the marked ultraviolet difference spectra (260–310 nm) induced by Mn^{2+} or Mg^{2+} or by Zn^{2+} or Co^{2+}, saturating concentrations of these divalent cations produced no detectable changes in the optical rotatory dispersion spectrum (217–270 nm) of unadenylylated glutamine synthetase. Circular dichroism measurements from 200 to 240 nm indicated that the native or the divalent cation-free enzyme has an α-helical content of 36%, with an approximated 24% of the configuration in β-pleated-sheet structures. The tryptophanyl and tyrosyl residues perturbed in the metal ion induced conformational change (activation) of glutamine synthetase apparently are located near the enzyme surface so that no major net changes occur in the structural configuration of the protein in this process. Circular dichroism measurements also indicated that a loss in quaternary structure does not necessarily involve α-helical regions. Rather, a loss in β structures was observed after dissociation of the dodecamer into subunits by treatment of the enzyme with base, whereas 6 M guanidine-HCl treatment converts the subunits to the expected random-coil configuration.

This paper describes some kinetic studies of the interaction of specific divalent cations with glutamine synthetase from *Escherichia coli*. These studies are an extension of those reported in the accompanying paper of Hunt *et al.* (1972), to which the reader is referred for pertinent background information.

The ultraviolet spectral perturbation induced by Mn^{2+} or Mg^{2+} interacting with glutamine synthetase was first observed in the studies of Shapiro and Ginsburg (1968). This property has been used in our present studies to obtain kinetic constants specifically for the metal ion induced conformational change in the enzyme. The calorimetric studies (Hunt *et al.*, 1972) showed that two proton equivalents were released from the enzyme in the binding of each molar equiv of Mn^{2+} or Mg^{2+}. This has been confirmed in the direct measurements of proton release presented here.

An unusual interaction of glutamine synthetase with Zn^{2+} has been explored recently by Miller *et al.* (1972a,b). With one molar equiv of Zn^{2+} per enzyme subunit bound, Zn^{2+} (with 50 mM $MgCl_2$ present) quantitatively precipitates the enzyme, and this has been used to develop a simple purification procedure for glutamine synthetase (Miller *et al.*, 1972a). Zn^{2+} is capable also of partially activating the unadenylylated enzyme form in the γ-glutamyl transfer assay (Miller *et al.*, 1972b). Segal and Stadtman (1972a,b) have shown recently that Co^{2+} stabilizes yet another conformation of unadenylylated glutamine synthetase, and is effective also in activating this form of the enzyme. With Co^{2+} activation, it was possi-

† From the Laboratory of Biochemistry, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received April 10, 1972.*

‡ Department of Chemistry, The Catholic University of America, Washington, D. C. 20017. Recipient of a National Heart and Lung Institute Special Research Fellowship award, Sept 1970–May 1971.

ble to demonstrate heterologous interactions between unadenylylated and adenylylated subunits in hybrid glutamine synthetase molecules (Segal, 1971; Segal and Stadtman, 1972a). Measurements of proton release from the enzyme produced by the binding of Zn^{2+} or Co^{2+} to unadenylylated glutamine synthetase are reported here also.

Materials and Methods

Glutamine Synthetase Preparations. The preparation of unadenylylated glutamine synthetase ($E_{\bar{1}}$)[1] and the glutamine synthetase assays used are described in the accompanying paper of Hunt *et al.* (1972). The same procedures were used also for completely removing divalent cations from the taut enzyme, and for the following removal of EDTA from the relaxed (divalent cation free) enzyme preparation. The fully adenylylated enzyme (E_{12}) preparation was obtained by enzymatic adenylylation of glutamine synthetase *in vitro*, as described by Ginsburg *et al.* (1970). If necessary, enzyme solutions were filtered through Millipore filters (0.45 μ) before use. Protein concentrations were determined from absorbancy measurements at 290 and 280 nm (Ginsburg *et al.*, 1970). In calculations, the native or relaxed enzyme at pH 7.2 was assumed to have a molecular weight of 600,000 (Shapiro and Ginsburg, 1968); $^1/_{12}$th of this value (or 50,000) was assumed for the molecular weight of the subunits of glutamine synthetase (Valentine *et al.*, 1968).

Reagents. Reagent grade hydrated $MnCl_2$, $MgCl_2$, $CoCl_2$, $ZnCl_2$, and $ZnSO_4$ were the sources of the divalent cations used. Sodium dodecyl sulfate and Ultra Pure grade guanidine-HCl were obtained from Mann Research Laboratories. Buffers were prepared as indicated in Hunt *et al.* (1972). All other compounds used were reagent grade.

Difference Spectral Measurements. All spectra were recorded using a Cary Model 15 spectrophotometer equipped with the 0–0.1 and 0–1 absorbance slide-wires. The sample cell used in most experiments was a water-jacketed silica cell (1 ml) of 1-cm light path. The temperature of the cell was controlled to $\pm 0.1°$ by circulating water from a constant-temperature bath (Haake) and the temperature of the cell contents was monitored by measuring the resistance of an immersed Teflon-coated thermistor. In general, difference spectra were recorded using as the reference a relaxed enzyme solution of the same concentration as the sample. A base line for the difference spectrum was established immediately prior to the run by filling both sample and reference cells with the reference enzyme solution. With EDTA and/or guanidine-HCl, a tandem cell arrangement was used to null small absorption contributions from the solvent.

Kinetic Measurements of Absorbancy Changes. The water-jacketed cell described above served as the reaction vessel for the spectrophotometric measurement of the rates of divalent cation-induced tightening and EDTA-induced relaxation of glutamine synthetase. The synchronous motor of the Cary Model 15 was used to measure absorbancy changes as a function of time. The plastic tubing connecting the cell to the water bath had sufficient slack to allow the cell to be removed from the sample compartment for cleaning, drying, and filling. A 1-ml sample of the enzyme solution was pipetted into the cell and allowed to stand about 5 min to achieve temperature equilibrium. The effector solution (1–10 μl of the divalent cation or EDTA solution) was added by means of a micropipet, the Teflon stopper was replaced, and the solutions were mixed by inverting the cell. Recording of the absorbance of the sample was begun about 6 sec after the addition of the effector solution. In most kinetic runs, neutral density screens were used in the reference compartment in place of a reference enzyme solution.

pH Measurements. A Radiometer Model PHM 25 meter equipped with a scale expander and a fast responding, combination microelectrode (A. H. Thomas Co., no. 4858-L25) was used for pH measurements. A Honeywell Electronik 19 strip-chart recorder was used in conjunction with the pH meter to follow the pH changes accompanying the binding of divalent cations to glutamine synthetase. A pH change of 0.1 unit was displayed full scale on the recorder, and a change of 0.001 pH could be resolved easily. The reaction vessel used for the kinetics of pH change was a cylindrical, water-jacketed glass vessel fitted with a plastic stopper, through which the electrode and a hypodermic needle could be inserted. The contents of the vessel were stirred with a small Teflon-coated stirring bar; 1 ml of solution was required to cover the active surface of the electrode and allow for motion of the stirring bar. The temperature of the solution was kept constant to $\pm 0.1°$ by circulating water from a constant temperature bath. In measurements of the pH change accompanying the binding of divalent cations by glutamine synthetase, 1 ml of the relaxed enzyme solution was pipetted into the clean, dry reaction vessel, the electrode and stopper were inserted, and the solution was stirred for 5 min or longer to allow temperature and electrode equilibration at 37°. A steady pH reading was taken as evidence that equilibrium had been established, and the divalent cation solution was not added until a drift rate of less than 0.0005 pH unit in 5 min had been attained. Divalent cation solutions were added using Hamilton microsyringes, since it was discovered that the use of Lang–Levy pipets for this purpose introduced CO_2 into the enzyme solution, causing large pH changes as the CO_2 was first adsorbed and then desorbed.[2] The addition of the divalent cations solution and mixing of the solutions required less than 5 sec, as evidenced by the fact that the new equilibrium pH was established within this time after the addition of a similar aliquot of dilute acid to the enzyme solution in the reaction vessel.

It was found that solutions of Zn^{2+} prepared by dissolving $ZnCl_2$ or $ZnSO_4$ in the same Tris-chloride buffer with which the relaxed enzyme had been equilibrated gave a solution of pH significantly lower than that of the enzyme solution.[3] The pH of the Zn^{2+} solution was carefully adjusted to match that of the enzyme solution by the addition of base. Solutions of Mn^{2+}, Co^{2+}, and Mg^{2+} prepared by dissolving their salts in Tris or Hepes buffers differed by less than 0.01 pH

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: $E_{\bar{n}}$, for glutamine synthetase preparations containing the subscript, ($\bar{n}$), average number of equiv of 5′-adenylic acid groups covalently bound per mole (600,000 g) of enzyme, where the extent of adenylylation can vary from $n = 0$ to 12 (Kingdon *et al.*, 1967); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, is a buffering compound, the properties of which are described by Good *et al.* (1966).

[2] To determine whether dissolved CO_2 was having an effect on the pH change accompanying the addition of divalent cations to the enzyme, some experiments were performed using a nitrogen atmosphere and solutions which had been purged of CO_2 with N_2 gas. The results from these experiments did not differ significantly from those done without the use of a nitrogen atmosphere.

[3] At pH 7.3, the ratio of $[ZnOH^+]:[Zn^{2+}] \simeq 0.022$ is calculated from the stability constant (Sillén, 1964) reported for 25° and zero ionic strength. In the experiments reported here at about 0.1 ionic strength and 37°, $[ZnOH^+]$ formation is negligible.

