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STRUCTURAL CHANGES INDUCED IN GLYCOGEN PHOSPHORYLASE *b* BY THE BINDING OF GLUCOSE AND CAFFEINE

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Summary

The allosteric inhibitors glucose and caffeine casuse significant structural alterations in glycogen phosphorylase *b* (1,4-α-D-glucan:orthophosphate α-D-glucosyltransferase, EC 2.4.1.1). Both cause a masking of two sulfhydryl groups and a reduction of binding affinity for AMP. Caffeine produces an alteration in the microenvironment of the binding site for 1-anilinnaphthalene-8-sulfonate, resulting in a decrease of quantum yield of fluorescence and a change in spectral distribution. The binding of glucose is exothermic with an enthalpy of binding of —6.0 kcal/mol. Glucose causes a change in the molecular ellipticity in the pyridoxal-5′-phosphate region. The implications of these results are discussed.

Introduction

The enzyme glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-D-glucosyltransferase, EC 2.4.1.1) has a position of central importance in carbohydrate metabolism as the agent for the mobilization of glycogen reserve carbohydrate and as a major element in the overall regulatory mechanism [1]. The recent X-ray crystallographic studies by Fletterick and coworkers [2—5] have made possible the location within the tertiary structure of the sites involved in catalysis and allosteric regulation. While the studies of Fletterick et al. were made upon phosphorylase *a*, the covalently activated form, it is probable that they can serve as a basis for discussion of the corresponding structural features of phosphorylase *b*.

Glycogen phosphorylase *b* is a dimer of molecular weight 195 000, which is

composed of two identical monomer units [1,6]. It is covalently activated by phosphorylation of Ser 14 and may be non-covalently activated by the binding of AMP. The AMP binding sites are located at the interface between two monomer units and incorporate elements of both polypeptide chains [2—5]. While phosphate and glucose 1-phosphate are also bound at the primary AMP site, the catalytic site where the conversion of these substrates occurs, is located about 30 Å away, in proximity to the site where pyridoxal-5′-phosphate is attached [4]. The negative allosteric effector glucose is also bound at a site in proximity to the pyridoxal-5′-phosphate [2]. High concentrations of glucose 1-phosphate replace glucose at this site. A second negative effector site, which preferentially binds nucleosides, including caffeine, adenosine, and inosine, is located about 10 Å from the glucose site [2]. In the case of phosphorylase *a*, a synergistic inhibition by caffeine, or nucleoside, and glucose exists [2,7].

The glucose effector site is probably not important in the regulation of muscle phosphorylase, since muscle contains no free glucose. However, it may be important for the liver enzyme, since this organ is freely permeable to serum glucose. The allosteric response to glucose may be a major element in the basic control mechanism for glucose homeostasis [2].

A currently popular model for the regulation of liver phosphorylase *a* is based upon a proposed synergistic inhibition by glucose and a second ligand, which binds at the nucleoside site [2]. The combined action of the two promotes the inactive T conformation, which is susceptible to the activity of phosphorylase phosphatase. The latter converts the enzyme to phosphorylase *b*. This form releases bound phosphatase, which subsequently dephosphorylates and activates glycogen synthetase.

The present paper will describe changes in properties of glycogen phosphorylase *b* induced by several allosteric regulators, including glucose and caffeine, as well as their synergistic activity.

Experimental

Materials. Glycogen phosphorylase *b* was isolated from frozen rabbit muscle (Pel-Freeze, Inc.) by the method of Fischer and Krebs [8] and was recrystallized three times from 0.03 M cysteine (pH 7.0), 1 mM AMP, and 10 mM $Mg(CH_3COO)_2$, at 0° C. Solutions were prepared by dissolution of the crystals in 0.05 M Tris-HCl, 0.1 M KCl, 0.005 M β-mercaptoethanol, pH 7.0, and were freed from AMP by passage first through a 1 × 40 cm Sephadex G-25 column eluted with buffer and then through a 0.5 × 4 cm column of Sephadex G-25 plus activated charcoal. The 260/280 nm absorbance ratio of the solutions employed here was normally in the range of 0.50—0.53.

