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Conformational Changes and Local Events at the AMP Site of Glycogen Phosphorylase *b*: A Fluorescence Temperature-Jump Relaxation Study†

B. Vandenbunder,* M. Dreyfus, and H. Buc

ABSTRACT: The relationship between nucleotide binding kinetics and the activation process of glycogen phosphorylase *b* has been studied with two fluorescent AMP analogues, 1,N_6-etheno-AMP (ϵAMP), an activator of phosphorylase *b*, and 1,N_6-etheno-2′-deoxy-AMP (ϵdAMP). This latter probe binds to the same nucleotide site but does not activate the enzyme. A major relaxation process consistent with a bimolecular association is observed after perturbation of the phosphorylase *b*-ϵAMP or ϵdAMP complex in 50 mM glycylglycine buffer. Residence times of these two nucleotides are of the same order of magnitude ($\simeq$500 μs at 11 °C). After addition of the anionic substrates, orthophosphate or glucose 1-phosphate, to the enzyme-nucleotide complex, a rapid chase ($\leq$3 ms) of ϵAMP and ϵdAMP is observed, which presumably reflects competition for the nucleotide binding site. This rapid chase is followed by a slow uptake of ϵAMP on the enzyme. This process is not observed when ϵAMP is replaced by ϵdAMP and reflects an isomerization of the enzyme in the 10-min range at 11 °C. Temperature-jump relaxation studies have shown that this isomerization is accompanied by a concomitant change of the binding kinetics of ϵAMP, a new binding process in the 20-ms time range becoming predominant. The residence time of ϵAMP on the corresponding enzyme conformation is 28 ms at 11 °C: this is very close to the value observed with phosphorylase *a* without any added substrate (11 ms at 24 °C). In addition to phosphate and glucose 1-phosphate, a variety of anionic effectors (sulfate, citrate) or cationic effectors (magnesium, spermine), as well as 5′-AMP, were found to produce the transition from the "fast-binding" to the "slow-binding" enzyme forms. In the light of these findings, we finally discuss the role of the nucleotide and of these various effectors in the activation of phosphorylase *b*, in the context of current models for allosteric transitions.

Glycogen phosphorylase is at the center of all regulation mechanisms controlling glycogen degradation. The molecular basis of regulatory effects on phosphorylase activity has been interpreted in terms of conformational changes, the more active states being stabilized either by covalent phosphorylation or through AMP binding.

On the basis of binding and activation studies, several groups (Madsen and Shechosky, 1967; Buc, 1967) suggested that the allosteric regulation of phosphorylase *b* conforms to the two-state concerted model (Monod et al., 1965). However, additional conformational states have been postulated to explain the activity of phosphorylase *b* at low AMP concentrations (Kastenschmidt et al., 1968) or the steady-state enzymatic response to AMP analogues (Black and Wang, 1968; Morange et al., 1976). Other conformational states have been proposed in order to account for the changes of protein structure upon the addition of ligand, detected by paramagnetic and spectroscopic reporter groups (Birkett et al., 1971; Madsen et al., 1976). However, the precise interpretation of most static measurements in terms of conformational changes is obscured by the fact that only conformation-averaged properties are recorded. Moreover, it is often difficult in spectroscopic work to decide whether the observed effects reflect gross changes in the tertiary or quaternary structure of the protein or minor structural modifications in the vicinity of the spectroscopic probe.

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On the other hand, in favorable cases, fast kinetics offer the possibility of discriminating between the various conformations present, by studying their ligand-binding properties separately. Furthermore, it is also possible to study the rate of interconversion of these conformations. Previous work from this laboratory has shown that the fluorescence of the AMP analogues 1,N_6-etheno-AMP (ϵAMP) and 1,N_6-etheno-2′-deoxy-AMP (ϵdAMP) is quenched upon binding to the AMP site (Vandenbunder et al., 1976). We have now used this property to investigate the nucleotide-binding kinetics on the various conformations of phosphorylase *a* and *b* present in solution, as well as their interconversion rates.

Materials and Methods

Glycogen phosphorylase *b* was purified from rabbit skeletal muscle according to Krebs et al. (1964), using 2-mercaptoethanol instead of cysteine. Phosphorylase *b* was converted into phosphorylase *a* as described by Krebs and Fischer (1962). 1,N_6-etheno-AMP (ϵAMP) and 1,N_6-etheno-2′-deoxy-AMP (ϵdAMP) were purchased from Sigma. Procedures for the determination of enzymatic activity and for equilibrium dialysis have been previously described (Vandenbunder et al., 1976).

Relaxation studies, in the 10 μs–100 ms range, have been carried out with a temperature-jump apparatus for fluorescence measurements, built at the Max-Planck Institute, Göttingen (Rigler et al., 1974; Jovin, 1975). Appropriate filters were set on the emission beam to ensure that the protein fluorescence signal was negligible ($\leq$5%) with respect to the nucleotide fluorescence. The temperature-jump magnitude was calibrated using the temperature-dependent pH change of the glycylglycine buffer, monitored with the indicator Bromothymol blue. A temperature jump of 8.5 °C resulted from a 30-kV discharge, using a 0.05-μF capacitor. The relaxation signal after perturbation was stored in the waveform recorder Biomation Model 805 and displayed on a chart recorder. Analysis of the curves was made on semilogarithmic paper.