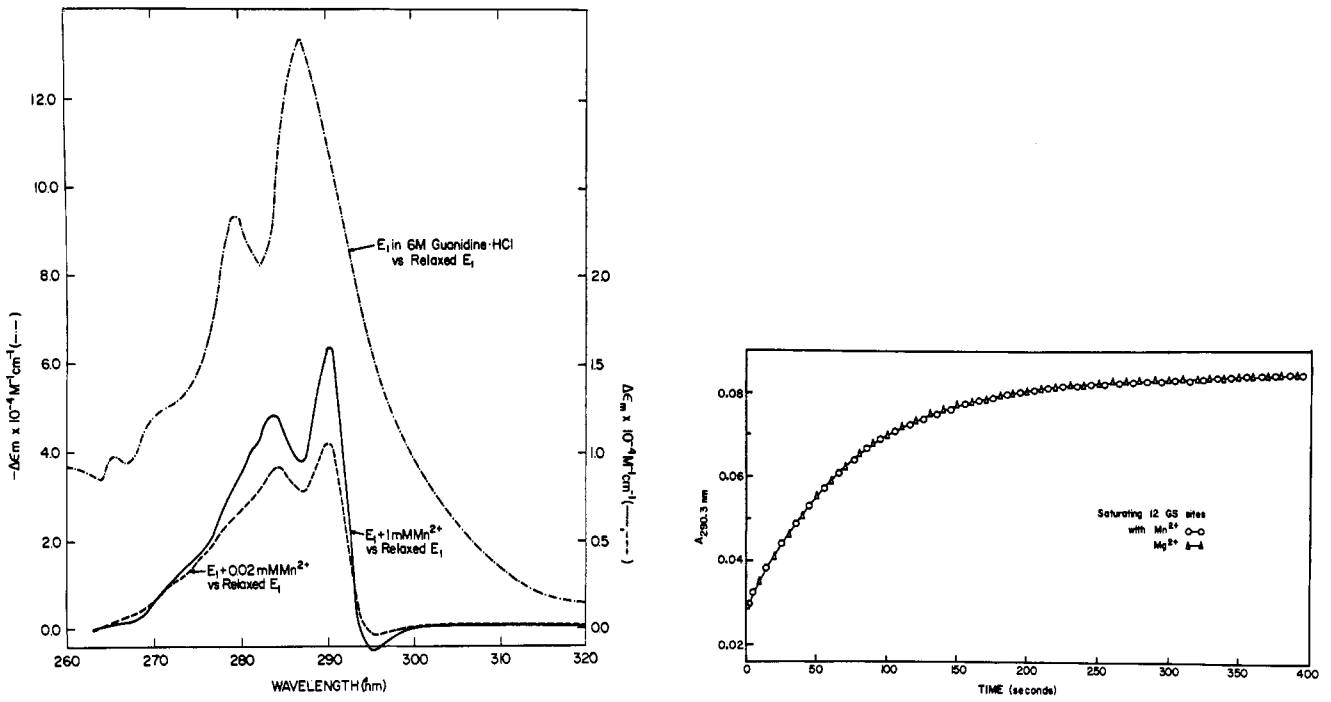

FIGURE 1: (a, left) Ultraviolet difference spectra at 25° for unadenylylated glutamine synthetase (E_1) with Mn^{2+} added to relaxed enzyme and for the relaxed enzyme in 6 M guanidine-HCl *vs.* the relaxed enzyme at the same concentrations. The concentration of the enzyme in the tightening experiments with Mn^{2+} added was 1.67 mg in 1 ml of 0.05 M Tris–0.1 M KCl buffer at pH 7.2. The difference spectrum for E_1 plus a saturating (1 mM Mn^{2+}) or subsaturating (0.02 mM Mn^{2+}) concentration of $MnCl_2$ is shown by the solid or dashed curve, respectively. The curve (–·–·–·–·) shows the difference spectrum of E_1 (0.25 mg/ml) in 6 M guanidine-HCl *vs.* divalent cation-free enzyme. Note the opposite sign of the changes in the molar extinction coefficients, which were calculated per mole of enzyme of 600,000 molecular weight, and the different scales on the ordinates. The difference spectra are shown after completion of time-dependent spectral changes (Shapiro and Ginsburg, 1968). (b, right) The time course of the absorbancy change at 290.3 nm ($A_{290.3\ nm}$) for the tightening of a relaxed preparation of unadenylylated glutamine synthetase (GS) at 31° and pH 7.2 with either Mn^{2+} (1 mM) or Mg^{2+} (4.5 mM). About 6 sec before the recording of changes in $A_{290.3\ nm}$, either Mn^{2+} (○) or Mg^{2+} (△) was added to the relaxed enzyme (2.0 mg of E_1 in 1 ml of 0.05 M Tris–0.1 M KCl buffer at pH 7.2).

from the enzyme solution, so that it was not necessary to adjust the pH of these solutions.

Treatment of Kinetic Data. First-order rate constants were obtained from the absorbance–time and pH–time data by means of a nonlinear least-squares computer fit of the data to a first-order rate equation. The absorbance–time data were fitted to the rate equation $A_t = (A_0 - A_\infty)e^{-kt} + A_\infty$, where A_t is the absorbance value measured at the time t, A_0 is the absorbance value at zero time, A_∞ is the absorbance value at infinite time, and k is the first-order rate constant. The computer program used treats A_0, A_∞, and k as adjustable parameters. The absorbance–time data and initial approximations of A_0, A_∞, and k (which need not be exact) are furnished to the computer, and the computer calculates the set of values of A_0, A_∞, and k which give the best fit between the data and the rate equation. The rate equation used to obtain first-order rate constants from the pH–time data was $(pH)_t = [(pH)_\infty - (pH)_0]e^{-kt} + (pH)_\infty$, where the subscripts have the same meaning as above.[4]

Optical Rotatory Dispersion and Circular Dichroism Measurements. Optical rotatory dispersion and circular dichroism (CD) spectra were recorded using a Cary Model 60 spectropolarimeter equipped with a Model 6001 CD attachment. All spectra were recorded at 27°, using fused silica cells, usually of 1 or 2-mm light path. A base line was established immediately prior to the recording of each spectrum, using the same cell and buffer solution as used in recording the spectrum.

Sedimentation Velocity Measurements. A Spinco Model E ultracentrifuge equipped with a photoelectric scanning absorption optical system (Schachman and Edelstein, 1966) was used at 290 nm in conjunction with a Kel-F-coated double-sector cell for the sedimentation of adenylylated glutamine synthetase (0.8 mg/ml) in 6 M guanidine-HCl at 24° (60,000 rpm). The values of Kawahara and Tanford (1966) for the viscosity and density of 6 M guanidine-HCl were used in preparing solutions and in corrections of the sedimentation coefficients.

Results

Ultraviolet Spectral Perturbations Induced by Mn^{2+} or Mg^{2+}. Previous studies of Shapiro and Ginsburg (1968) showed that the addition of Mn^{2+} or Mg^{2+} to relaxed (divalent cation free) glutamine synthetase produces the difference spectrum illustrated by the solid curve in Figure 1a. Relaxation caused by adding EDTA to this tightened form or to native enzyme produces the inverse difference spectrum when the reference is a solution of the native enzyme. A conformational change in the enzyme structure involving aromatic chromophores is indicated by the kinetics of the Mn^{2+}- or Mg^{2+}-induced special perturbation, as discussed below (Figure 1b and Table I). The shape of the difference spectrum suggests that approximately equal numbers of tryptophanyl and tyrosyl residues are shifted from a relatively polar to nonpolar en-

[4] This treatment involves the approximation that the observed pH change is directly proportional to the concentration of released protons, an approximation which seemed valid over the small pH interval involved in these experiments. Doubling the quantity of dilute strong acid added to the enzyme solution doubled the accompanying pH change. The same approximation was used in calculating the number of protons released per divalent cation bound to the enzyme.

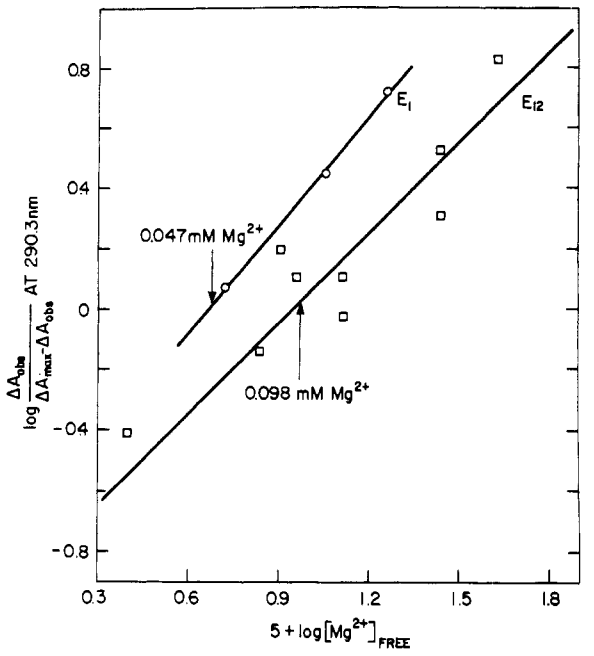

FIGURE 2: Hill plots of the spectrophotometric titrations of relaxed unadenylylated ($E_{\bar{I}}$) or adenylylated (E_{I2}) glutamine synthetase with Mg^{2+}. The free concentration of Mg^{2+} was calculated assuming that the fraction of enzyme bound to Mg^{2+} was equal to $\Delta A_{obsd}/\Delta A_{max}$, where ΔA_{obsd} and ΔA_{max} are the observed change in absorbancy at 290.3 nm at a given Mg^{2+} concentration and the maximum change in absorbancy observed at saturating concentrations of Mg^{2+}, respectively. The ratio of enzyme-metal ion complex to free enzyme is assumed to be the ratio: $\Delta A_{obsd}/(\Delta A_{max} - \Delta A_{obsd})$. Apparent half-saturating concentrations of free Mg^{2+} with $E_{\bar{I}}$ and E_{I2} are indicated at the arrows. The data were obtained at 37° for $E_{\bar{I}}$ and at 25° for E_{I2}, with about 2-mg/ml concentrations of enzyme in 0.05 M Tris–0.1 M KCl at pH 7.2 in both cases.

vironment in the tightening process. The ultraviolet difference spectrum produced by relaxation or by adding Mn^{2+} or Mg^{2+} to relaxed enzyme is independent of the extent of adenylylation of the glutamine synthetase preparation (Ginsburg *et al.*, 1970). In the present studies, it has been established that the difference spectral change is associated with the binding of just 12 molar equiv of Mn^{2+} or of Mg^{2+}; adding an excess of either of these metal ions to relaxed enzyme produces no further change in the difference spectrum.

Relaxation of glutamine synthetase produces a very small blue shift from about 278.0 to 277.5 nm in the peaks of the ultraviolet absorption spectrum. Comparing the difference spectra of unadenylylated enzyme that are produced by subsaturating and saturating Mn^{2+} concentrations (Figure 1a), there appears to be a slight red shift in the maximum absorbance change at 290 nm with increasing saturation with Mn^{2+}. This suggests that some subunit interaction occurs. With less than 12 molar equiv of Mn^{2+} or Mg^{2+} (not shown) bound to the enzyme, however, the ratio of the absorbance change at the peak (290 nm) to that at the shoulder (284 nm) is the same as it is with saturating concentrations of these metal ions.

In order to compare the difference spectrum produced by tightening to that obtained by a complete normalization of tyrosyl and tryptophanyl residues (Edelhoch, 1967), the spectrum of the relaxed enzyme in 6 M guanidine-HCl *vs.* the relaxed enzyme is shown also in Figure 1a. (Note that the difference extinction coefficients are opposite in sign and are plotted on different scales in Figure 1a.) Removing Mn^{2+} or Mg^{2+} from glutamine synthetase gives about 10% of the maximum perturbation obtained by denaturing the enzyme with 6 M guanidine-HCl. The spectrum of the denatured enzyme had a maximum absorbance (λ_{max}) at 275.5 nm and a minimum absorbance (λ_{min}) at 249.5 nm, which are to be compared to λ_{max} = 277.5 nm and λ_{min} = 250.0 nm for the relaxed enzyme. The number of tryptophanyl residues was calculated to be 2.7 by the procedure of Edelhoch (1967), using either the absorption spectrum of the enzyme in 6 M guanidine-HCl or the difference spectrum produced by 6 M guanidine-HCl (Figure 1a). The amount of tryptophanyl residue exposure brought about by the removal of Mn^{2+} or Mg^{2+} from the enzyme in the relaxation process therefore cannot involve more than a partial exposure equivalent to about 30% of one tryptophanyl residue per subunit. Estimation of the tyrosyl contribution to the difference spectra of Figure 1a is more difficult.