Tris, glucose, glucose 1-phosphate, unlabelled AMP, 5,5′-dithiobis(2-nitrobenzoic acid), and all enzymes other than phosphorylase were purchased from Sigma. Tritiated AMP was obtained from the New England Nuclear Corp. The fluorescent label *N*-(iodoacetyl-aminoethyl)-5-naphthylamine-1-sulfonate was bought from Molecular Probes. The spin label 3-(2-iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl was obtained from Synvar. All other chemicals were reagent or analytical grade. Glass-redistilled water was used for the preparation of all solutions.

Methods. Enzymic assays for glycogen phosphorylase *b* were carried out for the glycogen phosphorolysis reaction, employing the glucose 6-phosphate dehydrogenase coupled reaction in the procedure described by Helmreich and Cori [9]. Assays were normally done at 23°C.

Measurements of the spectral distribution of fluorescence intensity were made with a Jasco spectrofluorometer, whose emission spectra are compensated for the wavelength variation of the photomultiplier response.

Measurements of circular dichroism (CD) spectra were made with a Jasco circular dichroism apparatus.

Measurements of the binding of AMP were made by equilibrium dialysis, employing Spectrum dialysis tubing of controlled porosity. ^{3}H-Labeled AMP was used: in two parallel series, equivalent amounts of AMP containing [^{3}H]-AMP were placed inside or outside the sacs for a set of AMP levels, so that equilibrium was approached from either direction. At the conclusion of dialysis, radioactivities of the internal and of the external solutions were measured and averaged for each AMP level for the two series. The attainment of equilibrium, as judged by the equivalence of radioactivities within the sacs for equilibrium approached from either direction, required 3 h with agitation. Counting was done as described elsewhere [10]. The number of molecules of AMP which were bound per phosphorylase dimer, $\bar{\nu}$, was computed from the difference in radioactivities inside and outside the sacs, as described in an earlier publication [10].

The binding data were analyzed in terms of consecutive stepwise binding constants by means of the method described elsewhere [10]. If a function λ is defined for the presence case of two strong binding sites per dimer by

$$\lambda = 1 + k_1[\text{AMP}] + k_1k_2[\text{AMP}]^2 \qquad (1)$$

where k_1 and k_2 are the stepwise binding constants, then λ may be obtained from

$$\ln \lambda = \int \frac{\bar{\nu}}{[\text{AMP}]} \, d[\text{AMP}] \qquad (2)$$

By least-squares fitting of $(\lambda-1)/[\text{AMP}]$ versus [AMP], according to Eqn. 1, k_1 and k_2 may be computed.

Measurements of the fast phase of the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with sulfhydryl groups were made with an Aminco stopped flow apparatus and were monitored by the increase with time of absorbance at 412 nm. The data were displayed in digital form with the use of the Dasar unit supplied by Aminco.

The determinations of the heat of binding of glucose were made with an LKB batch microcalorimeter. For each glucose level the heat of dilution of glucose was subtracted instrumentally, as described elsewhere [11]. In each case the heat of dilution of the protein solution was measured in a separate run and subtracted from the total heat.

A fluorescence or spin label structural probe was placed on the rapidly reacting sulfhydryl by the following procedure. The probe which was employed was an iodoacetate derivative. In order to place a label exclusively upon the reactive sulfhydryl, the enzyme (~10 mg/ml) was dissolved in 0.05 M Tris-HCl,

0.1 M KCl, 0.01 M cysteine, (pH 7.0) and then freed from AMP and cysteine by passage through a 1 × 40 cm Sephadex G-25 column equilibrated with 0.05 M Tris-HCl 0.1 M KCl, pH 7.0. The protein was reacted with 1—5% of its weight of label in the presence of 0.1 M glucose for 30 min at 6°C. The conjugate was then freed from excess label by passage through a 1 × 40 cm Sephadex G-25 column equilibrated with 0.05 M Tris, 0.1 M KCl, 0.005 M β-mercaptoethanol.

