Manganese Ion Interactions with Glutamine Synthetase from *Escherichia coli*: Kinetic and Equilibrium Studies with Xylenol Orange and Pyridine-2,6-dicarboxylic Acid†

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ABSTRACT: Xylenol orange (a metal ion binding dye) has been used in equilibrium and kinetic studies of Mn^{2+} interactions with the structural n_1 sites of unadenylylated glutamine synthetase from *Escherichia coli*. The following properties of xylenol orange make it a useful reagent for studying protein–Mn^{2+} interactions: (1) Formation of a 1:1 complex between xylenol orange and Mn^{2+} at pH 7.2 (25 °C) with $K_A' = (1.6 \pm 0.1) \times 10^5$ M^{-1} produces a considerable increase in the molar extinction coefficient ($\Delta\epsilon = 1.9 \times 10^4$ M^{-1} cm^{-1} at 585 nm). (2) Xylenol orange does not bind to glutamine synthetase. (3) Stopped-flow measurements at pH 7.2 and 15 °C of xylenol orange interaction with Mn^{2+} give a second-order rate constant of 4.1×10^6 M^{-1} s^{-1} for complex formation and a first-order rate constant of 29 s^{-1} for Mn^{2+} dissociation from the dye. Xylenol orange was used spectrophotometrically at 25 °C (pH 7.2) to measure a time-dependent increase in the glutamine synthetase binding constant for Mn^{2+} that occurs during the Mn^{2+}-promoted conformational change with a half-time of 110 s at 25 °C. The increase from $K_A' \simeq 1.5 \times 10^5$ to 1.9×10^6 M^{-1} gives an estimate of $\Delta(\Delta G') \simeq -18$ kcal (mol of enzyme)$^{-1}$ for the conformational change at 25 °C (pH 7.2) of the manganese–protein complex. In stopped-flow experiments, xylenol orange was used to measure the off rate of Mn^{2+} from n_1 sites of the enzyme (after completion of the Mn^{2+}-promoted conformational change); Mn^{2+} dissociation was first order with a half-time of 0.22 s at 15 °C. Studies are described also utilizing pyridine-2,6-dicarboxylic acid (dipicolinic acid), which forms a 2:1 complex with Mn^{2+} with a small absorbance increase at 290 nm and binds Mn^{2+} without proton release so that it can be coupled to a pH-indicator dye. Experiments with dipicolinate in the absence and presence of bromothymol blue verified the off rate of Mn^{2+} from glutamine synthetase (which is coincident with a fast proton uptake) and, furthermore, showed that the slow proton uptake after the removal of Mn^{2+} from the enzyme (which is synchronous with the protein conformation change) is the result of a pK_a change through perturbation of an ionizable amino acid side chain not directly involved in Mn^{2+} binding. Thus, one proton binds at the rate of Mn^{2+} dissociation (presumably to a group within the Mn^{2+} binding cluster), and another proton per subunit binds after Mn^{2+} release as the protein undergoes the conformational transition. A scheme for Mn^{2+} binding to n_1 sites of glutamine synthetase in two states (a low- and a high-affinity conformation) fits the data presented.

Divalent cations have a structural as well as a catalytic role in glutamine synthetase from *Escherichia coli*. Each of the 12 subunits of this enzyme contains two noninteracting (independent) Mn^{2+} binding sites which must be filled to make that subunit active (Hunt et al., 1975). Mn^{2+} at the lower affinity (n_2) subunit site has been identified as that involved in chelating the nucleotide substrate at the catalytic site (Hunt et al., 1975; Villafranca et al., 1976). As reviewed previously (Ginsburg, 1972), the binding of Mn^{2+} to high-affinity, structural (n_1) sites of the dodecamer promotes a transition from an inactive, *relaxed*, to an active, *tightened*, conformation. Mn^{2+} at the subunit n_1 site also may have a catalytic role in binding the γ-carboxyl group of L-glutamate through an intervening (immobilized) water molecule during biosynthetic catalysis; Villafranca (1978) has measured a distance of 4.5 Å between Mn^{2+} at the n_1 site and the γ-carboxyl carbon of L-glutamate, and NMR and other data are consistent with Mn^{2+} at the n_1 site interacting with L-glutamate (Villafranca et al., 1975; Hunt & Ginsburg, 1980). The intrasubunit distance between n_1 and n_2 Mn^{2+} binding sites is reduced from ≥12 to ~8 Å by nucleotide binding (Hofmann & Glausinger, 1978; Balakrishnan & Villafranca, 1978).

The studies of this paper were designed to obtain new information on the interactions of Mn^{2+} with glutamine synthetase from *E. coli*. The approaches described are generally applicable to other Mn^{2+}–protein interactions, and Mn^{2+} is an activator of many enzymes from different sources (Mildvan, 1970). The binding of Mn^{2+} to protein (citing references on glutamine synthetase from *E. coli*) usually is measured by utilizing radioactive $^{54}Mn^{2+}$ (Denton & Ginsburg, 1969), atomic absorption (Hunt et al., 1975), or EPR (Villafranca & Wedler, 1974). The first two of these methods measure total Mn^{2+} so that a prior separation of the protein and the solution with which it is in equilibrium is necessary. The EPR technique often does not require such a separation, but it still does not provide an instantaneous measure of the extent of Mn^{2+} binding to a protein, such as is needed for following the kinetics of Mn^{2+} binding to or release from an enzyme. As an alternative to these methods, we have investigated the use of a metallochromic indicator, xylenol orange, in the present studies and have found that this reagent has a number of desirable properties for measuring protein–Mn^{2+} interactions. We have reinvestigated the stoichiometry of the Mn^{2+}–xylenol orange interaction and have measured the effective binding constant of xylenol orange for Mn^{2+} at pH 7.2, because there was doubt concerning the nature of the Mn^{2+}–dye complex (Cabrera-Martin et al., 1973; Bogachuk & Sheka, 1976), and there were large discrepancies in the reported dissociation constants of the Mn^{2+}–dye complex (Murakami et al., 1967; Bogachuk & Sheka, 1976; Kornev & Kardapolova, 1977; Saraswat et al., 1979).

Another reagent which has been of unique utility in this study is pyridine-2,6-dicarboxylic acid, commonly known as dipicolinic acid. This chelating agent is completely depro-

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tonated at pH 7.2 so that the formation of its complexes with divalent cations does not involve proton release.[1] This property has made it possible to remove Mn^{2+} from glutamine synthetase with dipicolinate and follow the accompanying rapid and slow reprotonations of the enzyme (Hunt & Ginsburg, 1972) with a pH-indicator dye.