Preparations of unadenylylated glutamine synthetase which had lost Mg^{2+}-dependent biosynthetic activity exhibited a proportionate decrease in the magnitude of the ultraviolet difference spectrum obtained by relaxation and tightening of the relaxed enzyme. Accordingly, the results reported here are only from spectral studies with enzyme that was fully active after tightening.

A time-dependent absorption increase at 290 nm in the tightening process previously was found to be specific for Mn^{2+}, Mg^{2+}, or Ca^{2+} (Shapiro and Ginsburg, 1968). These are the same divalent cations which were found to be effective in activating the relaxed enzyme (Kingdon *et al.*, 1968). Figure 1b shows the time course at 31° of the absorbance change at 290.3 nm which occurs when sufficient Mn^{2+} or Mg^{2+} is added to the relaxed enzyme to saturate 12 high-affinity sites. With the same enzyme concentration for the tightening experiments of Figure 1b, it is evident that the data points for the two metal ions are fit very well by a single curve. Thus, both the total absorbance change and the time course of the change are the same for the two metal ions at saturating concentrations.

The absorbance change at 290.3 nm has been used also to construct the Hill plots of Figure 2, from which the binding constant for the interaction of Mg^{2+} with the high-affinity sites of the enzyme was obtained. In the construction of the plots of Figure 2 it is assumed that the extent of absorbance increase upon addition of the metal ion is directly proportional to the quantity of metal ion bound to the high-affinity sites. Although the points for the adenylylated enzyme (E_{I2}) preparation in Figure 2 fit a slope of 1, there is considerable scatter in the points and therefore a possible error in the slope. Better data were obtained at 37° with the unadenylylated enzyme ($E_{\bar{I}}$) preparation, and these points fit a slope of 1.2. Obtaining a slope greater than one in this case suggests that there is some cooperativity in the Mg^{2+}-induced spectral perturbation of the unadenylylated enzyme. However, the half-saturation value of 0.047 mM Mg^{2+} will not be influenced significantly by this small degree of cooperativity.

The time course of the absorbance increase at 290.3 nm during the binding of Mg^{2+} and Mn^{2+} was used to obtain the first-order rate constants for the conformational change responsible for the absorbance increase (see Methods). The half-times for the conformational change are given in Table I for three temperatures and for saturating and half-saturating concentrations of the divalent cations. As already noted above, the half-times for the tightening of the enzyme are the same for saturating concentrations of Mn^{2+} and Mg^{2+}. However, the tightening of the unadenylylated ($E_{\bar{I}}$) enzyme is substantially slower when half-saturating, rather than saturating,

TABLE I: Kinetics of Conformational Change of Glutamine Synthetase on Addition and Removal of Metal Ions.[a]

Glutamine Synthetase Prepn	Temp (°C)	Metal Ion Added (Tightening Process)	$t_{1/2}$ (sec)[b] At Half-saturating Concn of Metal Ion	At Saturating Concn of Metal Ion
Unadenylylated glutamine synthetase ($E_{\bar{1}}$)	25	Mg^{2+}	178	107 ± 5
		Mn^{2+}	155	110 ± 5
	31	Mg^{2+}	77	55 ± 5
		Mn^{2+}	64	53 ± 5
	37	Mg^{2+}	47	28 ± 2
		Mn^{2+}	37	29 ± 2
Adenylylated glutamine synthetase (E_{12})	25	Mg^{2+} Mn^{2+}	133[c]	
	31	Mg^{2+} Mn^{2+}	56[c]	
	37	Mg^{2+} Mn^{2+}	34[c]	
		Relaxation with EDTA[d]		
Unadenylylated glutamine synthetase ($E_{\bar{1}}$)	25			~44
	31			~30
	37			~9

[a] From absorbancy change at 290.3 nm with 2 mg of glutamine synthetase per ml of 0.05 M Tris-Cl–0.10 M KCl buffer (pH 7.2). [b] Each half-time value is an average calculated from first-order rate constants determined from analyses of several experimental curves (see Figure 1b) by the nonlinear least-squares computer program (see Methods). For half-saturation with metal ion, $Mg^{2+} \simeq 0.075$ mM and $Mn^{2+} \simeq 0.020$ mM; for saturation with metal ion, $Mg^{2+} = 7.5$ mM and $Mn^{2+} \simeq 1.0$ mM. [c] With E_{12}, there was no apparent dependence of $t_{1/2}$ on the amount of either Mg^{2+} or Mn^{2+} present. [d] Approximately threefold excess EDTA was added to freshly tightened enzyme in each case. Relaxation rates were more variable than were rates of tightening (see text).

concentrations of the divalent cations are used, although the process is still first order. The effect of using half-saturating, rather than saturating, concentrations is somewhat more pronounced for Mg^{2+} that for Mn^{2+}.

In contrast to the rates observed with the unadenylylated enzyme, the half-time for tightening the fully adenylylated (E_{12}) at the different temperatures appears to be independent of the divalent cation concentration. This also was observed in previous tightening experiments with Mn^{2+} additions to a partially adenylylated enzyme ($E_{\bar{9}}$) preparation (Shapiro and Ginsburg, 1968). From the entries in Table I, the half-times for tightening the adenylylated enzyme appear to be slightly longer, but no more than 30% longer, than those observed for $E_{\bar{1}}$ at saturating concentrations of the divalent cation.

Half-times for the relaxation of the enzyme by EDTA treatment, which were obtained from first order decreases in the absorbancy at 290.3 nm, are given also in Table I. There was considerably more variability in the relaxation rates than in tightening rates, with the variations seeming to depend both on the enzyme preparation and on the length

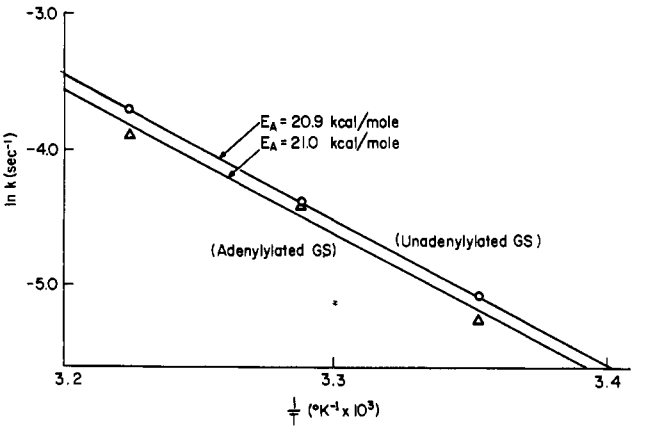

FIGURE 3: Arrhenius plots of the data presented in Table I with saturating concentrations of divalent cation present during the tightening of unadenylylated (○) or adenylylated (△) glutamine synthetase (GS). The logarithm of first order rate constants measured at 25, 31, and 37° are plotted against the reciprocal of the absolute temperature. The activation energy calculated from the slope is indicated by E_A.

of 4° storage of the enzyme–metal ion complex. The entries are for freshly tightened, fully active enzyme ($E_{\bar{1}}$) which gave fairly reproducible rates of relaxation upon EDTA additions.

All of the data of Table I were obtained using Tris buffer at pH 7.2. Measurements of tightening rates in Hepes and imidazole buffers at about the same pH as used in Table I gave half-times for the tightening process very similar to those listed in Table I. Arrhenius plots of the data from Table I for the tightening process are shown in Figure 3.

Proton Release during the Binding of Mn^{2+} or Mg^{2+}. Figure 4 shows the time course of the pH change accompanying the binding of Mn^{2+} or Mg^{2+} to unadenylylated enzyme in dilute Tris buffer (3 mM) at pH ~7.2 and 37°. In the absence of enzyme, the addition of Mn^{2+} to this buffer produced a negligible pH change as shown by the top curve of Figure 4. The addition of protons to the enzyme solution (24 moles of

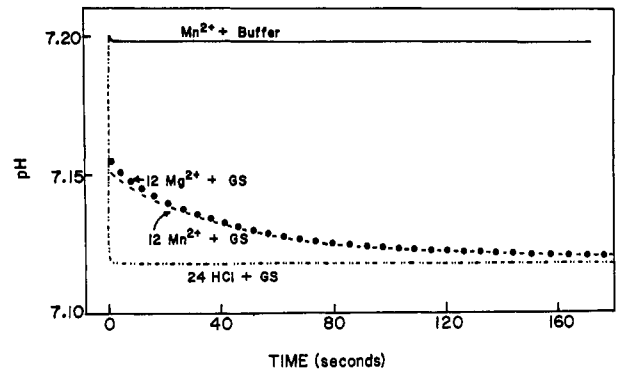

FIGURE 4: Proton release during the binding of Mn^{2+} or Mg^{2+} to the 12 high-affinity sites of glutamine synthetase at 37°. A relaxed preparation of unadenylylated glutamine synthetase (2.3 mg of $E_{\bar{1}}$ in 1 ml of 3 mM Tris–0.1 M KCl buffer) was equilibrated at 37° in a water-jacketed vessel with magnetic stirring until a constant pH was recorded. In separate experiments, 5 μl of 1.18×10^{-2} M $MnCl_2$ (----), 5 μl of 0.10 M $MgCl_2$ (●), or 5 μl of 1.84×10^{-2} M HCl (-·-·-·-) was injected into the protein solution with a calibrated syringe at zero time, and the time course of the pH change was followed with a Honeywell Electronik Model 19 recorder. An experiment in which the same amount of $MnCl_2$ was injected into 1 ml of buffer without enzyme is shown by the top solid curve.