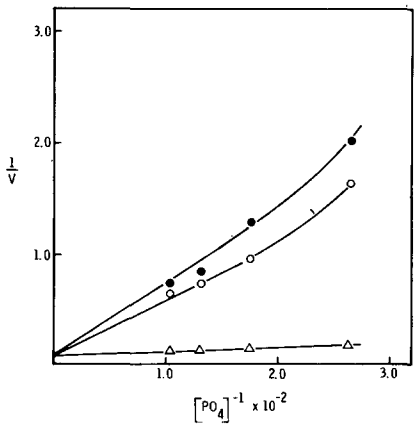
In each case the degree of labeling was computed from the ultraviolet absorption spectrum, assuming a molar extinction coefficient of $6.1 \cdot 10^3$ at 366 nm for *N*-(iodoacetyl-aminoethyl)-5-naphthylamine-1-sulfonate conjugates. The concentration of protein was computed assuming an absorbance at 380 nm equal to 1.32 for a 1 mg/ml solution.

Results

Enzymic activity

Figs. 1 and 2 summarize the effects of glucose and caffeine upon the rate of phosphorolysis of glycogen by phosphorylase *b*. In agreement with earlier results obtained for phosphorylase *a* [7], glucose has a pronounced inhibitory effect upon the enzymic reaction, which is reversed by high concentrations of either the substrate, inorganic phosphate, or the allosteric activator, AMP. As in the phosphorylase *a* case, glucose (0.1 M) interferes primarily with the binding of substrate; *V* is almost unchanged (Fig. 1).

The nucleoside adenosine has also a significant inhibitory effect. It moreover substantially enhances the observed inhibition by glucose; glucose and adenosine in combination have a greater effect than would be predicted from that of either acting singly. For example, 0.1 M glucose and 50 mM adenosine



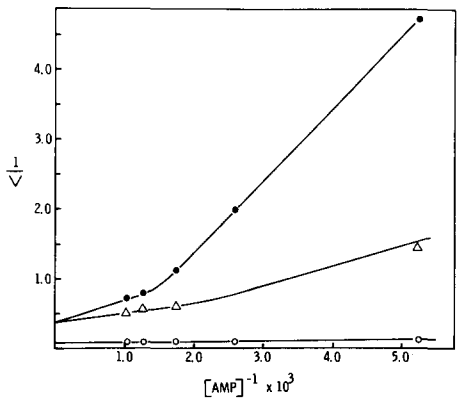


Fig. 1. A double reciprocal plot comparing the rate of phosphorolysis of glycogen (μmol/min per mg) as a function of phosphate concentration in the absence (△) and presence of 0.1 M glucose (○) or 2 mM caffeine (●) AMP is present at a level of 1 mM.

Fig. 2. A double reciprocal plot comparing the rate of phosphorolysis of glycogen (μmol/min per mg) as a function of AMP level in the absence (○) and presence of 0.1 M glucose (△) or 2 mM caffeine (●). The concentration of phosphate is 7.5 mM.

separately reduce the relative activity (5 mM phosphate, 1 mM AMP) from 1.00 to 0.25 and 0.53, respectively. In combination, they reduce it to 0.03.

The effect of caffeine is qualitatively analogous to that of adenosine, as previously reported for the case of phosphorylase *a* [2]. In both cases a synergistic effect with glucose is observed. For the above conditions, 1 mM caffeine reduces the relative activity to 0.63. In 1 mM caffeine and 0.1 M glucose, the relative activity is reduced to 0.02.