Experimental Procedures

Materials. Solutions of $MnCl_2$, $CoCl_2$, and $MgCl_2$ were standardized by titration against EDTA (Schwarzenbach & Flaschka, 1969). A solution of $MgCl_2$ was prepared by reacting $MgCO_3$ with excess HCl to minimize contamination of Mg^{2+} with Ca^{2+}. Xylenol orange (XO)[2] was purified by the method of Nakada et al. (1977), using a Waters C_{18} high-performance liquid chromatography (high-performance LC) column and a model 500 preparative high-performance liquid chromatograph. As judged from the spectra of commercial and high-performance LC purified dye samples, xylenol orange from either Fisher Scientific Co. or J. T. Baker Chemical Co. was ≥95% pure as supplied. Stock solutions of the purified XO and the commercially obtained XO were passed through a column of Chelex-100 resin (100–200 mesh), which was obtained from Bio-Rad Laboratories, at pH 7.2 to remove contaminating divalent or trivalent cations. After such treatment, the dye samples gave no absorbance decrease upon the addition of EDTA, and the above-mentioned commercial preparations of XO were quite adequate for use in our studies. However, alternate methods for purifying XO have been described (Murakami et al., 1967; Yamada & Fujimoto, 1976; Sato et al., 1977). Dipicolinic acid was used as obtained from Sigma Chemical Co. All other chemicals were of analytical grade.

Buffers also were treated with Chelex-100 resin to remove contaminating divalent cations. For purification of Tris buffer, the Chelex resin was acidified to pH 1 with HCl, washed with deionized water, and then neutralized with Tris base to pH 7.2 to place Tris-H^+ cations on the resin. This resin then could be used to purify Tris-HCl buffers at pH 7.2 (containing no K^+ or Na^+) without pH change. Chelex-100 in the K^+ form was used to treat Hepes–KOH buffers and KCl solutions (Hunt & Ginsburg, 1980).

Unadenylylated glutamine synthetase was purified from *E. coli* and stored as before (Shrake et al., 1977). The enzyme was fully active (106 and 55 units/mg, respectively) in the pH 7.57 saturating γ-glutamyl transfer assay at 37 °C of Hunt et al. (1975) and in the Mg^{2+}-supported biosynthetic assay at 30 °C of Ginsburg et al. (1970). The average state of adenylylation of the enzyme preparation was 1.2 equiv of covalently bound 5′-adenylic acid groups per mol of glutamine synthetase, as determined by assay (Stadtman et al., 1979) and UV spectral (Ginsburg et al., 1970) methods. Protein concentrations were obtained from UV spectrophotometric measurements by using a light scattering correction and published extinction coefficients (Ginsburg et al., 1970). An enzyme subunit molecular weight of 50 000 was assumed (Ginsburg, 1972). Relaxed (metal ion free) glutamine synthetase was prepared as previously described, using Chelex-100 resin to remove Mn^{2+} from the protein (Hunt & Ginsburg, 1980).

Measurement of Binding Constants. After a XO solution was passed through Chelex-100 resin, the concentration of xylenol orange was determined by spectrophotometric titration with standardized Co^{2+} with which the dye forms a very stable 1:1 complex. Aliquots of 1×10^{-3} M Co^{2+} were added to ~2 $\times 10^{-5}$ M XO solution in a cuvette, and the absorbance at 585 nm was measured after each addition of Co^{2+}. The absorbance attained a constant value after an excess of Co^{2+} had been added; the end point was determined from a plot of absorbance vs. total $[Co^{2+}]$. Such a plot is slightly nonlinear at low concentrations of total Co^{2+}, but then increases linearly with increasing total $[Co^{2+}]$ and displays a sharp break at the plateau for saturation of XO with Co^{2+}.

Spectrophotometric titrations at 585 nm of xylenol orange (4.00×10^{-5} M) in 20 mM Hepes–KOH–100 mM KCl buffer at pH 7.2 with $MnCl_2$ (5×10^{-3}M) were used to determine the apparent association constant of XO for Mn^{2+}. For each dye solution, the ratio of [MnXO]/[XO] is obtained from the absorbance of the Mn^{2+}-free dye (A_0), the absorbance of the Mn^{2+}-saturated dye (A_s) obtained by adding a large excess of 0.5 mM Mn^{2+} at the end of the titration, and the measured absorbance (A) at subsaturating Mn^{2+} concentration. With the assumption that only a 1:1 complex (MnXO) is formed between Mn^{2+} and XO:

$$[MnXO]/[XO] = (A - A_0)/(A_s - A) = K_A'[Mn^{2+}] \quad (1)$$

where $[Mn^{2+}]$ is the free concentration of Mn^{2+} and [XO] represents the free concentration of dye without regard to the state of protonation. Since the free-acid form of XO has six dissociable protons (H_6XO), xylenol orange at pH 7.20 would represent an approximate 3:1 mixture of H_2XO^{4-} and H_3XO^{3-}, based on the value of $pK_4 = 6.7$ (Murakami et al., 1967; Sato et al., 1977). If $[XO]_T$ and $[Mn^{2+}]_T$ are the total concentrations of dye and Mn^{2+}, respectively, then $[MnXO] = [XO]_T(A - A_0)/(A_s - A_0)$ and $[Mn^{2+}] = [Mn^{2+}]_T - [MnXO]$.

The stoichiometry of the Mn^{2+}–xylenol orange interaction was determined by Job's method of continuous variations [see Vosburgh & Cooper (1941) and Hammes et al. (1970)]. For measurement of the absorption changes at either 585 or 630 nm, the mole fraction of each component was varied from 0.0 to 1.0 while keeping the total mole fraction of XO plus Mn^{2+} equal to 1.0. For measurements at 585 nm, the wavelength at which complex formation produces the largest absorbance increase (Figure 1A), a series of solutions were used for which the total molarity of XO + Mn^{2+} = 2×10^{-5} M. The absorbance of each solution and the absorbance of a solution with the same concentration of XO without Mn^{2+} present were measured, and the difference between the absorbances with and without Mn^{2+} present (ΔA) was calculated. Similar measurements were made at 630 nm with the total molarity of XO + Mn^{2+} = 4×10^{-4}. Absorbances of XO solutions followed Beer's law. Solutions of xylenol orange appeared to be quite stable for several days at neutral pH, since no loss in the ability to bind Mn^{2+} or a change in spectral properties occurred.

Stopped-Flow Measurements. The stopped-flow instrument used in this study has been described by Rhee & Chock (1976). For the avoidance of artifacts due to bubble formation in the observation cell, solutions were degassed (under vacuum) prior to loading into the storage syringes of the instrument.

Measurements of Proton Release and Uptake. A dilute Tris-HCl (3 mM) buffer containing 100 mM KCl was used

[1] For the dissociation of the last H^+ from dipicolinic acid (pyridine-2,6-dicarboxylic acid), $pK_a = 4.6$ (Martell, 1964). This reference also gives stability constants of dipicolinic acid for various metal ions: for Mn^{2+}, $\log K_1 = 5.01$ and $\log K_2 = 3.48$; for Mg^{2+}, $\log K_1 = 2.4$.