Temperature-jump experiments are routinely performed as follows: the temperature-jump cell is filled with 1 mL of degassed sample solution and then allowed to stand in its thermostated holder until it reaches thermal equilibrium, as indicated by a thermistance inserted in the upper electrode. Two to five temperature-jump traces are then recorded, allowing suitable delays between individual experiments for temperature reequilibration. Finally, AMP is added to a final concentration of 1 mM, in order to chase all the bound fluorescent nucleotide: this allows the measurement of the fluorescence signal corresponding to the total concentration of etheno nucleotide released in solution, under inner-filter effect conditions strictly identical to those prevailing in the actual kinetic experiment. In all cases,we checked that relaxation signals were no longer observed after AMP had been added.

With buffer consisting of 50 mM glycylglycine, pH 6.9, at 25 °C (pH 7.5 at 4 °C), 100 mM KCl, 0.1 mM EDTA,[1] and 10 mM 2-mercaptoethanol or 1 mM dithiothreitol, the loss of enzyme activity after a series of ten 8.5 °C temperature jumps is less than 5%. Within experimental error, the results are reproducible for different phosphorylase preparations.

Temperature-jump experiments have been carried out at various temperatures. At low temperature, near 4 °C, cavitation problems are minimized, thereby allowing a higher precision in the measurements and an analysis of relaxation data over a wider range of enzyme and ligand concentrations. Therefore, for phosphorylase *b*, we first analyzed data obtained after jumping from 3 to 11 °C.

Treatment of the T-jump Data

Simple Binding Process. Consider a one-step binding process of nucleotide to enzyme:

$$E + F \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EF \qquad (1)$$

in which E, F, and EF refer, respectively, to the free enzyme, free nucleotide, and nucleotide–enzyme complex; k_1 and k_{-1} represent rate constants. The dynamic behavior of the system after a temperature jump is adequately described by a single exponential process with a relaxation time (τ) given by:

$$\tau^{-1} = k_1([E] + [F]) + k_{-1} \qquad (2)$$

(see, e.g., Bernasconi, 1976). While this is strictly valid only for small temperature changes, eq 2 still approximately describes the reequilibration process even for fairly large perturbations of the system, provided that one of the components (E or F) is present in significant excess over the other (pseudo-first-order conditions), a condition always fulfilled in our experiments. The equilibrium concentrations, [E] and [F], are calculated from the equilibrium constant obtained independently from equilibrium dialysis experiments. Thus, the variation of τ^{-1} as a function of [E] + [F] allows the determination of k_1 and k_{-1}.

Case Where Several Conformations Are Present. Equations 1 and 2 are only valid if the various enzyme conformations which exist in solution bind the ligand with identical kinetic parameters and/or if their interconversion is much faster than the binding process being studied. If neither condition is met, the different conformations will behave as independent species with respect to the binding process. Nevertheless, the binding on each conformation will not generally result in an independent relaxation process, because of competition for the available nucleotide; i.e., kinetic coupling will occur between the different binding modes (Kirschner et al., 1971). Even if a single relaxation process is observed, suggesting that only one conformation (or group of rapidly interconverting conformations) can bind the ligand, eq 2 cannot be readily used to interpret the results, because the concentration of the "nucleotide-binding" enzyme form to be used in place of [E] in eq 2 is generally not known. None of these difficulties occur if the ligand is used in large excess with respect to the slowly interconvertible enzyme species. In this case, the different binding processes to each conformation will be uncoupled, and the corresponding relaxation times will be given by expressions similar to eq 2, where $[F]_{tot}$ is used in place of [E] + [F]. Thus, plotting the observed relaxation times vs. the total ligand concentration allows the determination of the individual kinetic parameters for ligand binding to the different conformations and thus their individual affinities.

Results

Steady-State Kinetics and Equilibrium Dialysis. The dependence of the initial velocity of glycogen phosphorylase *b* activity upon the substrate concentration has been determined at saturating concentrations of ϵAMP, ϵdAMP, and AMP. Figure 1 shows that ϵAMP activates phosphorylase *b*, whereas ϵdAMP does not. The kinetic response with ϵAMP is sigmoïdal: plateau activity reaches the same value as that found with AMP.

Characterization of the phosphorylase *b*–nucleotide interaction has been carried out in 50 mM glycylglycine buffer, pH 6.9 (Vandenbunder et al., 1976). The two fluorescent AMP analogues were found to have similar affinities for the enzyme

[1] Abbreviation used: EDTA, (ethylenedinitrilo)tetraacetic acid.

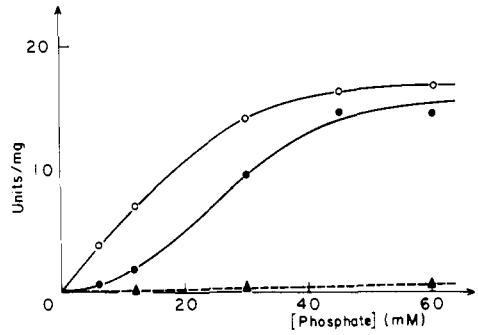

FIGURE 1: Dependence of phosphorylase *b* activity upon phosphate concentration. Enzyme was assayed at 11 °C, in 0.1 mM EDTA, 50 mM glycylglycine (pH 6.9) buffer, with 0.25% glycogen, in the presence of either 1 mM 5′-AMP (○), 0.34 mM ϵAMP (●) or 0.5 mM ϵdAMP (▲).

