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# Regulation of *Escherichia coli* Glutamine Synthetase. Evidence for the Action of Some Feedback Modifiers at the Active Site of the Unadenylylated Enzyme†

F. W. Dahlquist* and D. L. Purich

ABSTRACT: The interaction of unadenylylated form of *Escherichia coli* glutamine synthetase with several substrates and effectors has been examined by magnetic resonance techniques. These studies show that two manganese ions bind per enzyme subunit. From the dramatic line broadening observed in the alanine spectra in the presence of manganese and enzyme, it is concluded that the binding of alanine occurs at a site nearer one of the two manganese sites. Electron spin resonance (ESR) titration experiments suggest apparent dissociation constants of 20 and 120 $\mu M$ for manganese to these sites in the presence of 1.0 m$M$ magnesium ion. The manganese concentration dependence of the broadening of alanine suggests an affinity of 30 $\mu M$ for the manganese closest to the alanine binding site. This suggests that alanine binds closer to the more tightly bound manganese ion. Glutamate appears to displace the alanine and also appears to bind close to the strongly bound manganese ion. It is proposed that alanine and glutamine bind competitively and in the same site. The binding of alanine and ATP is shown to thermodynamically interact such that the presence of one ligand increases the affinity of the enzyme for the other ligand. The presence of ATP dramatically sharpens the alanine line width when manganese and glutamine synthetase are present. Addition of ADP or phosphate alone has little effect on the alanine line width but the addition of both ADP and phosphate shows the same dramatic sharpening as the addition of ATP alone, suggesting an induced fit conformational change in the enzyme induced by ATP or by both ADP and phosphate. A binding scheme is proposed in which all feedback inhibitors of the enzyme bind in a competitive fashion with substrates.

The structure and regulation of the catalytic activity of the enzyme glutamine synthetase isolated from *Escherichia coli* have been studied rather extensively in recent years (Stadtman and Ginsburg, 1974). The enzyme has been shown to consist of 12 polypeptide chains of identical or nearly identical primary sequence (Woolfolk et al., 1966), which are apparently arranged in two hexagonal rings (Valentine et al., 1968). Each polypeptide chain can exist in either of two chemically distinct forms depending upon the specific covalent modification of a single tyrosine residue by reaction with ATP to form *O*-adenylyltyrosine. Interesting-

† From the Molecular Biology Institute, University of Oregon, Eugene, Oregon 97403, and the Department of Chemistry, University of California, Santa Barbara, California 93106. *Received August 2, 1974.* This research was supported in part by BioMed Support Grant RR-07099-06 and Research Grants GM-18994 and GM-47874 from the National Institutes of Health, U.S. Public Health Service.

ly, the unmodified and adenylylated forms of the enzyme differ with respect to catalytic properties and susceptibility to inhibition by a number of compounds (Stadtman et al., 1968).

The biosynthetic reaction catalyzed by the enzyme requires glutamate, ATP, and ammonia to form glutamine, ADP, and phosphate. The enzyme-catalyzed reaction involves a divalent ion. The enzyme is specific for magnesium in the unmodified form and for manganese in the adenylylated form (Woolfolk and Stadtman, 1964; Woolfolk et al., 1966; Wulff et al., 1967). The manganese supported $\gamma$-glutamyl transfer reaction is catalyzed by either enzyme form (Kingdon and Stadtman 1967a,b; Kingdon et al., 1967; Shapiro et al., 1967; Wulff et al., 1967; Stadtman et al., 1968).

Woolfolk and Stadtman (1967) observed that glutamine synthetase is partially inhibited by saturating amounts of a variety of compounds which are biosynthetically derived from glutamine. The inhibition is cumulative such that the presence of high concentrations of all the inhibitors results in nearly complete inhibition of the enzyme. This suggested a complex allosteric mechanism of feedback inhibition with several distinct allosteric binding sites for the inhibitors. Other studies have shown that adenylylation increases susceptibility of the enzyme to inhibition by these inhibitors (Wulff et al., 1967; Kingdon and Stadtman, 1967a,b,; Kingdon et al., 1967). The state of adenylylation used in the initial studies of Woolfolk and Stadtman (1967) was not defined, and this fact may modify the requirement for numerous allosteric binding sites (Stadtman and Ginsburg,1974). The complexity of this system suggests that criteria other than steady-state kinetic observations need to be developed to help elucidate the nature of the binding sites for each of the proposed feedback inhibitors and their relationship to the overall regulatory mechanism of the enzyme.

To date the enzyme has been studied by a variety of what can be considered macroscopic probes. However, to more fully understand the detailed mechanism of ligand binding and its effects on catalysis we have endeavored to employ a technique which can provide microscopic information as well. In this respect Chock et al. (1973) have initiated studies utilizing a covalently bound, environment sensitive, fluorescent probe at the adenylylated site. However, it is not always possible to make unambiguous inferences using such approaches. On the other hand, magnetic resonance techniques are rather well suited for such studies. Unfortunately, direct high resolution nuclear magnetic resonance (NMR) studies of a protein of 600,000 molecular weight are difficult; yet there are a number of useful alternate approaches which can yield valuable microscopic information. One such technique involves the use of paramagnetic ions and their interaction with nuclear spins as observed by magnetic resonance methods to probe the environments of both the ions and their immediate surroundings in macromolecules (Mildvan and Cohn, 1970). We report here the use of Mn(II) and its effect on the proton magnetic resonance spectrum of ligands of glutamine synthetase. This information and the previously unpublished thermodynamic interaction with ATP and glutamate sites to establish criteria for the binding of ligands in the glutamate and ATP sites of the unadenylylated form of glutamine synthetase of *E. coli*. These criteria suggest that glutamate and alanine bind at the same site in a competitive fashion. Further, it is demonstrated that ATP and ADP plus phosphate most probably bind in the same site, but that ATP and ADP, while binding in the same site, do not behave in identical fashions.

Finally, these studies suggest that there may be a limited number of binding sites, all of which are most probably at the active center. We propose that the active site region consists of a number of binding subsites which effectors may span in various combinations and thereby provide a means for enzymic regulation.

## Materials and Methods

L-Alanine, L-glutamate, ATP, and ADP were obtained from Sigma Chemical Company in the highest purity available. All other chemicals were of at least reagent quality, and double glass distilled water was used throughout.

Glutamine synthetase was prepared from *E. coli* W cells grown on 0.66 *M* glycerol–0.02 *M* $NH_4Cl$ in a basic salt media and harvested approximately 3 to 4 hr after the onset of the stationary growth phase (Woolfolk et al., 1966). The enzyme was purified by the method of Woolfolk et al. (1966) through the third acid ammonium sulfate precipitation (step 7). Repeated cycling through the acid ammonium sulfate step resulted in an enzyme preparation having a specific activity of 95 using the dimethyl glutarate buffer system. Measurements of the state of adenylylation by the enzymic activity procedure of Stadtman et al. (1970) indicated that there were an average of 1.5 adenylyl groups/dodecamer. On the other hand, a value of 0.8 was obtained spectrally (Shapiro and Stadtman, 1970). Considering these two procedures to be of equal accuracy, we take a value of approximately 1.2 as the average state of adenylylation. Disc gel electrophoresis at pH 8.6 in a Tris-glycine buffer system revealed a single protein band and serves as a further indication of purity. Enzyme concentration was calculated using the procedure of Shapiro and Stadtman (1970) which makes an appropriate correction for the light scattering contribution.