[2] Abbreviations used: XO, xylenol orange (for the chemical structure, see the Eastman Organic Chemicals catalog); GS, glutamine synthetase; GS^R, divalent cation free glutamine synthetase in the relaxed, inactive conformation; $MnGS^T$, tightened, active glutamine synthetase with Mn^{2+} bound to n_1 binding sites; dipicolinic acid, pyridine-2,6-dicarboxylic acid; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

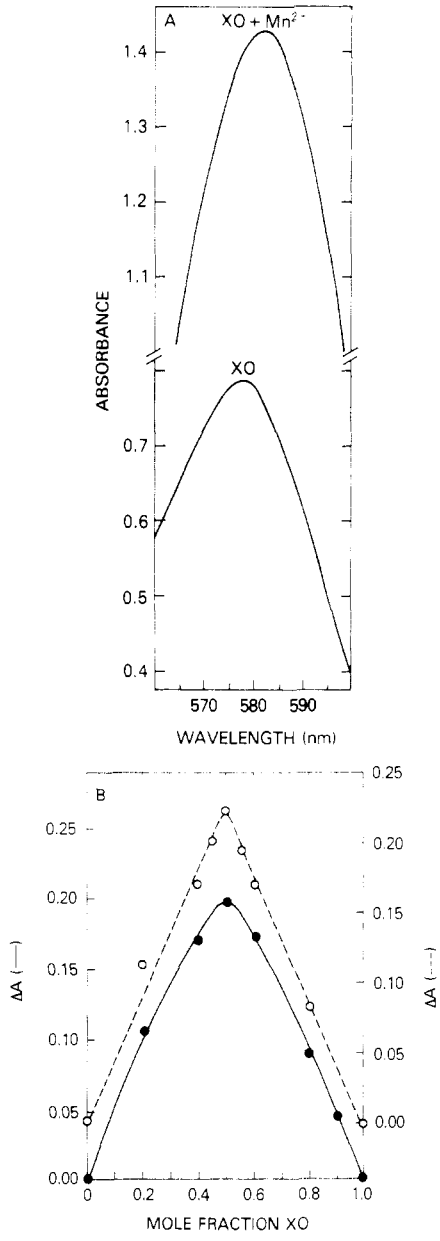

FIGURE 1: Spectrophotometric characterization of the Mn^{2+}–xylenol orange complex at pH 7.2 and ~25 °C. (A) Absorption maxima of a Chelex-treated xylenol orange (XO) solution at 3.2×10^{-5} M concentration and of a 3.2×10^{-5} M xylenol orange and 5×10^{-4} M $MnCl_2$ solution (XO + Mn^{2+}) in a cuvette with a 1.00-cm light path. (B) Job continuous variation plots for the Mn^{2+}–xylenol orange complex. Data for the upper plot (O---O) were obtained at 630 nm with the sum of the total concentrations of Mn^{2+} and xylenol orange equal to 4×10^{-4} M. Data for the lower plot (●—●) were obtained at 585 nm with the sum of the total concentrations of Mn^{2+} and xylenol orange equal to 2×10^{-5} M. ΔA is the difference in absorbance for the Mn^{2+}–XO solution and a XO solution of the same concentration containing no Mn^{2+}. Note that different scales are used for ΔA; left is for (●—●) and right is for (O---O). The buffer was 20 mM Hepes–KOH–100 mM KCl at pH 7.2 in both (A) and (B).

in the pH experiments to improve sensitivity (Hunt & Ginsburg, 1972). Solutions were degassed in a vacuum flask to remove CO_2 and then were adjusted to pH 7.20 with dilute HCl or KOH. For measurement of proton release during Mn^{2+} binding to glutamine synthetase, Mn^{2+} was added by a microliter syringe to a spectrophotometer cell containing the relaxed enzyme and 3×10^{-5} M bromothymol blue in 3 mM Tris-HCl–100 mM KCl buffer at pH 7.20. The absorbance change at 616 nm was recorded until the tightening reaction was completed, and then the solution was titrated back to the original absorbance value with standardized KOH to quantitate the proton release in the Mn^{2+} binding reaction. The removal of Mn^{2+} from the tightened enzyme was effected by adding excess dipicolinate at pH 7.20 to the Mn^{2+}–enzyme–bromothymol blue solution in the spectrophotometer cell; the absorbance change at 616 nm was recorded until the relaxation of the enzyme was complete. The solution then was titrated back to the original absorbance value with standard HCl to determine the number of protons taken up by the enzyme in the relaxation process.

Enzyme Dialysis against Xylenol Orange. For determination of the extent to which xylenol orange binds to the divalent cation free and manganese forms of glutamine synthetase, solutions of the relaxed and tightened enzymes (2 mg/mL) were dialyzed overnight at 4 °C against 2×10^{-5} M xylenol orange in 20 mM Hepes–KOH–100 mM KCl buffer (pH 7.2) without and with 1.0 mM $MnCl_2$ present. After 18 h, the absorbancies of the protein and dialysate solutions at 585 nm were identical, indicating that there is no significant binding of the dye to the relaxed or tightened enzyme forms under the conditions of our experiments.

Results

Characterization of the Interaction between Xylenol Orange and Mn^{2+}. Upon binding Mn^{2+} at neutral pH, xylenol orange undergoes a striking red to blue color change. Partial spectra of the metal ion free xylenol orange and of the xylenol orange–Mn^{2+} complex at pH 7.2 are shown in Figure 1A. Formation of the Mn^{2+}–dye complex results in a small shift in the absorption maximum and a considerable increase in the molar extinction coefficient; $\Delta\epsilon = 1.9 \times 10^4$ M^{-1} cm^{-1} at 585 nm and pH 7.2. The Job method of continuous variations for determining the stoichiometry of equilibrium binding was applied to the Mn^{2+}–xylenol orange interaction, and the results are shown in Figure 1B. The Job plots in Figure 1B were obtained from spectrophotometric data at two wavelengths and total concentrations of dye + Mn^{2+} (see Experimental Procedures); for each case, the mole fractions of xylenol orange and Mn^{2+} were reciprocally varied between 0.0 and 1.0, while keeping the total mole fraction of both components equal to 1.0. In this method, the concentration of the Mn^{2+}–dye complex has a maximum value when Mn^{2+} and xylenol orange are mixed so that their mole fractions correspond exactly to their combing ratio in the complex (Vosburgh & Cooper, 1941). Both plots in Figure 1B show a maximum absorption difference (complex formation) at mole fraction 0.5, indicating that the dominant species is a 1:1 complex between Mn^{2+} and the dye at xylenol orange concentrations of 10^{-5}–10^{-4} M. The apparent association constant of xylenol orange for Mn^{2+} was determined to be $(1.6 \pm 0.1) \times 10^5$ M^{-1} from spectrophotometric titrations of the dye with Mn^{2+} at pH 7.2 and 25 °C.[3]