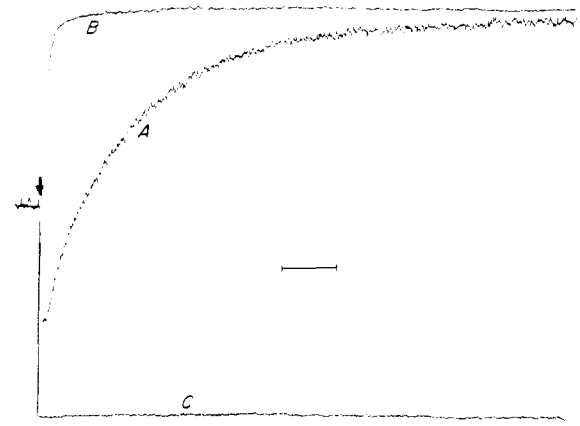

FIGURE 2: Example of relaxation signals obtained with the phosphorylase *b*-ϵAMP system. The total concentrations of phosphorylase *b* subunits and ϵAMP were, respectively, 139.5 and 142.5 μM. The arrow indicates the time at which an 8.6 °C temperature jump was triggered. The final temperature was 11 °C. The scale represents 200 μs for curve A and 4 ms for curves B and C. The major relaxation time is equal to 320 μs and its amplitude is 15% of the steady-state signal (curve A). Curve B shows the existence of a slower process ($\tau \simeq 4.8$ ms). After the addition of AMP, to a final concentration of 1 mM, no relaxation occurs (curve C).

and to bind competitively with respect to AMP. The saturation curve of phosphorylase *b* by ϵAMP was weakly cooperative, while with ϵdAMP it was nearly hyperbolic. In contrast, we have now found that, in the presence of phosphate (60 mM), the binding curve of ϵAMP is very cooperative ($K_1 = 600$ μM; $K_2 = 40$ μM), while the affinity of ϵdAMP for the enzyme is weak. Both saturation functions (not shown) are qualitatively similar to the previously reported plots of enzyme activity vs. nucleotide concentration in the presence of 60 mM phosphate and 0.25% glycogen (Vandenbunder et al., 1976).

The saturation curve of phosphorylase *a* by ϵAMP is hyperbolic (Vandenbunder et al., 1976). The affinity of phosphorylase *a* for ϵAMP has been reestimated as 6 μM at 24 °C. Under these conditions, the affinity of ϵdAMP as determined by a chase of trace amounts of radioactive AMP is 180 μM. The same large difference in affinity is observed for AMP and 2′-dAMP binding to phosphorylase *a* ($K_d = 1.8$ and 50 μM, respectively).

Binding Kinetics, in the Absence of Substrate. Phosphorylase *b*. Figure 2 shows typical relaxation curves observed in the 10 μs to 100 ms range, after a temperature jump, with solutions containing fluorescent nucleotide and phosphorylase, in 50 mM glycylglycine buffer, pH 6.9, at 11 °C. A fast decrease occurs simultaneously with the temperature change, followed by an increase of fluorescence of the solution. This increase is absent in solutions containing only either phosphorylase or fluorescent nucleotide. Addition of 1 mM AMP chases the bound fluorescent nucleotide and abolishes the relaxation signal (Figure 2C).

The nature of the major relaxation process of the phosphorylase *b*-ϵAMP system (Figure 2A) was further investigated by studying the dependence of the relaxation time upon enzyme and nucleotide concentration. Plotting τ^{-1} as a function of the sum of the concentrations of free enzyme and free nucleotide yields a straight line for ligand saturation ranging from 0.1 to 0.85 (Figure 3), thereby suggesting that the observed relaxation phenomenon is mainly due to a simple binding process (eq 1). Further evidence for this came from experiments in which the concentrations of enzyme and nucleotide were permuted: thus, as expected from eq 2, the same relaxation time was observed whether nucleotide or enzyme is in excess. From Figure 3, we obtain the following parameters for the binding process of ϵAMP on the enzyme at 11 °C: $k_1 = 1.1 \times 10^7$ M^{-1} s^{-1} and $1/k_{-1} = 670$ μs (Table I).

While these results suggest, at 11 °C, a one-step mechanism for ϵAMP binding to phosphorylase *b*, it must be pointed out that the "kinetic" dissociation constant, $k_{-1}/k_1 = 140$ μM, is larger than the overall dissociation constants obtained by

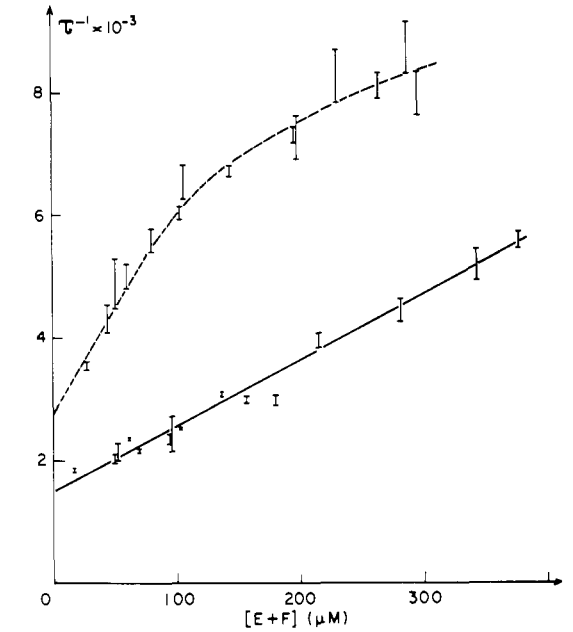

FIGURE 3: Plot of the inverse relaxation time (τ^{-1}) vs. the sum of concentrations of free phosphorylase *b* subunits and free nucleotide ϵAMP (full line) or ϵdAMP (broken line). [E] + [F] has been evaluated assuming a dissociation constant of 90 μM for ϵdAMP and 80 μM for ϵAMP derived from equilibrium dialysis. The buffer was 50 mM glycylglycine (see text). The final temperature was 11 °C.

equilibrium dialysis ($K_1 = 85$ μM; $K_2 = 70$ μM), suggesting the existence of a minor form which binds the nucleotide more efficiently. In fact, an additional small relaxation signal is obtained, in the 10-ms range (Figure 2B), which possibly arises from nucleotide binding to this form. A quantitative study of this relaxation effect was not possible because of its weak amplitude.