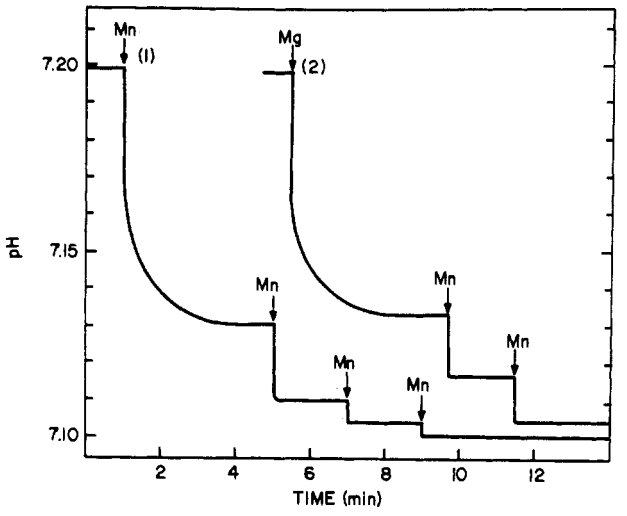

FIGURE 5: Proton release during the saturation of a first and second set of 12 binding sites of glutamine synthetase with Mn^{2+} at 37° (curve 1). These experiments were performed as described in the legend to Figure 4, with the same amount of unadenylylated enzyme (4.6×10^{-5} M E_I subunits) in curves 1 and 2. The first aliquot of metal ion added in curve 1 or 2 was just sufficient to saturate the 12 high-affinity sites of the enzyme. In curve 1, 5 μl of 1.18×10^{-2} M $MnCl_2$ was injected into the relaxed enzyme solution at 1 min; after an equilibrium pH was attained at 5 min, a second 5-μl aliquot of the stock $MnCl_2$ solution was added. Successive 5-μl aliquots of the same $MnCl_2$ solution were added to the protein at 7 and 9 min. In curve 2, 5 μl of 0.10 M $MgCl_2$ was first added to the relaxed enzyme; after 4 min and 6 min, 5-μl aliquots of 1.18×10^{-2} M $MnCl_2$ were injected into the protein solution, as indicated by the arrows.

HCl/mole of enzyme in the bottom curve of Figure 4) produced a rapid drop in pH, the final pH being attained within the time of mixing.[5] Upon the addition of only sufficient Mn^{2+} or Mg^{2+} to ensure at least 96% saturation of the 12 high-affinity sites of the enzyme molecule, there was a rapid decrease in pH which accounted for about 55% of the total pH change. This was followed by a slow first-order decay to the final pH value shown in Figure 4. It is evident from Figure 4 that the pH change accompanying the binding of 12 molar equiv of Mn^{2+} (or Mg^{2+}) to the relaxed enzyme was nearly as great (>95%) as that caused by the addition of 24 molar equiv of H^+. These results indicate that approximately two protons are released from the enzyme for each Mn^{2+} or Mg^{2+} ion bound, one proton being released in a step complete within the time of mixing (<3–5 sec) whereas the second proton is released in a slow, first-order step. The quantities of metal ions added in the experiments illustrated in Figure 4 were not sufficient to bind to other than the 12 activating metal ion binding sites of the glutamine synthetase molecule.

Although the curves of Figure 4 indicate slightly different kinetics with Mn^{2+} or Mg^{2+} the time course of the pH changes averaged for a number of experiments at 37° was the same for the binding of either Mn^{2+} or Mg^{2+} to the high-affinity sites of glutamine synthetase. The average half-time observed for the slow pH change during the binding of Mn^{2+} or Mg^{2+} to the unadenylylated enzyme at 37° was 31 sec.

Experiments exactly analogous to those illustrated for the Tris buffer in Figure 4 were performed at 37° in dilute Hepes buffer (3 mM Hepes–0.1 M KCl at pH 7.2), and the results were strikingly similar to those obtained in the Tris buffer. The binding of the first 12 Mn^{2+} or Mg^{2+} gave about 96% of the total pH change expected if two protons are released for each divalent cation bound. About 55% of the pH change occurred within the time of mixing, and the rest of the pH change was slow and first order with a half-time of about 30 sec at 37°.

There was a direct correlation between the potential Mg^{2+}-dependent biosynthetic activity of E_I and the magnitude of of the pH change produced by Mn^{2+} or Mg^{2+} binding. With one-third of the potential activity lost, only two-thirds equiv of H^+ per subunit was released in each a fast and a slow step when Mn^{2+} or Mg^{2+} was added to saturate each enzyme subunit. It appears that the catalytically inactive protein is without high-affinity binding sites for metal ions. This alone can account for activity loss, since the activation of glutamine synthetase depends upon Mn^{2+} or Mg^{2+} binding to these sites (Kingdon *et al.*, 1968; Denton and Ginsburg, 1969). A partial loss of metal ion binding sites was observed also in equilibrium dialysis experiments at 24° with a E_I preparation at pH 7.7, at which pH relaxed glutamine synthetase is unstable (Denton and Ginsburg, 1969). Since metal ions do not bind to catalytically inactive protein, the magnitude of the ultraviolet difference spectrum obviously will be affected also. A fully active enzyme therefore is required for quantitative measurements involving the stoichiometry of metal ion binding to this protein.

The pH change accompanying the binding of a second set of twelve Mn^{2+} ions to glutamine synthetase was measured at 37° also. The results are illustrated in Figure 5. In curves 1 and 2, Mn^{2+} or Mg^{2+} was added (as in Figure 4) at the indicated times in sufficient amounts to barely saturate 12 sites of the enzyme. After an equilibrium pH value had been attained, a second aliquot of Mn^{2+} equal in quantity to the first aliquot was added in curve 1. The pH decreased rapidly with more than 95% of the pH change occurring within the time of mixing. Successive additions of Mn^{2+} at 7 and 9 min again produced rapid decreases in pH with no evidence of slow pH changes, but each addition of Mn^{2+} produced a smaller change in pH than did the preceding one. Further addition of Mn^{2+} beyond that illustrated in Figure 5 (curve 1) produced an asymptotic approach to a pH value expected if a total of three protons were released in the binding of the first two Mn^{2+} ions per enzyme subunit (or 36 moles of H^+ released in binding the first 24 moles of Mn^{2+}/mole of enzyme). In fact, the pH changes observed upon the addition of the second, third, and fourth aliquots of Mn^{2+} in curve 1 are exactly that expected if one proton is released during the binding of a second Mn^{2+} ion to each subunit with an apparent association constant of $\sim 2 \times 10^3$ M^{-1} (pH 7.1). This binding constant estimated from the pH changes is in excellent agreement with the value obtained from equilibrium dialysis experiments for the binding of a second set of 12 Mn^{2+} to glutamine synthetase (Denton and Ginsburg, 1969).

Curve 2 of Figure 5 illustrates an interesting kinetic phenomenon. After tightening the enzyme with Mg^{2+}, the subsequent additions of Mn^{2+} produced pH changes that indicate that Mn^{2+}, as in curve 1, binds to a second set of twelve metal ion binding sites of the enzyme. Competition experiments in equilibrium dialysis measurements indicated that Mn^{2+} and Mg^{2+} bind to the same high-affinity sites of glutamine synthetase (Denton and Ginsburg, 1969). There is, however, about two orders of magnitude difference in the affinity at these sites for Mn^{2+} and Mg^{2+}. With unadenylylated enzyme

[5] The enzyme had some buffering effect since the addition of HCl to the buffer alone caused a slightly greater pH change than did its addition to the protein solution. It is quite possible that the relaxed and tightened enzyme forms have somewhat different buffering capacities.

at pH 7.2 having $K_A' \simeq 2 \times 10^6$ M^{-1} in binding the first 12 molar equiv of Mn^{2+}, the first addition of Mn^{2+} to the enzyme tightened with Mg^{2+} in curve 2 of Figure 5 should have competitively displaced about 90% of the bound Mg^{2+}. While not significantly changing the free Mg^{2+} concentration, such a displacement of bound Mg^{2+} by Mn^{2+} would lower the concentration of free Mn^{2+} to a level insufficient for saturation of the second lower affinity Mn^{2+} binding sites. In this case, neither proton release nor uptake would be observed since Mg^{2+} and Mn^{2+} displace the same number of proton equivalents in binding to the high-affinity sites. This is what is observed in the displacement of Co^{2+} by Mn^{2+} (see Figure 7a below). Contrary to expectation, the results of Figure 5 (curve 2) suggest that once the enzyme is in the tightened configuration produced by either Mn^{2+} or Mg^{2+}, a second set of metal ion binding sites reacts preferentially with freshly added Mn^{2+}. However, all of the high-affinity sites of glutamine synthetase must be first saturated with Mn^{2+} or Mg^{2+} before the second binding site of each subunit begins to be saturated with Mn^{2+}, since the stoichiometry of the proton release in Figure 4 was almost exactly two H^+ for each metal ion bound. Presumably, the pH changes recorded after Mn^{2+} was added to the Mg^{2+}-tightened enzyme in curve 2 of Figure 5 would be slowly reversed as the system equilibrated. Instrument drift precluded a check on this point.

Interaction of Zn^{2+} and Co^{2+} with Glutamine Synthetase. In following the time course and stoichiometry of the pH change produced by the interaction of Zn^{2+} or Co^{2+} with relaxed glutamine synthetase, some interesting differences from the observations with Mn^{2+} and Mg^{2+} (Figure 4) were apparent.

Figure 6 shows the pH change observed upon the addition of Zn^{2+} to a relaxed preparation of unadenylylated enzyme ($E_{\bar{1}}$) in dilute Tris buffer at pH ~7.2 and 37°. In the experiments of Figure 6, each aliquot of metal ion added at the arrows provided one-third equivalent of divalent cation per mole of enzyme subunits present. Similar additions of Zn^{2+} to the buffer without enzyme caused a change in pH of <0.006. Thus, in curve 1 of Figure 6, the three aliquots of Zn^{2+} add up to a total of 1 molar equiv of Zn^{2+}/enzyme subunits present. The dashed horizontal line in Figure 6 marks the pH obtained by the addition of 12 molar equiv of HCl to the relaxed enzyme. The stoichiometry of the proton release per Zn^{2+} added, as well as other studies of Miller *et al.* (1972a), suggest that Zn^{2+} almost quantitatively reacts with relaxed enzyme in this study ($K_A' \geq 10^6$ M^{-1}). Accordingly, it was concluded that one proton is released from glutamine synthetase during the binding of each of 12 Zn^{2+} ions.

The pH change accompanying the binding of Zn^{2+} to the relaxed enzyme was rapid, but not instantaneous. About 90% of the total pH change occurred within the 3–5 sec required for mixing, but there appeared to be a slow stage in the reaction with a half-time of about 5 sec. The mixing technique unfortunately was not designed to allow an accurate measurement of the time course of the pH change caused by Zn^{2+} binding.

