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A Calorimetric Study of the Interaction of Mn^{2+} with Glutamine Synthetase from *Escherichia coli*†

J. B. Hunt,‡ P. D. Ross, and A. Ginsburg*

ABSTRACT: Mn^{2+} is a specific activator and stabilizer of the dodecameric structure of glutamine synthetase from *Escherichia coli*. The interaction of Mn^{2+} with the unadenylylated form of this enzyme has been investigated calorimetrically in the present study. To measure proton release from the enzyme during metal ion binding at pH 7.17 (37°), Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and Tris-chloride buffers were used since these buffering compounds have quite different heats of protonation ($\Delta H = -4.8$ and -11 kcal per mole of H^+, respectively). Although complex thermokinetic curves were observed, the results are consistent with the following interpretation. During the binding of each of the first 12 equiv of Mn^{2+} ($K_{eq}' = 2 \times 10^6$ M^{-1}), two protons are displaced from the enzyme per mole of subunit of ~50,000 molecular weight (with measured values of ΔH differing by 13 kcal/mole subunit in the two buffers). One proton is released instantaneously and the second proton is released in a slow first-order process that has an apparent half-time at 37° of ~55 sec and at 25° of ~230 sec. The heats observed in the slow reaction are less than that due to protonation of either buffer, suggesting an endothermic process. The slow thermal process may be attributed to a conformational change in the protein, with the endothermic contribution not resolved from the associated slow release of one proton equivalent. Since very little net heat is associated with the interaction of Mn^{2+} (or Mg^{2+}) with glutamine synthetase, the binding of these divalent cations to the enzyme involves a rather large entropy increase. For the binding of each Mn^{2+} to unadenylylated glutamine synthetase at 37° and pH 7.2, the following thermodynamic parameters are indicated: $\Delta G' = -8.9$ kcal mole^{-1} (standard state for hydrogen ions at activity of $10^{-7.2}$ M), $\Delta H \simeq +3$ kcal mole^{-1}, and $\Delta S' \simeq +38$ cal deg^{-1} (mole of subunit-Mn)$^{-1}$.

Glutamine synthetase from *Escherichia coli* is a dodecameric aggregate of 12 apparently identical subunits (Woolfolk *et al.*, 1966) arranged molecularly in two superimposed hexagons (Valentine *et al.*, 1968). The enzyme has a molecular weight of 600,000 (Shapiro and Ginsburg, 1968).

A dominant control of glutamine synthetase activity in *E. coli* occurs through enzyme-catalyzed adenylylation and deadenylylation reactions, which in turn are regulated by intracellular metabolite concentrations (Stadtman *et al.*,

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1968a,b; Holzer *et al.*, 1967; Holzer, 1969; Shapiro and Stadtman, 1970a). The site of adenylylation in glutamine synthetase is a specific tyrosyl residue within the subunit polypeptide structure of ~50,000 molecular weight which, upon adenylylation, becomes a very stable 5′-adenylyl-*O*-tyrosyl derivative (Shapiro and Stadtman, 1968; Heinrikson and Kingdon, 1971). Adenylylation, which can occur to the extent of 12 equiv of 5′-adenylyl groups attached per mole of glutamine synthetase (Kingdon *et al.*, 1967), markedly affects various catalytic parameters of the enzyme (Shapiro and Stadtman, 1970a). Among the properties affected are the interaction of the enzyme with divalent cations (Kingdon and Stadtman, 1967; Denton and Ginsburg, 1969; Stadtman *et al.*, 1968a) and the molecular stability of the enzyme (Stadtman *et al.*, 1970).

Earlier studies have shown that the removal of Mn^{2+} from glutamine synthetase causes a conformational change (*relaxation process*) that leads to an exposure of sulfhydryl groups (Shapiro and Stadtman, 1967) and, without change in molecular weight, to an exposure of tryptophanyl and tyrosyl residues and a decrease of ~0.6 S in $s^0_{20,w}$ (Shapiro and Ginsburg, 1968). A *tightening* of the relaxed enzyme by the addition of Mn^{2+} or Mg^{2+} activates glutamine synthetase in catalyzing the biosynthesis of L-glutamine from L-glutamate, ATP, and ammonia (Kingdon *et al.*, 1968). The stoichiometry of the binding of Mn^{2+} by glutamine synthetase in the *tightening* or activation process was found to be 12 Mn^{2+}/enzyme molecule or one Mn^{2+} bound per subunit (Denton and Ginsburg, 1969). The apparent intrinsic association constant (K_{eq}) for Mn^{2+} binding to 12 apparently equivalent and independent enzyme sites was found to be $\sim 2 \times 10^7$ to 1×10^5 M^{-1}, depending upon pH (pH 7.0–7.7) and the extent of adenylylation of the enzyme preparation. In the same studies, a second set of 12 equiv of Mn^{2+}/mole of glutamine synthetase were found to be bound with $K_{eq} \simeq 2 \times 10^4$ M^{-1}. These two Mn^{2+} binding sites per enzyme subunit are believed to be involved in the catalytic mechanism.

This paper reports on calorimetric studies of the interaction of Mn^{2+} with unadenylylated glutamine synthetase. Since it was anticipated that there would be a contribution to the heat evolved by protons released from the enzyme in the metal binding process, two buffers with quite different heats of protonation, but about the same pK, were employed in these experiments. Some kinetic studies on divalent cation–glutamine synthetase interactions are presented in the following paper (Hunt and Ginsburg, 1972).

