Site–Site Interactions in Glycogen Phosphorylase *b* Probed by Ligands Specific for Each Site†

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ABSTRACT: Three ligand binding sites on glycogen phosphorylase *b* which were originally described by kinetic and physicochemical means, and more recently located and defined in molecular terms by X-ray crystallography, have been probed by ligands specific for each site. Kinetic analyses, supplemented by X-ray crystallographic binding studies, permit assignment of each ligand to a primary binding site, as well as determination of its dissociation constant and interaction with ligands binding to the other sites. 8-Anilino-1-naphthalenesulfonate binds most strongly to the activator site, in competition with adenosine 5′-phosphate, presumably because its sulfonate group interacts with several arginine residues, and binds only weakly to the hydrophobic inhibitor site, possibly because of charge repulsion. It is itself a weak activator and decreases binding affinities for compounds specific for the inhibitor site. Our results with 8-anilino-1-naphthalenesulfonate are not consistent with predictions of its expected behavior and suggest caution in the use of this reagent as an indicator of hydrophobicity. Our second major probe, caffeine, binds primarily to the inhibitor site, shows competitive inhibition with substrate binding to the catalytic site, and decreases the affinity for the activator at the activator site. The catalytic site was probed with two different types of ligand. Glucose, known to stabilize the inactive T conformation of the enzyme, competes with the substrate α-D-glucose 1-phosphate for the catalytic site and decreases the affinity of adenosine 5′-phosphate for the activator site. Glucose also improves the binding affinity of caffeine for the inhibitor site by 3–5-fold, both compounds synergistically stabilizing the inactive T conformation. On the other hand, the substrate analogue uridine diphosphoglucose also competes with substrate for the catalytic site, but in doing so, it tends to stabilize the active R conformation. As a result, it improves the binding affinity for adenosine 5′-phosphate at the activator site but prevents the binding of caffeine to the inhibitor site. The derived interaction constants quantify the relationships discussed above.

Determination of the three-dimensional structure of glycogen phosphorylases *a* and *b* by X-ray crystallography has permitted the identification and characterization of several discrete ligand binding sites (Fletterick et al., 1976a,b; Fletterick & Madsen, 1980; Weber et al., 1978). These have been probed by a variety of techniques, and an understanding of the specificity and interactions between these sites is gradually emerging. Thus, the activator site in the N-terminal domain is near the serine-14 phosphate and has a preferred specificity for adenosine 5′-phosphate (AMP)[1] but will also accept IMP as an activator (Black & Wang, 1968; Rahim et al., 1976). Crystallographic studies on phosphorylase *b* have demonstrated that the allosteric inhibitors glucose-6-P and ATP bind here, accounting for their competition with AMP, and stabilize the inactive T conformation of this enzyme (Johnson et al., 1979; Lorek et al., 1980). At the interface between the N and C domains, 30 Å away, the catalytic site contains the coenzyme, pyridoxal phosphate, and binds the substrate glucose-1-P or P_i. Two types of inhibitors are recognized here: glucose or its analogues which compete with glucose-1-P but stabilize the inactive T conformation (Sprang et al., 1982b) and analogues of glucose-1-P which stabilize the R conformation Withers et al., 1982). The latter include UDPG and glucose cyclic 1,2-phosphate, both of which have been demonstrated to bind to the catalytic site by crystallographic means (Yang, 1979; Jenkins et al., 1981; Withers et al., 1982).

A third site whose existence was recognized by calorimetric and kinetic means before an understanding of its significance was gained (Ho & Wang, 1973; Morange et al., 1976) was shown by X-ray crystallography to be located 10 Å from the active site (Fletterick et al., 1976; Kasvinsky et al., 1978b). So far as we know, compounds binding here are inhibitory, and a detailed characterization suggests that this prediction will prove true for all compounds which bind at this locus (Sprang et al., 1982a). A wide variety of compounds bind to this site but usually have in common a condensed ring system which intercalates (stacks) between the aromatic side chains of Phe-285 and Tyr-612. The major source of binding energy is "hydrophobic" in nature (including the stacking energy as a hydrophobic contribution) and correlates with the loss of solvent-accessible surface of the conjugated ring system of the bound ligand. A more detailed discussion of the thermodynamic and structural parameters of the binding is given by Sprang et al. (1982a).