In solutions of ϵdAMP and phosphorylase *b*, only one relaxation time, in the time range 100–500 μs at 11 °C, is observed. The dependence of τ^{-1} upon free enzyme and free nucleotide concentrations is shown in Figure 3. Clearly, the curve deviates from linearity and would presumably reach a plateau for very high [E] + [F] values, thus indicating more

TABLE I: Reaction Rate Parameters, Reaction Enthalpies, and Equilibrium Constants Calculated from Kinetic Data.

system	temp (°C)	k_1[a] ($\times 10^{-7}$ M^{-1} s^{-1})	$1/k_{-1}$[a] (ms)	$K_d = k_{-1}/k_1$ (μM)	K_d[b] (μM)	ΔH (kJ·M^{-1})
phosphorylase *a* + ϵAMP	24	1.2	11.5	7	6	47 ± 5
phosphorylase *a* + ϵdAMP	24	3.0	0.74	45	180	
phosphorylase *b* + ϵAMP	24	3.7	0.22	120	140	60 ± 4
phosphorylase *b* + ϵAMP	11	1.1	0.67	140	$K_1 = 85$ $K_2 = 70$	56 ± 4
phosphorylase *b* + ϵdAMP	24	7.2	0.11	130	150	71 ± 4
phosphorylase *b* + ϵdAMP	11	4.5	0.45	50	90	
phosphorylase *b* + ϵAMP + 60 mM phosphate	11	0.09	28.0	40	$K_1 = 600$ $K_2 = 40$	
phosphorylase *b* + ϵAMP + 10 mM spermine	11	0.35	25.0	11		

[a] See eq 1 for definition. [b] Determined by equilibrium dialysis. Cooperative binding curves were fitted with two binding constants, K_1 and K_2 (see Vandenbunder et al., 1976).

complex behavior than the ϵAMP-phosphorylase *b* system. Yet, the observed relaxation time was still found invariant upon permutation of the concentrations of enzyme and nucleotide, thus ruling out the possibility that only a minor population of the enzyme participates in the observed process. In fact, the simplest model which fits the results is the following two-step scheme, which has been worked out by several authors (see, e.g., Bernasconi, 1976).

$$E + F \underset{\text{fast}}{\overset{K}{\rightleftharpoons}} ef' \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} EF \qquad (3)$$

This model yields the following expression for the associated relaxation time:

$$\tau^{-1} = k_{-2} + k_2\left[\frac{K([E] + [F])}{1 + K([E] + [F])}\right] \qquad (4)$$

In agreement with eq 4, a plot of $(\tau^{-1} - k_{-2})^{-1}$ vs. $([E] + [F])^{-1}$ is found to yield a reasonably straight line, from which the values $1/K = 200\ \mu M$, $k_2 = 9 \times 10^3\ s^{-1}$, $k_{-2} = 2.2 \times 10^3\ s^{-1}$, and $[EF]/[EF'] = k_2/k_{-2} \simeq 4$ are obtained. If this model is accepted, it can be easily shown that the data corresponding to the linear part of the τ^{-1} vs. [E] + [F] plot can be analyzed according to eq 2 to yield kinetic parameters corresponding to the overall reaction from [E] + [F] to EF. The corresponding data have been included in Table I.[2]

Relaxation Experiments on Glycogen Phosphorylase a. These experiments have been carried out at a final temperature of 24 °C because of the low solubility of phosphorylase *a* at lower temperatures. Furthermore, smaller temperature jumps (3 °C) were used. The inverse of the relaxation time is found to be a linear function of [E] + [F] for values up to 40 μM, and the dissociation constant estimated from kinetic data (7 μM) agrees well with the overall dissociation constant deduced from fluorescence titrations (6 μM).

Relaxation observed with the ϵdAMP-phosphorylase *a* system may also be associated with a single-step binding process. The residence time of ϵdAMP on phosphorylase *a* differs from that of ϵAMP by more than one order of magnitude (see Table I). However, the form which binds ϵdAMP is probably not predominant, since there is a discrepancy between the kinetic dissociation constant (50 μM) and the overall dissociation constant deduced from equilibrium dialysis (180 μM).

Determination of Binding Enthalpies from Relaxation Amplitudes. In all cases where the dependence of the relaxation time upon concentration indicates a one-step binding process, the analysis of the amplitude was found consistent with such an interpretation (Jovin, 1975; Thusius, 1972). Concerning the ϵdAMP-phosphorylase *b* system for which the nucleotide binding is coupled to an isomerization step (eq 3 and 4), only the amplitudes corresponding to the linear part of the τ^{-1} vs. [E] + [F] plot were analyzed to yield an estimation of the overall enthalpy change from E + F to EF (see Thusius, 1972). The corresponding thermodynamic data are included in Table I.

Kinetics of Nucleotide Binding in the Presence of Anionic Substrates. As seen above, fast kinetic experiments in glycylglycine buffer show that phosphorylase *b*, unlike phosphorylase *a*, makes no clear distinction between ϵAMP and ϵdAMP (Table I). On the other hand, such discrimination does exist in the presence of the enzyme substrates, as indicated by steady-state enzymatic kinetics (Figure 1). This prompted us to investigate the influence of anionic substrates, phosphate and glucose 1-phosphate, on the binding of both nucleotides.