Materials and Methods

Glutamine Synthetase Preparations. Glutamine synthetase was purified to homogeneity from *E. coli* previously grown on glycerol and limiting ammonia, as described by Woolfolk *et al.* (1966) and Shapiro and Stadtman (1970b). The enzyme preparations used here contained an average of 1.0 equiv of covalently bound 5′-adenylyl groups per mole (600,000 g) of glutamine synthetase. The enzyme which had been used in metal-binding experiments was repurified by subjecting it to a series of at least three ammonium sulfate precipitations in 0.01 M $MnCl_2$–0.01 M imidazole buffer, as described by Woolfolk *et al.* (1966). The enzyme preparation was stored at 4° as a precipitate in *ca.* 50% saturated ammonium sulfate, from which samples were collected as needed by centrifugation.

In general, protein concentrations were obtained from absorbancy measurements at 290 nm, corrected for turbidity of the solutions by fourth-power extrapolation to 290 nm of the 340-nm absorbance. In the calculations of protein concentration the absorbancy indices $A^{0.1\%}_{290\,nm} = 0.356$ for the relaxed (metal free) enzyme and $A^{0.1\%}_{290\,nm} = 0.385$ for the Mn^{2+}- or Mg^{2+}-saturated enzyme were assumed (Shapiro and Stadtman, 1970b). Protein concentrations determined in this manner agreed quantitatively with those obtained from absorbancy measurements at 280 nm (Ginsburg *et al.*, 1970). For the calculations of molar concentration of enzyme, a molecular weight of 600,000 (~50,000 molecular weight per subunit) was assumed.

Divalent cations were removed from the enzyme preparations by treatment with a tenfold excess of EDTA to divalent cation (~0.01 M EDTA) for 30 min at *ca.* 25° followed by either gel filtration through Sephadex G-25 equilibrated with the chosen buffer or dialysis for at least 24 hr against *ca.* 1000 volumes of the buffer. Passage of the enzyme–EDTA mixture twice through a 1.5 × 22 cm column of G-25 Sephadex freed the relaxed enzyme solution of EDTA and brought the enzyme solution to the pH of the chosen buffer. When gel filtration was not used, the relaxed enzyme solution was dialyzed exhaustively against the chosen buffer. Relaxed enzyme solutions were tested for complete removal of EDTA and divalent cations by measuring the absorbance changes accompanying the addition of EDTA or of Mn^{2+} or Mg^{2+}. Divalent cation-free solutions of glutamine synthetase showed only the absorbance change expected for dilution upon the addition of EDTA, and enzyme solutions containing EDTA gave less than the expected absorbance increase upon the addition of one Mn^{2+} ion per enzyme subunit of ~50,000 molecular weight.

Glutamine Synthetase Assays. The Mg^{2+}-activated biosynthetic activity of the unadenylylated enzyme was measured routinely by the coupled spectrophotometric assay as described previously (Ginsburg *et al.*, 1970). Results reported here were obtained with enzyme having activity ≥38 units/mg at room temperature. Prolonged dialysis or storage at pH 7.8 of the relaxed enzyme sometimes caused a loss in biosynthetic activity and in these cases the enzyme preparation was not used.[1]

The extent of adenylylation of the enzyme was determined by spectral analysis (Shapiro and Stadtman, 1970b) and from measurements of γ-glutamyl transferase activities at pH 7.15 with Mn^{2+} and with Mn^{2+} + Mg^{2+} (Stadtman *et al.*, 1970). The two methods gave $\bar{n} = 0.9$ and $\bar{n} = 1.2$ for the enzyme preparation used here.

Reagents. Reagent grade hydrated $MnCl_2$ and $MgCl_2$ were the sources of the divalent cations used. The stock solution of $MnCl_2$ was assayed spectrophotometrically as MnO_4^- after oxidation with sodium bismuthate. Stock solutions of imidazole were treated with activated charcoal and filtered to remove any yellow color. Reagent grade KOH, KCl, and 38% HCl were used as received for the preparation of solutions. Tris (as Trizma base) and Hepes[2] were obtained from Sigma Chemical Co., and solutions of the latter were filtered before use to remove a small amount of insoluble matter.

[1] An apparent correlation between activity in the coupled assay and the ability of the enzyme to bind Mn^{2+} and Mg^{2+} was observed. A sample of relaxed enzyme which had lost about one-third of its activity gave only two-thirds of the normal absorbance increase at 290.3 nm and only two-thirds of the normal proton release upon saturation with Mn^{2+} (Hunt and Ginsburg, 1972).

[2] Good *et al.* (1966) have described the physical properties of the buffers used here: Tris, tris(hydroxymethyl)aminomethane (pK^{20} = 8.3; $\Delta pK_a/°C = -0.031$); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pK^{20} = 7.55; $\Delta pK_a/°C = -0.014$). Both buffers have negligible metal ion–buffer binding constants for Mn^{2+} or Mg^{2+}.