Curve 2 of Figure 6 was obtained by adding to relaxed enzyme one-third equivalent of Zn^{2+} per mole of subunits, followed by two successive additions of Mn^{2+}, each of which supplied one-third equivalent of Mn^{2+} per enzyme subunit. The total pH change produced by the Zn^{2+} and first Mn^{2+} additions was almost exactly that expected for the release of one proton equivalent per enzyme subunit present. This is as expected if one-third of the subunits present bind Zn^{2+} with the release of one H^+ per Zn^{2+} bound and if one-third

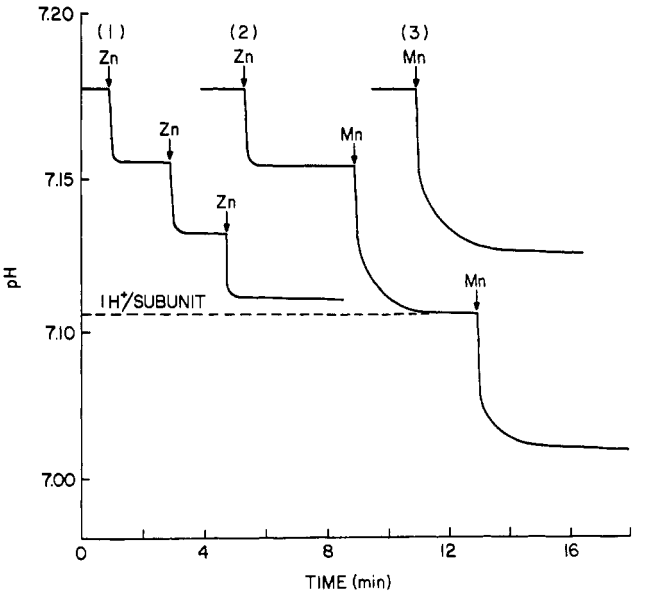

FIGURE 6: Proton release during the binding of Zn^{2+} to unadenylylated glutamine synthetase at 37°. The experiments were performed as described in the legend to Figure 4 with 4.0 mg of relaxed enzyme ($E_{\bar{1}}$) in 1 ml of 3 mM Tris–0.1 KCl buffer. At the times indicated in curves 1 and 2, the Zn^{2+} addition at each arrow was 50 μl of 6×10^{-4} M $ZnCl_2$ (the $ZnCl_2$ solution had been previously adjusted to the pH of the relaxed enzyme solution). Mn^{2+} addition at each arrow indicated in curves 2 and 3 was 50 μl of 6×10^{-4} M $MnCl_2$. After three injections of metal ion, there was one-eighth excess divalent cation to enzyme in each case. The horizontal dashed line indicates the pH obtained upon the addition of 1 molar equiv of HCl (0.080 μmole of H^+) to relaxed glutamine synthetase.

of the subunits present bind Mn^{2+} with the release of two H^+ per Mn^{2+} bound. The time course of the pH change accompanying the binding of Mn^{2+} with Zn^{2+} already present was about the same as that seen for the binding of Mn^{2+} in the absence of Zn^{2+} (curve 3) with a half-time of 31–33 sec. It then appears that bound Zn^{2+} does not interfere with the normal binding of Mn^{2+} to glutamine synthetase when the total quantity of the two divalent cations present does not exceed one equiv of metal ion per mole of subunits. Unfortunately, the tendency of glutamine synthetase to precipitate in the presence of Zn^{2+} (Miller *et al.*, 1972a) limited the extent to which competition between Mn^{2+} and Zn^{2+} could be studied by pH measurements.

Curve 1 of Figure 7a shows the pH changes accompanying the addition of Co^{2+} to relaxed glutamine synthetase ($E_{\bar{1}}$), with the arrows marking the times at which aliquots of Co^{2+} were added. Each aliquot of Co^{2+} provided one-third equivalent of Co^{2+} per mole of subunit. The experiments of Figure 7a were performed at 37° in dilute Tris buffer, but a lower enzyme concentration was used than that in the experiments of Figure 6 so that the total pH change per H^+ released was smaller. The pH change accompanying the addition of Co^{2+} to the relaxed enzyme was complete within the time of mixing, and no further change in pH was observed over a period of 15 min. The pH change produced by the Co^{2+} additions decreased steadily with each succeeding aliquot of Co^{2+}. The total pH change brought about by the addition of seven aliquots of Co^{2+} in curve 1 was about 95% of that expected for the release of two protons per enzyme subunit. This is more easily seen in Figure 7b, in which the dashed horizontal line marks the total pH change obtained by the addition of

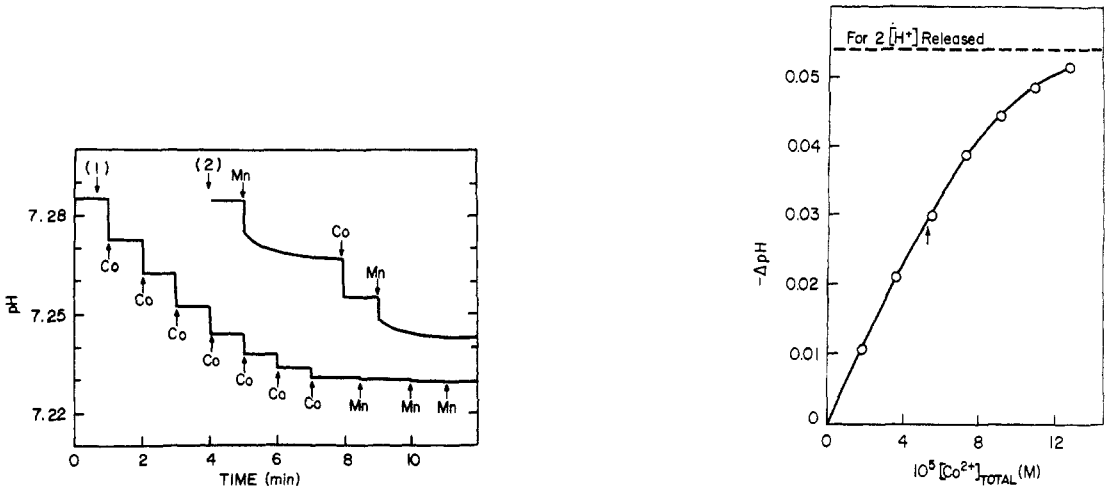

FIGURE 7: Proton release during the binding of Co^{2+} to unadenylylated glutamine synthetase at 37°. In part a (left) the experiments were performed as described in the legend to Figure 4 with 2.7 mg of relaxed enzyme (E_i) in 1 ml of 3 mM Tris–0.1 M KCl buffer. At the indicated times in curves 1 and 2, Co^{2+} addition at each arrow was 4.7 μl of 3.9×10^{-3} M $CoCl_2$; 4.7 μl of 3.9×10^{-3} M $MnCl_2$ was added at each arrow in curves 1 and 2. In part b (right) the results from expt 1 of part a are replotted as the change in pH ($-\Delta$pH) *vs.* the total concentration of Co^{2+} present. The arrow marks the midpoint of the saturation function, at which point a free concentration of $Co^{2+} = 1.9 \times 10^{-5}$ M is calculated. The dashed line shows the pH change obtained upon the addition of 2 molar equiv of HCl (0.108 μmole of H^+) per enzyme subunit present.

24 molar equiv of HCl to the relaxed enzyme. Curve 1 of Figure 7a was used to construct the plot of ΔpH *vs.* the total concentration of Co^{2+} shown in Figure 7b. The stoichiometry of the Co^{2+} addition in curve 1 of Figure 7a was such that the resultant saturation function in Figure 7b can be described by 12, but not 24, molar equiv of Co^{2+} binding to glutamine synthetase with $K_A' \simeq 5 \times 10^4\ M^{-1}$.

The addition of Mn^{2+} to the enzyme that was nearly saturated with Co^{2+}, as in the later part of curve 1 of Figure 7a, caused very little further change in pH. The three aliquots of Mn^{2+} added provided a total of 1 equiv of Mn^{2+}/enzyme subunit. This quantity of Mn^{2+} was sufficient to cause significant binding to the second set of Mn^{2+} binding sites (Figure 5), if the Mn^{2+} was not used up in some other manner. The lack of a substantial pH change in this case indicates that Mn^{2+} displaced Co^{2+} from the enzyme without further release or uptake of protons or that Co^{2+} inhibited the binding of Mn^{2+} to the second set of sites. The second experiment of Figure 7a (curve 2) shows the pH changes produced by successive additions at the arrows of one-third molar equivalent of each Mn^{2+}, Co^{2+}, and Mn^{2+}. The presence of subsaturating Mn^{2+} did not inhibit the binding of Co^{2+}, whereas subsaturating Co^{2+} decreased the magnitude but did not influence the time course of the pH change produced by the second Mn^{2+} addition. This latter effect is due to the partial displacement of Co^{2+} by Mn^{2+}.

Ultraviolet spectral measurements indicated that Mn^{2+} had replaced Co^{2+} from most of the 12 high-affinity sites of the enzyme by the end of expt 1 in Figure 7a. This technique was useful because the Co^{2+}-stabilized conformation of unadenylylated glutamine synthetase (Segal and Stadtman, 1972b) exhibits only a small ultraviolet difference spectrum (solid curve of Figure 8B) in comparison to that produced by Mn^{2+} or Mg^{2+} binding (Figure 1a). At zero time in Figure 8A, an aliquot of Co^{2+} was added to relaxed enzyme which was equivalent to the total of seven aliquots in Figure 7b and therefore sufficient to nearly saturate the enzyme with Co^{2+}. A rapid decrease in absorbancy at 290.3 nm (beyond that caused by dilution of the enzyme) occurred. This was followed by a slow increase in absorbance ($A_{290.3\,nm}$) which took about 18 min at 25° for a return to $\Delta A_{290.3\,nm} \simeq 0$, with a half-time of about 5 min. At 19 min, when the Co^{2+}-induced slow spectral change was completed, the indicated amount of Mn^{2+} was added to the Co^{2+}-saturated enzyme. A slow change in absorbancy at 290.3 nm ensued with an approximate half-time of 3 min. The total $\Delta A_{290.3\,nm}$ produced by the first Mn^{2+} addition in Figure 8A was equal to about 70% of the total $\Delta A_{290.3\,nm}$ which is produced by a saturating concentration of Mn^{2+} by itself. When the sample at the end of expt 1 of Figure 7a was treated with excess EDTA to metal ion, the decrease in the absorbance at 290.3 nm also was 70% of the magnitude expected if 12 molar equiv of Mn^{2+} had been bound to the high-affinity sites of the enzyme. These observed changes in absorbancies at 290.3 nm are within 20% of the changes predicted, assuming a direct competition between Co^{2+} and Mn^{2+} at the same site of the enzyme subunit, with $K_A' = 5 \times 10^4\ M^{-1}$ for Co^{2+} and $K_A' = 2 \times 10^6\ M^{-1}$ for Mn^{2+}. It should be noted too that the difference spectrum of the enzyme to which both Co^{2+} and Mn^{2+} had been added (as in the dashed curve of Figure 8B) is the same as that produced by Mn^{2+} alone (Figure 1a).