Proton magnetic resonance spectra were obtained using a Varian XL-100 spectrometer operating in fluorine external lock mode. Longitudinal relaxation rate measurements were performed using a 180°, $\tau$, 90° pulse sequence with computer controlled accumulation. The probe preamplifier was detuned to ensure linear response of the subsequent amplifiers when water relaxation rates were performed.

ESR spectra were obtained with a Varian E-4 spectrometer equipped with a Varian Model V-4350 variable temperature accessory. For quantitative measurements, samples were introduced into the cavity by placing exactly 100 $\mu$l of solution in a disposable micropipet which was then placed inside a quartz ESR tube repositioned exactly in the cavity for each measurement. Instrument settings were the same (except for receiver gain) for each determination.

Fluorescence measurements were performed using a Schoeffel double monochrometer fluorimeter. Both incident light intensity and fluorescence intensity were observed simultaneously. Correction of the observed fluorescence intensity for fluctuations in incident light intensity were performed digitally using a Varian 620i laboratory computer. Averaging of the signal intensity to reduce random error was performed by boxcar integration using the computer.

All measurements were performed in 0.01 *M* imidazole buffer (pH 6.7) (25°) containing 0.1 *M* KCl, $10^{-3}$ *M* $MgCl_2$, and $10^{-4}$ *M* $MnCl_2$ unless otherwise noted.

The concentration of magnesium and manganous ion in the various enzyme solutions was determined by atomic absorption on a Varian atomic absorption spectrometer cali-

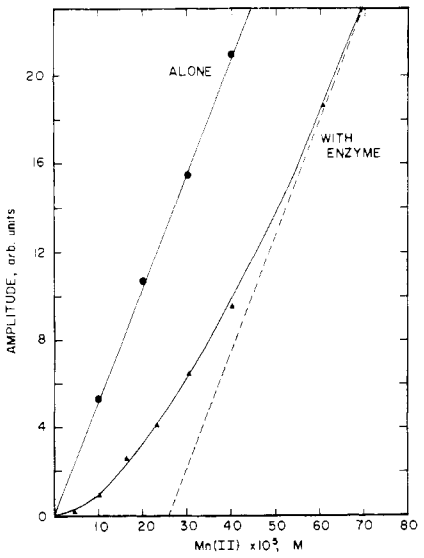



FIGURE 1: A plot of the amplitude of the ESR signal of Mn(II) hexaquo complex vs. total Mn(II) in solution in the absence (●) and in the presence (▲) of $1.3 \times 10^{-4}$ *N* glutamine synthetase at 25°. The dashed line represents an extrapolation to the total concentration of manganese bound.

brated with suitable divalent metal ion standards. Such measurements indicated that the enzyme preparation was free of manganous ion after extensive dialysis against buffers containing $10^{-3}$ *M* $Mg^{2+}$.

## Results and Discussion

*The Binding of Manganese(II) to Glutamine Synthetase.* The ESR spectrum of manganese(II) hexaquo complex in solution consists of six relatively sharp resonances. In most cases, when Mn(II) is bound to a macromolecule, the derivative spectrum is broadened beyond detection at room temperature. For a mixture of free and bound manganese(II), the normal six-line spectrum is observed, with the amplitude of the spectrum being proportional to only the concentration of free Mn(II) aquo complex. In such a situation, comparison of the observed amplitude with suitable standards allows the determination of the concentration of free Mn(II) ion. The concentration of bound Mn(II) can be calculated as the difference between the total amount added and the amount free. Figure 1 shows a plot of the manganese signal intensity as a function of manganese concentration in the presence and absence of the nonadenylylated form of glutamine synthetase. The experiment was performed in the presence of 1 m*M* $MgCl_2$ and 0.1 *M* KCl to minimize nonspecific binding. Complete removal of the metal ions has been shown to result in a less stable, "relaxed" form of the enzyme (Shapiro and Ginsburg, 1968; Kingdon et al., 1968) which undergoes aggregation at the concentrations used in this study. For this reason we always looked at the replacement of magnesium by manganese ion. Fortunately, direct metal ion binding has been studied by a variety of techniques by Ginsburg and coworkers (Denton and Ginsburg, 1969, 1970; Shapiro and Ginsburg, 1968; Ginsburg, 1969, 1972; Ross and Ginsburg, 1969; Hunt and Ginsburg, 1972) and by Kingdon et al. (1968). Therefore the displacement reactions could be performed with confidence. The figure shows the saturating value of two Mn(II) ions bound per protein subunit. This stoichiometry agrees with that determined by Hunt and Ginsburg (1972) by equilibrium dialysis techniques. The shape of the curve shown in Figure 1 suggests that the two ions do not bind with identical apparent affinity. It should be clear that, under these conditions, the experiment really involves competition between magnesium and manganese for these two sites. Thus one of the two bound manganese ions appears to be a more effective competitor than the other. The apparent constants used to generate the solid line shown in the figure were $2 \times 10^{-5}$ and $12 \times 10^{-5}$ *M*, respectively, for the two divalent ion sites.

Table I: Water Proton Relaxation Rates.[a]

| Species Present | $1/T_1$ ($sec^{-1}$) | $1/T_2$ ($sec^{-1}$) |
|---|---|---|
| Buffer + 130 $\mu N$ enzyme | 0.42 | 8.95 |
| Buffer + 130 $\mu N$ enzyme + 100 $\mu M$ Mn(II) | 2.70 | 22.3 |

[a] $1/T_{1p} = 2.3 \pm 0.2$ $sec^{-1}$; $1/T_{2p} = 13 \pm 1$ $sec^{-1}$; $T_{1p}/T_{2p} = 5.8 \pm 1$.

