A Calorimetric Study of the Binding of Two Feedback Inhibitors to the Glutamine Synthetase from *Escherichia coli**

Philip D. Ross and Ann Ginsburg

ABSTRACT: The apparent heats of binding of two feedback inhibitors, alone or mixed, at saturating concentrations (in 0.02 M imidazole, 0.1 M KCl, and 0.001 M $MnCl_2$, pH 7.07), to the Mn^{2+}-activated form of the glutamine synthetase from *Escherichia coli* have been measured by calorimetry at 25°. Where ΔH is expressed as calories per mole of subunit of 50,000 molecular weight, $\Delta H = -7350$ and $\Delta H = -2000$ for L-tryptophan and adenosine 5′-monophosphate binding, respectively. For a saturating mixture of L-tryptophan and adenosine 5′-monophosphate, a ΔH value of -9600 cal/mole was measured which is about equal to the sum of the enthalpies of the binding of the individual effectors to the enzyme. The additivity of the measured ΔH values demonstrates that the Mn^{2+} form of glutamine synthetase, when saturated with 12 equiv of each effector/mole of enzyme, has separate binding sites for L-tryptophan and adenosine 5′-monophosphate. The ability to measure reaction heats in the 0.2–5-mcal range suggests that calorimetry will be useful in studying the interaction between this enzyme and even more weakly, or strongly, bound effectors. There is no correlation between the measured ΔH values and the apparent free energies of binding of either L-tryptophan or adenosine 5′-monophosphate to glutamine synthetase in the same buffer system. Apparent entropy changes of about -11 and $+11$ cal deg^{-1} (mole of subunit)$^{-1}$ were estimated for the binding of L-tryptophan and adenosine 5′-monophosphate, respectively. These entropy changes are 12 times greater for the entire glutamine synthetase molecule, and are therefore quite large. When both effectors are bound simultaneously to the enzyme, the accompanying total apparent entropy change is nearly zero.

The glutamine synthetase of *Escherichia coli* is composed of 12 similar, if not identical, subunits that are molecularly arranged in two superimposed hexagons (Woolfolk *et al.*, 1966; Shapiro and Stadtman, 1967; Valentine *et al.*, 1968). The enzyme is isolated as a homogeneous protein that is activated by Mn^{2+} or Mg^{2+} (Kingdon *et al.*, 1968; Denton and Ginsburg, 1969), and, in the native active form, behaves hydrodynamically as a spherical particle of ~600,000 molecular weight (Shapiro and Ginsburg, 1968).

Glutamine synthetase catalyzes the formation of L-glutamine from L-glutamate, ATP, and ammonia. Since L-glutamine in microorganisms is the biochemical precursor of at least six chemically different end products, this enzyme occupies a key position in a highly branched biosynthetic pathway (Stadtman *et al.*, 1968a). Consequently, the cellular regulation of glutamine synthetase is important, and several mechanisms of control for this activity in *E. coli* and other microorganisms have been described (Stadtman *et al.*, 1968a,b; Holzer *et al.*, 1967, 1968). As demonstrated with the *E. coli* enzyme, an important regulation of glutamine synthetase activity can occur through feedback inhibition by the multiple end products of L-glutamine metabolism (Woolfolk and Stadtman, 1964, 1967). The cumulative inhibition observed kinetically with the addition of combinations of these inhibitors at saturating levels suggested that each inhibitor acts independently in partially inhibiting the enzyme. Direct binding studies have indicated more recently that interactions can occur between binding sites (Ginsburg, 1969). However, the binding results with AMP and L-tryptophan have shown also that the enzyme molecule binds 12 equiv of each of these feedback inhibitors. It was suggested, in view of the dodecameric structure of the enzyme, that each subunit of glutamine synthetase possessed a single, unique binding site for each inhibitor. In this report, the apparent thermodynamics of the binding of AMP and L-tryptophan to glutamine synthetase has been studied calorimetrically under saturating conditions in order to obtain further information about the nature of these allosteric effector sites.