Given the hydrophobic nature of the inhibitor site, one might predict that other hydrophobic compounds would bind there. Of considerable interest in this respect is ANS, which Stryer (1965) suggested to be a ligand of choice for probing hydrophobic binding sites in proteins since binding to such sites enhanced its fluorescence. Since then, it has been used widely as an indicator of hydrophobic binding sites. Seery & Anderson (1972) enlarged on Stryer's observation that ANS binds to phosphorylase by demonstrating that there is at least one binding site for each monomer and that substrates and modifiers decrease the affinity of these sites, perhaps in a noncompetitive manner. Steiner & Greer (1977) evaluated the simultaneous binding of AMP and ANS by determining the stepwise association constants. Although their data can be

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[1] Abbreviations: AMP, adenosine 5′-phosphate; IMP, inosine 5′-phosphate; glucose-1-P, α-D-glucose 1-phosphate; glucose-6-P, α-D-glucose 6-phosphate; ATP, adenosine 5′-triphosphate; P_i, inorganic phosphate; UDPG, uridine diphosphoglucose; ANS, 8-anilino-1-naphthalenesulfonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate.

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interpreted as indicating direct competition between AMP and ANS, they also discussed other possibilities. We therefore chose to study the binding of ANS by using both crystallographic and kinetic approaches, believing that we would find it to bind to the inhibitor site and that this would explain the observations in the literature. Our discovery that its primary binding site is elsewhere has implications for the interpretation of a variety of studies which purport to use it as a probe for hydrophobic sites.

Along with ANS, we studied the interaction of other ligands specific for the various sites on the phosphorylase *b* monomer, thus building up a quantitative description of the heterotropic site–site interactions on each monomer. Conditions were chosen to minimize the homotropic interactions between sites on the two monomers of the molecule, a topic treated elsewhere (Madsen & Shechosky, 1967) and reviewed extensively by Madsen et al. (1976). The fourth major site on each monomer, that for glycogen storage, was ignored by conducting the kinetic studies at saturating glycogen concentrations.

Materials and Methods

Most biochemicals were purchased from Sigma Chemical Co., except DTT which was obtained from Bio-Rad Laboratories. ANS was purchased from Pierce Chemical Co. Phosphorylase *b* (EC 2.3.1.1) was prepared from rabbit muscle by the method of Fischer & Krebs (1962), using DTT instead of cysteine, and recrystallized at least 3 times before use. Phosphorylase *a* and crystals thereof were prepared as described previously (Fletterick et al., 1976).

The crystallographic analysis of crystals soaked in various ligands and the preparation of difference electron density maps were carried out as described previously (Kasvinsky et al., 1978a). Kinetic analyses were conducted at 30 °C in 0.5-mL incubation mixtures containing 1% glycogen, 3 mM sodium glycerophosphate, 0.3 mM EDTA, 0.5 mM DTT, and 5–8 μg of phosphorylase *b* with concentrations of AMP, glucose-1-P, and other compounds as indicated, all at pH 6.8. Inorganic phosphate produced by the phosphorylase reaction was converted to standard enzyme units as described previously (Engers et al., 1970). Data presented as Lineweaver–Burk plots were analyzed by the method of Wilkinson (1961) while the Dixon plots were analyzed by the method of least squares by a standard statistics program.

Results and Discussion

Kinetic Model. Figure 1 represents the phosphorylase dimer with the chief ligand binding sites and their major interactions both heterotropic and homotropic, positive and negative, as discussed in an earlier publication (Madsen et al., 1981). Indicated also are the primary binding sites for the ligands employed in this study. The primary binding sites for all these ligands were not necessarily known in advance of the kinetic and binding data presented below, but we anticipate the conclusions in order to have at hand a clear and simple model for discussion of the results.

The kinetic scheme in Figure 2 is derived in part from the binding site model shown in Figure 1, but also from the results of the kinetic studies below. Thus, the scheme predicts exclusive or nonexclusive binding of various combinations of ligands, but in some cases, the kinetic model was derived from the data, as will become apparent. The rate equation shown in the legend for Figure 2 was derived by the methods given in Segel (1975), and the treatment of the data and its interpretation also followed Segel. To simplify the scheme and rate equation, we have used the symbol G to denote both glucose and UDPG. However, it is well-known that glucose stabilizes