These apparent constants are actually *competition constants* much weaker than the real affinity of manganese ion for these sites. Since magnesium ion is present at a concentration of 1 m*M*, the binding of manganese ion is actually a competition between manganese and magnesium for the divalent metal ion sites. If the sites behave in an independent fashion, we may relate the apparent constants for manganese to the sites to intrinsic affinities of manganese and magnesium by the relationship

$$K_{1\,app} = K_{Mn1}[1 + ([Mg]/K_{Mg1})]$$

$$K_{2app} = K_{Mn2}[1 + ([Mg]/K_{Mg2})]$$

where $K_{Mn1}$, $K_{Mg1}$, $K_{Mn2}$, and $K_{Mg2}$ refer to the intrinsic affinities of manganese and magnesium for the first and second sites. Direct binding studies of manganese and magnesium to the "relaxed" (ion free) form of unadenylylated glutamine synthetase have shown two manganese sites with affinities of $5 \times 10^{-7}$ and $5 \times 10^{-5}$ *M*, respectively (Hunt and Ginsburg, 1972). Direct magnesium binding studies have shown one site with an affinity of $5 \times 10^{-5}$ *M*. Presumably a second magnesium site exists with less strong affinity. These earlier studies suggest that the magnesium competes with the tighter manganese site. At 1.0 m*M* MgCl, the apparent affinity for the first site should be $K_{1app} = 10^{-5}$ *M*. This is in reasonably good agreement with our value of $2 \times 10^{-5}$ *M* under slightly different conditions. The second apparent manganese dissociation constant is $12 \times 10^{-5}$ *M*. This value is a factor of two higher than that observed for manganese binding in the absence of magnesium. Thus magnesium does not effectively compete for this site under our conditions. This suggests that the value of the intrinsic magnesium ion dissociation constant for this second site is $10^{-3}$ *M* or weaker.

The effect of manganese ion on the spin-lattice and spin-spin relaxation times of water protons has often been employed to examine the nature of the bound manganese ions (Mildvan and Cohn, 1970). The results of the determination of these relaxation times for $1.0 \times 10^{-4}$ *M* Mn(II) in the presence and absence of $1.2 \times 10^{-4}$ *M* glutamine synthetase subunit concentration are summarized in Table I. All determinations were carried out at 100 MHz at 27°.

The values of the relaxation rates, $1/T_{1p}$ and $1/T_{2p}$, due to the presence of bound manganese, both increase dramatically as compared to free ion. Since $T_{1p}$ and $T_{2p}$ are significantly different and do not vary significantly with tempera-

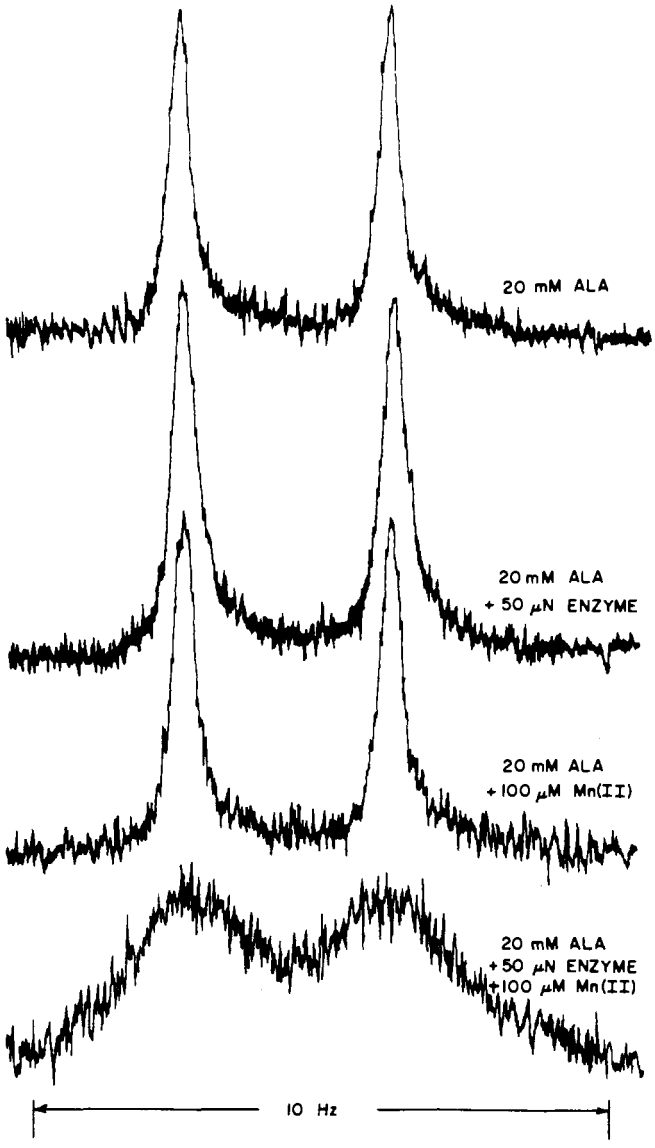



FIGURE 2: The 100-MHz $^1H$ NMR spectra of the alanine methyl doublet; alone, in the presence of enzyme, in the presence of manganous ion, and in the presence of both manganous ion and enzyme.

ture, the exchange of water between solution and the manganese ion hydration sphere must be rapid. Under these conditions, the ratio $T_{1p}/T_{2p}$ can be shown (Navon, 1970) to depend on the correlation time for the interaction between the water proton and the paramagnetic electrons of the manganese ions. For many enzyme systems, the correlation time for the interaction is controlled by the electron spin relaxation time of the bound manganese ion. Under these conditions, the correlation time is related to the $T_{1p}/T_{2p}$ ratio, $R$, by the equation (Bigbee and Dahlquist, 1974):

$$\tau_c = \left[\frac{R - 1.19}{0.69}\right]^{1/2} \frac{1}{\omega_I} \quad (1)$$

where $\omega_I$ is the proton resonance frequency in radians per second. Since there are two possible sites for the manganese ion, this correlation time must be considered to be an "average" value. This average is rather ill defined if the correlation times differ significantly for the two sites. Since about 80% of the bound manganese ions occupy the tighter site under these conditions, the average should realistically represent the correlation time for manganese bound to the tighter divalent ion site. Using eq 1, a value of $4 \times 10^{-9}$ sec

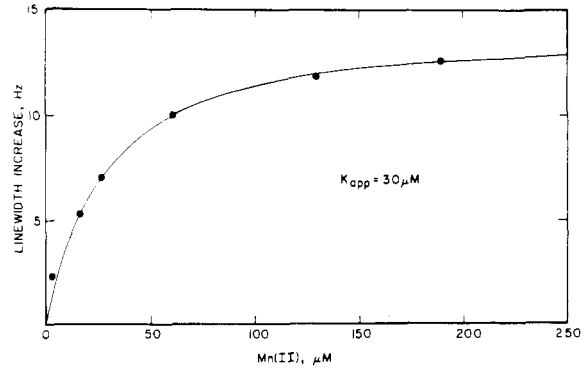



FIGURE 3: Dependence of the increase in alanine (40 m$M$) line width on the concentration of free manganous ion in the presence of $1.2 \times 10^{-4}$ $N$ glutamine synthetase. Free manganese concentration was determined by ESR intensity measurements as shown in Figure 1. The solid line is a theoretical curve for a single apparent dissociation constant of $3 \times 10^{-5}$ $M$ and a total observed line width of 13 Hz.

is obtained for the correlation time. This is about two orders of magnitude faster than predicted on the basis of rotational diffusion arguments for a globular molecule of 600,000 molecular weight. This supports the notion that the correlation time is dominated by the electron spin relaxation time, $\tau_e$. This value is comparable to other values of $\tau_e$ determined in this fashion. For example, manganese ions bound to carboxypeptidase A, thermolysin, and pyruvate kinase give values of 2.3, 2.0, and $3 \times 10^{-9}$ sec, respectively (Bigbee and Dahlquist, 1974). For the purposes of analyzing effector interactions on the enzyme, however, it is important to recognize that only an order of magnitude estimate of the correlation time is required.