The rate of formation of the Mn^{2+}–xylenol orange complex was measured by following the absorbance increase at 585 nm in a stopped-flow apparatus. The reaction was first order in each reactant with a second-order rate constant of 4.1×10^6

[3] The apparent association constant of xylenol orange for Mn^{2+} at pH 7.2 which we have measured ($K_A' = 1.6 \times 10^5$ M^{-1}) approximates the value of 1.3×10^5 M^{-1} reported by Bogachuk & Sheka (1976) for the reaction $H_2XO^{4-} + Mn^{2+} \rightleftarrows MnH_2XO^{2-}$. However, using also their value of $pK_4 = 6.9$ for the dye, a value of $K_A' = 7 \times 10^4$ M^{-1} at pH 7.2 is calculated from their data. If the value of $pK_4 = 6.7$ reported by Murakami et al. (1967) and Sato et al. (1977) is used instead, a value of $K_A' = 1.0 \times 10^5$ M^{-1} at pH 7.2 is calculated from the data of Bogachuk & Sheka (1976). The discrepancy between this value and our equilibrium and kinetic determinations of the K_A' value for xylenol orange binding Mn^{2+} at pH 7.2 may be due to a variation in the quality of the commercial dye used.

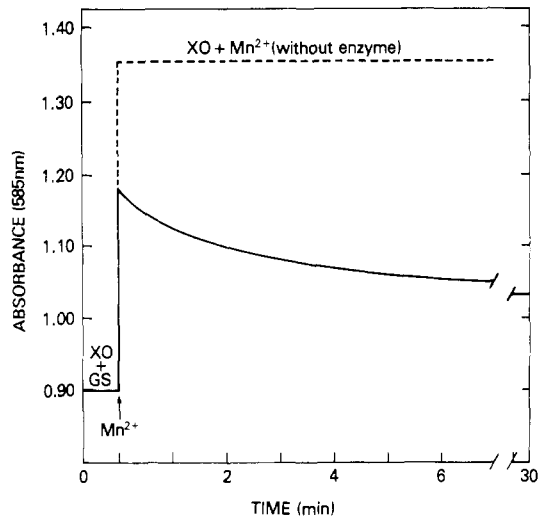

FIGURE 2: Absorbance change at 585 nm as a function of time produced by the addition of Mn^{2+} to divalent cation free (relaxed) glutamine synthetase in the presence of xylenol orange (solid line). At zero time, 5 μL of 7.5 mM $MnCl_2$ was added to a spectrophotometric cell containing 1.02 mL of 1.75 mg/mL relaxed glutamine synthetase (35 μM subunits), 40 μM xylenol orange, 100 mM KCl, and 20 mM Hepes–KOH buffer at pH 7.2. The absorbance change was complete by 30 min. In the control experiment (dashed line), 5 μL of 7.5 mM $MnCl_2$ was added to an identical solution of xylenol orange prepared without enzyme present. Solutions were at ~25 °C.

M^{-1} s^{-1} at 15 °C. The first-order rate constant for dissociation of the Mn^{2+}–dye complex is 29 s^{-1} at 15 °C, as measured by dilution of the complex with 20 mM Hepes–KOH–100 mM KCl buffer at pH 7.2 in the stopped-flow spectrophotometer. The apparent equilibrium association constant calculated from these rate constants is $K_A' = 4.1 \times 10^6$ M^{-1} $s^{-1}/29$ $s^{-1} = 1.4 \times 10^5$ M^{-1}, which is in excellent agreement with the K_A' value obtained by spectrophotometric titration of xylenol orange with Mn^{2+} at pH 7.2 and 25 °C.

Time-Dependent Increase in the Apparent Affinity of n_1 Enzyme Sites for Mn^{2+}. Figure 2 illustrated absorbance changes at 585 nm which follow the addition of Mn^{2+} (1 equiv of Mn^{2+}/mol of subunit) to relaxed (metal ion free) glutamine synthetase in the presence of xylenol orange. The events of this figure occur on a time scale much longer than that of the stopped-flow results to be given below. The measurements of Figure 2 are not complicated by xylenol orange binding to the protein; equilibrium dialysis of glutamine synthetase against 2×10^{-5} M xylenol orange in the absence and presence of Mn^{2+} indicated a lack of dye binding to the enzyme in either case. Upon Mn^{2+} addition to the enzyme, there is an immediate sharp increase in the absorbance produced by some binding of Mn^{2+} to xylenol orange, and this is followed by a slow decrease in absorbance. The slow absorbance decrease is synchronous with the slow absorbance increase at 290 nm which occurs during the tightening process due to a perturbation of aromatic protein residues (Hunt & Ginsburg, 1972); ΔA_{290nm} was followed independently in a parallel experiment to that shown in Figure 2. Note that the same amount of Mn^{2+} added to xylenol orange in the absence of protein (shown by the dashed line in Figure 2) gives a higher absorbance that is time independent. Thus, the protein competes with xylenol orange for Mn^{2+} from the outset.

At zero time in Figure 2, xylenol orange and both the n_1 and n_2 Mn^{2+} binding sites of the relaxed enzyme compete for Mn^{2+}. A reasonable explanation for the subsequent time-dependent absorbance changes in Figure 2 is that these result from a conversion of the relaxed enzyme to a tightened conformation with a higher affinity for Mn^{2+}. Accordingly, the slow absorbance change in Figure 2 occurs as the concentration of free Mn^{2+} decreases during the tightening reaction of the enzyme, which results in a dissociation of some of the Mn^{2+}–dye complex formed initially and in a consequent absorbance decrease at 585 nm.

From the stoichiometry of the solution components in Figure 2, the absorbance measured at zero time, and the apparent association constants at pH 7.2 for xylenol orange binding Mn^{2+} and for n_2 sites of the enzyme binding Mn^{2+} ($K_A' = 2 \times 10^4$ M^{-1}; Denton & Ginsburg, 1969; Hunt & Ginsburg, 1972), an apparent equilibrium constant for the binding of Mn^{2+} to n_1 sites of the relaxed enzyme was calculated. From this analysis, $K_A' = (1.5 \pm 0.5) \times 10^5$ M^{-1} for the binding of Mn^{2+} to n_1 sites of the enzyme in the relaxed conformation.[4] This value is ~13-fold lower than the value of $K_A' = (1.9\text{–}2.0) \times 10^6$ M^{-1} previously measured for the equilibrium binding of Mn^{2+} to the unadenylylated (tightened) enzyme (Hunt et al., 1975; Villafranca et al., 1976). The plateau in the absorbance decrease in Figure 2 after 30 min corresponds approximately to that anticipated from the published K_A' value for Mn^{2+} binding to n_1 sites of the tightened enzyme in the high-affinity conformation.