Experimental Section

Calorimetric measurements of the heat of reaction upon mixing were made at 25° in a LKB Model 10700 batch type microcalorimeter (Wadsö, 1968). The calibration of this instrument is described elsewhere (Scruggs and Ross, 1969). Usually, 2 ml of solution containing effector was combined with 3 ml of dialysate containing glutamine synthetase in the reaction vessel. In the balance calorimeter vessel, 2 ml of effector was mixed with 3 ml of dialysate to eliminate the heat of dilution of the effector. The solutions were loaded into the calorimeter cell volumetrically by means of calibrated syringes fitted with Teflon needles.

The glutamine synthetase used in these studies is a homogeneous protein preparation that has been described previously (Shapiro and Ginsburg, 1968; Denton and Ginsburg, 1969). As isolated from *E. coli* by Denton, using the procedure of Woolfolk *et al.* (1966), it contains an average of 2.3 equiv of covalently bound 5′-adenylyl groups (Shapiro *et al.*, 1967) per mole (600,000 g) of glutamine synthetase. However, the adenylylation state of glutamine synthetase was found to not influence the binding of either AMP or L-tryptophan;

* From the Laboratory of Molecular Biology, National Institute of Arthritis and Metabolic Diseases, and the Laboratory of Biochemistry, Section on Enzymes, National Heart Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received July 25, 1969.*

TABLE I: Calorimetric Results.[a]

I. L-Tryptophan + glutamine synthetase			
Final L-tryptophan concentration = 0.01 M			
Initial protein concentration (mg/ml):	10.87	5.63	3.95
Tryptophan + enzyme (mcal):	−4.65	−2.095	−1.425
Enzyme dilution heat (mcal):	+0.19	+0.391	+0.295
Total heat (mcal):	−4.84	−2.486	−1.720
Q (mcal/mg of enzyme)	−0.148	−0.147	−0.145
Average Q_I = −0.147 mcal/mg of enzyme			
II. AMP + glutamine synthetase[b]			
Initial protein concentration = 5.44 mg/ml; final AMP concentration = 9.72×10^{-4} M			
Q_{II} = −0.040 mcal/mg of enzyme			
III. AMP + L-tryptophan + glutamine synthetase			
Initial protein concentration = 3.95 mg/ml; final AMP and L-tryptophan concentration, each 0.01 M			
Q_{III} = −0.192 mcal/mg of enzyme			
Summation of results			
$Q_I + Q_{II}$ = −0.187 mcal/mg of enzyme $\simeq Q_{III}$ = −0.192 mcal/mg of enzyme			

[a] In addition to effector and enzyme concentrations indicated, all solutions at 25.0° contained 0.02 M imidazole-chloride, 0.1 M KCl, and 0.001 M $MnCl_2$ (pH 7.07). [b] Q_{II} calculated for 100% saturation of the enzyme with AMP (see text).

further, this same enzyme preparation was used in the direct binding studies to which the calorimetric results are correlated (Ginsburg, 1969). Before use, protein solution was dialyzed exhaustively at 4° over a 3-day period against three changes of 1000 volumes of buffer containing 0.02 M imidazole-chloride, 0.1 M KCl, and 0.001 M $MnCl_2$, and then clarified by centrifugation. The last dialysate (pH 7.07 at 25°) was saved and used for all dilutions. The dialyzed protein solution was stored at 4° until equilibrated at 25° for the calorimetry experiments, under which conditions the enzyme is stable. After the calorimetric experiments, the enzyme was recovered and the combined fractions (~200 mg) were repurified through the acetone and ammonium sulfate steps of Woolfolk *et al.* (1966). The glutamine synthetase concentrations were determined from absorbancy measurements at 280 mμ (Shapiro and Ginsburg, 1968). Bovine serum albumin (a three-times-crystallized preparation from Pentex Corp.) was dialyzed as described above and the specific absorbancy factor, $A^{0.1\%} = 0.667$ at 279 mμ, determined by Foster and Sterman (1956) was used. Concentrations of AMP and L-tryptophan were determined from the spectral extinction coefficients of Boch *et al.* (1956) and Beaven and Holiday (1952), respectively.