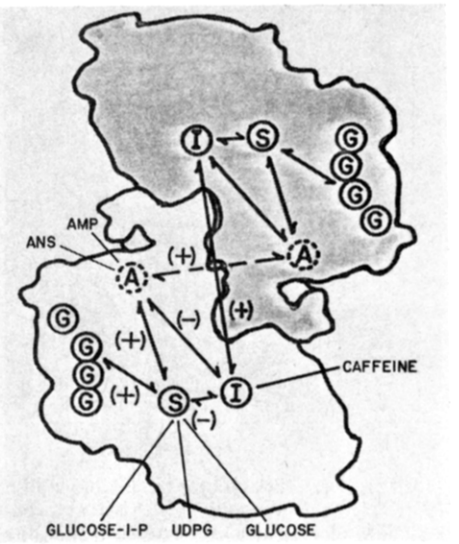

FIGURE 1: Major ligand binding sites on the phosphorylase *b* dimer are depicted together with some of the interactions (positive or negative) between them. In addition, the primary binding sites of the ligands discussed in this paper are indicated: A, activator site; S, catalytic site; I, inhibitor or negative effector site; GGGG, glycogen storage site. Note that the interactions are more complex than depicted here since the interaction signs for linking the substrate site S with the other sites depend on whether an R-promoting or T-promoting ligand is bound. Thus, the inhibitor glucose is positively cooperative with site I ligands, but the inhibitor UDPG is negatively cooperative with site I ligands.

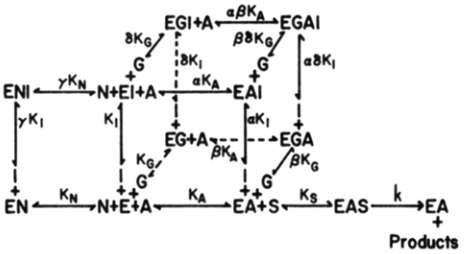

FIGURE 2: Kinetic scheme for the interaction of the sites on the phosphorylase *b* monomer as probed by ligands specific for each site: E = enzyme–glycogen complex, N = ANS, I = caffeine, A = AMP, S = glucose-1-P, and G = glucose or UDPG. The complete rate equation is $V_M/v = K_AK_S/([A][S]) + K_S/[S] + K_AK_S[I]/([A][S]K_I) + K_AK_S[G]/([A][S]K_G) + K_AK_S[N]/([A][S]K_N) + K_AK_S[G][I]/([A][S]\delta K_GK_i) + K_S[I]/([S]\alpha K_I) + K_S[G]/([S]\beta K_G) + K_AK_S[N][I]/([A][S]\gamma K_NK_I) + K_S[G][I]/([S]\alpha\beta\delta K_GK_I) + 1$.

the inactive T conformer of phosphorylase while UDPG stabilizes the active R conformer, although both bind to the same site in the catalytic site. Both these effects are reflected in the results of the kinetic analysis given below. In addition, some compounds used in this study, such as ANS, bind to a second site, but this secondary binding does not usually manifest itself in the kinetic patterns and has been ignored in the formulation of the kinetic scheme.

Binding Site for ANS. ANS is a good inhibitor for phosphorylase *b* and exhibits competitive inhibition when AMP concentration is varied at constant substrate concentration (Figure 3). A replot of the slopes is slightly curved, but a dissociation constant of 0.05 mM may be estimated. When the binding data in Figure 1 of Steiner & Greer (1977) are plotted in reciprocal form, a pattern is obtained which indicates competition between AMP and ANS for the same two sites on the phosphorylase *b* dimer and a dissociation constant for ANS of 0.066 mM. Steiner & Greer (1977) calculated the two stepwise dissociation constants for the binding of ANS to phosphorylase *b* to be 0.032 and 0.119 mM, indicating very little cooperativity, from which an intrinsic dissociation con-

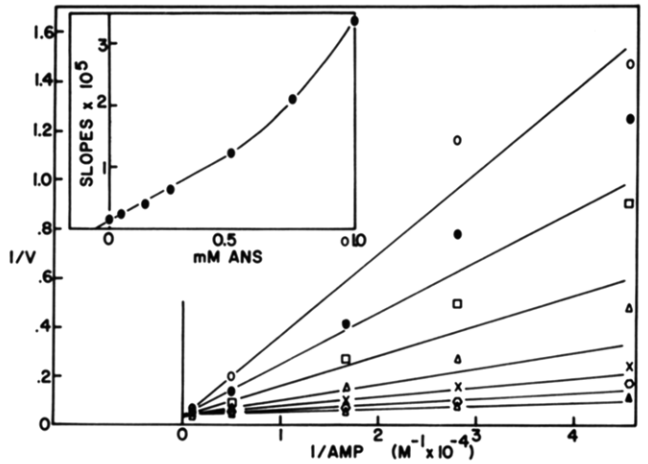

FIGURE 3: Lineweaver–Burk plot for the inhibition of phosphorylase *b* by ANS at varying concentrations of AMP at a constant concentration of 2.5 mM glucose-1-P. ANS concentrations from the lowest to the highest line are 0, 0.5, 1.0, 1.5, and 2.0 mM. $1/V$ in this and all other figures is the reciprocal of the initial velocity in micromoles per minute per milligram.