*Interaction with* L-*Alanine.* It has been proposed that alanine is a potent feedback inhibitor of the enzyme. Kinetic studies of adenylylated and unmodified enzyme suggest that alanine is a competitive inhibitor of glutamate (Woolfolk and Stadtman, 1967). It is clear that kinetic studies cannot distinguish between two possible alternatives—direct competition for the same site or mutually exclusive binding at topologically distinct sites. It is recalled that kinetic studies suggested that CTP behaves as a competitive inhibitor in the *E. coli* aspartate transcarbamylase reaction (Gerhart and Pardee, 1962), but numerous subsequent studies have detailed the interaction of this effector not only at a site away from the active center but on a distinct regulatory protein (Gerhart and Schachman, 1965). Because alanine offers a resolved, easily interpreted first-order spectrum, its interaction with the enzyme would be particularly easy to study by NMR methods.

The NMR spectra of the alanine methyl doublet are shown in Figure 2 in the presence of (a) buffer, (b) buffer plus 50 $\mu M$ unadenylylated glutamine synthetase, (c) buffer plus $1.0 \times 10^{-4}$ $M$ $MnCl_2$, and (d) buffer plus $1.2 \times 10^{-4}$ unadenylylated glutamine synthetase plus $1.0 \times 10^{-4}$ $M$ $MnCl_2$. The dramatic broadening seen in Figure 2d of the alanine methyl doublet suggests that alanine is binding to the enzyme surface at a position near one or both divalent metal ion binding sites.

To distinguish which divalent ion site was near the bound alanine, a series of alanine methyl line width measurements was performed with varying concentrations of manganese ion added to a constant concentration of enzyme and alanine. The results of these experiments are summarized in Figure 3. The alanine line width is shown as a function of free manganese ion concentration determined by ESR am-

Table II: Water Line Widths in the Presence of Glutamine Synthetase and Various Ligands.

| Species Present | Line Width (Hz) |
|---|---|
| Buffer + 130 $\mu N$ enzyme | 3.0 |
| Buffer + 130 $\mu N$ enzyme + 100 $\mu M$ Mn(II) | 8.4 |
| + 40 m$M$ alanine | 8.2 |
| + 200 $\mu M$ ADP | 7.8 |
| + 40 m$M$ P | 10.6 |
| + 200 $\mu M$ ADP + 40 m$M$ P | 7.8 |
| + 200 $\mu M$ ATP | 8.0 |

plitude measurement. The solid line corresponds to a single hyperbolic binding curve with a value equal to $K_{diss} = 3.0 \times 10^{-5}$ $M$. Comparison of this value with those obtained for the two manganese sites by ESR titration strongly suggests that the more tightly bound manganese ion is near the bound alanine and that the second manganese ion is bound at a site relatively distal to that effector site.

A possible alternative explanation of this result is that the alanine actually binds near the weaker manganese ion binding site but the binding of the ion to this site is strengthened in the presence of alanine. Thus more manganese should be bound in the presence of alanine. As shown in Table II, the relaxation rate of water caused by the enzyme in the presence of 100 $\mu M$ manganese is not increased by the presence of alanine. Therefore no more water accessible manganese is bound to the enzyme as a result of the presence of alanine at these concentrations. However, it is possible that since alanine binding is apparently quite weak, no demonstrable increase in the amount of manganese bound would be observed at these alanine concentrations. Therefore we cannot rigorously rule out the alternative explanation. In the absence of conflicting data, however, we shall assume that the agreement of apparent manganese affinities by direct ESR observation and from alanine broadening experiments is evidence for alanine binding near the stronger manganese site.

It should be possible to measure the dissociation constant, $K_S$, of enzyme complex by observation of the alanine concentration dependence of the methyl line width in presence of a constant amount of enzyme and manganese using the relationship (Dahlquist and Raftery, 1968)

$$[S]_0 = [E]_0(T_{2p}/T_{2B}) - K_S$$

where $[S]_0$ is the total alanine concentration and $[E]_0$ is the total enzyme site concentration. The quantities of $1/T_{2p}$ and $1/T_{2B}$ correspond to the observed paramagnetic contributions to the observed line width and to the bound line width.

Interestingly, the line width was nearly independent of alanine concentration over the range 5–100 m$M$. This implies that $K_S$ is substantially greater than $[S]_0$ at all concentrations used. Thus $K_S$ must be greater than 100 m$M$. This conclusion follows from the fact that the observed line width is the population average of the free line width and bound contribution. The lack of an alanine concentration dependence in the line width at constant enzyme and manganese concentration implies that the fraction of the total alanine which is bound does not change significantly as the alanine concentration varies. The only way that this can occur is if the number of alanine molecules bound increases linearly with alanine concentration. Thus the alanine dissociation constant must be greater than the concentration of alanine employed. The value of $K_S$ for alanine must be greater than 100 m$M$ for the site near the tight manganese ion binding site.

The bound contribution to the line width, $1/T_{2B}$, is itself made up of two terms

$$1/T_{2MB} = 1/(T_{2M} + \tau)$$

where $T_{2M}$ corresponds to the relaxation time of the bound species and $\tau$ is essentially the lifetime of the alanine–enzyme–manganese complex. If exchange is fast, $\tau \ll T_{2M}$, the bound contribution, $1/T_{2B}$ is equal to $1/T_{2M}$. For slower exchange rates, $1/T_{2B}$ is always less than $1/T_{2M}$. One method for determining if exchange rates are limiting is to examine the temperature dependence of the observed line width. The lifetime should decrease with temperature and observed contribution to the line width should therefore increase. In contrast to this result the observed alanine line width in the presence of the enzyme–manganese complex was seen to sharpen as the temperature was increased. This suggests that alanine is in fast exchange between the enzyme surface and solution. This interpretation is not completely unambiguous, however. Therefore, we can only use the observed broadening to set a lower limit on the bound relaxation time.

The concentration dependence of the alanine line width implies an alanine dissociation constant in excess of 100 m$M$. This poor affinity suggests that the alanine could be binding near the metal ion, but nonspecifically. However, as shown in Figure 4, L-glutamate when added to a solution of alanine, enzyme, and manganese, sharpened the alanine resonance and the concentration dependence could be interpreted as competition between alanine and glutamate for the site near the metal ion. A dissociation constant of 3–5 m$M$ could be calculated for the glutamate–enzyme complex. Therefore, although alanine binds very weakly, it appears to be specifically competitive with glutamate for its site near the metal ion.