Solutions of 0.025 and 0.05 M L-tryptophan were prepared by diluting the anhydrous compound from Nutritional Biochemical Corp. in the protein dialysate. Stock solutions of 0.05 M AMP were prepared by titrating adenosine 5′-monophosphoric acid (Grade V from Sigma Chemical Corp.) in water at about two-fifths of the final volume with KOH to pH 7.02, and then after adjusting the concentrations of imidazole-chloride (pH 7.1) and $MnCl_2$ to those of the protein dialysate, three-fifths volume of dialysate was added. The final pH generally required little or no adjustment to pH 7.07. This neutralization procedure approximately balances the ionic activity of the potassium salt of the ionized phosphate group of AMP to that of KCl in the dialysate, before dilution with dialysate. The stock AMP was diluted further with the protein dialysate or with the 0.05 M L-tryptophan solution for the calorimetry experiments.

Deionized water with a conductivity $\leq 1.7 \times 10^{-6}$ ohm^{-1} was obtained from a Model DJ-128 water deionizing unit from the Crystal Research Laboratory, Inc., and this water was used exclusively for the preparation of solutions. A Model PHM 25 radiometer equipped with a scale expander and a Leeds and Northrup no. 124138 microelectrode assembly was used for pH determinations at 25°. A Cary 15 recording spectrophotometer was used for spectra.

Results

The calorimetric results are presented in Table I. In all the studies contained in Table I, it was necessary to correct the measured heat of reaction between effector and enzyme for the heat of dilution of the enzyme. The heat of dilution of glutamine synthetase was determined as a function of protein concentration, and from the resultant curve the values for the dilution heats shown in the upper portion of Table I were obtained. The dilution of the enzyme was a slightly endothermic process, which was largest at a protein concentration of ~7 mg/ml. Possibly, a very small per cent of aggregates of dodecameric units in the enzyme solution that melt out upon dilution could account for the smaller endothermic dilution heats observed at higher protein concentrations. Aggregates of the native dodecameric protein cannot represent any appreciable fraction of the total protein since the viscosity and sedimentation studies do not reveal their presence (Shapiro and Ginsburg, 1968). The physical studies also show that the Mn^{2+}-activated dodecameric enzyme does not dissociate into subunits at neutral pH. It was decided to carry out the calorimetric work at low concentrations of enzyme in order to minimize complications arising from possible

aggregate formation. For comparative purposes, bovine serum albumin at an initial concentration of 11.76 mg/ml was diluted in the same proportion and buffer system (see Methods). The dilution heat observed was −0.027 mcal/mg of protein, and the dilution of this protein appeared to be an uncomplicated exothermic process. The results obtained with bovine serum albumin do indicate that the endothermic dilution heats measured for glutamine synthetase, although small in magnitude, are probably significant.

In the first section of Table I, the heat of the binding of L-tryptophan to glutamine synthetase is reported. The final concentration of L-tryptophan in each calorimetric experiment was 0.01 M which is in considerable excess of the saturating concentration of approximately 3×10^{-3} M determined from direct binding studies (Ginsburg, 1969). A heat of −0.147 mcal/mg of enzyme was found for the interaction of saturating amounts of L-tryptophan with glutamine synthetase. This value was independent of protein concentration, indicating the attainment of saturating conditions in all cases. The average deviation of less than 1% in these energies, in which each result represents the combination of two independent calorimetric experiments yielding measured heats in the range of 0.2–5 mcal, demonstrates the excellent performance of this calorimeter at these very low heat levels.

In the second section of Table I, the calorimetric results for the binding of AMP to glutamine synthetase are reported. For technical reasons, the AMP concentration used in these experiments corresponded to 88% saturation of the enzyme at the final free concentration of AMP (0.918 mM). The heat reported (Q_{II}) is corrected to 100% saturation of the enzyme (*i.e.*, −0.0356/0.88 mcal per mg of enzyme), and is a low exothermic value. In view of the large apparent intrinsic association constant for the binding of AMP to the enzyme and the very small measured heat, it immediately follows that the binding interaction between AMP and glutamine synthetase is largely entropically driven (see Discussion and Table II below).