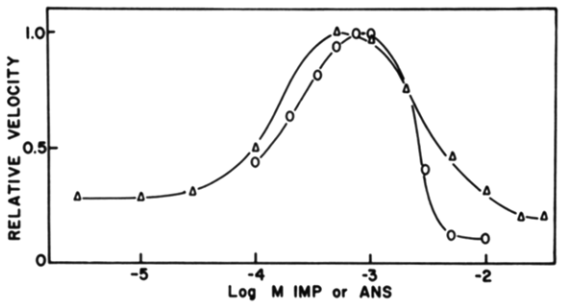

FIGURE 4: Effect of varying concentrations of IMP or ANS on the enzymatic activity of phosphorylase *b*. (Δ) IMP concentration is varied with glucose-1-P concentration fixed at 1.5 mM; relative velocity of 1 was 0.2 μmol min^{-1} mg^{-1}. (O) ANS concentration is varied with glucose-1-P concentration fixed at 7 mM; relative velocity of 1 was 1.3 μmol min^{-1} mg^{-1}.

stant of 0.062 may be derived, in good agreement with that calculated from the reciprocal plot of their data, and reasonably similar to the kinetically derived constant in this paper. ANS also shows competitive inhibition with respect to the substrate, glucose-1-P, at a constant concentration of AMP (data not shown).

These results are consistent with a model in which ANS binds at the same site as does AMP, thus interfering by direct physical interference, while glucose-1-P binds only to enzyme already liganded with AMP (see Figure 2). Steiner & Greer (1977), however, obtained evidence for an additional binding site for ANS which would not be reflected in our kinetic inhibition experiments. Our control experiments suggested a slight activation by ANS, and further investigation revealed that, in the absence of AMP, low concentrations of ANS activated phosphorylase *b* while higher concentrations reversed this activation. Figure 4 illustrates these results. For comparison, the effect of IMP, demonstrating both activation and inhibition, is depicted on the same plot. A result similar to this for IMP was reported earlier for phosphorylase *a* (Kasvinsky et al., 1978b) and was interpreted to mean that IMP bound first to the AMP (activator) site and second, with less affinity, to the inhibitor site. One may suggest the same interpretation for the binding of ANS. The apparent K_d for binding to the inhibitor site is 4 mM for IMP and approximately 3 mM for ANS. A Lineweaver–Burk plot for the activation by ANS yields an apparent K_m of 0.14 mM at 75 mM glucose-1-P (V_M = 5% of that with AMP) and an apparent K_m of 0.2 mM at 7 mM glucose-1-P (V_M = 4% of that with AMP).