During Mn^{2+} binding studies with unadenylylated glutamine synthetase, Villafranca et al. (1976) observed that the amplitude of the EPR signal for free Mn^{2+} (at pH 7.0 and 25 °C) slowly decreased when samples were recorded within 1 min of preparation. This effect was not analyzed further, but, as in the case of Figure 2, it can be attributed to the concentration of free Mn^{2+} decreasing during a transition of the enzyme from a relaxed to a tightened conformation.

Proton Release and Uptake by Glutamine Synthetase during the Binding and Dissociation of Mn^{2+}. Absorbance changes at 616 nm in the acid–base indicator bromothymol blue at ~pH 7.2 were used in the experiments of Figure 3 to measure the time courses of proton release and uptake from glutamine synthetase during the binding and removal of Mn^{2+}, respectively, at 25 °C. Absolute absorbance changes of the pH-indicator dye were unimportant since standard base or acid was used to calibrate proton changes in each experiment of Figure 3. In the solid curve of Figure 3A, sufficient Mn^{2+} was added to saturate both the n_1 and n_2 Mn^{2+} binding sites of the relaxed enzyme; the dashed curve shows the proton release when n_1 sites are filled before n_2 sites. One proton per subunit site is released rapidly as Mn^{2+} binds to n_1 sites, and another proton is released rapidly as Mn^{2+} binds to n_2 sites of the relaxed enzyme. A third proton equivalent per subunit is released slowly during the protein conformational change promoted by Mn^{2+} binding to n_1 sites of the relaxed enzyme. The dashed curve of Figure 3A confirms results previously obtained by direct pH measurements (Hunt & Ginsburg, 1972).

In Figure 3B, the process of Figure 3A is reversed by the removal of Mn^{2+} from the enzyme with excess dipicolinate at pH 7.2. Two protons per subunit return rapidly to the enzyme as the Mn^{2+} bound to n_1 and n_2 sites is removed. A third proton equivalent per subunit is slowly taken up by the enzyme in exact synchronization with the UV spectral change at 290 nm which accompanies the relaxation reaction (Hunt &

[4] The generous estimate of the error in K_A' for the binding of Mn^{2+} to n_1 sites of the relaxed enzyme is based on assuming as much as a 50% error in K_A' for Mn^{2+} binding to the n_2 sites of the enzyme (Shrake et al., 1977) and a 25% error in K_A' for Mn^{2+} binding to xylenol orange. The reproducibility in the measurement was actually better than ±10%, but the calculation of K_A' for Mn^{2+} binding to n_1 sites is sensitive to the values of the other two equilibrium constants.

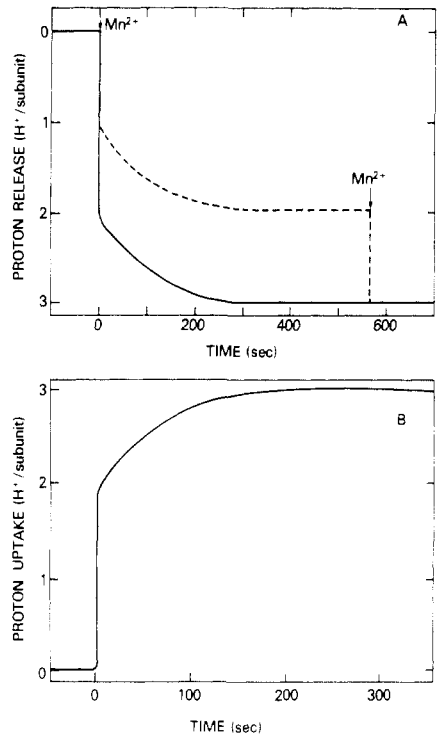

FIGURE 3: Proton release during the binding of Mn^{2+} to divalent cation free (relaxed) glutamine synthetase (A) and proton uptake accompanying the removal of Mn^{2+} from tightened glutamine synthetase (B). (A) At zero time, 5 μL of 8.5 mM Mn^{2+} (dashed curve) or 5 μL of 100 mM Mn^{2+} (solid curve) was added to 1.00 mL of 2.02 mg/mL relaxed glutamine synthetase (40.4 μM subunits) in a spectrophotometric cell of 1.0-cm light path. The Mn^{2+} and enzyme solutions at pH 7.2 contained 3×10^{-5} M bromothymol blue, 3 mM Tris-HCl, and 100 mM KCl. The absorbance change at 616 nm was monitored on the 0.1 absorbance scale of a Cary Model 15 spectrophotometer. A second aliquot of Mn^{2+} (5 μL of 100 mM $MnCl_2$) was added for the dashed curve at 570 s. At the end of each experiment, the enzyme solution was titrated back to the original pH (absorbance value) with 8 mM KOH, using a calibrated microliter syringe, to determine the number of proton equivalents released per enzyme subunit. (B) Mn^{2+} was added to relaxed glutamine synthetase as in the solid curve of (A), and the solution was allowed to equilibrate at ~25 °C for 15 min. At zero time, 10 μL of 0.25 M dipicolinate at pH 7.2 was added to the tightened enzyme (40.2 μM subunits) containing 3×10^{-5} M bromothymol blue, and the absorbance change at 616 nm was monitored. When the absorbance change was complete, the solution was titrated back to the zero-time absorbance value (pH value) with 8 mM HCl to determine the proton equivalents taken up per enzyme subunit. All experiments were conducted at room temperature (~25 °C), and all solutions were degassed under vacuum to minimize the effects of dissolved CO_2. Absorbance changes could be reproduced by alternative additions of KOH and HCl.

Ginsburg, 1972). The fact that this latter proton equivalent returns to the enzyme long after the Mn^{2+} has been removed by dipicolinate (Figure 4B) is strong evidence that this proton is released after Mn^{2+} binding due to a conformationally linked pK_a change of some amino acid side chain that does not participate in forming the Mn^{2+} binding cluster at the n_1 site of the subunit. The stoichiometry of the slow proton release or uptake at pH 7.2 (1.0 equiv of H^+/subunit) is consistent with the perturbation of a single ionizable residue per subunit, although partial contributions from two or more subunit side chains could lead to the same stoichiometry.

Relaxation of the glutamine synthetase previously was induced by the addition of EDTA to the manganese enzyme (Shapiro & Ginsburg, 1968; Hunt & Ginsburg, 1972). A half-time of ~44 s at 25 °C was measured from the absorbancy change at 290 nm produced by adding a 3-fold excess of EDTA to the Mn^{2+}-tightened enzyme (Hunt & Ginsburg, 1972). Similar kinetics for the relaxation of the Mn^{2+}-tightened enzyme were obtained in the present studies by adding excess xylenol orange or dipicolinate at pH 7.2 and following ΔA_{290nm} of the protein conformational change as a function of time. The time course of the first-order relaxation reaction of glutamine synthetase therefore is independent of the presence of EDTA, dipicolinate (Figure 3B), or xylenol orange, indicating that these reagents do not interfere with the relaxation process by interacting with the protein.