The third section of Table I shows the results of the combined experiment in which L-tryptophan and AMP both at final concentrations of 0.01 M were mixed with the enzyme. It was previously found that the binding of L-tryptophan to glutamine synthetase in the imidazole–KCl buffer was not significantly influenced by the presence of AMP. However, the binding of AMP, for which the enzyme has a higher affinity in the absence of other added effectors, was found to be decreased by L-tryptophan (see Discussion below). Therefore, the calorimetric experiment with the mixture utilized high concentrations (0.01 M) of both components that were well beyond the individual saturating concentrations of AMP and L-tryptophan in order to ensure the complete binding of AMP to the Mn^{2+} form of the enzyme. The result obtained for the combined binding experiment was −0.192 mcal/mg of enzyme. This measured value is in excellent agreement with the sum of the measured heats of interaction of glutamine synthetase with each component separately, as shown in the last line of Table I. The agreement is quite remarkable since the experimental errors of six independent calorimetric studies, each of approximately 1% accuracy, are involved in this summation. The additivity of the measured heats shown at the bottom of Table I suggests that glutamine synthetase has separate and apparently independent binding sites under these conditions for each of the two inhibitors, AMP and L-tryptophan.

The heat of dilution of each effector was roughly determined in order to approximate the entropy of dilution under the conditions of the calorimetric studies. Combining these heat values with the calculated free-energy change of dilution (assuming activity coefficients to be unity), ΔS values of +1.86 and +2.5 cal deg^{-1} $mole^{-1}$ were estimated for the dilution of L-tryptophan and AMP, respectively, in these experiments. These entropy changes are small compared with the very large entropy change accompanying the reaction of either of these effectors with glutamine synthetase (see Discussion and Table II below).

Discussion

It is possible to combine the measured heats of Table I with the results of previous equilibrium binding studies (Ginsburg, 1969) to calculate apparent thermodynamic quantities characterizing the interaction between effector and each enzyme subunit. These calculations are based on the previously determined molecular weight of 600,000 for glutamine synthetase (Shapiro and Ginsburg, 1968) and a structure consisting of 12 subunits (Valentine *et al.*, 1968). Since the enzyme binds a total of 12 equiv of either AMP or L-tryptophan at saturating concentrations, a subunit is considered to contain one binding site for each effector. Thus, the thermodynamic parameters may represent average values for the binding of effectors to each subunit. With 1 mg of enzyme equivalent to 2×10^{-8} mole of subunit, the measured heats (Q_I, Q_{II}, and Q_{III}) shown in Table I are converted into ΔH values, expressed in calories per mole of subunit, in Table II. These values of ΔH were then combined with the average apparent association constants obtained in the equilibrium binding studies to calculate apparent entropy changes accompanying binding, ΔS, expressed in cal/deg-mole of subunits in Table II. Values of $[S]_{0.5}$ are listed in Table II also to describe the free concentration of each effector required to half-saturate the respective 12 binding sites of the enzyme under the different conditions, and these values were estimated from the binding studies (Ginsburg, 1969). The parameter $[S]_{0.5}$ is useful in the cases in which the binding of effectors does not follow the law of mass action.

We wish to stress the apparent nature of the thermodynamic parameters listed in Table II, since they may include energetic contributions from protein conformational changes accompanying the binding of effectors in addition to the intrinsic enthalpy and entropy of binding at the interaction sites of the macromolecule. This may very likely be the case for L-tryptophan which exhibits a cooperative type of binding curve (Ginsburg, 1969) that is suggestive of a conformational change in the enzyme being effected through the binding of this inhibitor (Monod *et al.*, 1965; Wyman, 1967). In contrast, the interaction of AMP with glutamine synthetase was shown to be more straightforward. The glutamine synthetase molecule was found to have 12 equivalent and independent AMP binding sites described by a single apparent intrinsic association constant of ~8000 $mole^{-1}$ (Ginsburg, 1969). Therefore, the small exothermic enthalpy change measured for the interaction of AMP with the enzyme in Table II shows that this binding is largely an entropically driven process. For L-tryptophan, ΔS is of opposite sign to that found for AMP. The entropy change involved in the interaction of L-tryptophan with the enzyme serves to moder-

TABLE II: Apparent Thermodynamic Parameters.