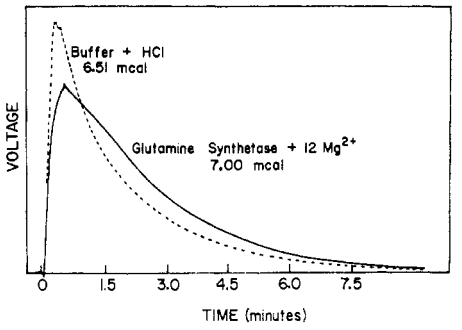

FIGURE 1: Microcalorimetric (batch type) experiments at 37° with the dashed curve showing heat release during ~2% neutralization of Hepes buffer and the solid curve showing the heat release produced upon glutamine synthetase binding 12 molar equiv of Mg^{2+}/mole of enzyme in 0.05 M Tris-C–0.10 M KCl buffer at pH 7.19. The heat release in each case is proportional to the area under the voltage curve which returns to a base line of 0 volts in ~20 min at 37°; only the initial major heat bursts are illustrated here. For the buffer neutralization, 1.8 ml of 8.4×10^{-4} M HCl in 0.10 M KCl was added to 3.8 ml of 0.05 M Hepes–0.10 M KCl buffer at pH 7.2 in the calorimeter. For the enzyme-binding experiment, sufficient Mg^{2+} was added in 1.8 ml to 3.8 ml of glutamine synthetase (5.6 mg/ml) in the calorimeter to give a final concentration of 1.0 mM $MgCl_2$ (0.93×10^{-3} M Mg^{2+} unbound).

Deionized water prepared by passing ordinary distilled water through a Crystal Research Laboratory Model DJ-128 deionizing unit was used in the preparation of all solutions.

Calorimetric Measurements. Calorimetric measurements of the heats of reaction between glutamine synthetase and Mn^{2+} or Mg^{2+} were made in a LKB Model 10700 batch-type microcalorimeter (Wadsö, 1968), calibrated as described elsewhere (Scruggs and Ross, 1970). Usually 1.8 ml of buffer[2] solution containing the Mn^{2+} or Mg^{2+} was mixed with 3.8 ml of the same buffer containing relaxed glutamine synthetase (4 mg/ml) in the reaction vessel. In the tare calorimeter vessel, 1.8 ml of the buffer solution containing metal ion was mixed with 3.8 ml of the same buffer to cancel out mechanical heating effects (chemical heating effects arising from the heat of dilution of the metal ion were negligible with Mn^{2+} and $\leq 3\%$ correction with Mg^{2+}). The heat of dilution of the relaxed enzyme was measured by mixing it with divalent cation-free buffer in the calorimeter. The solutions were loaded into the calorimeter cell volumetrically by means of calibrated glass syringes fitted with Teflon needles.[3] The calorimeter was equipped with a Keithley Model 149 millimicrovoltmeter and Texas Instruments Co. Servowriter II recorder with a Disc integrator, which provided a record of the time course of the thermopile output (expressed in volts) arising from the difference in temperature between the sample and reference cells. The integrator section of the recorder recorded the difference in heat production in the two cells.

The reactants were allowed to stand in the calorimeter for at least 1 hr prior to mixing to assure thermal equilibrium with the surroundings (environmental room set at either 37 or 25°), the criterion for which was a persistent voltage difference (<2 μV) between the two thermopiles. After mixing the reactants, the duration of experiments was 20–25 min at 37° and at least 30 min at 25°, at the end of which time calorimeter runs were terminated upon reattainment of the baseline voltage.

Spectral Measurements. All spectra were recorded using a Cary Model 15 spectrophotometer equipped with 0–0.1 and 0–1 slide-wires. Spectra at temperatures other than ambient were obtained using a water-jacketed sample cell through which water from a constant-temperature bath was circulated. Prior to difference spectral measurements, enzyme solutions were usually passed through 0.85 μ Millipore filters to remove lint. Sample temperatures were measured by means of a thermistor probe (Yellow Springs Instrument Co. no. 44103) and an Idustrial Instruments Model RC 16B2 conductivity bridge.

pH Measurements. A Radiometer Model PHM 25 meter equipped with a scale expander and combination microelectrode (A. H. Thomas Co., no. 4858-L25) was used for pH measurements. The pH of solutions used for calorimetry was measured at the temperature of the run before and after mixing, using a water-jacketed vessel to maintain sample temperature. Such pH measurements provided assurance of equilibration of the enzyme solutions with the buffer. Relaxed enzyme solutions differing by more than 0.02 pH unit from the solution of buffer containing divalent cation were not used for calorimetric runs.

Results

In Figure 1, a typical record of the thermopile output voltage as a function of time for the reaction of relaxed glutamine synthetase with the divalent cation Mg^{2+} is contrasted to the voltage *vs.* time record for an instantaneous reaction involving nearly the same total heat production in neutralization of a buffer. The heats produced in the reactions are proportional to the areas under the voltage curves, which have been truncated for illustrative purposes. In Figure 1 the initial voltage rise is representative of less than 10^{-3}° maximum increase in temperature during either reaction in the calorimeter. At the time of the initial rise in voltage, mixing is estimated to be ~90% complete and then 100% complete at the beginning of the decay in voltage as the drum with the cells completes its rotation. Mixing was always found to be complete as evidenced by the absence of further heat evolution upon stirring at the end of the voltage decay, which took 20–25 min at 37° (see Methods).