As seen in Figure 4A, fluorescence titration curves indicate a striking difference in the binding of ϵAMP and ϵdAMP to the enzyme in the presence of phosphate. While the strength of the ϵdAMP-phosphorylase *b* interaction steadily decreases when the phosphate concentration is increased, the corresponding curve for the phosphorylase *b*-ϵAMP system is clearly biphasic. Upon increasing the phosphate concentration, the fluorescence of the solution first increases, indicating a loosening of the nucleotide-enzyme interaction. However, for larger phosphate concentrations (>20 mM) the fluorescence decreases, showing that the affinity of the enzyme toward ϵAMP increases and/or the fluorescence quantum yield of the bound nucleotide decreases. In fact, equilibrium dialysis confirms that, at 60 mM phosphate, ϵAMP regains an appreciable affinity for the enzyme, while the ϵdAMP-enzyme interaction is very weak. A similar biphasic curve has been already reported for the binding of AMP to phosphorylase *b* at room temperature upon increasing orthophosphate concentration (Chachaty et al., 1978).

[2] It should be emphasized that, despite formal differences, the mechanism for binding of ϵAMP and ϵdAMP may be quite similar: the kinetic results obtained for the binding of ϵAMP on the enzyme could equally well be fitted by eq 4, provided $K([E] + [F]) \ll 1$; i.e., the concentration of the intermediate fast-binding species EF′ is negligible with respect to E and F. In fact, it has been pointed out by several authors (see, e.g., Hammes and Schimmel, 1970) that mechanism 3 may be of quite general validity for enzyme-ligand binding processes. In many cases, the concentration of EF′ is indetectably small, thus resulting in a "simple binding process" kinetic law: the only indication of an existing fast preequilibrium is the observation of forward binding rate constants (i.e., k_1) far smaller than expected for a diffusion-controlled bimolecular reaction (10^8–10^9 M^{-1} s^{-1}), as indeed observed here.

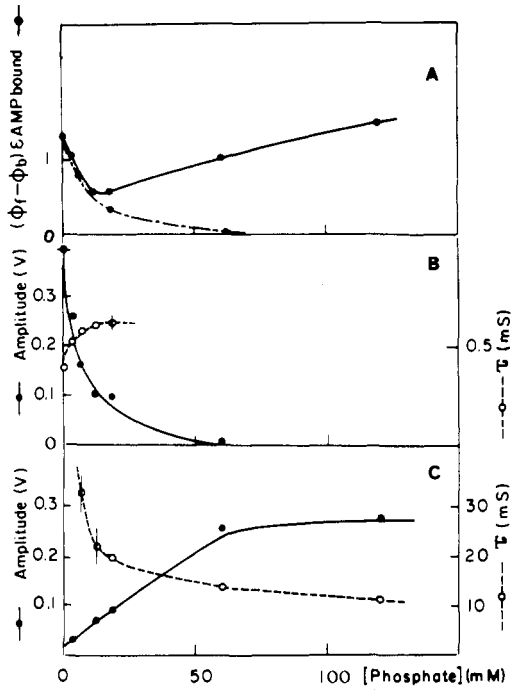

FIGURE 4: (A) Solid line. Sequential release and uptake of ϵAMP by glycogen phosphorylase *b* as the orthophosphate concentration is increased. Titration is followed by fluorescence on a solution containing 85 μM enzyme and 27 μM ϵAMP at 3 °C in glycylglycine. Dashed line: Same experiment using ϵdAMP instead of ϵAMP. (B) The disappearance of the fast relaxation phenomenon. (C) The appearance of the slow relaxation process as a function of orthophosphate concentration for the same ϵAMP–phosphorylase *b* system.

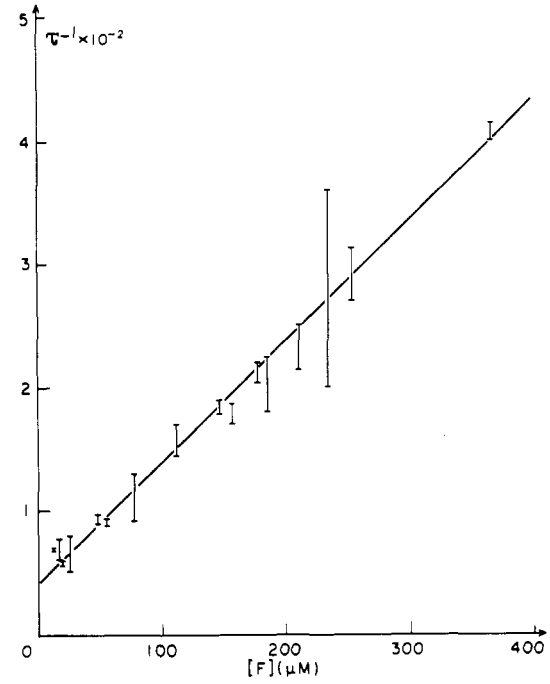

FIGURE 5: The inverse of the relaxation time (τ^{-1}) corresponding to the slow-binding mode of ϵAMP on phosphorylase *b* is plotted as a function of the *free* nucleotide concentration (glycylglycine buffer with 60 mM added orthophosphate; final temperature was 11 °C). In a first set of experiments, the nucleotide was used in large excess, and τ^{-1} was plotted vs. the total nucleotide concentration (see text). This yielded a kinetically determined affinity of ca. 40 μM for the enzyme form responsible for this process. Since this affinity is much higher than the overall enzyme affinity (K_1 = 600 μM; K_2 = 40 μM), this enzyme form should be a very minor species in the unliganded enzyme. Equation 2 then predicts that, even when ϵAMP is no longer in large excess with respect to the enzyme, τ^{-1} should be a linear function of the free nucleotide concentration, as indeed observed here.