In experiments not shown, advantage was taken of the relatively low affinity that dipicolinate has for Mg^{2+}; $K_A' \simeq 250\ M^{-1}$ for Mg^{2+} as compared to $\sim 10^9\ M^{-1}$ for Mn^{2+}, with Mg^{2+} forming a 1:1 complex with dipicolinate whereas the dominant complex with Mn^{2+} at pH 7.2 is a Mn^{2+}–(dipicolinate)$_2$ species.[1] At the end of the absorbance increase in Figure 3B, the enzyme could be retightened by adding 1 mM $MgCl_2$ to the spectrophotometric cell containing relaxed glutamine synthetase, bromothymol blue, dipicolinate, and some Mn^{2+}–(dipicolinate)$_2$ complex. Upon Mg^{2+} addition to the relaxed enzyme in Figure 3B, two proton equivalents per subunit are released (one fast and one slow as in Figure 3A for Mn^{2+} binding to n_1 sites). This is the same result as that obtained previously by calorimetric and pH measurements (Hunt et al., 1972; Hunt & Ginsburg, 1972).

Kinetics of the Dissociation of Mn^{2+} from Glutamine Synthetase. Figure 4A shows a typical stopped-flow absorbance–time trace obtained at pH 7.2 by mixing xylenol orange with glutamine synthetase which has had a prior equilibration with sufficient Mn^{2+} to fill about 70% of the n_1 binding sites. Under such conditions, >97% of the Mn^{2+} is bound to the enzyme, and only about 4% of the n_2 sites are filled. A computer-drawn first-order rate plot derived from the absorbance–time curve is shown also. From experiments such as that shown in Figure 4A, the first-order rate constant for the dissociation of Mn^{2+} from n_1 sites of the tightened enzyme is $3.1\ s^{-1}$ (half-time, $t_{1/2}$, = 0.22 s at 15 °C). The half-time for the pseudo-first-order binding of Mn^{2+} by 2.5×10^{-4} M xylenol orange at 15 °C and pH 7.2 is about 1 ms so that Mn^{2+} would be bound by the dye as rapidly as it is released from the enzyme.

That the slow process responsible for the time course of the absorbance change in Figure 4A is the release of Mn^{2+} from the enzyme was confirmed by measuring the dissociation of Mn^{2+} from the protein in two other ways. The absorbance–time trace of Figure 4B was obtained by mixing a sample of the same glutamine synthetase solution used in Figure 4A (containing 0.7 equiv of Mn^{2+}/mol of subunit) with excess dipicolinate and observing the reaction at 290 nm, a wavelength at which formation of the Mn^{2+}–(dipicolinate)$_2$ complex results in an absorption increase. In Figure 4C, the enzyme solution of Figure 4A was mixed with excess dipicolinate in the presence of bromothymol blue, and the absorbance increase at 616 nm associated with the rapid proton uptake by glutamine synthetase (Figure 3B) was observed. Since the appearance of the released Mn^{2+} as a complex with two different chelating agents (xylenol orange and dipicolinate) and the uptake of the fast proton all occur at the same rate, it seems likely that the slow step for all three reactions is the step common to the three, namely, the release of Mn^{2+} from the tightened enzyme.

The fact that the rate of uptake of the "fast" proton (Figure 4C) corresponds to the rate of Mn^{2+} release from the n_1 sites of the enzyme (Figure 4A,B) suggests that the amino acid side chain being protonated forms part of the Mn^{2+} binding cluster at the n_1 subunit site. This is in contrast to the case of the "slow" proton uptake which follows Mn^{2+} removal from n_1

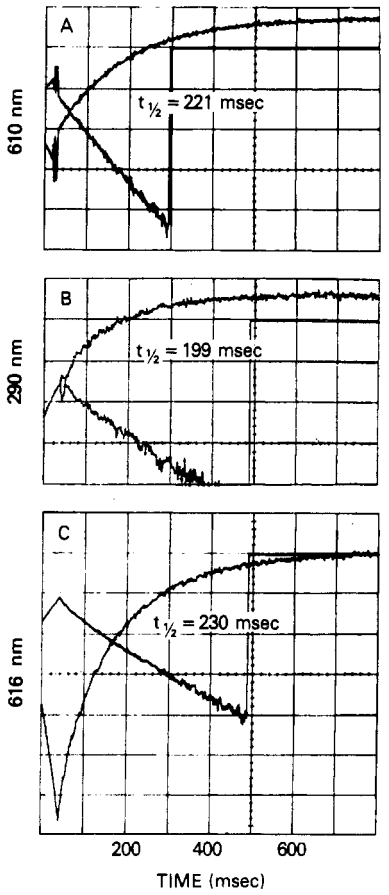

FIGURE 4: Oscilloscope tracings recorded during stopped-flow spectrophotometry of the removal of Mn^{2+} from glutamine synthetase produced by xylenol orange or dipicolinate. Prior to the experiments in (A–C), relaxed glutamine synthetase (1.9 mg/mL; 38 μM subunits) was allowed to equilibrate for 30 min at 24 °C with 27 μM $MnCl_2$ in the presence of 3 mM Tris-HCl–100 mM KCl buffer at pH 7.2. The solution containing 0.71 equiv of Mn^{2+} per enzyme subunit then was degassed and equilibrated at 15 °C for 15 min before mixing with an equal volume of either 0.5 mM xylenol orange (A) or 2.5 mM dipicolinate (B and C) in the stopped-flow instrument. In (C), both the enzyme solution and the dipicolinate solution also contained 30 μM bromothymol blue. In (A), the absorbance increase at 610 nm is due to the formation of a 1:1 Mn^{2+}–xylenol orange complex (Figure 1); in (B), the absorbance increase at 290 nm is due to the formation of a 2:1 dipicolinate–Mn^{2+} complex; in (C), the absorbance increase at 616 nm is due to deprotonation of bromothymol blue. The curves in (A–C) represent absorption increases with scales of 200, 100, and 50 mV per division, respectively. The straight lines in (A–C) represent plots of ln $(A_\infty - A_t)$ vs. time where A_∞ and A_t are absorptions at infinite and at time t, respectively. The half-time ($t_{1/2}$) values were calculated from the corresponding first-order plots.

sites of the enzyme (Figure 3B) and involves other ionizable protein residues perturbed during the conformational transition of the enzyme.