The acid–base neutralization reaction (buffer + HCl) is expected to be complete within the time of mixing having a second-order rate constant of *ca.* 10^{10} M^{-1} sec^{-1}. The curve for this latter reaction in Figure 1 shows a rapid voltage rise followed by a decay back to the base voltage as heat is dissipated from the reaction vessel to its surroundings. The shape of the curve for the instantaneous reaction is highly reproducible, as is the decay time of the calorimeter for such a reaction. The calorimeter used in the present study requires 90 ± 5 sec after the initial heat burst for the decay of the voltage difference to $1/e$ of its initial value, regardless of whether the heat burst is provided electrically or by a rapid reaction (*e.g.*, acid–base neutralization or sucrose dilution).

The curve of Figure 1 for the enzyme–metal ion reaction differs in two important respects from the curve for the instantaneous buffer–HCl reaction. The maximum voltage attained is substantially lower in the case of the metal ion–enzyme reaction, and the time required for decay back to the original voltage is substantially longer for the metal ion–enzyme reaction. A reasonable explanation for this difference in behavior is that the metal ion–enzyme reaction is not instantaneous, so that

[3] Enzyme solutions were also retrieved from the calorimeter the same way. Sterile plastic disposable syringes were found to contaminate the enzyme solutions with an impurity which absorbed strongly in the ultraviolet region.

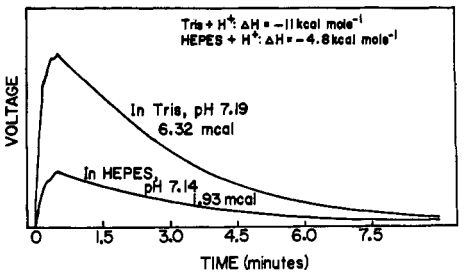

FIGURE 2: Initial heat release during the binding of 12 molar equiv of Mn^{2+} to glutamine synthetase (15.2 mg) at 37°. As indicated, the calorimetric experiments were performed either in 0.05 M Tris-Cl–0.10 M KCl or in 0.05 M Hepes–0.10 M KCl buffer at pH ~7.17. In the calorimeter in each case, 3.46×10^{-4} mmole of Mn^{2+} in 1.8 ml was added to 3.8 ml of glutamine synthetase (4.0 mg/ml) to produce a final concentration of 7.5×10^{-6} M unbound Mn^{2+} with at most 4% of the enzyme-binding sites unsaturated. The total heat release in millicalories in the two experiments, each of ~25 min in duration, and the heats of protonation of the two buffers (see Table I) are indicated as inserts to the figure.

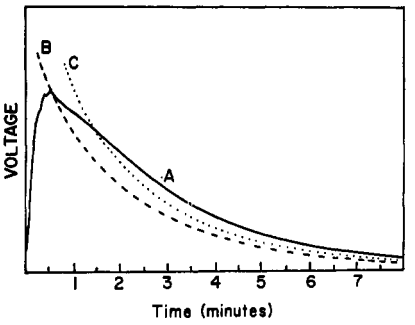

FIGURE 3: Illustration for the analysis of the observed calorimetric decay curves obtained in the initial major heat release (in volts) produced by the binding of 12 molar equiv of Mg^{2+} or Mn^{2+} to glutamine synthetase. Curve A (——) is reproduced from Figure 2 for the voltage curve obtained with Mn^{2+} + glutamine synthetase in Tris-chloride buffer at 37° (pH 7.19). Curves B and C (– – –, . . .) are *normal* calorimeter decay curves for voltage crossover at 0.5 and 1.5 min, respectively, as obtained for Hepes buffer + HCl in Figure 1 (see text). The difference between the areas under curves A and B and curves A and C is the unreleased heat (ΔQ) for the enzyme-metal ion interaction at 0.5 and 1.5 min, respectively.

some heat of reaction is still being produced in the reaction cell after dissipation of heat to the surroundings has set in. A discussion of this phenomenon will follow (see below).

Mn^{2+}, rather than Mg^{2+}, was used in most of the calorimetric experiments because the binding constants for Mn^{2+} were known from the studies of Denton and Ginsburg (1969), and the amount of Mn^{2+} necessary to saturate the twelve high-affinity metal-binding sites of glutamine synthetase could be quantitated.

The voltage–time record for the reaction of Mn^{2+} with high-affinity sites on glutamine synthetase was very similar to that already shown for the reaction with Mg^{2+}. For a given total heat production, the maximum voltage attained was lower and the decay back to the equilibrium voltage was slower for the Mn^{2+}–enzyme reaction than for a rapid acid–base reaction. In Figure 2 are shown typical voltage *vs.* time curves for the reaction of the same quantities and concentrations of Mn^{2+} and glutamine synthetase in two different buffers, Tris and Hepes at the same pH and temperature. It is seen that the shapes of the curves are very similar, but that the quantity of heat evolved during the reaction in Tris buffer is much larger. Since the only significant difference between the two experiments of Figure 2 is the difference in the heats of protonation of the buffers (Table I below), the difference in the two curves suggests that protonation of the buffer accounts for most of the heat evolved during the metal ion–enzyme interaction.