Effector (Present at Saturating Concentrations[a])	$[S]_{0.5}$[b] (M × 10^4)	$\Delta G'$ (cal $mole^{-1}$) (from Binding Measurement[a,c])	ΔH (cal $mole^{-1}$) (from Calorimetry at 25.0°)	ΔS (cal deg^{-1} $mole^{-1}$) (Calcd[d])
L-Tryptophan	9.1	~−4150	−7350	−10.7
AMP	1.25	−5330	−2000	~+11
Mixture of L-tryptophan	9.1	~−9200	−9600	~−1.3
AMP	~2			

[a] At saturation, 12 equiv of each effector is bound per mole of glutamine synthetase (or 1 equiv/subunit of 50,000 mol wt). The buffer contains 0.02 M imidazole-chloride, 0.1 M KCl, and 0.001 M $MnCl_2$ at pH 7.07. [b] $[S]_{0.5}$ is the concentration of effector required for half-saturation of the enzyme, and this quantity is only equivalent to the reciprocal of the apparent intrinsic association constant (k'_A) in the case of AMP binding since the binding of L-tryptophan to glutamine synthetase is cooperative with a variable k'_A function (Ginsburg, 1969). [c] Binding measurements were at 4° in the buffer system given in *a*; $\Delta G'$ is expressed for 298°K, assuming no temperature dependence; $\Delta G' = -RT \ln (1/[S]_{0.5}) \simeq -RT \ln k'_A$. [d] Calcd from: $\Delta S = (\Delta H - \Delta G)/T$, for $T = 298$°K.

ate the rather large contribution of ΔH to the apparent free energy of this reaction. It is evident from the quantities shown in Table II that there is no immediately obvious correlation between the average apparent association constant ($1/[S]_{0.5}$) and ΔH or ΔS. We call attention to the large absolute values of ΔS of 11 cal deg^{-1} (mole of subunits)$^{-1}$ for L-tryptophan or AMP since the entropy change involved for a single protein molecule would be 12 times as great or 132 eu. Whether accidental or of some undetermined significance, it is of interest that these large entropy changes are approximately canceled in the combined interactions of L-tryptophan and AMP with the enzyme.

The measured heat and free energy of the enzyme reaction with effectors are thermodynamic parameters of the whole system so that a possible dismutation of protons between the buffer environment and the protein during the binding of effectors must be considered also. Although the imidazole buffer (used at 0.02 M, ~0.12 ionic strength, and the same pH in the calorimetric and binding studies) has a large heat of ionization, this buffer is preferred because it has an as yet undetermined specific effect in stabilizing glutamine synthetase during purification and storage.[1] Inorganic phosphate or cacodylate buffers that ionize in the correct pH range with smaller heats accompanying ionization markedly inhibit the enzyme. Without knowing the structural changes induced in the enzyme by phosphate or cacodylate ions, it would be undesirable to employ either of these buffers in the calorimetric studies. The fact that the enzyme has the capacity to bind 12 equiv or 1 full equiv per subunit of either AMP or L-tryptophan under the conditions used argues against a proton competing at either of the two types of sites in the binding of these effectors at pH 7.0–7.5. (If an amino acid residue of the protein participating in an electrostatic binding mechanism is only partially in an ionized or protonated form, the apparent maximum number of binding sites with similar affinities for the ion will reflect this fact (Saroff, 1957; Lewis and Saroff, 1957).) Further, the binding of AMP to glutamine synthetase appears to involve hydrophobic rather than electrostatic forces in the specific binding mechanism (Ginsburg, 1969). It is possible that in the binding of L-tryptophan, or of AMP, a conformational change is induced in the enzyme that influences the ionization of a protein group removed from the immediate binding site. If a proton is added to or removed from the buffer environment during the binding of either effector, this becomes a coupled reaction to the binding event.[2] Thus, it cannot be said with certainty that identical binding parameters would be obtained in another buffer system at the same pH. However, our results do describe the apparent thermodynamic parameters for the binding of the effectors to glutamine synthetase in the imidazole buffer employed.