In a single experiment analogous to that of Figure 4C with bromothymol blue present, dipicolinate was used to induce the release of Mn^{2+} from a glutamine synthetase sample with about 1.75 equiv of Mn^{2+} bound per mol of subunit. From the rapidity of the proton uptake by n_2 sites, which was faster than and partially resolved from the rate of proton uptake by n_1 sites, an upper limit of 0.05 s is set for the half-time for Mn^{2+} dissociation from n_2 sites at 15 °C. Thus, the dissociation of Mn^{2+} from n_2 sites is at least 4-fold faster than that from n_1 sites of the enzyme.

Discussion

Complex formation between xylenol orange and Mn^{2+} produces a considerable increase in the absorbance of the dye ($\Delta\epsilon = 1.9 \times 10^4$ M^{-1} cm^{-1} at 585 nm), which makes xylenol orange a sensitive reagent for measuring Mn^{2+}. Our results show that the dominant and perhaps exclusive complex of xylenol orange with Mn^{2+} is a 1:1 complex over the most useful range of Mn^{2+} and xylenol orange concentrations. The apparent association constant[3] of 1.6×10^5 M^{-1} at pH 7.2 for Mn^{2+} makes xylenol orange a good reagent for measuring Mn^{2+}–protein binding constants over the approximate range of 2×10^4 to 5×10^6 M^{-1}. The rate constant for formation of the Mn^{2+}–xylenol orange complex also makes xylenol orange a convenient reagent for indicating by stopped-flow spectrophotometry the rate of Mn^{2+} release from manganese proteins. A significant transfer of Mn^{2+} from a protein–Mn^{2+} complex to xylenol orange will occur on mixing for proteins having apparent association constants for Mn^{2+} in the approximate range of 10^4–10^7 M^{-1}, for which half-times for dissociation are expected to vary from ~10 ms to 10 s. A xylenol orange concentration of 10^{-4} M can be used by working at about 610 nm, which will give a half-time of about 2 ms for the pseudo-first-order formation of the Mn^{2+}–xylenol orange complex.

The range of protein–Mn^{2+} binding constants over which a dye is useful is larger for stopped-flow experiments than for protein binding constant measurements. For the measurements of protein apparent association constants for metal ions, the extents of conversion of both the protein and the metal ion binding dye to their metal ion complexes should be in the range of 10–90%; otherwise, the concentration of either the metal ion bound or the metal ion free species is poorly defined. For kinetic measurements, however, use can be made of even the very small absorption change which occurs when the dye is mixed with a protein–metal ion complex for which the stability constant is ~100-fold greater than that for the dye–metal ion complex. Furthermore, a small, slow absorbance change is easily resolved from a large, fast absorbance change in a fast reaction kinetic experiment. For example, the latter situation would be encountered in measuring the release of Mn^{2+} from a protein to which it is bound weakly. In such a case, the metal ion binding dye would react rapidly upon mixing with the relatively large amount of free Mn^{2+}, and then would react more slowly with the small amount of protein-bound Mn^{2+} as it was released from the protein.

The fact that xylenol orange does not bind to either metal ion free or tightened manganese glutamine synthetase means that kinetic and equilibrium results of this study are not complicated by an interaction between the dye and protein. Hopefully, this will be the case for other protein–Mn^{2+} systems as well.

From equilibrium dialysis measurements, apparent association constants for Mn^{2+} binding to n_1 and n_2 independent subunit sites of glutamine synthetase are 1.9×10^6 and 2×10^4 M^{-1}, respectively, in the absence of effectors at pH 7.2 and 25 °C (Hunt et al., 1975; Shrake et al., 1977). The rapid release of two protons per subunit on the addition of excess Mn^{2+} to the relaxed enzyme is evidence that n_2 sites exist prior to the protein conformational change promoted by the binding of Mn^{2+} to n_1 sites. The spatial distance between the enzyme subunit n_1 and n_2 metal ion binding sites has been estimated to be ≥12 Å in the absence of effectors and ~8 Å in the presence of nucleotide (Hofmann & Glaunsinger, 1978; Balakrishnan & Villafranca, 1978), but in either case the n_1 and n_2 sites of a subunit act independently in binding Mn^{2+} (Hunt et al., 1975; Denton & Ginsburg, 1969).

A reasonable explanation for the stabilization of an active form of glutamine synthetase by Mn^{2+} binding to n_1 sites is that one or more additional protein side-chain ligands are

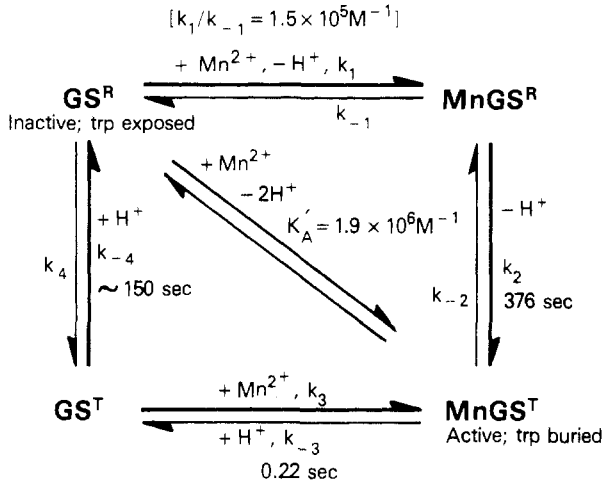

FIGURE 5: Scheme for the binding of Mn^{2+} to the structural (n_1) sites of glutamine synthetase from *E. coli*. The directions of spontaneous reactions are shown by the heavier arrows; all reactions are thermodynamically reversible. The stoichiometry of Mn^{2+} binding to n_1 sites of the enzyme is 1.0 equiv of Mn^{2+} per subunit; the conformational change promoted by adding Mn^{2+} to the divalent cation free, relaxed enzyme (GS^R) is associated with the saturation of 12 Mn^{2+} binding sites per dodecamer (Hunt & Ginsburg, 1972). The relaxed enzyme (GS^R) has an inactive conformation and can be activated (tightened) by Mn^{2+} addition, forming $MnGS^T$ in an active conformation (Kingdon et al., 1968). Tryptophanyl, tyrosyl, and sulfhydryl residues are relatively more exposed to solvent in the R than in the T conformation (Shapiro & Ginsburg, 1968). GS^T is the divalent cation free enzyme having the conformation of $MnGS^T$, and $MnGS^R$ is the Mn^{2+} complex of the low-affinity, relaxed enzyme. The association constant (K_A') and proton release value for the binding of Mn^{2+} to GS^R on the diagonal are from previous studies at pH 7.2 and ~25 °C (Hunt & Ginsburg, 1972; Hunt et al., 1972, 1975); k_2 and k_{-4} are first-order constants with $t_{1/2}$ values given for 15 °C, calculated from corresponding $t_{1/2}$ values at 25 °C and an Arrhenius activation energy of 20.9 kcal/mol for tightening and relaxation processes, respectively (Hunt & Ginsburg, 1972). Values from this study are $k_1/k_{-1} = 1.5 \times 10^5$ M^{-1} at ~25 °C (Figure 2) and $k_{-3} = 3.2$ s^{-1} with $t_{1/2} = 0.22$ s at 15 °C (Figure 4). The proton release or uptake (with negative or positive signs, respectively) is shown for the various reactions.