An attempt was made to quantitatively analyze the slow heat release. Curve A of Figure 3 is a typical voltage *vs.* time plot for the Mn^{2+}–glutamine synthetase reaction in Tris at 37°. Curve B is a segment of a decay curve for an instantaneous reaction, hereafter called a normal calorimeter decay curve, which has been constructed to intersect curve A at time = 0.5 min. If the Mn^{2+}–glutamine synthetase reaction were already complete at 0.5 min so that no more heat was produced after this point in time, then decay of the voltage along curve B would be expected. The area under curve B after the intersection at 0.5 min is the heat which has already been produced but has yet to be dissipated in decay back to the equilibrium voltage. The area between curves A and B is the heat which is produced in the calorimeter after 0.5 min. Curve C of Figure 3 is a normal calorimeter decay curve constructed to intersect curve A at 1.5 min. The area between curves C and A is the heat produced in the calorimeter after 1.5 min. It is seen that much less heat is produced after 1.5 min than after 0.5 min, so that a significant fraction of the heat-producing reaction must occur in the interval between these times.

It is evident that the heat produced in the calorimeter after any time t (denoted by the symbol ΔQ) could be obtained in a similar manner from the difference in areas between the reaction curve and a normal decay curve. In practice, ΔQ was obtained by subtracting from the number of integrator counts observed after time t for the metal–enzyme reaction the number of integrator counts expected for an instantaneous reaction after the calorimeter had attained the observed voltage.

In Figure 4, ΔQ is shown plotted on a logarithmic scale as a function of time for the Mn^{2+}–glutamine synthetase re-

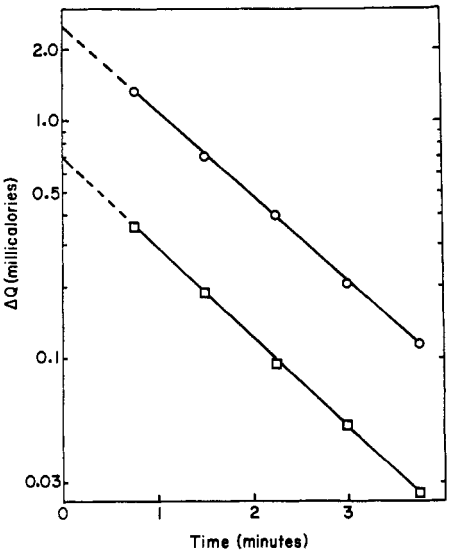

FIGURE 4: A kinetic representation of the slow thermal process. The unreleased heat (ΔQ) is plotted on a logarithmic scale *vs.* time for the interaction of glutamine synthetase with 12 molar equiv of Mn^{2+}/mole of enzyme in Tris-C (○) or in Hepes (□) buffer at pH 7.17 at 37° (see text).

TABLE I

Calorimetric Expt at 37°	ΔH (kcal mole^{-1}) Tris, pH 7.19[a]	Hepes, pH 7.14[a]	$\Delta(\Delta H)$ (kcal mole^{-1})
	Part a		
Buffer + H^+	−11.0[b]	−4.8	−6.2
Glutamine synthetase + Mn^{2+}	−19.7	−6.3	−13.4
Fast process	−10.6	−4.0	−6.6
Slow Process	−9.1	−2.3	−6.8
	Part b		
Endothermic contribution (GS + Mn^{2+}) − (buffer + $2H^+$) =	+2.3	+3.3	

[a] Values are the average of at least six different calorimetric experiments, with precision estimates given in the text. The *total heats* (see Figure 2) for glutamine synthetase + Mn^{2+} (expressed in kcal per mole of subunit or kcal per Mn^{2+} bound) have been corrected for a small exothermic contribution from dilution of the protein. Experimentally, −0.35 and −0.20 mcal were measured in Tris and Hepes buffers for the dilution of glutamine synthetase from 4.0 to 2.7 mg per ml in the calorimeter (conditions as specified in the legend to Figure 2). The heat of dilution of glutamine synthetase under these conditions is calculated to be −13.8 and −7.9 kcal per mole of enzyme in Tris and Hepes buffers, respectively. [b] Literature value (Izatt and Christensen, 1968).

action in both the Tris and Hepes buffers. These plots are expected to be linear if a single first-order reaction is responsible for the slow production of heat. It is seen that the plots are indeed linear and moreover, data plots of the type shown in Figure 4 were generally found to exhibit first-order behavior from the first measurement at ~60% to >90% of the reaction. Since the slopes of the plots in Figure 4 are nearly the same, the half-times for the slow thermal reaction in the two buffers must be similar. Extrapolation of the linear plots back to the mixing time ($t = 0$) gives the total heat associated with the slow reaction, if it is assumed that the first order process is occurring from the mixing time at $t = 0$. For the run in Tris buffer illustrated in Figure 4, the heat produced in the slow reaction was 2.6 mcal, whereas the total heat produced in this run was 6.2 mcal. The difference between these heats must then have been produced in some rapid reaction. The Mn^{2+}–glutamine synthetase reaction must then involve two steps, a fast heat-producing step and a slow heat-producing step.

Based on plots such as shown in Figure 4 for a number of runs in the two buffers, the half-time for the slow heat-producing reaction with glutamine synthetase + Mn^{2+} in Tris at 37° was found to be 55 ± 5 sec and the half-time in Hepes was found to be ~54 sec.