The apparent entropy changes of Table II are not readily interpretable. However, we have some information on the mechanism of AMP binding to glutamine synthetase. Neither the ionized phosphate group of AMP nor the divalent cation bound to the enzyme participate in the binding mechanism and the purine 6-amino group of AMP is an important structural feature, with the enzyme having equal to greater affinities for adenosine and adenine and a fivefold lower affinity for GMP and none for IMP at the AMP binding sites (Ginsburg, 1969). Without electrostatic forces involved in the binding of AMP to the enzyme, the positive entropy change (Table II) could reflect a disorientation of water or of some hydrophobic protein group at the AMP binding site. Not enough is

[1] The stabilizing effect of imidazole on glutamine synthetase may relate to the fact that this compound is a structural analog of L-histidine, one of the feedback inhibitors of the enzyme (Woolfolk and Stadtman, 1964).

[2] It seems unlikely that as much as one proton per subunit is exchanged with the buffer in a secondary coupled reaction, since this would yield a heat of $\sim \pm 0.17^6$ mcal for 1 mg of enzyme. A small fractional change in the protein subunit charge induced by binding (*i.e.*, binding influencing the pK of an already partially ionized amino acid residue) would be more reasonable from the magnitude of heats observed in Table I. However, at pH 7.1, the most probable amino acid residue to ionize would be that of histidine so that a proton exchange between buffer and protein imidazole groups would yield very little net heat. No pH changes (ΔpH = 0.00 ± 0.01 pH unit) were observed during binding measurements using up to 10 mg of enzyme/ml.

known about the binding of L-tryptophan to the enzyme to examine the possible origin of this negative entropy change.

Previous equilibrium binding studies have indicated that there is an interaction between the AMP and L-tryptophan binding sites of glutamine synthetase at subsaturating AMP concentrations (Ginsburg, 1969). Throughout the concentration range of L-tryptophan binding, the binding of this effector was not noticeably influenced by the presence of about half-saturating concentrations of AMP. However, the binding of AMP was significantly decreased under these conditions. The effect of L-tryptophan in decreasing the apparent affinity of the enzyme for AMP was much less marked with the Mn^{2+} than with the Mg^{2+} form of the enzyme. In contrast, the binding of AMP alone to glutamine synthetase was found to be the same in the absence or presence of either Mg^{2+} or Mn^{2+}. In the calorimetric studies of Tables I and II, only Mn^{2+} was employed as the activating and stabilizing divalent cation. Extrapolations of the binding data for AMP with the Mn^{2+} enzyme form in the presence of saturating concentrations of L-tryptophan, indicate that there is a sharpening of the AMP saturation function. The apparent nonreciprocal relations observed experimentally for the L-tryptophan and AMP binding functions are anomolous if considered in terms of the fundamental linkage relationships proposed by Wyman (1964, 1967). Possibly, the measurements of AMP and L-tryptophan binding are not descriptive of the whole system. If the effects of divalent cation, imidazole buffer,[1] protons, KCl, AMP, and L-tryptophan on the glutamine synthetase molecule are all taken into account by the thermodynamic potentials defined by Wyman, it is possible that reciprocally linked functions could be demonstrated. Obviously, the allosteric linked functions in the case of the glutamine synthetase from *E. coli* are complex, and we do not yet know enough about this system to theoretically analyze our observations on the interactions of combined effectors with this enzyme. It should be recognized also that little is known in general about linked functions which can give rise to apparently unidirectional pathways.

As shown in Table II, there is only a slight increase in the $[S]_{0.5}$ value for AMP due to the effect of L-tryptophan decreasing the affinity of the Mn–enzyme for AMP. If all of this change in the average apparent association constant for AMP is assumed to be a change in ΔH, this would only amount to 280 cal, or a difference in Q_{II} of Table I of 0.0056 mcal which is only $\sim 3\%$ of the AMP contribution to the total heat of reaction of the mixed inhibitors binding to the enzyme. It is doubtful that this effect of L-tryptophan on the binding of AMP by glutamine synthetase could be detected by the calorimetric technique. It will be recalled that ΔH is proportional to the logarithm of k'_A so that ΔH is a less sensitive parameter of interaction between binding sites than are the binding functions. If L-tryptophan makes the AMP binding cooperative, and this has not been definitely established, there is a real possibility that an additional entropy change is introduced in the binding of AMP to the enzyme in the presence of L-tryptophan. The data of Table II suggest that a small, but definitely negative, additional entropy change is associated with the simultaneous binding of both L-tryptophan and AMP to the enzyme.