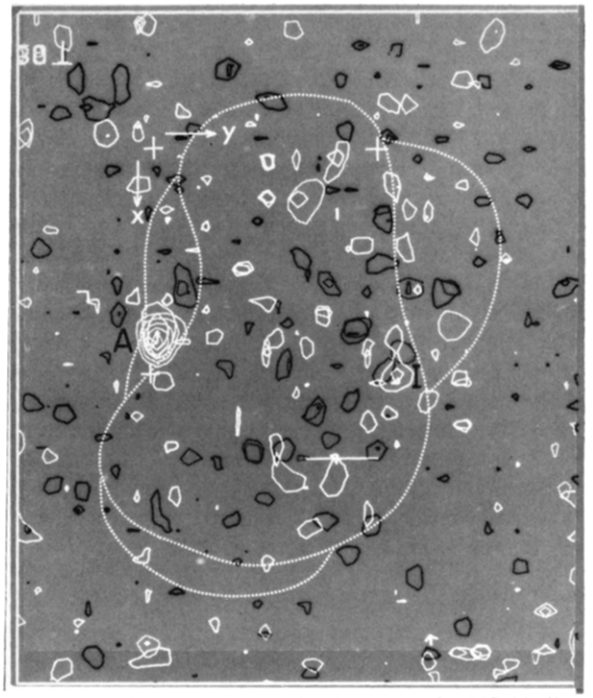

FIGURE 5: Difference electron density map of ANS binding to phosphorylase *a* at 4.5-Å resolution. *x* and *y* coordinates at 0 and 0.25 are indicated by (+). Dotted lines are shown about the molecular outlines at the two regions along *z* that include the activator, catalytic, and inhibitor sites (*z* sections are 0.08 Å thick, centered on 0.4 for the activator and 0.24 for the latter two sites). The horizontal bar represents 10 Å. Changes in electron density which are positive from the native enzymes are represented by white contour lines, negative by black. A crystal of phosphorylase *a* was washed free of glucose and soaked in the crystallization buffer containing 0.79 mM ANS. The figure indicates a high occupancy of the activator site, denoted A, and a low occupancy of the inhibitor site, denoted I.

When a crystal of phosphorylase *a* was soaked in a solution of ANS and then subjected to X-ray diffraction analysis, the difference electron density map shown in Figure 5 was obtained. By comparison with the results of previous studies (Kasvinsky et al., 1978b), the primary binding of ANS may be assigned to the activator site which normally binds AMP. A limited amount of binding has also occurred at the inhibitor site. Again, the results parallel those obtained for the binding of IMP to phosphorylase *a* in the crystal, as seen in Figure 7a of Kasvinsky et al. (1978b).

The kinetics of ANS inhibition of phosphorylase *a* are more difficult to analyze. ANS is noncompetitive with substrate in the absence of AMP but competitive when AMP is present at saturating concentrations. This suggests inhibition by binding at both the activator and inhibitor sites in the absence of AMP and at the inhibitor site in the presence of AMP. Compounds which bind at the inhibitor site (e.g., caffeine) are competitive with glucose-1-P.

Caffeine. Caffeine inhibition of phosphorylase *a* has been thoroughly characterized (Kasvinsky et al., 1978b,c). Caffeine binds primarily to the inhibitor site, with weak binding to the activator site, in crystals of phosphorylase *a*. Binding to the inhibitor site causes competitive inhibition with respect to glucose-1-P because caffeine stabilizes a protein conformation (T state) in which certain amino acid residues (Asp-283 in particular) occupy part of the glucose-1-P position. The inhibition is synergistic with glucose with an interaction constant of 0.3.

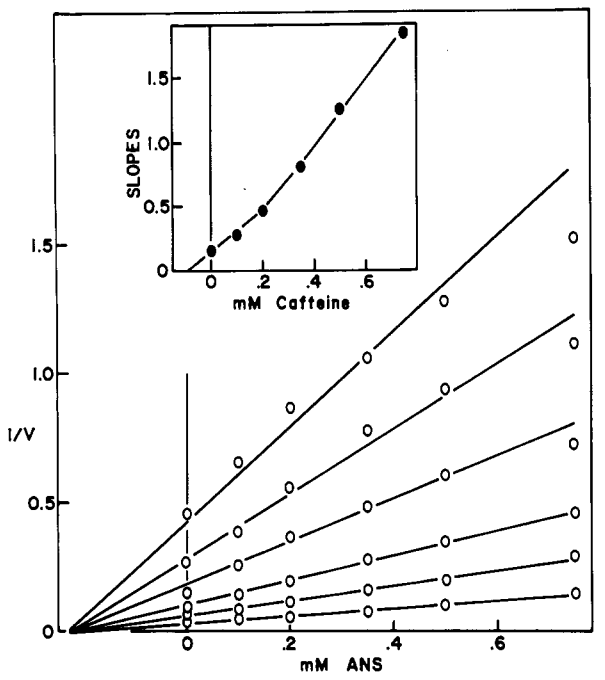

FIGURE 6: Dixon plot for the inhibition of phosphorylase *b* by caffeine and ANS at constant concentrations of AMP and glucose-1-P of 0.1 and 7 mM, respectively. Caffeine concentrations from the lowest to the highest line are 0, 0.1, 0.2, 0.35, 0.5, and 0.75 mM.