Calorimetry

Fig. 3 displays the heats of binding as a function of the free glucose concentration, as determined by microcalorimetry. The reaction is exothermic, a limiting heat of -6.3 ± 0.5 kcal/mol being attained at high glucose levels. One glucose binding site has been identified per monomer unit [2]. If, as an approximation, the binding by the two sites per dimer unit is treated as proceeding independently, then the average value $\overline{K}$ of the equilibrium constant for the binding of glucose by a single site is given by

$$K = \frac{\alpha}{(1-\alpha)[\mathrm{glc}]}$$

where $\alpha = \Delta H/\Delta H_t$ and [glc] is the molar concentratin of free glucose. Here ΔH is the heat evolved per mol of phosphorylase monomer units and ΔH_t ($= -6.3$ kcal) is the limiting heat evolved per monomer unit under saturation conditions. From the slope of a linear plot of $\alpha/(1-\alpha)$ versus the concentration of free glucose a value of $\overline{K}$ of $1.0 \cdot 10^3 \pm 0.4 \cdot 10^3$ is obtained.

AMP binding

Fig. 4. compares the binding isotherms for AMP in the presence and absence of glucose. A significant reduction in binding is evident in the presence of glucose, saturation being attained by a glucose level of 0.1 M. Computation of the two consecutive stepwise equilibrium constants for the binding of AMP by the phosphorylase dimer, according to Eqns. 1 and 2, indicates that both are reduced in magnitude in the presence of glucose as was found earlier for different conditions of pH [12]. The constants k_1 and k_2 fell from $0.75 \cdot 10^4$ and $0.43 \cdot 10^4$, respectively, to $0.50 \cdot 10^4$ and $0.22 \cdot 10^4$, respectively, in the presence of 0.1 M glucose. Since the glucose effector site is located 30 Å from the AMP allosteric site, this suggests a transmitted modification of the microenvironment of the latter. A parallel effect has already been reported for the phosphorylase *a* case [7].

Caffeine, either alone or in the presence of 0.1 M glucose, greatly reduced the binding of AMP (Fig. 4), having a much more pronounced effect than glucose. In 0.05 M caffeine, the binding of AMP is virtually abolished. The combined effect of caffeine and glucose was much greater than predicted for the additive individual effects of the two ligands (Fig. 4). Thus, in the absence of glucose, k_1 and k_2 fell to $0.24 \cdot 10^4$ and $0.11 \cdot 10^4$, respectively, in 10 mM caffeine. With both 0.1 M glucose and 10 mM caffeine, present they both fell to $<0.05 \cdot 10^4$.

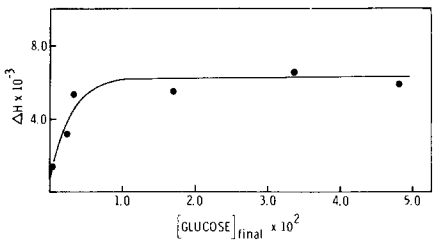


Fig. 3. The heat per mole of phosphorylase *b* monomer which is evolved upon mixing with glucose solutions to the indicated final level of glucose.

CD spectra

A comparison of the ultraviolet CD spectra for phosphorylase *b* in the presence and absence of 0.1 M glucose indicated that there was no important effect in the peptide (190—240 nm) or the aromatic (250—300 nm) spectral regions. Any changes were small (<1%) and probably within experimental uncertainty. The implication is that this level of glucose is without major effect upon the secondary structure, or upon the microenvironments of the aromatic groups.

A significant effect is however observed in the pyridoxal-5′-phosphate region (300—350 nm), with a perceptible shift of the band (Fig. 5). The state of the buried pyridoxal-5′-phosphate group thus appears to be responsive to the binding of glucose, whose locus of binding is in proximity to the site [2,4]. In contrast, 0.1 M caffeine produced no significant effect upon the spectrum.