Finally, it should be mentioned that an unusual regulation of glutamine synthetase activity occurs through enzymatic adenylylation and deadenylylation, as discussed by Stadtman *et al.* (1968b). It was demonstrated that adenylylation of glutamine synthetase changes the kinetic response of the enzyme to inhibitors (Kingdon and Stadtman, 1967; Kingdon *et al.*, 1967; Shapiro *et al.*, 1967). However, the extent of adenylylation of the enzyme did not influence the individual binding of AMP or L-tryptophan (Ginsburg, 1969). Therefore, it is most likely that the calorimetric results obtained here under the conditions of the binding studies will apply to other enzyme preparations containing differing amounts of covalently bound 5′-adenylyl groups.

In summary, the thermodynamic results presented in this paper suggest that glutamine synthetase has separate sites for AMP and L-tryptophan that interact with saturating concentrations of these effectors to yield apparently additive heats of binding. It appears that calorimetry will be a useful tool for the investigation of the binding of both weakly or strongly interacting effectors to this protein, or to other allosteric enzymes. In addition, the calorimetric technique offers a special advantage in studying the multiple interactions of different effectors with an enzyme. Saturating levels of effectors with quite different association constants, both alone and in combination with the enzyme, will each yield a heat of binding per mole of a similar order of magnitude.

Acknowledgments

We wish to express our appreciation to Dr. E. R. Stadtman for suggesting to us the use of calorimetry to study the combined interactions of effectors with glutamine synthetase under saturating conditions, and for a continued stimulating interest in this work. Also, we wish to acknowledge that exploratory calorimetric measurements were in collaboration with Dr. W. W. Forrest, who was a guest scientist in the Laboratory of Technical Development, National Heart Institute, National Institutes of Health, while on leave from CSIRO, Division of Nutritional Biochemistry, Adelaide, Australia.

References

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.

Boch, R. M., Ling, N. S., Morell, S. A., and Lipton, S. H. (1956), *Arch. Biochem. Biophys. 62*, 253.

Denton, M. D., and Ginsburg, A. (1969), *Biochemistry 8*, 1714.

Foster, J. F., and Sterman, M. D. (1956), *J. Am. Chem. Soc. 78*, 3656.

Ginsburg, A. (1969), *Biochemistry 8*, 1726.

Holzer, H., Mecke, D., Wulff, K., Leiss, K., and Heilmeyer, L., Jr. (1967), *Advan. Enzyme Reg. 5*, 211.

Holzer, H. Schutt, H., Masek, F., and Mecke, D. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 721.

Kingdon, H. S., Hubbard, J. S., and Stadtman, E. R. (1968), *Biochemistry 7*, 2136.

Kingdon, H. S., Shapiro, B. M., and Stadtman, E. R. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1703.

Kingdon, H. S., and Stadtman, E. R. (1967), *Biochem. Biophys. Res. Commun. 27*, 470.

Lewis, M. S., and Saroff, H. A. (1957), *J. Am. Chem. Soc. 79*, 2112.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Saroff, H. A. (1957), *J. Phys. Chem. 61*, 1364.
Scruggs, R. L., and Ross, P. D. (1969), *J. Mol. Biol.* (in press).
Shapiro, B. M., and Ginsburg, A. (1968), *Biochemistry 7*, 2153.
Shapiro, B. M., Kingdon, H. S., and Stadtman, E. R. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 642.
Shapiro, B. M., and Stadtman, E. R. (1967), *J. Biol. Chem. 242*, 5069.
Stadtman, E. R., Shapiro, B. M., Ginsburg, A., Kingdon, H. K., and Denton, M. D. (1968b), *Brookhaven Symp. Biol. 21*, 378.
Stadtman, E. R., Shapiro, B. M., Kingdon, H. S., Woolfolk, C. A., and Hubbard, J. S. (1968a), *Advan. Enzyme Reg. 6*, 257.
Valentine, R. C., Shapiro, B. M., and Stadtman, E. R. (1968), *Biochemistry 7*, 2143.
Wadsö, I. (1968), *Acta Chem. Scand. 22*, 927.
Woolfolk, C. A., Shapiro, B. M., and Stadtman, E. R. (1966), *Arch. Biochem. Biophys. 116*, 177.
Woolfolk, C. A., and Stadtman, E. R. (1964), *Biochem. Biophys. Res. Commun. 17*, 313.
Woolfolk, C. A., and Stadtman, E. R. (1967), *Arch. Biochem. Biophys. 118*, 736.
Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.
Wyman, J. (1967), *J. Am. Chem. Soc. 89*, 2202.