A single calorimetric experiment shown in Figure 1 measured the heat released upon the binding of Mg^{2+} to glutamine synthetase in Tris-Cl buffer at pH 7.19 (37°). The final Mg^{2+} concentration was sufficient to 98% saturate the 12 high-affinity Mn^{2+} binding sites of the unadenylylated enzyme (Denton and Ginsburg, 1969), which induces a conformational change in the enzyme structure (Shapiro and Ginsburg, 1968; Hunt and Ginsburg, 1972). In the experiment of Figure 1, a total heat production of 17 kcal/mole of subunits present was measured, of which ~7 kcal/mole was associated with a slow first-order process having $t_{1/2} \simeq 54$ sec.

The calorimetric data for the binding of the first 12 molar equiv of Mn^{2+} to glutamine synthetase at pH ≃ 7.17 and 37° are summarized in Table I. Of particular importance is the last column of data, the values for $\Delta(\Delta H)$, which is the difference in heat production in the two buffers. Using a value obtained here at 37° for the heat of protonation of Hepes buffer, which is in fair agreement with the values reported by Hinz *et al.* (1971), the heats of protonation of the two buffers differ by 6.2 kcal/mole. The total heat produced in the two buffers during the Mn^{2+}–glutamine synthetase reaction differs by 13.4 kcal/mole of Mn^{2+} bound, which is approximately that expected if two protons were released to react with the buffer for each Mn^{2+} ion bound. It is apparent that the heat production associated with the fast process differs by 6.6 kcal/mole of Mn^{2+} bound and that associated with the slow process differs by 6.8 kcal for each mole of Mn^{2+} bound. The latter differences are approximately the differences expected if one proton is released in the fast process and one proton is released in the slow process for each Mn^{2+} bound to glutamine synthetase. The total heat produced in each of the buffers in somewhat less than that expected from the protonation of 2 moles of buffer. This suggests that there is a small endothermic contribution to the total heat, amounting to about 2.3–3.3 kcal/mole of enzyme subunit. The lower absolute heat released in the slow process indicates that the endothermic reaction is associated principally with the slow rather than the fast process.

The heat release associated with the slow process (obtained by extrapolation to zero time as in Figure 4) was quite reproducible (±5%). There was, however, some variation in the observed total heat release, which was greater in Hepes than in Tris buffer. It was generally found that in experiments in which the calorimeter returned precisely to the initial equilibrium voltage, the resultant heat was nearly exactly the value obtained by averaging all similar runs (Table I). The value of $\Delta(\Delta H)$ will be affected by at most 10% by the observed variations in values of ΔH. The endothermic contribution, of course, is more uncertain.

The conditions of the experiments on which Table I is based were such as to ensure the binding of more than 0.95, but less than 1.00, molar equiv of Mn^{2+} ion per subunit of enzyme. Treatment of the enzyme from the calorimeter cell with EDTA gave >95% of the absorbance decrease at 290.3 nm expected for an enzyme saturated with Mn^{2+} at the highest affinity binding sites, and the enzyme was fully active in the biosynthetic assay.[1]

At 25° and pH 7.2, approximately the same total heat release as observed at 37° was obtained for the interaction of Mn^{2+} with glutamine synthetase in Tris or Hepes buffer. In Tris buffer (five runs at 25°, but as otherwise described in the legend to Figure 2), an average total heat of −23 ± 4 kcal per molar equiv of Mn^{2+} bound to each enzyme subunit (of which −8 ± 0.3 kcal/mole was associated with the slow proton release) was observed. A half-time of 228 ± 48 sec was estimated for the slow process at 25°. The observed half-times for the first-order slow reactions at 25 and 37° indicate an activation energy of ~22 kcal/mole (enzyme subunit-Mn^{2+}) for this process.

From the 25 and 37° calorimetric data obtained with Tris buffer, an apparent heat-capacity change ($\Delta C_p'$) was approxi-

mated to be -110 ± 65 cal deg^{-1} $mole^{-1}$ for the total heat evolved in the slow thermal process. A negative value for $\Delta C_p'$ has several possible origins (Hinz *et al.*, 1971), the most important of which in this case are alterations in protein structure induced by the binding of metal ions. The precision in measurements of the total heat of binding was $\pm 17\%$ standard deviation from the mean. Consequently, $\Delta C_p'$ for the overall process of metal ion binding to glutamine synthetase is uncertain, with calculated values of $\Delta C_p'$ varying from 0 to ± 330 cal deg^{-1} $mole^{-1}$. A value of $\Delta C_p' \approx 0$ for the binding of Mn^{2+} to glutamine synthetase is suggested, however, by an apparently small temperature dependence of the binding constant. Both the *low* total heat of the binding reaction and the similar stoichiometry of Mn^{2+} binding at 37 and 25° suggest that the binding constant is very nearly the same at these two temperatures.