To further support this point of view, the NMR spectrum of glutamate was observed in the presence and absence of enzyme–manganese complex as shown in Figure 5. It is difficult to draw quantitative conclusions from these spectra since the glutamate has a complicated second-order proton magnetic resonance spectrum. A slight broadening of the glutamate resonances was observed in the presence of $2 \times 10^{-5}$ $M$ manganese ion alone which corresponds to the free concentration of manganese in the experiments of Figure 5. From the broadening observed in the presence of enzyme, however, it is clear that glutamate binds near the metal ion site. Thus one may surmise that glutamate is competitive with the weakly binding alanine observed by line broadening and further that the glutamate also binds near the paramagnetic manganese binding site.

It is possible to estimate the distance between the manganese site and the alanine binding site. Although the observed broadening of the alanine methyl resonance is relatively small, only a very small fraction of the alanine is bound. Since the observed line width is essentially the population average of the line widths due to the free and bound state, it is clear that the bound line width in the presence of manganese is very great indeed. If we estimate the alanine dissociation constant as 300 m$M$ (see below), then under the conditions used to obtain Figure 4, the bound line width would correspond to 40 KHz. This is much too large to be due to merely binding to the enzyme. It must reflect the

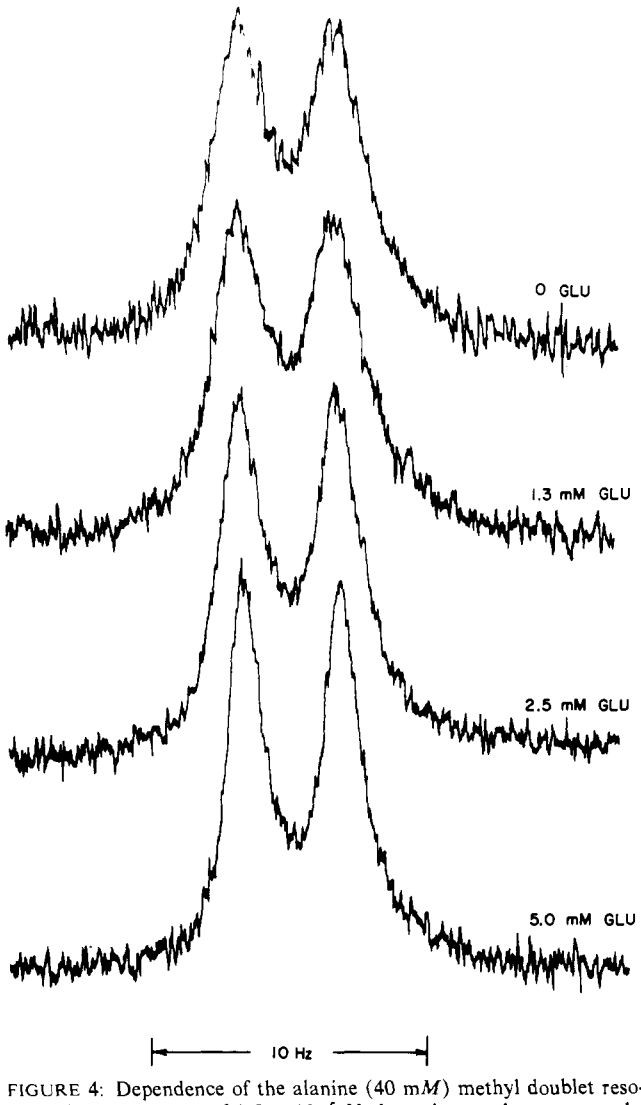



FIGURE 4: Dependence of the alanine (40 m$M$) methyl doublet resonance in the presence of $2.5 \times 10^{-5}$ $N$ glutamine synthetase upon the addition of glutamate to the solution at 25°.

proximity of the alanine site to the manganese site. The distance between the sites, $r$, can be estimated using the relationship: $r = 36.4(T_{2M})^{1/6}$ Å, which is derived from the Solomon (1955) equation which describes the dipolar induced transverse relaxation rate of nuclei in the presence of paramagnetic ions. This equation only holds for $\tau_c = 4 \times 10^{-9}$ sec which we estimated from the water relaxation data and a proton resonant frequency of 100 MHz. For the alanine methyl resonance $T_{2m} = 8 \times 10^{-6}$ sec and this gives an approximate value of $r$ of 5 Å. This distance is based on the assumption that the dissociation rate of the alanine is substantially greater than $1/T_{2M}$ or $10^5$/sec. If this condition is not met the actual distance will be less than our estimate of 5 Å.

Since both the glutamate and alanine appear to be within this short distance of the manganese ion we feel that it is most likely that the binding sites are indeed at same site or are at least overlapping. This nicely accounts for the mutually exclusive binding of glutamate and alanine seen previously by kinetic methods and now by magnetic resonance techniques.

Villafranca and Wedler (1974) have recently concluded that the high affinity manganese site participates in the binding of glutamate in the *adenylylated* form of the enzyme. While it is possible that the two forms of the enzyme are completely different in their binding site configurations, this seems unlikely. Thus it seems reasonable to conclude that alanine and glutamate bind in essentially the same site in both adenylylated and unadenylylated forms of the enzyme.

*Multiple Ligand Complexes and Effector Induced Interactions.* The increase in alanine line width, associated with binding to the enzyme–manganese complex, can be employed to monitor the interactions of the enzyme–manganese–alanine complex with other ligands. Figure 6 shows the effects of the addition of ATP, ADP, phosphate, and ADP plus phosphate on the alanine methyl resonance in the presence of enzyme and manganese. Addition of 1 equiv of ATP per subunit results in a dramatic sharpening of the alanine resonance. A similar addition of ADP or of an excess of phosphate results in a slight sharpening and moderate broadening of the alanine methyl resonance, respectively. However, a dramatic sharpening in line width is observed when both ADP and phosphate are added together. Qualitatively, it is clear that the effect of addition of both ADP and phosphate is not simply the sum of the effects associated with the addition of either ligand separately. Further-

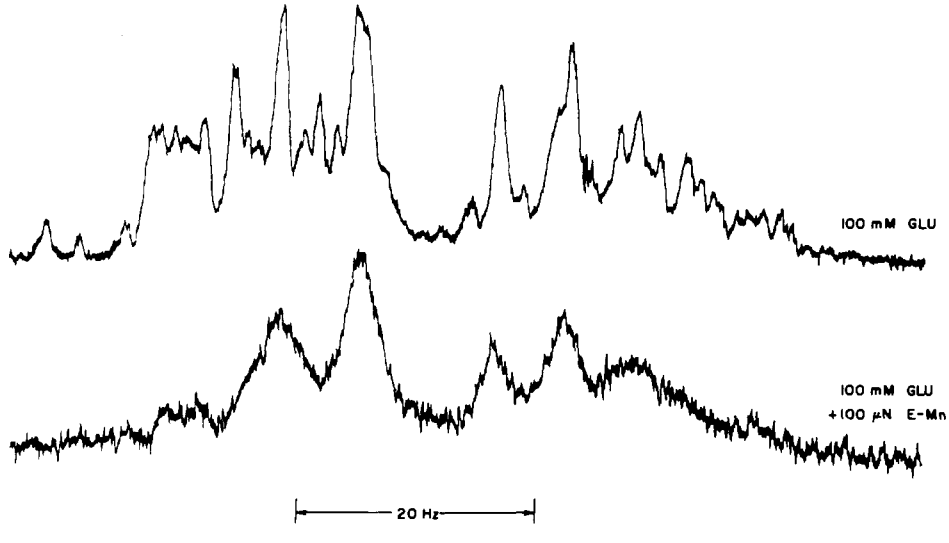



FIGURE 5: The 100-MHz $^1$H NMR spectra of 100 m$M$ glutamate alone and in the presence of $1.0 \times 10^{-4}$ $N$ enzyme–manganous ion complex. Free manganous ion was approximately $1 \times 10^{-5}$ $M$ under these conditions. The temperature was 25°.