For phosphorylase *b*, also, we find that caffeine is competitive with glucose-1-P (data not shown). At constant substrate concentration, the inhibition pattern is noncompetitive with AMP, thus ruling out a model in which caffeine cannot bind to enzyme liganded with AMP (either by direct competition to the same site or because a conformational change has rendered its primary site inoperable). The primary plot (not shown) demonstrates noncompetitive inhibition of caffeine vs. AMP (when glucose-1-P concentration is held constant at 7 mM) and also yields a K_A for AMP of 0.025 mM. The slope replot yields a K_I for caffeine of 0.08 mM. The slope replot of the Dixon plot of these data yields an αK_A value of 0.143 mM from which we calculate that the interaction constant α is 5.7.

When caffeine and ANS concentrations are varied together at constant AMP and glucose-1-P concentrations, the Dixon plots produce lines which intersect at or below the abscissa (Figure 6), indicating synergistic inhibition with negative cooperativity. The interaction constant was estimated at 2. Thus, ANS, being a weak activator, stabilizes a conformation which provides poor binding for caffeine, but the interaction constant of 2 is significantly less than the 5.7 observed between AMP and caffeine binding.

Glucose and UDPG. As was previously demonstrated for phosphorylase *a* (Helmreich et al., 1967), glucose is a competitive inhibitor with respect to glucose-1-P in the case of phosphorylase *b* [data not shown, but see Cori & Cori (1940)]. Glucose also tends to exhibit competitive inhibition with respect to AMP in the case of phosphorylase *a* because it binds almost exclusively to the T state with, at the most, 5% as much affinity to the R form stabilized by AMP (Helmreich et al., 1967). With phosphorylase *b*, on the other hand, the inhibition pattern is noncompetitive when AMP concentration is varied (data not shown), indicating that AMP and glucose can bind to the same form of the enzyme and are not exclusive, as we indicate in Figure 2. A replot of the slopes is curved, indicating cooperativity of glucose binding, but may be extrapolated to a minimal value of 3.4 mM glucose. This value is equal to $([S]/K_S + 1)K_G$ from which, using [S] = 7 and K_S = 2.2, the minimal value of K_G = 0.8 mM. This compares well with the value of 1 mM obtained by Steiner et al. (1980) from calorimetric measurements. The experiment in which glucose and glucose-1-P concentrations were varied at constant AMP concentration also yielded K_G = 0.8 mM.

The points of intersection of the lines in the primary plot of this experiment are scattered, due to the glucose cooperativity, but an average value of 0.15 mM may be determined for the intersections and may be assigned to βK_A. Since K_A from the same experiment is 0.024 mM, the interaction constant β is approximately 6.6, a measure of the negative effects which AMP and glucose have on each others binding affinities.

When glucose and caffeine concentrations are both varied at constant concentrations of AMP and glucose-1-P (not shown), the resulting Dixon plot exhibits intersecting lines, indicating synergism. This phenomenon had been found earlier with phosphorylase *a* (Kasvinsky et al., 1978c). The slope replots are straight lines which yield values for δK_G and δK_I times a function incorporating K_A, [A], α, and β. By using the known values for these constants, one may calculate a value for the interaction constant of 0.2, which is only very approximate. However, there is little doubt that glucose and caffeine improve their respective binding affinities by stabilizing the same form of the enzyme, as was true for phosphorylase *a* where the interaction constant was 0.3 (Kasvinsky et al., 1978c). The synergistic cooperative effects of glucose and caffeine on various measures of conformational changes have been amply documented earlier (Withers et al., 1979; Steiner et al., 1980).

UDPG has previously been demonstrated to be a competitive inhibitor with respect to glucose-1-P for all phosphorylases examined so far (Madsen, 1961). In the case of phosphorylase *b*, an apparent inhibition constant of 1.4 mM may be calculated from a reciprocal plot with glucose-1-P concentration varied and various fixed concentrations of UDPG. Since AMP was present at 1 mM, in excess of its K_A of 0.026 mM, the apparent inhibition constant approximates βK_G (=1.4 mM). When AMP concentration is varied, with substrate concentration held constant, the inhibition by UDPG is noncompetitive (data not shown), although the lines tend toward being parallel. The slope replot yields a value for K_G of 5.5 so that by comparison with the βK_G the interaction constant β is 0.25. Thus, in contrast to the other active site bound inhibitor, glucose, UDPG is bound more tightly by the AMP-liganded enzyme than by the free enzyme. This is the behavior expected for an analogue of the substrate, glucose-1-P, which would be expected to bind almost exclusively to the AMP-promoted R-state conformation. Exclusive binding would have yielded uncompetitive inhibition, a result we anticipated and even observed in preliminary, less detailed experiments. The final results are more in keeping with the previous finding of slight activity with glucose-1-P when AMP is absent (Engers & Madsen, 1968).