Tryptophan and pyridoxal-5′-phosphate fluorescence

Neither 0.1 M glucose nor 0.01 M caffeine was observed to produce any

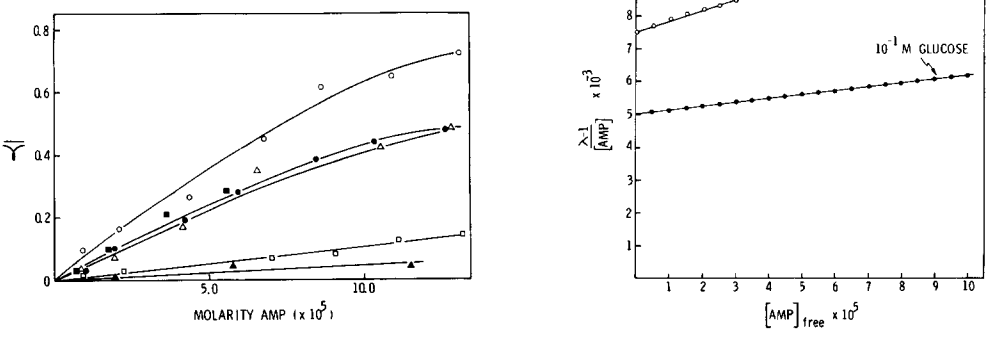


Fig. 4. (Left) The dependence of $\bar{\gamma}$, the average number of AMP molecules bound per phosphorylase dimer upon the concentrations of free AMP, in the presence and absence of glucose or caffeine. ○, no additive; ●, 0.1 M glucose; ■, 0.4 M glucose; △, 0.01 M caffeine; □, 0.05 M caffeine; ▲, 0.01 M caffeine plus 0.1 M glucose. (Right) The dependence of [(λ − 1)/AMP] upon [AMP] in the presence and absence of 0.1 M glucose.

significant effect in the intensity or spectral distribution of tryptophan or pyridoxal-5′-phosphate fluorescence.

Sulfhydryl reactivity

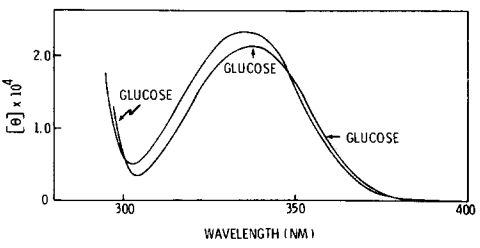
There is some apparent disagreement in the literature as to the number of rapidly reacting —SH groups per phosphorylase monomer [13—19]. However, two groups have found that, for phosphorylase *b* which has been recrystallized from a cysteine-containing medium, one rapidly reacting sulfhydryl is present on each monomer unit, with indication of two or more slowly reacting sulfhydryls [15,19]. The apparent presence of a single rapidly reacting sulfhydryl stems from the partial loss of the two fast sulfhydryls cysteine-171 and cysteine-317 by reaction with cysteine [20], leaving about one fast sulfhydryl per monomer unit, on the average.

In agreement with Battell et al. [15], and with Sanner and Tron [19], a stopped-flow study indicated the presence of approximately one —SH group per monomer unit whose reaction with 5,5′-dithiobis(2-nitrobenzoic acid) was complete within 10 s (Fig. 6). No significant change in rate was observed in the presence of 0.1 M glucose, suggesting that the microenvironment of this group was essentially unchanged by glucose binding (Fig. 6).

If the reaction was monitored for longer times it was found, in agreement with Battell et al. [15] that an additional two —SH groups reacted over a period of 1—2 h. The presence of 0.1 M glucose had in this case a profound effect, blocking the reaction of these sulfhydryls almost quantitatively (Fig. 7). In contrast, glucose 1-phosphate had almost no effect (Fig. 7).

While no loss of activity accompanied the reaction of the single rapidly reacting sulfhydryl in the presence or absence of glucose, the reaction (1 mM reagent for 1 h) of the two more slowly reacting sulfhydryls caused a 90% loss of activity. The presence of 0.1 M glucose completely protected the enzyme from deactivation.