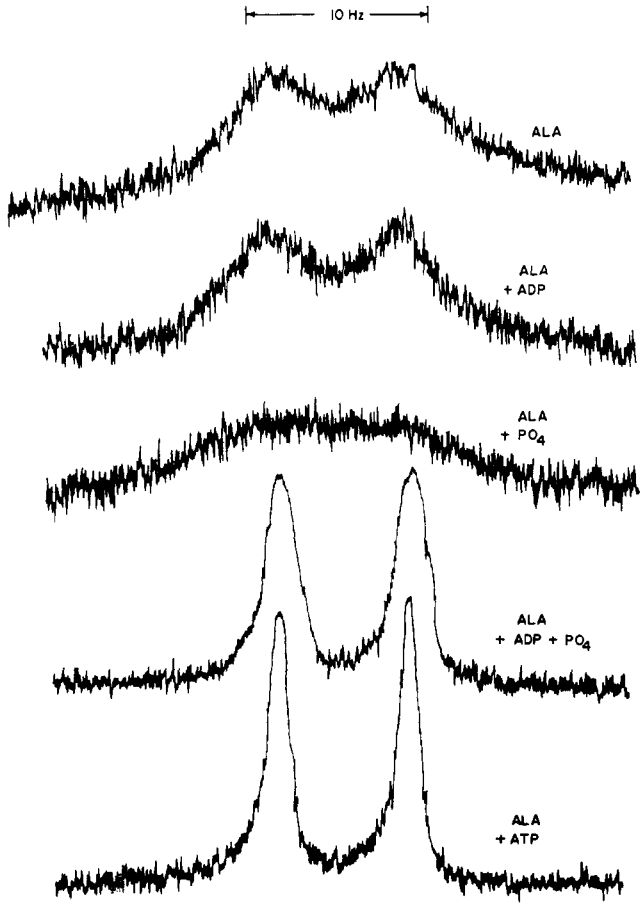



FIGURE 6: Effect of various nucleotides on the alanine methyl group line width. In each case, $5.0 \times 10^{-5}$ *N* glutamine synthetase, 40 m*M* alanine, and $1.0 \times 10^{-4}$ *M* manganous ion were included in ADP and ATP final concentrations where $1.0 \times 10^{-4}$ *M* while phosphate was added to a final concentration of 5.0 m*M*.

more the presence of both ADP and phosphate results in an identical effect to the addition of ATP alone. This may provide an excellent example of a series of enzyme-ligand interactions which demonstrate the "induced fit" hypothesis proposed by Koshland (1958). Thus decrease in the interaction of alanine with bound manganese ion is induced by ATP binding which can only be reproduced by the binding of both ADP and phosphate to the ATP site and not by the binding of either ligand separately. This observation offers a possible explanation for the observed requirement of ADP and phosphate and a divalent ion in the exchange reaction catalyzed by either form of glutamine synthetase:

$$NH_2OH + \text{glutamine} \underset{Me^{2+}}{\overset{ADP,P_i}{\rightleftharpoons}} NH_3 + \gamma\text{-glutamylhydroxamate}$$

While ADP and phosphate are required for catalysis, neither appears to necessarily participate chemically in the exchange reaction (Stadtman, 1971). The observed NMR results suggest that both ADP and phosphate together are necessary to induce the same state as that induced by ATP binding. Apparently this ATP induced state is required for catalytic activity of any kind.

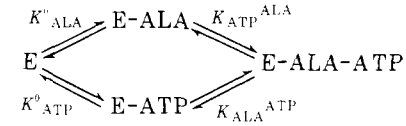
In contrast to the effects observed for the the alanine methyl resonance, the water proton relaxation rate caused by the enzyme bound manganese ions is largely unaffected by the addition of alanine, ATP; ADP, and phosphate either singly or in combination. These results are summarized in Table II. The water line width behaves very similarly to the alanine line width in the presence of ADP and phosphate. Both line widths decrease slightly in the presence of ADP and increase about 50% in the presence of phosphate. However, the addition of ATP or ADP and phosphate together gives the same line width as the addition of ADP alone. This suggests that the addition of these other ligands does not significantly alter the number of water accessible manganese atoms bound or the correlation time for the enzyme bound manganese-water interaction.

There are at least three immediate alternative explanations for the observed changes in the alanine methyl resonance line width induced by ATP binding to glutamine synthetase. The observed line width due to the presence of enzyme-manganese complex is given by (Luz and Meiboom, 1964)

$$\frac{1}{T_{2p}} = \frac{[ES]}{[S]_0} \frac{1}{T_{2M} + \tau} \tag{2}$$

The binding of ATP could: (i) decrease the affinity of alanine for the enzyme-manganese complex, thereby decreasing [ES]; (ii) ATP binding could increase the value of $\tau$, the bound lifetime of alanine, perhaps by making the affinity between alanine and the enzyme-manganese complex greater; and (iii) ATP binding could increase the value of $T_{2M}$, perhaps by causing a conformation change which changes the distance from the bound alanine methyl group to the bound manganese. A fourth possibility is that ATP binding displaces the manganese ion from the enzyme surface. This can be immediately discarded since no increase in ESR signal is detected in the presence of ATP and the water relaxation rates are also only slightly affected by the addition of ATP to the enzyme-manganese complex. Effects due to this latter possibility most likely minimized by the presence of magnesium ion at a tenfold higher level than total manganous ion. Further, consideration of the relative affinities of ATP for these divalent ions (O'Sullivan, 1969) supports this view.