presented to Mn^{2+} during the protein conformational change. This, and the mere fact that the Mn^{2+}–enzyme combination must be more stable after the conformational change, since this transition occurs spontaneously, leads to the expectation that the binding constant for Mn^{2+} increases during the protein conformational change. We have indeed observed an increase in the apparent association constant for Mn^{2+} from ~1.5×10^5 to 1.9×10^6 M^{-1} during the conformational change of the enzyme. This increase in the Mn^{2+} binding constant corresponds to a difference in free energy $\Delta(\Delta G')$ of ~−1.5 kcal (mol of subunit)$^{-1}$ or ~−18 kcal (mol of enzyme)$^{-1}$ at pH 7.2 and 25 °C. It should be noted that this $\Delta(\Delta G')$ value is for the transition of the liganded species $MnGS^R \rightarrow MnGS^T$ in Figure 5, which certainly is more negative than $\Delta(\Delta G')$ for the conformational transition of the unliganded enzyme $GS^R \rightarrow GS^T$.

Earlier studies (Hunt & Ginsburg, 1972) did not preclude the possibility that the proton released synchronously with the slow burial of aromatic protein residues was derived from a new protein ligand presented to Mn^{2+} at the subunit n_1 site as a result of the conformational change. Since the removal of Mn^{2+} from the protein by excess dipicolinate is very rapid (Figure 4B), the ensuing slow proton uptake in Figure 3B must occur as a result of a pK_a change in one or more subunit amino acid residues outside the n_1 binding cluster. The slow proton release promoted by Mn^{2+} binding to n_1 sites of the enzyme would be a reversal of this process. Thus, the Mn^{2+}-promoted conformational transition of the enzyme causes a perturbation of ionizable residues, probably as a result of changes in solvent exposure.

Stopped-flow experiments with dipicolinate in the absence and presence of bromothymol blue showed that there is a fast proton uptake coincident with Mn^{2+} dissociation from n_1 sites of glutamine synthetase. This suggests that the amino acid side chain being rapidly protonated forms part of the Mn^{2+} binding cluster at the n_1 subunit site. Thus, one proton binds at the rate of Mn^{2+} dissociation from the enzyme (probably to a group within the n_1 binding site), and another proton per subunit binds after Mn^{2+} release as the protein undergoes the conformational transition.

In discussing the binding of Mn^{2+} to n_1 sites of glutamine synthetase (GS), it is convenient to use the scheme presented in Figure 5. The enzyme is a dodecamer composed of apparently identical subunits, each of which has separate n_1 and n_2 binding sites for Mn^{2+} (Ginsburg, 1972). The scheme of Figure 5 does not take into account the saturation of the lower affinity n_2 sites with Mn^{2+}, which are involved in binding the nucleotide substrate at the subunit catalytic site (Hunt et al., 1975). However, Mn^{2+} binding to n_2 sites does not influence the reactions shown in Figure 5, since n_1 and n_2 subunit sites are independent (noninteracting).

In the scheme of Figure 5, the enzyme can exist in two conformations; the relaxed enzyme, GS^R, dominates in the absence of divalent cations, and the tightened enzyme, $MnGS^T$, dominates with Mn^{2+} bound to n_1 sites. In the relaxed, *inactive* state, tryptophanyl, tyrosyl, and sulfhydryl groups of the protein are more accessible to solvent (exposed) than in the tightened, *active* state (Shapiro & Ginsburg, 1968; Ginsburg, 1972). The intermediate $MnGS^R$ in Figure 5 is required to explain the binding of Mn^{2+} to n_1 sites prior to the conformational change. It is reasonable to assume that the intermediate GS^T is formed by treatment of $MnGS^T$ with excess dipicolinate, EDTA, or xylenol orange to induce the dissociation of Mn^{2+} from $MnGS^T$.

The rates of proton release or uptake which have been measured in Figure 5 are in these three cases much slower than expected for rate-limiting proton transfer (Eigen & De Maeyer, 1963). In each instance, the actual proton-transfer step is coupled to some slower rate-limiting step, i.e., an enzyme conformational change in the case of k_2 and k_{-4} and a cleavage of a Mn^{2+}–protein bond (substitution reaction at Mn^{2+}) in the case of k_{-3}.

Prior to the present study, only the rate constants k_2 and k_{-4} and the apparent association constant for Mn^{2+} binding to n_1 sites of unadenylylated glutamine synthetase at pH 7.2 (K_A' on the diagonal of Figure 5) had been measured. Half-time values at 15 °C shown in Figure 5 for the corresponding first-order rate constants k_2 and k_{-4} were calculated from k_2 and k_{-4} values at 25 °C and an Arrhenius activation energy of 20.9 kcal/mol for the tightening and relaxation processes, respectively (Hunt & Ginsburg, 1972). Because of the uncertainty in the Arrhenius activation energy for the relaxation process, the value of k_{-4} at 15 °C (Figure 5) is an approximation.

The apparent association constant of GS^R for Mn^{2+} which we have measured now provides the ratio of rate constants $k_1/k_{-1} \simeq 1.5 \times 10^5$ M^{-1} at 25 °C (pH 7.2). This study also has provided the first-order rate constant $k_{-3} = 3.1_5$ s^{-1} at 15 °C (pH 7.2) with a corresponding half-time of 0.22 s. This rate is ~680-fold faster than k_{-4} for the conformational change in the relaxation process.

Of the remaining rate constants to be determined in Figure 5, probably k_{-2} and k_4 cannot be measured directly, since there

is no obvious means of relaxing the enzyme with Mn^{2+} present at n_1 sites or of tightening the enzyme in the absence of Mn^{2+}. However, k_{-2} may be calculated from data at 25 °C (pH 7.2) by using eq 2 where $k_2 = 6.3 \times 10^{-3}$ s^{-1} at 25 °C (Hunt & Ginsburg, 1972). From this computation, $k_{-2} = 4.97 \times 10^{-4}$ s^{-1} at 25 °C (with a corresponding half-time of 1393 s), which is 12.7-fold slower than k_2. Studies are in progress to measure k_1, k_{-1}, and k_3 of Figure 5.

$$(k_1/k_{-1})(k_2/k_{-2}) = K_A = 1.9 \times 10^6\ M^{-1} \qquad (2)$$

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