We investigated whether this sharp discrimination was also reflected in the relaxation spectrum of the enzyme–nucleotide complex. Indeed, upon the addition of phosphate, the amplitude of the binding process previously observed in the 500-μs range steadily decreases to zero, being reduced to half of its value for a phosphate concentration of ca. 5 mM (Figure 4B). On the other hand, when ϵAMP was used, a new relaxation process was observed, in the 10-ms time range. The associated amplitude increases steadily for phosphate concentrations up to 60 mM (Figure 4C). The nature of this slow relaxation was investigated at 11 °C under conditions for which the "fast binding" process is no longer observed, i.e., at a phosphate concentration of 60 mM. Since, under these conditions, the equilibrium binding curve is strongly cooperative ($K_1 \simeq 600$ μM; $K_2 \simeq 40$ μM), several enzyme forms, having different affinities for the nucleotide, must exist in solution, and therefore large nucleotide to enzyme concentration ratios were used. Nevertheless, by varying the enzyme concentration it was possible to measure the relaxation time over a wide saturation range (from 0.05 to 0.85). Under these conditions, the reciprocal relaxation time was found to be a linear function of the total nucleotide concentration, as expected from eq 2 (Figure 5). This demonstrates that the observed relaxation phenomenon (subsequently referred to as the "slow-binding process") is associated with the binding of the nucleotide to one of the enzyme forms present in solution.

We have attempted to study the kinetics of the transition from the "fast-binding" to the "slow-binding" situation by rapidly adding phosphate (60 mM) to the phosphorylase *b*–ϵAMP system preincubated at 11 °C in glycylglycine buffer and recording the relaxation spectrum as a function of time. Small temperature jumps (3 °C) were applied every 4 to 5 min, so that the average temperature of the solution was barely affected. Furthermore, a large nucleotide to enzyme concentration ratio was used; this ensures that the binding processes on the various conformations of the enzyme are kinetically uncoupled and that the saturation ratio of each of these conformations is independent of time. Under these conditions, it is easily shown that the relaxation amplitude corresponding to each binding step will be proportional to the concentration of the enzyme species from which it arises (see, e.g., Thusius, 1972), thereby providing a convenient means for following the variation of this concentration as a function of time.

Within 30 s after the addition of phosphate, at 11 °C, the amplitude of the fast relaxation is reduced to zero, and the new relaxation signal in the 10-ms range is seen. Simultaneously, a rapid increase of the total fluorescence of the solution is observed (Figure 6). Supplementary experiments performed with a spectrofluorimeter fitted with a Durrum stopped-flow device show that this increase takes place within the mixing time of the two solutions (3 ms). Subsequently, the amplitude of the slow relaxation process increases exponentially toward its final value, with a time constant of about 10 min. A concomitant decrease of the total fluorescence signal is observed, with an identical time constant (Figure 6), indicating that the formation of the "slow-binding" species causes an uptake of nucleotide onto the enzyme. For a constant nucleotide concentration, the time constant associated with this transition showed no systematic dependence upon enzyme concentration (Figure 7). This suggests that the rate-limiting step in the formation of the "slow-binding" form of the enzyme is an isomerization rather than an aggregation–disaggregation phenomenon.

A similar experiment was performed with the ϵdAMP–phosphorylase *b* complex: here the addition of phosphate

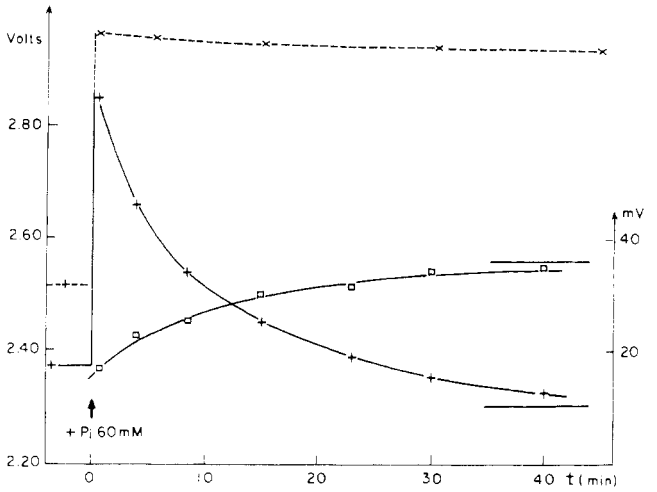

FIGURE 6: (+) Time dependence of the fluorescence of the ϵAMP phosphorylase *b* system after the rapid addition of orthophosphate (final concentration 60 mM) at 11 °C in glycylglycine buffer. (□) Concomitant amplitude change of the relaxation signal corresponding to the slower binding process. Both curves are exponential and can be fitted using the same decay time (13 min). (×) Same experiment as (+) using ϵdAMP instead of ϵAMP (nucleotide concentration 220 μM; enzyme concentration 117 μM). The left ordinate scale corresponds to curves (+) and (×), and the right one to (□). Total fluorescence signal after complete chase by AMP corresponds to 3.13 V in these two experiments.