*Thermodynamic Interactions of Ligands.* In order to further distinguish among the above possibilities, the effect of ATP on the binding of alanine was examined. Consider the thermodynamic cycle:




The enzyme E can bind either or both ligands ALA and ATP. The quantities $K_{ALA}{}^0$, $K_{ATP}{}^0$, $K_{ATP}{}^{ALA}$, and $K_{ALA}{}^{ATP}$ correspond to the formation binding constants of ALA for free enzyme, ATP for free enzyme, ATP for enzyme bound with ALA, and ALA for enzyme bound with ATP, respectively. Any three of the four affinity constants are independent such that

$$K_{ALA}{}^0/K_{ATP}{}^0 = K_{ALA}{}^{ATP}/K_{ATP}{}^{ALA}$$

Thus, if ATP binding strengthens alanine binding, it follows that alanine binding *must* strengthen ATP binding. Unfortunately, there is no simple, precise, method to measure the affinity of alanine for glutamine synthetase. However, the intrinsic protein fluorescence of glutamine synthetase is enhanced and slightly shifted to the blue in the presence of ATP. This provides a method for precise determination of

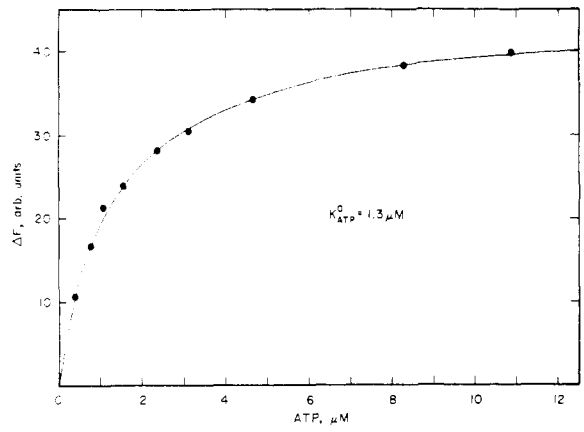



FIGURE 7: The ATP concentration dependence of glutamine synthetase fluorescence, with excitation at 295 nm and emission at 340 nm. The enzyme was 1.0 with $10^{-7}$ $N$ in subunits. The solid line is a theoretical curve for a single dissociation constant of 1.3 $\mu M$ for the ATP–enzyme complex.

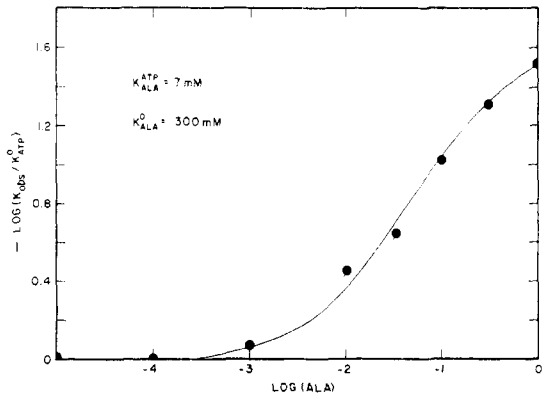



FIGURE 8: The alanine concentration dependence of the apparent dissociation constant for ATP. The line is a theoretical curve using $K_{ALA}{}^0$ equal to 300 m$M$ and $K_{ALA}{}^{ATP}$ equal to 7 m$M$. See text for definition of these constants. The enzyme concentration was $1 \times 10^{-7}$ $N$ for each determination.

the ATP affinity to the enzyme. A plot of the observed relative change in protein fluorescence as a function of free ATP concentration is shown in Figure 7. The experiments were performed in the absence of alanine. The solid line shown in the figure is the theoretical hyperbolic binding curves with a dissociation constant of $1.3 \times 10^{-6}$ $M$. In the presence of alanine the apparent ATP dissociation constant, $K_{app}$, is related to the dissociation constants $K_{ALA}{}^0$ and $K_{ALA}{}^{ATP}$ by the relationship (Dixon, 1953)

$$K_{app} = K_{ATP}{}^0 \left[1 + \frac{[ALA]}{K_{ALA}{}^0}\right] \Big/ \left[1 + \frac{[ALA]}{K_{ALA}{}^{ATP}}\right] \quad (3)$$

Therefore, a study of the alanine concentration dependence of $K_{app}$ should allow the calculation of both $K_{ALA}{}^0$ and $K_{ALA}{}^{ATP}$. The results of such a study have been summarized in Figure 8 as a plot of $K_{app}/K_{ATP}{}^0$ vs. the logarithm of alanine concentration. The solid line is a theoretical curve using eq 3 and values of $K_{ALA}{}^0$ and $K_{ALA}{}^{ATP}$ of 300 and 7 m$M$, respectively. The agreement between the observed and calculated lines is quite good, although the fit is not as sensitive to the value of $K_{ALA}{}^0$ as of $K_{ALA}{}^{ATP}$ since saturation with alanine is not quite achieved. The estimated errors in the two values are about 20% for $K_{ALA}{}^{ATP}$ and about 50% for $K_{ALA}{}^0$. Thus ATP and alanine interact in a positive fashion, with ATP increasing alanine binding by a factor of about 40 and vice versa. This clearly demonstrates a thermodynamic interaction between ATP and alanine binding. Importantly, the fluorescence change due to ATP binding was the same, independent of the presence of alanine, implying that the fluorescence change is a true measure of ATP binding.

The observation that ATP increases the affinity of alanine for the enzyme rules out the possibility that the observed decrease in alanine line width in the presence of ATP is caused by a displacement of alanine from the enzyme. It is interesting that we have observed that both ATP and glutamate cause sharpening in the methyl group resonance of alanine. Yet we have concluded that the ATP binding strengthens alanine while glutamate displaces the alanine. The magnetic resonance data alone do not allow such a distinction to be drawn. However, it seems clear that glutamate and alanine binding are mutually exclusive from kinetic evidence. The fluorescence binding study of ATP and alanine interactions discussed here justify the alternative conclusion drawn concerning the reason for the dramatic sharpening of the alanine resonance in the presence of ATP. In any study of this sort, line widths changes can be due to changes in fraction bound, exchange rate changes, or to bound relaxation rate (changes usually attributed to a change in the distance between the paramagnetic center and the observed nucleus when bound). These possibilities cannot be easily distinguished by magnetic resonance methods alone. Our evidence suggests that ATP must dramatically slow the rate of exchange or must cause a significant change in the manganese alanine methyl group distance in the bound state. These remaining possibilities are not easily distinguished.

The binding interactions of ADP and alanine or glutamate with the enzyme suggest that a distance change between manganese and the methyl group of bound alanine is responsible for the dramatic sharpening of the alanine resonance shown in Figure 6. The presence of either alanine or glutamate strengthens the binding of ADP to the enzyme in a fashion similar to ATP binding. The more complicated quantitative picture results from what appears to be a slight positive cooperativity in ADP binding. (This will be discussed in more detail in another communication.) On the other hand, the qualitative view clearly involves a similar positive interaction between ADP and glutamate or alanine. Again, this supports the idea that alanine and glutamate reside at the same subsite of the active center. Despite the similar thermodynamic interaction between alanine and ADP or ATP, the line width results are quite different. This implies that strengthening of the alanine affinity due to nucleotide binding alone is not sufficient to explain the line width results. It is our view that an increase in the bound manganese alanine distance induced by ATP binding is the most likely explanation for the observed decrease in alanine line width seen in Figure 6. We feel that it is this conformation change which is also induced by the binding of ADP and phosphate together.