Another feature of the results shown in Figures 7a and 8A deserve comment. The binding of Co^{2+} to unadenylylated glutamine synthetase is rapid, as judged by the proton displacement in Figure 7a. There is, however, first a rapid and then a slow secondary small change in the absorbancy at 290.3 nm for which the most reasonable explanation is that Co^{2+} produces both a rapid and slow conformational change in the enzyme structure affecting the microenvironment of some aromatic residues. The slow spectral change brought about by Co^{2+} is not associated with a release or uptake of protons by the enzyme. Likewise, the binding of Mn^{2+} to the enzyme that has been presaturated with Co^{2+} induces a slow ultraviolet spectral perturbation, which occurs at a rate similar to that of the absorbancy change accompanying the direct binding of Mn^{2+} to the relaxed enzyme (Table I). However, the conformational change produced by the reaction of Mn^{2+} with relaxed enzyme is synchronized with the release of one proton equivalent from the enzyme whereas the binding of Mn^{2+} to the Co^{2+}–enzyme has no accompanying

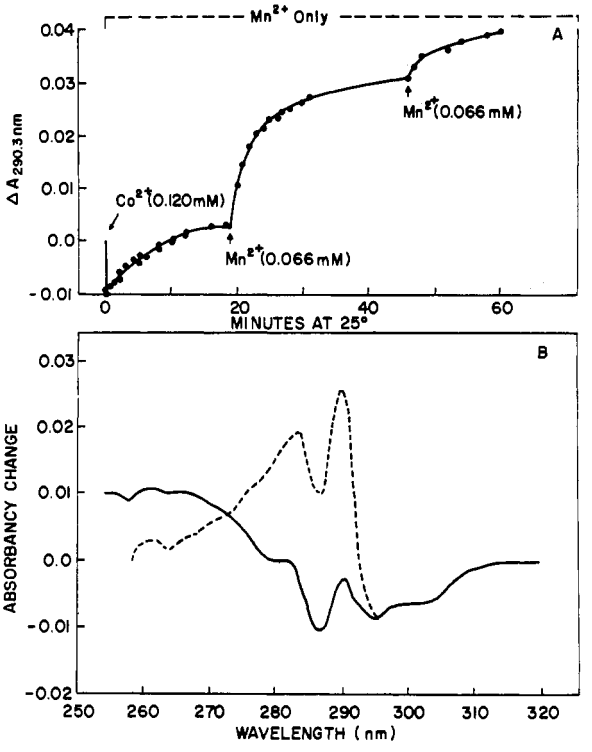

FIGURE 8: Absorbancy changes produced by the interaction of Co^{2+} and of Co^{2+} plus Mn^{2+} with unadenylylated glutamine synthetase (0.0407 mM E_1 subunits) in 1 ml of 4 mM Tris–0.1 M KCl buffer at 25°. The reference solution was the relaxed enzyme at the same concentration; before adding a volume of effector to the sample cell, an equal aliquot of buffer containing no divalent cation was added to the reference solution. In part A at zero time, 10 μl of 12.1 mM $CoCl_2$ was added to relaxed enzyme at pH 7.2. After 19 min and 46 min, 5-μl aliquots of 13.46 mM $MnCl_2$ were added to the enzyme solution containing 0.12 mM $CoCl_2$. The dashed line shows the absorbancy change at 290.3 nm obtained by adding 0.067 μmole of $MnCl_2$ at zero time to the relaxed enzyme (without Co^{2+}) at pH 7.2. In part B, the solid curve is the difference spectrum of the relaxed enzyme plus Co^{2+} as in part A, but with the enzyme solutions at pH 6.7 instead of at pH 7.2. The dashed line is the difference spectrum recorded at the end of the time-dependent absorbancy change at 290.3 nm after the addition of 0.066 mM $MnCl_2$ to the enzyme at pH 6.7 containing 0.12 mM $CoCl_2$. The changes in absorbancy produced by the addition of either Co^{2+} or Co^{2+} plus Mn^{2+} to the relaxed enzyme were lower in magnitude in part B than in part A, because the enzyme solution was at pH 6.7 in part B instead of at pH 7.2 as in part A and $K_A' = K/[H^+]^2$ for the binding of either divalent cation to the enzyme.

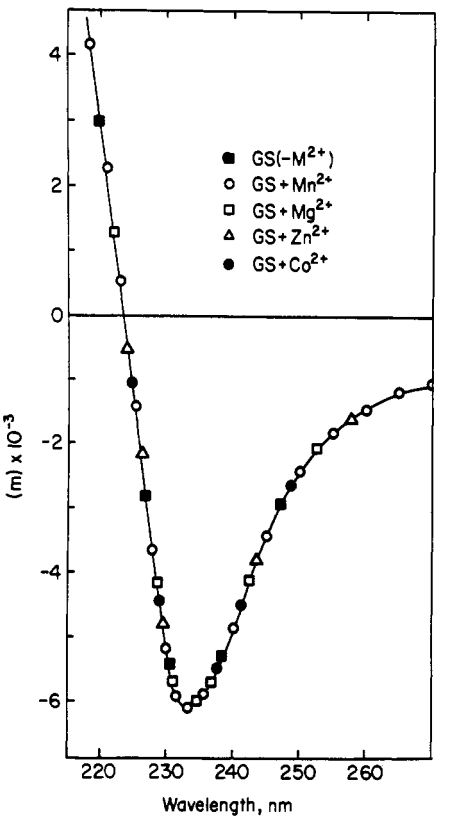

FIGURE 9: Optical rotatory dispersion curve for unadenylylated glutamine synthetase (GS) in the present or absence of metal ions. Measurements were made at 27° with *ca.* 0.55 mg/ml of enzyme (E_1) in 0.02 M Tris-Cl and 0.1 M KCl at pH 7.2 in a 0.1-cm silica cell without divalent cation present (■) or with 1 mM $MnCl_2$ (○), 1 or 60 mM $MgCl_2$ (□), 0.5 mM $ZnCl_2$ (△), or 0.8 mM $CoCl_2$ (●) present. The measured rotations were normalized to [*m*] in the units: (deg cm²)/dmole of residues assumed to have a mean residue weight of 114, without correcting for the refractive indices of the media.

proton release. Some implications of these observations will be discussed below.

Optical Rotatory Dispersion and Circular Dichroism Spectra of Native and Dissociated Enzyme Forms. The optical rotatory dispersion curve from 217 to 270 nm for unadenylylated glutamine synthetase in Tris buffer at pH 7.2 and 27° is illustrated in Figure 9. Optical rotatory dispersion measurements do not distinguish between unadenylylated and adenylylated glutamine synthetase forms (Wulff *et al.*, 1967). Figure 9 shows that the removal or addition of specific divalent cations, which markedly affect other properties of the enzyme, also had no influence on the optical rotatory dispersion spectrum. The concentration of each Mn^{2+}, Mg^{2+}, Zn^{2+}, or Co^{2+} in the experiments of Figure 9 was sufficiently high to saturate at least 12 metal ion binding sites of the enzyme. Assuming a mean residue weight of 114 and a refractive index of 1.34, $[m] = -5320$ (deg cm²)/dmole at 233 nm. From the magnitude of this trough at 233 nm (Simmons *et al.*, 1961; Greenfield *et al.*, 1967), an α-helical content of about 39% for glutamine synthetase is estimated.

The circular dichroism spectra of unadenylylated glutamine synthetase and of some disaggregated enzyme forms are shown in Figure 10. As in the case of optical rotatory dispersion, the absence of divalent cations or the presence of Mg^{2+} or of Mn^{2+} (1 mM) in Tris-chloride–0.1 M KCl buffer at pH 7.2 had no perceptible influence on the circular dichroism spectrum of glutamine synthetase to a measurable 215 nm in the far-ultraviolet spectrum. The relaxed enzyme at pH 7.2 in the phosphate–NaCl buffer of Figure 10 had exactly the same circular dichroism spectrum to about 207 nm as did the enzyme with 1 mM $MgCl_2$ present. Using the method of Greenfield and Fasman (1969) which takes advantage of an isosbestic point for the circular dichroism spectra of β- and random-coil structures at 208 nm to calculate the per cent α-helix, glutamine synthetase was estimated to have 36% α-helical structures; $\theta_{208} = -14{,}300$ (deg cm²)/dmole for the relaxed enzyme in 0.01 M phosphate–0.1 M NaCl buffer at pH 7.2 and 27°. This latter value and the circular dichroism spectrum of the native enzyme shown in Figure 10 were approximately fitted to the standard curves of Greenfield and Fasman (1969) to estimate structural contents of about 24% β-pleated sheet and about 40% random-coil segments in the configuration of glutamine synthetase. The estimates of 36–

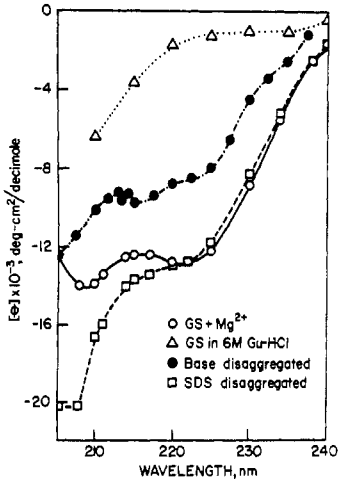

FIGURE 10: Circular dichroism spectra of unadenylylated glutamine synthetase (GS) and disaggregated glutamine synthetase at 27°. The enzyme concentration was 0.36 mg E_1^1/ml and a 0.1-cm light path was used. With Mg^{2+} (1 mM) or without divalent cations (○), the enzyme (GS) was in 0.01 M sodium phosphate–0.1 M NaCl buffer at pH 7.2. After removing divalent cations from the enzyme, it was dissociated with 6 M guanidine-HCl for either 15 min or overnight (△), dissociated by treatment with 1% sodium dodecyl sulfate (SDS) followed by overnight dialysis at 23° against 0.1% sodium dodecyl sulfate in 0.01 M sodium phosphate buffer (□), or disaggregated in base (●) by treatment with 3 mM carbonate–bicarbonate, 2 mM sodium phosphate, and 10 mM NaCl buffer at pH 9.50 for 15 min prior to the recording of the circular dichroism spectrum.

39% α-helical regions in the dodecameric enzyme by optical rotatory dispersion and circular dichroism measurements are in reasonably close agreement.

The near-ultraviolet spectral perturbations produced by the addition of Mg^{2+}, Mn^{2+} (Shapiro and Ginsburg, 1968), Co^{2+} (Segal and Stadtman, 1972b), or Zn^{2+} (Miller *et al.*, 1972a) to relaxed glutamine synthetase presumably are due to the movement of aromatic chromophores to another environment (Timasheff, 1970). Apparently, these conformational changes that are produced by the binding of the various activating divalent cations are cancelled or masked by other structural changes that are included in optical rotatory dispersion or molar ellipticity measurements. Likewise, the small shape and/or hydrodynamic volume change that is indicated by the decrease in sedimentation rate produced by relaxation of the native enzyme (Shapiro and Ginsburg, 1968) do not appear to involve changes in the secondary structure of the dodecamer.

Once divalent cations are removed from glutamine synthetase, a variety of mild denaturing conditions will cause dissociation of the enzyme into subunits (Woolfolk and Stadtman, 1967; Shapiro and Ginsburg, 1968). Divalent cations, and to a lesser extent monovalent cations, stabilize the quaternary structure of the dodecamer. Disaggregation of relaxed glutamine synthetase by 6 M guanidine-HCl, alkali, or sodium dodecyl sulfate produces some interesting differences in circular dichroism spectra (Figure 10).