Human Pituitary Growth Hormone. Studies of the Tryptophan Residue*

Jorge Brovetto-Cruz† and Choh Hao Li

ABSTRACT: The single tryptophan residue in the human growth hormone molecule has been quantitatively and specifically reacted with 2-nitrophenylsulfenyl chloride and 2-hydroxy-5-nitrobenzyl bromide using 50% acetic acid as reaction media. The derivatives have been characterized by biological, chemical, and biophysical techniques. The nitrophenylsulfenyl derivative (I) retains full growth-promoting activity and its physicochemical properties are similar to those of the native molecule. The monolabeled 2-hydroxy-5-nitrobenzyl derivative (II) possesses much less growth-promoting potency, while the doubly labeled derivative (III) is almost completely devoid of this biological activity. The relative looseness of the molecular structure and the lowered stability of these latter two derivatives (II and III) is shown by the higher rate of tryptic digestion and the irreversibility of their spectrophotometric titration in aqueous media as compared with the native hormone. When tested for lactogenic activity, all these derivatives were inactive. It may therefore be concluded that the tryptophan residue in the human growth hormone molecule is not essential for growth-promoting activity, but, in contrast, may play an important role in the lactogenic activity of the hormone, suggesting that there are two different "active sites" for the two biological activities.

The fact that the phenolic groups of the 2-hydroxy-5-nitrobenzyl moieties in derivatives II and III have an abnormal ionization behavior, and that total alkylation of the tryptophan residue occurs in 50% acetic acid, but not in 0.2 M acetic acid solution suggested that the tryptophan residue is "buried" in the interior of the hormone molecule, and is exposed in 50% acetic acid.

Recent studies on the reduction and alkylation of the disulfide bonds in HGH[1] (Dixon and Li, 1966; Bewley *et al.*, 1968, 1969) have shown that the tetra-*S*-carbamidomethylated hormone retains full growth-promoting and lactogenic activities and that its physicochemical properties are similar to those of the native hormone. The fact that the disulfide bridges are neither essential for the biological activity nor for the three-dimensional structure suggested that the conformation of the HGH molecule must be largely a consequence of noncovalent forces. Since tryptophan residues are known to play an important role in stabilizing the structure of proteins by hydrophobic interactions with other nonpolar residues (Kauzmann, 1959), it is of interest to determine the essentiality of the tryptophan residue for the maintenance of the biological property and structure of HGH.

In this paper we present investigations that have been carried out using two specific tryptophan reagents: NPS-Cl (Scoffone *et al.*, 1968) and HNB-Br (Koshland *et al.*, 1964).

* From the Hormone Research Laboratory, University of California, San Francisco, California. Paper XX in the Human Pituitary Growth Hormone Series. *Received June 2, 1969.* The support of the American Cancer Society (T-19), the Allen Foundation, and The Geffen Foundation of New York is acknowledged.

† International Postdoctoral Research Fellowship (F05 TW-1173-02) of National Institute of Health, U. S. Public Health Service, 1968–1970; on leave from the University of Uruguay, Montevideo, Uruguay.

[1] Abbreviations used are: HGH, human growth hormone; NPS-Cl, 2-nitrophenylsulfenyl chloride; HNB-Br, 2-hydroxy-5-nitrobenzyl bromide; HNB-OH, 2-hydroxy-5-nitrobenzyl alcohol.