Finally it is possible to estimate the magnitude of the distance change associated with ATP binding. The observed alanine line width decreases nearly a factor of 10 in the presence of ATP. Accounting for the increase in alanine affinity due to ATP binding, this corresponds to about a factor of 100 decrease in bound relaxation rate. Because of the inverse sixth power dependence of the dipolar interaction between the ion and methyl spins, only a factor of two increases in distance is required. Using the affinity of alanine

Table III: Possible Effector Interactions with the Active Site Region of *E. coli* Glutamine Synthetase.

| Inhibitor | Binding Subsites Occupied[a] |
|---|---|
| Alanine | A |
| AMP | B-1 |
| Carbamyl-P | C, D, E |
| CTP | B-3 |
| Glucosamine-6-P | C |
| Glycine | A |
| Histidine | A |
| Tryptophan | A |

[a] A, α-amine carboxylate region; B-1, B-2, corresponding to entire region spanned by nucleoside 5-mono- and diphosphate; C, γ-phosphate of ATP; D, ammonia; and E, γ-carboxylate of glutamate.

in the absence of nucleotide and the correlation time deduced from the water relaxation studies, a bound manganese alanine distance of approximately 5 Å may be deduced in the absence of ATP. Thus a 5 Å increase in that distance due to ATP binding is sufficient to account for all the observed effects.

## Conclusions

We have examined some of the interactions of six ligands of glutamine synthetase. It appears that the ATP site may be occupied by either ADP or phosphate since all three ligands cause a similar fluorescence change (F. W. Dahlquist and D. L. Purich, unpublished findings). The site may also bind AMP, which is thought to be an important feedback inhibition of the biosynthetic reaction and yet supports the γ-glutamyl transfer reaction when manganous and arsenate are present (Stadtman and Ginsburg, 1974). Thus the site may be considered to consist of series of subsites capable of binding phosphate or arsenate and AMP or ADP at the same time.

From the mutually exclusive nature of glutamate and alanine binding, their similar line width increases in the presence of manganous ion and the enzyme, and their similar thermodynamic interaction with ADP it seems clear that both amino acids bind to the same site in the active center. This suggests binding subsites which are specific for the α-amino and carboxyl region of the amino acids and yet another subsite interacting with the γ-carboxylate region of the glutamate site.

Using this notion of binding subsites, the various proposed effectors may be placed within the active site region, primarily interacting with one or more binding subsite. These proposals are summarized in Table III. Clearly some kinetic and equilibrium binding evidence remain in conflict with these assignments. Yet equilibrium binding studies of ligands with low affinity constants are inherently limited and difficult to quantitatively interpret and kinetic studies of inhibitors capable of spanning several adsorption pockets of the active site are not completely unambiguous. On the other hand, it may be necessary to amend our simplified model and it will, thus, remain to be shown which proposal is most justifiable by methods like the NMR approach outlined here.

While the present report deals with only a limited number of the many effector interactions, it appears that our approach should ultimately provide a rather unambiguous view of the role of effectors in the control of glutamine synthesis. It is important to recognize that while these studies point toward active site directed effects, they do *not* exclude the physiological importance of the inhibitors. Rather, they point out a mechanism by which the important metabolic signals of bacterial nitrogen metabolism may be expressed without the need for a large number of independent sites. In view of the emerging X-ray crystallographic evidence that a considerable amount of information is required to define the conformation of nucleotide binding sites in the dehydrogenases and the nucleotide-dependent kinases (Bränden et al., 1973; Blake and Evans, 1974), it is not too surprising that there is a real need for such conservative mechanisms of feedback inhibition utilizing the binding specificity elements of active site itself.

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# Interaction of Fluorescence Probes with Acetylcholinesterase. The Site and Specificity of Propidium Binding†

Palmer Taylor* and Shelley Lappi

ABSTRACT: A bis-quaternary fluorescence probe, propidium diiodide, has been found to exhibit a tenfold enhancement of fluorescence when bound to acetylcholinesterase from *Torpedo californica.* The complex is characterized by a high affinity, $K_D = 3.0 \times 10^{-7}$ *M,* and 1:1 stoichiometry with the 82,000 molecular weight subunit of acetylcholinesterase. A wide variety of other quaternary ammonium ligands such as decamethonium, gallamine, *d*-tubocurarine, tetraethylammonium, and tetramethylammonium will completely dissociate propidium from the enzyme as will monovalent and divalent inorganic cations. The competitive dissociation does not show cooperative behavior or a distinct requirement for occupation of multiple sites of different affinity to produce displacement. While a directly competitive relationship can be illustrated macroscopically, the various quaternary ligands show a different susceptibility toward inorganic cation displacement. The affinity of propidium relative to gallamine increases with ionic strength. This finding indicates that there is not complete equivalence in the negative subsites to which quaternary groups bind. Although edrophonium will also displace propidium from the enzyme, the dissociation constant obtained from this competitive relationship is 3.5 orders of magnitude greater than the constants obtained for inhibition of catalysis. By competitive displacement titrations it is shown that the primary binding site of edrophonium is distinct from that of propidium and a ternary complex with the two ligands can form on each subunit. In contrast to edrophonium, the binding of propidium is unaffected by methanesulfonylation of the active center serine and is uncompetitive with the carbamylating substrate, *N*-methyl-7-dimethylcarbamoxyquinolinium. Thus, it appears that propidium associates with a peripheral anionic center on the enzyme. Although propidium and edrophonium associate at separate sites on acetylcholinesterase, bis-quaternary ligands where the quaternary nitrogens are separated by 14 Å displace both ligands from the enzyme with equal effectiveness.

From steady-state kinetic studies using natural and synthetic substrates, it has become evident that various quaternary ammonium ligands and inorganic cations interact strongly with acetylcholinesterase at more than a single site (Changeux, 1966; Kitz et al., 1970; Wombacher and Wolf, 1971; Belleau et al., 1970). This evidence has been reinforced by nuclear magnetic resonance (Kato, 1972) and, more recently, fluorescence spectroscopic measurements of ligand association with acetylcholinesterase (Mooser et al., 1972; Mooser and Sigman, 1974). The latter approaches possess the inherent advantage that ligand association can be measured directly rather than relying upon the influence of the ligand on multiple catalytic parameters.

The monitoring of ligand–acetylcholinesterase complex formation by fluorescence, to date, has been based on ligands which exhibit greatly diminished quantum yields upon association with the enzyme (Mooser et al., 1972) or ligands whose absorption spectra are suitable to effect quenching of protein tryptophanyl fluorescence upon binding to the enzyme (Schnitzky et al., 1973; Taylor and Jacobs, 1974). In cases where the site of binding was analyzed, the above ligands were found to interact with the active center of acetylcholinesterase, although one end of the bis-quaternary ligands interact at a locus outside of the active center (Mooser et al., 1972; Taylor and Jacobs, 1974). We have recently observed that propidium (3,8-diamino-5,3′-diethylmethylamino-*n*-propyl-6-phenylphenanthridium) is a potent inhibitor of acetylcholinesterase catalysis and in vivo elicits competitive blockade at the neuromuscular junction

† From the Division of Pharmacology, Department of Medicine, University of California—San Diego, La Jolla, California 92037. *Received October 7, 1974.* Supported by Grant GM-18360 from the National Institutes of Health.