Initial calorimetric experiments on the binding of the first 12 Mn^{2+} to glutamine synthetase were performed at pH 7.8 and 37°, because the apparent affinity of the enzyme for Mn^{2+} is greatest at pH ≥ 7.7 (Denton and Ginsburg, 1969). Total heat production in two experiments was -7.6 and -9.6 kcal per mole of subunits present, with approximately 40% of the total heat released in a slow process in each case. Unfortunately, relaxed preparations of glutamine synthetase are unstable at this pH; the enzyme at the high pH was found later to have lost $\sim 50\%$ of its biosynthetic activity. It is quite probable therefore that the lower heat production at the higher pH was due simply to a diminished capacity of the enzyme to bind Mn^{2+}. Such a phenomenon has been observed in other kinetic experiments.[1]

Discussion

The calorimetric studies reported here have shown that with the interaction of the first 12 Mn^{2+} or Mg^{2+} with the *E. coli* glutamine synthetase, two protons are displaced per Mn^{2+} or Mg^{2+} bound. At pH 7.2 and 37 or 25°, approximately one proton is released rapidly and one proton is released in a slow thermal process. The slow process appears to include a small endothermic contribution, whereas nearly all the heat evolved in the fast process can be accounted for by the heat of protonation of the buffer used. The calorimetric results can be written in the following reaction sequence,

$$[GSH_2] + Mn^2 \overset{k_1\ (\text{fast})}{\rightleftharpoons} [GSH]\text{-}Mn^+ + H^+ : \Delta H_1 \approx 0 \quad (1)$$

$$[GSH]\text{-}Mn^+ \overset{k_2\ (\text{slow})}{\rightleftharpoons} [GS]\text{-}Mn + H^+ : \Delta H_2 \approx +3 \text{ kcal mole}^{-1} \quad (2)$$

which combine to give the overall binding reaction

$$[GSH_2] + Mn^{2+} \rightleftharpoons [GS]\text{-}Mn + 2H^+ \quad (3)$$

where [GS] is representative of 1 of 12 subunits of glutamine synthetase of mol wt 50,000. The discussion of this reaction sequence will be divided into parts A and B.

A. Thermodynamic Considerations. The enthalpies reported for reactions 1 and 2 have been corrected for the heat of protonation of the buffer species present. Since the experiments were performed at constant pH (pH 7.17 $\pm$ 0.03), the free energy change for reaction 3 ($\Delta G_{1,2}$) is constant and proportional to the logarithm of the apparent intrinsic association constant (K_{eq}'), which is equal to $K_{eq}/(H^+)^2$. Apparently, K_{eq}' for reaction 3 is independent of the buffer species present.

In previous studies (Denton and Ginsburg, 1969), the first 12 molar equiv of Mn^{2+} (or one Mn^{2+} per subunit of glutamine synthetase) were found to be bound to apparently independent and equivalent sites of the enzyme. The apparent association constant for this binding reaction was dependent on pH and on the adenylylation state of the enzyme, with this constant increasing with increasing pH and decreasing with increasing extents of adenylylation. As reaction 3 is written, K_{eq}' should vary inversely with the square of the hydrogen ion concentration. This relationship was found in direct binding measurements, but only in a limited pH range of pH 6.5 to about pH 7.8. At higher pH, K_{eq}' appeared to be independent of pH. This suggests that there is an independent effect of high pH on the enzyme conformation that is reflected in the value of K_{eq}'. An apparent association constant for reaction 3 of $K_{eq}' = 2 \times 10^6$ M^{-1} at pH 7.2 was extrapolated from the previous equilibrium binding measurements at 24° with $^{54}Mn^{2+}$ and unadenylylated glutamine synthetase at pH 8 (Denton and Ginsburg, 1969). This value of K_{eq}' is judged to be approximately correct from the stoichiometry of the proton release from glutamine synthetase at this pH (37 or 25°) upon the addition of an amount of Mn^{2+} that was quantitated on this basis.

Since very little net heat is associated with the interaction of Mn^{2+} (or Mg^{2+}) with glutamine synthetase, the binding of these divalent cations to the enzyme involves a rather large entropy increase. Combining the measured heats at 37° ($\Delta H_1 + \Delta H_2 = \Delta H_{1,2}$) for the enthalpy change in reaction 3 with $\Delta G_{1,2}' = -8.9$ kcal $mole^{-1}$ (standard state for hydrogen ions at activity of $10^{-7.2}$ M at 37°), a value of $\Delta S' \simeq +38$ cal deg^{-1} (mole of subunit-Mn)$^{-1}$ is calculated. In the single calorimetric experiment with Mg^{2+} added to glutamine synthetase, $\Delta H_1 \simeq 0$ and $\Delta H_2 \simeq +4$ kcal/mole of Mg^{2+} bound to each enzyme subunit. From data presented in the accompanying paper of Hunt and Ginsburg (1972), $\Delta G_{1,2}' = -6.1$ kcal/mole of Mg^{2+} bound to the enzyme at pH 7.2 (37°). Using these values for $\Delta G_{1,2}'$ and $\Delta H_{1,2}$, the thermodynamic quantity $\Delta S' \simeq +33$ cal deg^{-1} (mole of subunit-Mg)$^{-1}$ is calculated. Since each of the 12 subunits of glutamine synthetase appear to be involved in the binding of the first 12 Mn^{2+} or Mg^{2+}, the overall entropy change for the dodecamer will be 12 times greater or ~ 420 cal deg^{-1}.