In contrast to the rapid sulfhydryl, whose reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) resulted in no change in the CD spectrum, the reaction of the two slow sulfhydryls completely altered its appearance in the pyridoxal-5′-



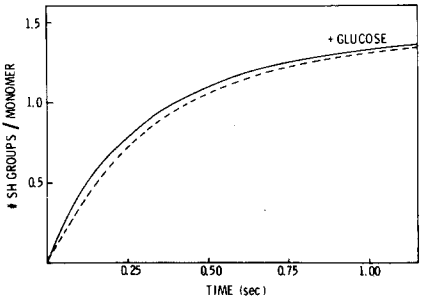


Fig. 5. A comparison of the CD spectra of phosphorylase *b* in the PLP region in the presence and absence of 0.1 M glucose.

Fig. 6. The reaction of DTNB (1 mM) with the reactive sulfhydryl of phosphorylase *b*, in the absence and presence of 0.1 M glucose, as monitored by a stopped-flow spectrophotometer.

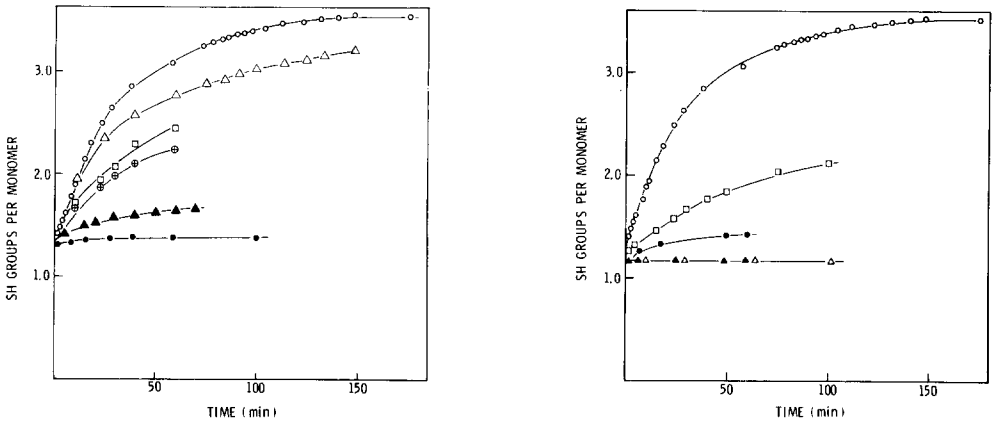


Fig. 7. (Left) the reaction of the two slowly reacting sulfhydryls of phosphorylase *b* with DTNB (1 mM) in varying levels of glucose, adenosine, and glucose 1-phosphate at 6°C. ○, no additives; △, 0.1 M glucose 1-phosphate; □, 0.05 M adenosine; ⊕, 0.01 M glucose; ▲, 0.05 M adenosine plus 0.01 M glucose; ●, 0.1 M glucose. (Right) the reaction of the two slowly reacting sulfhydryls of phosphorylase *b* in the presence of varying levels of caffeine and glucose at 6°C. ○, no additives; □, 0.01 M glucose; ●, 1 mM caffeine; ▲, 0.01 M glucose plus 1 mM caffeine; △, 0.01 M caffeine.

phosphate region (Fig. 8), the singly positive band being replaced by a biphasic spectrum with positive and negative bands. This effect was likewise abolished in the presence of 0.1 M glucose (Fig. 8).

It was also of interest to examine the effect of the nucleoside adenosine, both alone and in the presence of glucose (Fig. 7). Adenosine alone (0.05 M) depressed significantly the rate of reaction of the two slow sulfhydryls. However, the addition of a sub-saturating level of glucose (10 mM), which by itself produced only a modest retardation, almost abolished the reaction. The combined effect of glucose plus adenosine was greater than that of either modifier alone, in harmony with the synergistic action of the two observed elsewhere.