Since UDPG is a substrate analogue which competes with glucose-1-P and stabilizes the R state, while caffeine stabilizes the T state, we would expect that UDPG and caffeine would be mutually exclusive inhibitors and show parallel lines on a Dixon plot. This Dixon plot is shown in Figure 7, where in fact the lines not only are parallel to begin with but also show a divergence from parallelism in the opposite direction from any tendency toward intersection. If the data are plotted as velocity vs. caffeine concentration at various levels of UDPG, it is seen that the higher concentrations of UDPG tend to reverse the inhibition by the higher levels of caffeine. This

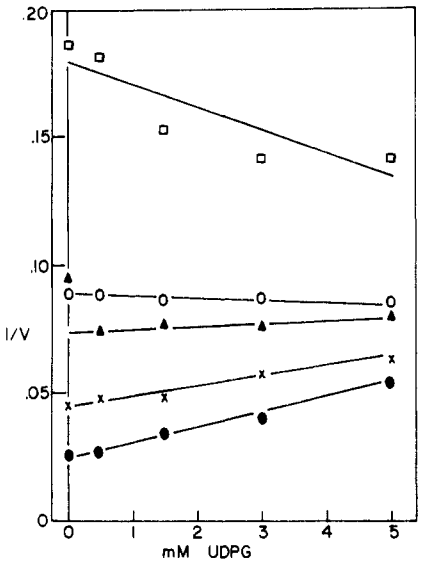

FIGURE 7: Dixon plot for the inhibition of phosphorylase *b* by caffeine and UDPG at constant concentrations of AMP and glucose-1-P of 1.0 and 7 mM, respectively. Caffeine concentrations from the lowest to the highest line are 0, 0.25, 0.5, 0.8, and 1.2 mM.

phenomenon, which accounts for the divergence from parallel lines seen in Figure 7, is caused by the cooperativity between active sites, such that UDPG bound to an active site in one monomer improves the binding of substrate to the active site of the symmetry-related monomer. We have earlier reported this effect by demonstrating the partial reversal of ATP inhibition by UDPG (Madsen & Shechosky, 1967), but this interaction with a ligand at the inhibitor site was, of course, unknown then.

When UDPG and glucose concentrations are both varied at constant substrate and activator concentrations (data not shown), the Dixon plots resemble that shown in Figure 7, as would be expected for two inhibitors which occupy the same site.

General Discussion. There have been many studies of the interactions between various substrates, activators, and inhibitors of glycogen phosphorylase. In 1963 and 1964, Helmreich et al. demonstrated the reciprocal effect of AMP on the binding of substrates (glycogen, phosphate, or glucose-1-P) as well as vice versa (Helmreich et al., 1963; Helmreich & Cori, 1964). The homotropic cooperativity between the catalytic sites on adjacent subunits of the phosphorylase *b* dimer was discovered soon after (Madsen, 1964). A review published in 1976 (Madsen et al., 1976) summarized the state of our knowledge just before the availability of the three-dimensional molecular structure derived from X-ray crystallography permitted a more informed interpretation of the biochemical studies. Thus, it had been shown that AMP induces a conformational change in the environment of the coenzyme as determined by the fluorescence characteristics of the latter and that the binding of AMP improved the binding of substrate or competitive inhibitors of substrate such as UDPG. Other physicochemical probes also demonstrated qualitatively that there was direct site–site interaction between the activator, substrate binding, and coenzyme sites on each monomer and that this interaction could be both positive and negative. The location of these sites with respect to each other, however, either was unknown or was wrongly assumed, and new additional sites either were unknown or were only vaguely recognized.

The crystallographic studies have enabled us to locate exactly the various ligand binding sites on each monomer and their relationship to each other. The wide variety of control sites on the phosphorylase molecule presents an opportunity to test the interaction of various ligands specific for each site by the steady-state kinetic approaches of Segel (1975). Other enzymes have equal or more complex combinations of interacting ligand binding sites, but these have not yet been unambiguously defined by the delineation of their three-dimensional structures. The study outlined here may be of a type applicable to other complex allosteric enzymes. It should be noted that most studies of site–site interactions since the crystal structures of the phosphorylases became available have been with the *a* form. The *b* form is the major allosteric model since activity is almost totally dependent on AMP being present at the activator site. Studies of the *b* form therefore permit quantitative analysis of the effects of ligands bound to the activator site on the other major sites.