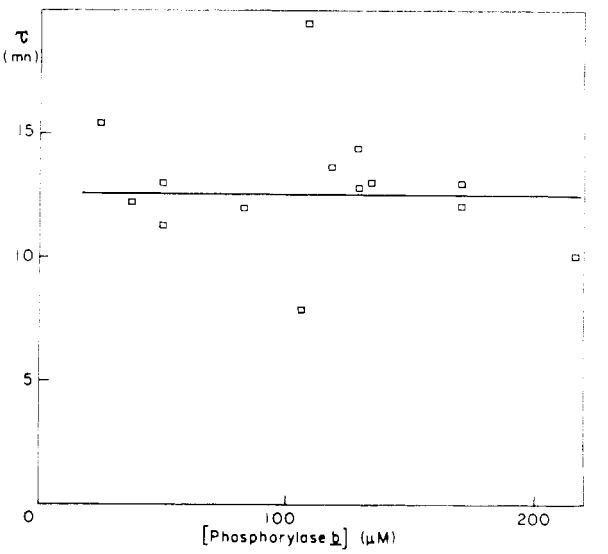

FIGURE 7: Dependence of the time constant of the slow fluorescence decay observed after adding 60 mM phosphate to the ϵAMP-phosphorylase *b* system (cf. Figure 6), as a function of total enzyme concentration. The final equilibrium concentration of the free nucleotide was 170 μM in all cases.

causes a similar disappearance of the "fast-binding" process with a concomitant rise in the total fluorescence (Figure 6), but no subsequent isomerization process was observed.

Effect of Other Activators on the Allosteric Transition. The orthophosphate concentrations required for the observation of the slow-binding process are large compared to the apparent dissociation constant of phosphate to phosphorylase *b* (cf. Busby and Radda, 1976). This suggests that the slow-binding conformation is stabilized by a process other than the binding of phosphate to the substrate site. This assumption has been already put forward by others, since various ions in the Hofmeister series (Engers and Madsen, 1968) or polyamines (Wang et al., 1968) can mimic the activating effect of orthophosphate. This prompted us to investigate the effects of these ions on the binding kinetics of etheno nucleotide to phosphorylase *b*.

In the presence of 10 mM spermine, 100 mM sulfate, 100 mM magnesium, or 100 mM glycero phosphate, at 11 °C, ϵAMP binding to phosphorylase *b* is characterized by a slow relaxation time; this behavior was not observed when ϵdAMP was used instead of ϵAMP. Therefore, these effectors mimic the effect of substrate anions with respect to ϵAMP fixation, except for two differences. Firstly, while the residence time of the nucleotide on the slow binding form of the enzyme was nearly independant of the effector used (25-30 ms at 11 °C), the *forward* rate constant for the nucleotide-enzyme binding process was found far higher when using magnesium or spermine rather than orthophosphate. This causes the affinity of the slow-binding form of the enzyme to approach that of the *a* form. Secondly, while upon the addition of spermine or magnesium to the enzyme-nucleotide complex the slow-binding form is still generated along a slow (≃10 min at 11 °C) isomerization process, the initial rise in total fluorescence which was seen immediately after the addition of orthophosphate is no longer observed.

Similar results were also found when AMP was added to the ϵAMP-phosphorylase *b* system. In these experiments, we used a large excess of enzyme to minimize kinetic coupling between the binding of the two nucleotides. It was found that when ϵAMP was used in trace amounts and the AMP concentration was progressively increased the amplitude of the fast-binding process was lowered, while that of the slow-binding process increased: this is simply interpreted as an increase of the concentration of the slow-binding form, although the quantitative interpretation of this experiment is difficult.

Discussion and Conclusions

Through the examination of the elementary binding steps of ϵAMP and ϵdAMP on glycogen phosphorylase *a* or *b*, three types of situations have been encountered.

Firstly, glycogen phosphorylase *b* binds ϵAMP and ϵdAMP in glycylglycine buffer in a simple way. The vast majority of enzyme conformations present in solution must be in rapid equilibrium with respect to the binding process and/or must be associated with closely similar kinetic parameters for binding. Both nucleotide-enzyme complexes are characterized by short lifetimes (~500 μs at 11 °C) and dissociation constants in the 100 μM range.

A second extreme situation is obtained in the same buffer with glycogen phosphorylase *a*; here again the enzyme behaves as a simple binding protein. Yet, ϵAMP binds more tightly with a long residence time (of the order of 30 ms). In contrast, ϵdAMP binding still takes place in the 500-μs range.

The third situation arises when phosphorylase *b* is incubated with any of a variety of activators which all reduce the nucleotide requirement for the enzyme activity. In this case, if the effector concentration is large enough, the fast-binding step of ϵAMP to the *b* enzyme is converted into a slower association process similar to the phosphorylase *a*-ϵAMP binary system. We have concentrated our study on the enzyme forms observed in the presence of orthophosphate, but a similar situation is encountered when either magnesium, spermine, sulfate, glycerophosphate, or AMP is added to the phosphorylase *b* ϵAMP complex. In contrast, if ϵdAMP, which does not activate the enzymatic reaction, is substituted for ϵAMP, the slow relaxation process is never seen.

Taken together, our results are fully compatible with the concerted model for allosteric transitions of Monod et al. (1965). We can operationally define in glycogen phosphorylase *b* a collection of conformations in fast equilibrium, called B_2.

In this state, etheno nucleotides form a complex of short lifetime with the enzyme. The second state, A_2, is favored by activators and substrates. It binds ϵAMP with a better affinity than the B_2 state and, like glycogen phosphorylase *a*, it strongly immobilizes this probe. Our arguments in favor of a major $A_2 \rightleftarrows B_2$ concerted process, similar in nature to the one described by Kirschner et al. (1971) in the case of yeast glyceraldehyde-3-phosphate dehydrogenase, are qualitative. They are essentially: (a) the fact that two, and only two, modes of binding of ϵAMP, characterized by very different residence times of the nucleotide on the enzyme, are found when different effectors are added; (b) the observation that cooperative interactions are generated through a single isomerization step, the rate constant of which does not depend on the nature of the activator used; (c) previous work of this laboratory showing that at 4 °C a similar isomerization process restores the homotropic interactions between 5′-AMP sites (Buc and Buc, 1975).