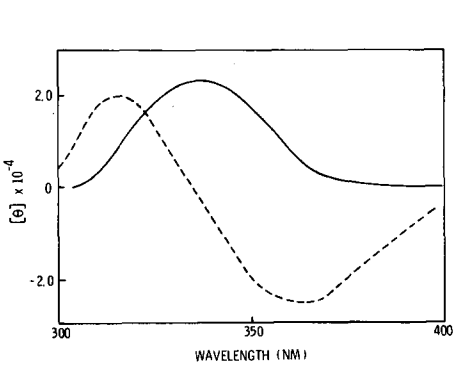
At the highest concentration of adenosine permitted by its solubility, the protection of the two slow sulfhydryls was only partial. However, 0.01 M caffeine completely blocked their reaction (Fig. 7). At sub-saturating levels of glucose and caffeine, a synergistic effect of the two in combination was observed (Fig. 7).

From the recent sequential studies of Koide et al. [6] the two slowly reacting sulfhydryls may probably be identified as Cys-108 and Cys-142.

Neither 0.1 M glucose nor 0.1 M caffeine produced any significant change in the fluorescence emission spectrum of an *N*-(iodoacetyl-aminoethyl)-5-naphthylamine-1-sulfonate label or in the electron spin resonance spectrum of the spin label attached specifically to the reactive sulfhydryls.

1-Anilino-naphthalene-8-sulfonate

Each monomer unit of glycogen phosphorylase *b* has been shown to bind one or more molecules of 1-anilino-naphthalene-8-sulfonate to yield fluorescent complexes [10,21]. The addition of glucose to phosphorylase plus 1-anilino-



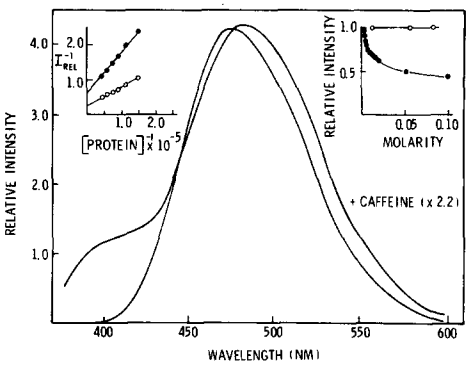


Fig. 8. The CD spectrum of phosphorylase *b* in the PLP region after treatment with 1 mM DTNB (2 h at 6°C) in the absence (- - - - -) and presence (———) of 0.1 M glucose.

Fig. 9. Fluorescence emission spectra of ANS (10 μM) plus phosphorylase *b* in presence and absence of 0.1 M caffeine. The excitation wavelength is 340 nm. Right inset: Relative fluorescence intensity at 470 nm as a function of the concentration of glucose (○) and of caffeine (●). Left inset: Double reciprocal plot of relative intensity versus molarity of phosphorylase for ANS (10 μM) in the presence (●) and absence (○) of 0.1 M caffeine.

naphthalene-8-sulfonate has only a marginal effect upon the intensity and spectral distribution of fluorescence, suggesting that the binding of glucose has no major influence upon either the binding equilibria or quantum yield (Fig. 9).

In contrast, the addition of caffeine results in a pronounced decrease in fluorescence intensity, as well as in a shift in emission maximum to longer wavelengths (Fig. 9). The implication is that the microenvironment of the binding site is modified as a consequence of the binding of caffeine.

A double reciprocal plot of intensity versus protein concentration (Fig. 9) indicates that the intercept, and hence the quantum yield is modified in the presence of caffeine.

Discussion

From the preceding results it is clear that the binding of glucose or caffeine by glycogen phosphorylase *b* results in significant conformational changes in solution and that these involve a substantial fraction of the tertiary structure. The effects of the two modifiers are non-equivalent.

Detailed structural information is available for phosphorylase *a* from the crystallographic studies of Fletterick and coworkers [2—5]. While it is uncertain to what extent the detailed structural features recur in phosphorylase *b*, it is probably reasonable to assume that the broader structural conclusions apply to the latter form as well.