In 6 M guanidine-HCl, the subunits assume a random-coil configuration (Greenfield and Fasman, 1969). After 15 min or overnight, the circular dichroism spectrum of the enzyme in 6 M guanidine-HCl (Figure 10) was the same. The difference in optical activity between the native enzyme and the enzyme treated with 6 M guanidine-HCl also was indicated dramatically in optical rotatory dispersion measurements. The optical rotation of the guanidine-treated enzyme became increasingly more negative below 230 nm, which is characteristic of the random-coil configuration (Greenfield *et al.*, 1967). The optical rotations of α-helical and β structures increase below 230 nm, as does also the optical rotation of the native enzyme (Figure 9). These observations agree with sedimentation velocity results in which the relaxed enzyme in 6 M guanidine-HCl has $s_{20,w} = 2.6$ S. Applying the empirical equation of Tanford *et al.* (1967) for polypeptide chains in a random coil configuration, the uncorrected value of 0.86 S for the enzyme in 6 M guanidine-HCl (see Methods) corresponds to a polypeptide chain of about 450 amino residues in length, or about 51,000 molecular weight if a mean residue weight of 114 is assumed. Sedimentation equilibrium of the enzyme in 5 M guanidine-HCl previously yielded a subunit molecular weight of about 50,000 (Woolfolk *et al.*, 1966).

Exposure of relaxed glutamine synthetase to Tris buffer at pH 9.5 has been shown by Ciardi *et al.*[6] to dissociate the enzyme within 12 sec to $^1/_{12}$th the original light-scattering value. Still, the circular dichroism spectrum measured 15 min after exposure of a relaxed preparation of unadenylylated enzyme to pH 9.5 showed that there were appreciable residual structures (Figure 10). The value of θ at 208 nm obtained for the base-disaggregated enzyme after 15 min was nearly identical with that of the native protein. This wavelength is the isosbestic point for the circular dichroism of β structures and the random-coil form (Greenfield and Fasman, 1969) so that the circular dichroism spectrum produced by base may be due to the conversion of some β structures to the random coil form. If so, dissociation appears to affect β structures rather than α-helical structures of the protein, and the residual structures at pH 9.5 must be ordered regions within the dissociated polypeptide chains of the subunits. The base-disaggregated, unadenylylated glutamine synthetase has been observed[6] to have a slightly faster sedimentation rate than do the dissociated subunits in 6 M guanidine-HCl or in 7 M urea. An aliquot of the base-disaggregated enzyme was diluted 1:1 after 15 min at pH 9.5 with 0.01 M phosphate–0.1 M KCl buffer in order to adjust the pH to 7.0. Under these conditions, reassociation of subunits and regain of a stable activity occurs,[6] and the circular dichroism spectrum was found to be about the same as that recorded for the native enzyme.

The anionic detergent, sodium dodecyl sulfate, causes an increase in the apparent α-helical content of glutamine synthetase (Figure 10), using θ at 208 nm as an index of the amount of α helix (Greenfield and Fasman, 1969). The configurations of the polypeptide chains apparently are not randomized by the detergent, even though polyacrylamide gel electrophoresis indicates that sodium dodecyl sulfate causes dissociation of the enzyme into subunits.[7] Visser and Blout (1971) have observed that several proteolytic enzymes, each of which is composed of a single polypeptide chain, show an apparent increase in α-helical structures in the presence of sodium dodecyl sulfate. In commenting on this effect of detergents, Tanford (1968) has hypothesized that the hydrophobic head, rather than the ionized head, of sodium dodecyl sulfate may interact with existing ordered regions or isolated

[6] J. E. Ciardi, S. Shifrin, and E. R. Stadtman (manuscript in preparation); personal communication from J. E. Ciardi.

[7] In polyacrylamide gel electrophoresis at pH 7.2 in 0.1% sodium dodecyl sulfate by the method of Shapiro *et al.* (1967), relaxed glutamine synthetase is disaggregated to a single band with a migration corresponding to a molecular weight of about 50,000 (J. Yeh and A. Ginsburg, unpublished data).

hydrophobic side chains of the polypeptide chain to create micelle-like, newly ordered regions.

Discussion

Proton release from unadenylylated glutamine synthetase caused by the binding of activating metal ions has been measured here directly at pH ~7.2 and 37°. The results are summarized in the following reactions (1–6), where [GS] represents a subunit of glutamine synthetase of 50,000 molecular weight and K' is the apparent intrinsic association constant for the binding reaction

$$[GSH_3] + Mn^{2+} \overset{\text{fast}}{\rightleftharpoons} [GSH_2]\text{-}Mn^{+} + H^{+} \quad (1a)$$

$$[GSH_2]\text{-}Mn^{+} \overset{\text{slow}}{\rightleftharpoons} [GSH]\text{-}Mn + H^{+}\text{: } K_{1ab}' = 2 \times 10^{6}\ M^{-1} \quad (1b)$$

$$[GSH]\text{-}Mn + Mn^{2+} \overset{\text{fast}}{\rightleftharpoons} [GS]\text{-}Mn_2^{+} + H^{+}\text{: } K_2' = 2 \times 10^{4}\ M^{-1} \quad (2)$$

$$[GSH_3] + Mg^{2+} \overset{\text{fast}}{\rightleftharpoons} [GSH_2]\text{-}Mg^{+} + H^{+} \quad (3a)$$

$$[GSH_2]\text{-}Mg^{+} \overset{\text{slow}}{\rightleftharpoons} [GSH]\text{-}Mg + H^{+}\text{: } K_{3ab}' = 2 \times 10^{4}\ M^{-1} \quad (3b)$$

$$[GSH]\text{-}Mg + Mn^{2+} \overset{\text{fast}}{\longrightarrow} Mn^{+}\text{-}[GS]\text{-}Mg + H^{+}\text{: nonequilibrium} \quad (3c)$$

The above stoichiometry in reactions 1 and 3 is observed only when the unadenylylated enzyme with Mn^{2+} or Mg^{2+} bound is fully active in a Mg^{2+}-dependent biosynthetic catalysis.

Similar kinetics were observed for the proton release in reactions 1b and 3b, or for the accompanying ultraviolet spectral perturbation, produced by the binding of either Mn^{2+} or Mg^{2+} to the 12 high-affinity sites of the enzyme. In previous equilibrium binding studies (Denton and Ginsburg, 1969), Mg^{2+} competed with Mn^{2+} at the high-affinity metal ion binding sites of glutamine synthetase. These two divalent cations also produce the same ultraviolet spectral perturbations when added to relaxed glutamine synthetase. It therefore is probable that Mn^{2+} and Mg^{2+} react at the same metal ion binding site of each enzyme subunit and cause the same protons to be displaced from the protein during binding.

The direct studies of proton displacement by Mn^{2+} or Mg^{2+} have corroborated the conclusions from calorimetric results reported in the preceding paper of Hunt *et al.* (1972). A bimolecular reaction (1a, 3a) between glutamine synthetase and Mn^{2+} or Mg^{2+} followed by a slow first-order reaction (1b, 3b) involving a conformational change in the enzyme structure is proposed. The conformational change in reactions 1b and 3b was followed spectrophometrically in these studies; at saturating concentrations of Mn^{2+} or Mg^{2+}, approximately the same first-order rate constants were measured at 37° for either the slow proton release or the ultraviolet spectral perturbation at 290.3 nm. The latter measurements at different temperatures indicated an Arrhenius activation energy of 21 kcal (mole subunit)$^{-1}$ for the binding of these divalent cations to unadenylylated or to adenylylated glutamine synthetase (Figure 3).

Although the slow proton release in reactions 1b and 3b was synchronized with the tyrosyl–tryptophanyl perturbations at saturating concentrations of Mn^{2+} or Mg^{2+}, the rates of $\Delta A_{290.3\ nm}$, but not apparently of $\Delta[H^+]$, were slower at half-saturating concentrations of these ions (Table I). Thus, the conformational change that involves changes in the microenvironment of tyrosyl–tryptophanyl residues is not necessarily coupled to the slow proton release during metal ion binding. In fact, as reactions 1 and 3 are written, binding of Mn^{2+} or Mg^{2+} precedes the conformational change, which mechanistically is similar to the induced-fit proposal of Koshland (1958). The slower rates in $\Delta A_{290.3\ nm}$ at subsaturating than at saturating concentrations of Mn^{2+} or Mg^{2+} suggest that there is some cooperativity in the conformational change of unadenylylated enzyme that is not apparent in binding measurements. Indeed, a plot of the spectral change of unadenylylated enzyme *vs.* Mg^{2+} concentration (Figure 2) indicated a Hill coefficient of about 1.2. This is interpreted to mean that subunit interactions can affect the induced spectral perturbation. Slightly more than one subunit metal ion binding site apparently needs to be saturated for the tryptophanyl–tyrosyl movement within each subunit. This small degree of cooperativity was not observed in studies of the interaction of Mn^{2+} or Mg^{2+} with the adenylylated enzyme.

Proton release during the binding of Mn^{2+} to a second set of 12 lower affinity binding sites of glutamine synthetase (Denton and Ginsburg, 1969) is indicated in reaction 2. Only a fast step was observed during the displacement of a single proton by each Mn^{2+} bound to the second metal ion binding site. There is no ultraviolet spectral perturbation accompanying reaction 2. It is probable that this second metal ion binding site, either with metal ion alone or with a metal ion–ATP complex, is functionally important in biosynthetic catalysis. Recent results of Segal and Stadtman (1972b) indicate that the conformation at the active site of glutamine synthetase is affected by at least two metal ion binding sites per subunit.

Reaction 3c represents the kinetic phenomenon observed when Mn^{2+} was added to enzyme which had been tightened with Mg^{2+}. Instead of completely displacing Mg^{2+} at the high-affinity sites, as would be expected from the difference in apparent equilibrium constants at these sites for Mg^{2+} and Mn^{2+}, the Mn^{2+} added was bound instantly to the lower affinity metal ion binding sites. Eventually, Mn^{2+} would be expected to replace Mg^{2+} at the high-affinity sites. In competition studies, Co^{2+} was displaced by Mn^{2+} from the Co^{2+}-saturated enzyme in the reaction:

$$[GSH]\text{-}Co + Mn^{2+} \overset{\text{fast}}{\rightleftharpoons} [GSH]\text{-}Mn + Co^{2+}\text{: } K_4' \simeq 40 \quad (4)$$

The fact that neither a release nor uptake of protons was observed during this reaction indicates that the displacement of Co^{2+} by Mn^{2+} is rapid (see Results). The actual replacement of Co^{2+} by Mn^{2+} was demonstrated spectrally at 25°. The enzyme tightened with Mn^{2+} or Mg^{2+} *vs.* the relaxed enzyme exhibits a substantial ultraviolet difference spectrum (Figure 1a), whereas the Co^{2+}-stabilized conformation does not (Figure 8B). Further, Kingdon *et al.* (1968) found that whereas Mg^{2+}, Mn^{2+}, or Ca^{2+} was effective, Co^{2+} or Zn^{2+} was ineffective, in activating glutamine synthetase in a Mg^{2+}-dependent biosynthetic assay. It is tempting to speculate that when Co^{2+} is at the high-affinity sites of the enzyme (reaction 5 below), a conformation is stabilized from which Co^{2+} can be readily exchanged. A slow rate of exchange of metal ion at the subunit high-affinity site when the enzyme is stabilized in the activated, tightened configuration would explain why Mn^{2+} does not instantly displace Mg^{2+} from these same sites (reaction 3c).