Table I: Site-Specific Inhibition Patterns with Phosphorylase *b*[a]

compound	effects at ligand binding sites		
	catalytic site	activator site	inhibitor site
ANS (N)	competitive with S	*b* competes with A $K_n = 0.05$ mM	synergistic with I negative cooperativity $\gamma = 2$
caffeine (I)	competitive with S	noncompetitive with A $\alpha = 5.7$	*b* $K_i = 0.08$ mM
glucose (G)	*b* competes with S $K_g = 0.8$ mM	noncompetitive with A $\beta = 6.6$	synergistic with I positive cooperativity $\delta = 0.2$
UDPG (G)	*b* competes with S $K_g = 5.5$ mM	noncompetitive with A $\beta = 0.25$	parallel lines on Dixon plot exclusive with I

[a] S denotes the substrate, glucose-1-P, and A denotes AMP.
[b] Primary binding site of each compound.

Table I summarizes the data obtained in this study with respect to the dissociation constants for each inhibitor and the interaction constants with other ligands. An interaction constant greater than 1 indicates a negative interaction between two ligands with respect to their binding strengths. Thus, ANS, competing with AMP for the activator site but being a weak activator itself, weakens the binding of caffeine to the inhibitor site. ANS and caffeine tend to stabilize two different conformers of the enzyme. For the same reason, caffeine weakens the binding of AMP and vice versa. Glucose, binding to the active site but stabilizing an inactive conformer, weakens the binding of AMP and strengthens that of caffeine. In contrast, UDPG also binds to the active site but, being an analogue of the substrate, stabilizes the same conformer as does AMP but antagonizes the binding of caffeine. These effects are quantified by the interaction constants which agree with that knowledge which was previously available (Madsen et al., 1976). Particularly interesting is the finding that UDPG and caffeine cannot bind simultaneously even though they go to different sites. We can assume that the reason is the same structural basis for the caffeine being a competitive inhibitor for glucose-1-P (Withers et al., 1982).

The results with ANS are significant with respect to the use of this compound as a probe for hydrophobic regions in proteins. The binding studies published previously by Steiner & Greer (1977), the kinetic analysis, the crystallographic results, and the weak activation are all consistent with its primary binding being at the activator site. It would appear that the main reason for this is the group of arginine residues (numbers 308, 309, and 242) which constitute a relatively nonspecific

anion binding site which will also bind phosphate, glucose-1-P, sulfate, arsenate, ATP, ADP, and glucose-6-P in addition to AMP. Arg-308 undergoes a conformational change upon phosphate loading of this phosphoryl site. Presumably, once the sulfonic acid group is "anchored" to the arginines, the aromatic rings can interact with other residues, such as Tyr-75, to stabilize the active R conformation. Thus, the requirements for a compound to be an activator are not too specific provided that it contains a suitable anionic group and a suitable ring system. As one tests structures which come closer to resembling AMP, the activation improves, as may be seen by studies with IMP (Black & Wang, 1968). On the other hand, one does not need an anionic group to be chelated by the arginines if there is some other way of anchoring an adenine analogue to the inner structure of the protein. Thus, the work of Graves and his colleagues (Anderson & Graves, 1973; Anderson et al., 1973) combined with the sequence studies of Titani et al. (1977) showed that when 8-[[*m*-[*m*-(fluorosulfonyl)benzamido]benzyl]thio]adenine was reacted with Tyr-155 a phosphorylase *b* derivative with 50% of the normal activity was obtained.

Returning to the nature of the binding sites for ANS, it is apparent that the ability of the three arginines to chelate the sulfonic acid residue can overcome the deficiencies in hydrophobic character of the activator site. On the other hand, the inhibitor site would appear to be tailor-made for ANS, with Tyr-612 and Phe-285 parallel to each other and 7–8 Å apart, thus forming a hydrophobic slot. Our previous studies demonstrated, however, that a strong negative charge on purine derivatives which would otherwise bind strongly to the inhibitor site caused a severe diminution in the strength of binding (Kasvinsky et al., 1978b; Sprang et al., 1982a).

Registry No. ANS, 82-76-8; UDPG, 133-89-1; AMP, 61-19-8; glucose-1-P, 59-56-3; IMP, 131-99-7; caffeine, 58-08-2; glucose, 50-99-7; glycogen phosphorylase *b*, 9012-69-5.

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