The glycogen phosphorylase monomer may be regarded as consisting of two distinct domains. The N-terminal domain consists of the first 489 residues from the N-terminus and contains both the glycogen storage locus and the AMP binding site. The C-terminal domain contains the remainder of the 841 residues

[2,6]. The catalytic site of phosphorylase is located in a cleft at the interface of the two domains. The pyridoxal-5′-phosphate group, which is attached to Lys-679, is in proximity to the catalytic site. The latter is the binding site for glucose. The binding of activating ligands by crystals of glucose-inhibited phosphorylase a causes distinct structural changes which are confined to the N-terminal domain. A shift of the protein chain occurs in the N-terminal region which forms the subunit interface. A second region of change is the locus of oligosaccharide binding, which is believed to be involved in the attachment of phosphorylase to the glycogen particle, while a third altered region occurs in the neighborhood of the catalytic site and the pyridoxal-5′-phosphate group.

It is likely that the binding of glucose or caffeine does not induce major changes in the secondary or tertiary structure of phosphorylase *b*. The absence of significant changes in the CD spectrum in the peptide and aromatic regions in the presence of saturating levels of glucose is consistent with this conclusion, as is the failure of caffeine to produce any significant alteration in the pyridoxal-5′-phosphate region. The only change observed which was outside of experimental uncertainty was a minor alteration in the pyridoxal-5′-phosphate spectrum induced by glucose. Neither ligand altered the tryptophan emission spectrum to a significant extent. There was also no observable effect upon a fluorescent or a spin label reporter group attached to the reactive sulfhydryl, which may probably be identified with Cys-171 or Cys-317 [6], with the implication that the microenvironment of this group is not modified to an important extent.

Both glucose and caffeine render unreactive the two slowly reacting sulfhydryls of phosphorylase *b*. Both of these are probably partially buried within the tertiary structure, as is suggested by their limited reactivity and by the reduced mobility of an attached spin label. It is likely that a relatively minor structural transition could convert these groups from a partially to a completely shielded state. The failure of glucose 1-phosphate to protect either site indicates that the effect of glucose does not arise solely from simple steric shielding. Since Cys-108 should be well separated from both the catalytic and nucleoside sites, it is probable that the alteration of tertiary structure accompanying the binding of glucose or caffeine extend over a considerable region of the N-terminal domain. The synergistic effect of the two ligands is very evident.

A major difference between the two ligands appears in their effect upon AMP binding. Saturating levels of glucose reduce, but do not abolish the binding of AMP, while leaving the cooperativity essentially unaltered. In contrast, sufficiently high levels of caffeine eliminate AMP binding almost quantitatively. The two ligands are highly synergistic in action. In the case of phosphorylase *a*, purines are bound preferentially at the nucleoside site, with some weak binding at the AMP activator site. The blocking of AMP binding may be attributed to the combined effect of an induced conformation change with some contribution from direct competition for binding at the primary AMP site.

A second major differences arises in the effects of the two ligands upon 1-anilino-naphthalene-8-sulfonate binding. Caffeine, but not glucose, causes a substantial decrease in the intensity of fluorescence developed in the presence

of phosphorylase *b*. That this does not result solely from a displacement of bound 1-anilino-naphthalene-8-sulfonate is indicated by the spectra shift observed in the presence of excess caffeine. The implication is that the microenvironment of the binding site is significantly altered as a consequence of the conformational change caused by caffeine binding. The primary site has not been definitely located within the structure of phosphorylase, but there is some indication that it is not greatly distant from the AMP activator site [10,21].

In summary, the binding of glucose or caffeine results in a detectable, although subtle, conformation change, whose effects are manifested at several widely separated points in the N-terminal domain of phosphorylase *b*. The binding of caffeine appears to result in more extensive changes than does that of glucose and may mimic the effect of a physiological modifier [2]. In the presence of glucose the effects of caffeine appear at lower concentrations of the latter, suggesting the existence of a heterotropic cooperativity of binding of the two ligands. The existence of these induced structural changes probably accounts for the enzyme inhibitor effect of caffeine and is a factor in that of glucose.

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