Studies of the interaction of Co^{2+} with unadenylylated

enzyme by following proton displacement showed the following

$$[GSH_3] + Co^{2+} \overset{\text{fast}}{\rightleftharpoons} [GSH]\text{–}Co^{2+} + 2H^+: \quad K_5' = 5 \times 10^4\ M^{-1} \quad (5)$$

The competitive displacement of Co^{2+} by Mn^{2+} (reaction 4) was almost exactly that anticipated from the difference in stability constants for these two ions. Accordingly, the simplest interpretation of these results is that Co^{2+} binds to the the same 12 high-affinity sites of the enzyme as does Mn^{2+}. However, Co^{2+} stabilizes a quite different conformation of glutamine synthetase. This difference in the conformations stabilized by Co^{2+} and Mn^{2+} (or Mg^{2+}) could relate to differences between these metal ions in electronic configuration and ionic radius. The instantaneous displacement of two proton equivalents by the binding of each Co^{2+} to the relaxed enzyme in reaction 5 is of interest. It suggests that the slow proton release during the binding of Mn^{2+} or Mg^{2+} to the enzyme could occur from an anticooperative force that moves a second ionizing group while possibly causing the tryptophanyl–tyrosyl residue burial. Of course, there is no way of knowing from these measurements whether the two protons displaced from the enzyme subunit by Co^{2+} are the same ones displaced by Mn^{2+} or Mg^{2+}. Moreover, whether or not the ionizing groups are in the metal ion binding cluster is unknown. If the slow proton release during the binding of Mn^{2+} or Mg^{2+} to the enzyme is an induced ionization of some group outside the metal ion binding site, Co^{2+} can somehow instantly achieve the same net release in proton equivalents as does Mn^{2+} (or Mg^{2+}) in reactions 1a,b (or 3a,b).

Secondary ultraviolet spectral perturbations produced by Co^{2+} binding to the enzyme are even slower than those produced by Mn^{2+}. Also, Mn^{2+} must instantaneously replace Co^{2+} for no release or uptake of protons to be seen, and yet the time course of $\Delta A_{290.3}$ after Mn^{2+} addition to the Co^{2+}-saturated enzyme was slow. Clearly, the binding of metal ions in reactions 4 and 5 precedes conformational changes reflected in the ultraviolet spectral perturbations, with a given equilibrium constant reflecting all steps in the binding process.

It has been discovered recently by Miller *et al.* (1972a) that 1.5 mM $ZnSO_4$ in the presence of 50 mM $MgCl_2$ at pH 5.9 has the capacity to quantitatively precipitate glutamine synthetase in crude extracts of *E. coli*. A simple method of purification of glutamine synthetase from *E. coli* has been developed, utilizing the specificity of the Zn^{2+} interaction and the insolubility of the Zn–enzyme–Mg complex. In this procedure, the Zn–enzyme–Mg complex can be repeatedly recrystallized by lowering the $MgCl_2$ concentration to 2.5 mM $MgCl_2$ to dissolve the Zn–enzyme precipitate and then raising the $MgCl_2$ concentration to 50 mM $MgCl_2$ to induce paracrystalline aggregate formation, which occurs in 10 min at 37°. After several recrystallizations without adding more Zn^{2+}, the enzyme was found to contain close to 1 molar equiv of Zn^{2+}/subunit. An association constant of 10^6–$10^7\ M^{-1}$ was estimated from the stoichiometry of the Zn^{2+}-induced precipitation of the enzyme in the presence of 50 mM $MgCl_2$ (Miller *et al.*, 1972a). The ultraviolet difference spectrum of relaxed enzyme to which 1 molar equiv of Zn^{2+}/subunit had been added was similar to that of the Co^{2+}–enzyme (Figure 8b). Doubling the Zn^{2+} concentration in these studies did not significantly alter the difference spectrum. The presence of $MgCl_2$ converted the difference spectrum to that of the Mg^{2+} or Mn^{2+}–enzyme (Miller *et al.*, 1972a).

The proton release during the binding of Zn^{2+}, which appeared to be almost stoichiometric with the amount of Zn^{2+} added, is represented in reaction 6. One, instead of two, equivalents of proton was released during the binding of each molar equivalent of Zn^{2+} (with the amount of Zn^{2+} added not exceeding the concentration of enzyme subunits). A slow step in reaction 6, with a half-time of about 5 sec at 37°, was observed. About 90% of the proton release occurred, however, within 3–5 sec. The species $[ZnOH]^+$ under the conditions of the Zn addition (Figure 6) will be less than 3% of the total Zn^{2+} present,[3] and therefore $[ZnOH^+]$ formation cannot account for the lower proton displacement observed in Zn^{2+} binding than that observed during the binding of other divalent cations (reactions 1, 3, and 5).

$$[GSH_3] + Zn^{2+} \overset{\text{slow?}}{\rightleftharpoons} [GSH_2]\text{–}Zn^+ + H^+: K_6' \geq 10^6\ M^{-1} \quad (6)$$

It is concluded that Zn^{2+} binds with high affinity to another site on the enzyme subunit, with Mg^{2+} or Mn^{2+} capable of simultaneously occupying the high-affinity Mg^{2+} or Mn^{2+} site. The insolubility of the Zn–enzyme limited the extent to which competition between Zn^{2+} and Mn^{2+} could be studied. At less than 1 molar equiv of Zn^{2+}/enzyme subunit, however, the binding of Mn^{2+} appeared to be independent of the Zn–enzyme present. In the studies of Miller *et al.* (1972a), the binding of Mg^{2+} to other sites on the enzyme must be required for paracrystalline aggregation of the Zn–enzyme.

Since only one proton equivalent is released by the binding of Mn^{2+} to a second lower affinity site of the enzyme subunit (reaction 2), it is possible that Zn^{2+} reacts preferentially with this site rather than at the high-affinity site for Mn^{2+} or Mg^{2+}. Alternatively, Zn^{2+} reacts at some other site and in so doing still allows Mg^{2+} or Mn^{2+} to react at the high-affinity sites, the saturation of which produces Mg^{2+}- or Mn^{2+}-induced ultraviolet spectral perturbations. Whatever the site of Zn^{2+} interaction, once Zn^{2+} is bound to glutamine synthetase it is difficult to remove Zn^{2+} from the enzyme. This suggests that Zn^{2+} binding causes a conformational change in the protein that precipitously decreases its rate of exchange with other divalent cations.

The optical rotatory dispersion or circular dichroism spectra (200–270 nm) of unadenylylated glutamine synthetase were not affected by the binding of either Mn^{2+} or Mg^{2+} to the relaxed enzyme. Optical rotatory dispersion measurements with sufficient Zn^{2+} or Co^{2+} to saturate 12 sites of the enzyme also were the same as those with the relaxed enzyme. The insensitivity of these techniques in detecting metal ion induced structural changes is surprising. A tightening of the relaxed enzyme with Mn^{2+} or Mg^{2+} activates glutamine synthetase (Kingdon *et al.*, 1968), causes an apparent burial of sulfhydryl groups (Shapiro and Stadtman, 1967) and of tryptophanyl–tyrosyl residues, decreases the hydrodynamic volume or frictional coefficient (Shapiro and Ginsburg, 1968) and stabilizes the quaternary structure of the enzyme (Woolfolk and Stadtman, 1967). The various structural changes produced by the binding of specific divalent cations to glutamine synthetase must be masked or somehow cancel in optical rotatory dispersion and circular dichroism spectral measurements.

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Kinetic Evaluation of Substrate Specificity in the Glyoxalase-I-Catalyzed Disproportionation of α-Ketoaldehydes†

David L. Vander Jagt,*‡ Liang-Po B. Han, and Charles H. Lehman

ABSTRACT: The dissociation constants, K_{diss}, of the adducts formed in the preenzymic reaction between substrates (α-ketoaldehydes) and coenzyme (glutathione, GSH) in the glyoxalase system have been determined for methylglyoxal, phenylglyoxal, and a series of meta- or para-substituted phenylglyoxals including *p*-CH_3, *p*-OCH_3, *m*-OCH_3, *p*-Br, *p*-Cl, *p*-OH, *p*-NO_2, and *p*-phenyl. For methylglyoxal, $K_{diss} = 3.0 \pm 0.5 \times 10^{-3}$ M in the pH range 5–9; for phenylglyoxal, $K_{diss} = 0.60 \pm 0.05 \times 10^{-3}$ M, increasing somewhat at the higher end of this pH range. At pH 7, all the substituted phenylglyoxals show K_{diss} *ca.* $1–3 \times 10^{-3}$ M. The lack of substituent effects on K_{diss} is reflected also in the similar rates of adduct formation, followed at pH 3. The consistent values observed for K_{diss} when the initial GSH and α-ketoaldehyde concentrations are varied suggest only 1:1 adducts are formed. The data support the idea that the adducts are hemimercaptals. Glyoxalase-I shows very broad specificity in the disproportionation of these adducts into the corresponding GSH thiol esters of the α-hydroxycarboxylic acids. In every case the hemimercaptal is the glyoxalase-I substrate; and all V_{max} values are within a factor of 4 of V_{max} for the methylglyoxal–GSH adduct, again showing complete insensitivity to ring substituents. However, the K_M values for the substituted phenylglyoxals–GSH adducts (2×10^{-4} to 2×10^{-5} M) are smaller than K_M for the methylglyoxal–GSH adduct (3×10^{-4} M). The glyoxalase-I reaction is inhibited competitively by free GSH, $K_i = 5 \times 10^{-3}$ M. Although the glyoxalase-I reaction is known to involve intramolecular hydride transfer, the marked insensitivity of the reaction to variations in the α-ketoaldehydes suggests that hydride transfer may not be the rate-determining step.

The disproportionation of α-ketoaldehydes into the corresponding α-hydroxycarboxylic acids, catalyzed by the glyoxalase system, appears to be a common intracellular reaction (Dakin and Dudley, 1913a,b; Neuberg, 1913; Lohmann, 1932; Knox, 1960) and is of interest for several reasons: (a) the biological importance of the glyoxalase system

† From the Departments of Biochemistry and Chemistry, University of New Mexico, Albuquerque, New Mexico 87106. This work was supported by U. S. Public Health Service, National Institutes of Health (Grant 1 R01 CA11850-01), and U. S. Atomic Energy Commission under Sandia Corp. Contract 51-1985. An equipment grant from the Research Corp. is gratefully acknowledged. A preliminary report of this work was presented at the Southwest Regional Meeting of the American Chemical Society, San Antonio, Texas, Dec 1971.

‡ Present address: Department of Biochemistry, University of New Mexico School of Medicine, Albuquerque, N. M. 87106.