In similar studies of another protein–metal ion interaction, Henkens *et al.* (1969) attributed the observed large increase in entropy to charge neutralization of solvated carboxylate ions upon complex formation with an attendant liberation of water. The entropy increase, together with the positive enthalpy change, observed for reaction 3 suggests that a charge neutralization of carboxylate ions possibly occurs also in the binding of Mn^{2+} (or Mg^{2+}) to glutamine synthetase. If so, the metal ion binding site of the subunit will be relatively less polar after chelation with Mn^{2+} or with Mg^{2+}.

The two protons displaced from the enzyme by Mn^{2+} at neutral pH seem likely to occur from the ionization of two nitrogeneous groups. These amino acid residues may form a part of the Mn^{2+} binding cluster or one or both groups may be structurally perturbed by Mn^{2+} binding to a nearby site so that there is an induced ionization. The instability of relaxed glutamine synthetase at pH ≥ 7.8 precluded calorimetric measurements that may have provided information on this point. Proton displacement from partially active ($\geq 50\%$) glutamine synthetase by Mn^{2+} at pH 7.8 occurred, however, as both a fast and slow proton release, although the stoichiometry of the proton displacement in either reaction was poor.[1]

B. Kinetic Considerations. The rapid proton release in reac-

tion 1 is written to represent a bimolecular reaction in which Mn^{2+} binds to the protein subunit while simultaneously displacing a proton. The bimolecular rate of metal ion–chelate formation generally is limited only by the characteristic rate of water substitution in the inner coordination sphere of the metal ion ($k \simeq 6 \times 10^6$ M^{-1} sec^{-1} for $Mn(H_2O)_6^{2+}$; $k \simeq 10^5$ M^{-1} sec^{-1} for $Mg(H_2O)_6^{2+}$; Diebler *et al.*, 1969). These rates are more than 10^6 times greater than the observed rate of the slow thermal process (see below) or of the slow proton release (Hunt and Ginsburg, 1972) in reaction 2. The slow thermal process in reaction 2, which is more complicated than written, is first order, and the slow proton release in this reaction appears to be coupled to a slow Mn^{2+}- or Mg^{2+}-induced conformational change of the enzyme (see below). $Mn(H_2O)_6^{2+}$ will be stripped of as many water molecules as there are amino acid residues of the subunit polypeptide chain involved in chelating the metal ion (Diebler *et al.*, 1969) and this could easily number more than the proton equivalents released.

In previous studies, the interaction of divalent cation-free preparations of glutamine synthetase with Mn^{2+} (or Mg^{2+}) was shown to activate the enzyme in catalysis (Kingdon *et al.*, 1968), to cause a burial of reactive sulfhydryl groups (Shapiro and Stadtman, 1967) and of tryptophanyl and tyrosyl residues (Shapiro and Ginsburg, 1968), and to increase slightly the sedimentation rate ($\Delta s^0_{20,w} = +0.6$ S). Consistent with these previous observations is the interpretation that the slow first-order thermal process observed here in the calorimetric studies of the enzyme–Mn^{2+} interaction also arises from a Mn^{2+}-induced conformation change in the protein. Further, it should be noted that in previous calorimetric studies of the interaction of glutamine synthetase with two feedback inhibitors (Ross and Ginsburg, 1969) a slow thermal process was not evident from the appearance of the thermopile voltage decay curves.

The first order rate plots of the slow thermal reaction, that presumably arises from the protonation of the buffer, were linear from the first measurable time at about 60% to more than 90% completion of the reaction (Figure 4). The endothermic process therefore is not resolved from the slow proton release. The slow thermal process in reaction 2 was found to have a half-time of ~55 sec at 37° and of ~230 sec at 25°. These data indicate an apparent activation energy of ~22 kcal (mole of subunit-Mn)$^{-1}$ for the slow thermal process. About the same activation energy has been found for the Mn^{2+}- or Mg^{2+}-induced ultraviolet spectral perturbation of glutamine synthetase (Hunt and Ginsburg, 1972). However, the half-time of the slow thermal process at either 37 or 25° is nearly twice that observed for the rate of proton release or of the ultraviolet spectral perturbation at the same temperature, the kinetics of which are presented in the accompanying paper (Hunt and Ginsburg, 1972). The discrepancy between the first-order rate constants obtained here and in the other kinetic studies of reaction 2 suggests that this reaction is more complex than written. The slower rates in the calorimetric measurements could be due to conformational changes in the enzyme structure that are reflected in the endothermic contribution, but are not measured in the direct studies of proton release or of tryptophanyl–tyrosyl perturbation. The previously observed sedimentation changes indicate that the binding of Mn^{2+} or Mg^{2+} to glutamine synthetase produces a gross change in the hydrodynamic behavior of the enzyme (Shapiro and Ginsburg, 1968). Nevertheless, the interaction of metal ions with glutamine synthetase does not produce changes in the secondary structure that can be detected by optical rotatory dispersion or circular dichroism spectral measurements (Hunt and Ginsburg, 1972).

A bimolecular reaction followed by a slower first-order reaction has been observed also in the binding of oxygen by hemocyanin (Brunori, 1971). Significantly, hemocyanin contains two atoms of Cu^{2+} per O_2 binding site, without interactions apparently occurring between oxygen binding sites of the protein. The movement of the second Cu^{2+} atom induced by O_2 binding might be analogous to the movement of a potential chelating group induced by the binding of Mn^{2+} (or Mg^{2+}) to glutamine synthetase.

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