However, several features are expected to complicate the interpretation of the relaxation spectra. The first one, which is partially understood (cf. below), is the pleiotropic action of orthophosphate. The second one is the existence of other fast conformational changes (Buc et al., 1973) and of tetramerization at low temperature. Indeed, analytical ultracentrifugation shows that, under our experimental conditions, the enzyme becomes largely tetrameric upon the addition of both ϵAMP and orthophosphate (60 mM). It may thus be questioned whether the slow-binding form is a tetrameric species. In fact, all that has been shown here is that the rate-limiting step in the formation of this form is an isomerization reaction. It is possible, however, that this newly created form rapidly aggregates to a considerable extent under our experimental conditions.

Finally, it should be stressed that the number of observable states may depend on the effector which is used to probe the system. Thus, it is impossible to detect with ϵdAMP the major B_2 to A_2 transition. Likewise, conformations stabilized by orthophosphate alone and having a poor affinity for the activator would not have been detected in our present experiments if they were formed during the isomerization process.

Comparison between AMP and ϵAMP Effects. From the present study it is concluded that both the etheno nucleotide and an additional highly charged effector are required to generate an "*a*-like" form of glycogen phosphorylase *b*. This is true both at low temperature (where most of our studies have been performed) and at 25 °C (results not shown). A similar conclusion also emerges from recent works in which different probes have been used to monitor AMP binding on the enzyme *at room temperature* (cf. Shimomura and Fukui, 1976; Feldmann and Hull, 1977; Chachaty et al., 1978). In particular, the work of Feldmann and Hull contains gross estimates for the residence time of the nucleotide on the various enzyme forms, which are in qualitative agreement with our direct measurements.

On the other hand, we have obtained some evidence that *at low temperature* AMP may behave differently from its fluorescent analogue, i.e., may on its own largely bring phosphorylase *b* to the slow-binding form without the help of any additional effector. This conclusion is based on the observation that when ϵAMP was used as an indicator we were able to generate a slow-binding signal upon the addition of AMP to the enzyme. Shimomura and Fukui (1976) also found from their CD studies that cooling the phosphorylase *b*–AMP system below 13 °C is sufficient to generate an *a*-like form of the enzyme. It seems, therefore, logical to conclude that ϵAMP and AMP differ mainly below the temperature transition of 13 °C (cf. Kastenschmidt et al., 1968). Under these conditions, the nucleotide site strongly immobilizes the activator either when AMP binds to it or when ϵAMP and orthophosphate are added together.

The Different Roles of Orthophosphate. It seems worthwhile to emphasize at this point the complexity of the effect of phosphate (or glucose 1-phosphate) on phosphorylase. Conceivably, the phosphate ion has three distinct roles: it can a priori compete with the nucleotide for the AMP allosteric site, because of its structural analogy with the ribose 5′-phosphate group. Secondly, it must bind to a stereospecific substrate-binding site. Finally, it presumably has a nonspecific effect, similar to that of highly charged ions. This pleiotropic role of orthophosphate presumably explains why different dissociation constants are observed when different probes are used to monitor its binding to the enzyme (Busby et al., 1976). Furthermore, recent X-ray diffraction studies (Sygusch et al., 1977; Weber et al., 1978) gave direct evidence for the first two effects and showed that the phosphate–nucleotide competition takes place directly at the nucleotide-binding site. Indeed, the initial rise in fluorescence seen in Figure 6 when orthophosphate is added to the enzyme–nucleotide complex is best explained by a phosphate–nucleotide competition on the B_2 state. Such a competition should take place within the lifetime of the nucleotide–enzyme complex (say 500 μs) and actually is found to occur within 3 ms after mixing. Furthermore, magnesium or spermine ions, which obviously have no structural relationship to a nucleotide, did not produce this fluorescence rise. A quantitative estimate of the competition in the initial state can be obtained from the dependence of the fast-binding relaxation time, upon phosphate concentration (Figure 4B). It yields a competitive inhibition constant of the order of 5 mM at 3 °C. On the other hand, it is also probable that orthophosphate competes with the nucleotide in the A_2 state; thus, the rate constant for the formation of the phosphorylase *b*–ϵAMP complex at 60 mM phosphate is more than an order of magnitude smaller than observed with the phosphorylase *a*–ϵAMP complex, while the lifetimes are comparable (Table I). This is presumably what would be expected from a direct phosphate–nucleotide competition. From this observation, the competitive inhibition constant of phosphate on the A_2 state can be similarly estimated to ca. 5 mM.

Since the affinity of phosphate for the nucleotide binding site is therefore not very different in the A_2 and B_2 states, it may be asked which of the two other possible phosphate effects provides the main driving force for the phosphate-dependent B_2 to A_2 transition. At present, we rather favor a nonspecific effect on the protein environment, since a variety of ions, some bearing no structural relationship with orthophosphate, were found able to induce the same transition. Indeed, these findings may be of even broader generality; thus, we have recently found that the addition to the buffer of a wide variety of organic compounds bearing hydropholic groups can induce the transition from the fast- to the slow-binding situation. Simultaneously, we observed that many of these compounds can activate phosphorylase *b* in the absence of any nucleotide (as much as 50% of the activity observed in the presence of saturating 5′-AMP was found in some cases). Thus, in this situation, as with phosphorylase *a*, a strong correlation exists between the immobilization of the bound nucleotide and the state of activity of the enzyme.

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