Influence of substrates on *in vitro* dephosphorylation of glycogen phosphorylase *a* by protein phosphatase-1

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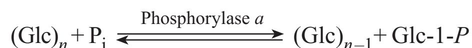
The kinetic theory of the substrate reaction during modification of enzyme activity has been applied to a study of the dephosphorylation of phosphorylase *a* by protein phosphatase-1 (ppase-1). On the basis of the kinetic equation of the substrate reaction in the presence of ppase-1, all the inactivation rate constants for the free enzyme and the enzyme–substrate(s) complexes have been determined. Binding of the allosteric substrate, glucose 1-phosphate, to one subunit of phosphorylase *a* protects completely against ppase-1 action on either the same subunit or the adjacent subunit, whereas binding of the non-allosteric substrate, glycogen, to one subunit protects this subunit partially, but has no effect on the modification on the neighbouring subunit. Analysis of the data suggests that the allosteric behaviour of phosphorylase *a* can be interpreted in terms of a modified concerted model. The present method also provides a novel approach for studying dephosphorylation reactions. Since the experimental conditions used resemble more closely the *in vivo* situation where the substrate is constantly being turned over while the enzyme is being modified, this new method would be particularly useful when the regulatory mechanism of the reversible phosphorylation reaction toward certain enzymes is being assessed.

Key words: enzyme kinetics, conformational change, co-operativity, inactivation, rate equation.

INTRODUCTION

Glycogen phosphorylase plays an important role in the regulation of glycogen metabolism in muscle [1–4]. It exists in two inter-convertible forms: phosphorylase *a* and *b*. Phosphorylase phosphatase converts 'active' phosphorylase *a* (EC 2.4.1.1) into 'inactive' phosphorylase *b* by cleavage of the phosphate group from Ser[14] of phosphorylase *a*. The reverse reaction, the conversion of phosphorylase *b* into *a*, is catalysed by phosphorylase *b* kinase. The substrate for phosphorylase phosphatase, phosphorylase *a*, exists as a dimer or a tetramer depending upon the temperature and the protein concentration [3]. In contrast with phosphorylase *b*, phosphorylase *a* retains almost total activity in the absence of AMP. The enzyme catalyses phosphorolytic cleavage of glycogen to produce glucose 1-phosphate (Glc-1-*P*):



$$(\mathrm{Glc})_n + \mathrm{P_i} \xrightleftharpoons{\text{Phosphorylase } a} (\mathrm{Glc})_{n-1} + \text{Glc-1-}P$$

Regulation of the activity of phosphorylase has been a topic of study for several decades. Much of the emphasis of those studies has been directed toward elucidation of the steps in the signal cascade. Recently, the search has been for an equivalent and complimentary sequence of regulatory reactions for the inactivation of phosphorylase *a* by its specific phosphatase, phosphorylase phosphatase-1 [5]. Protein phosphatases are an important group of enzymes involved in cellular regulation [6]. These include phosphoserine, phosphothreonine, phosphotyrosine, and phosphohistidine protein phosphatases. Phosphorylase phosphatase has served as a paradigm for the study of the mammalian protein phosphatases. One of these is known as protein phosphatase-1 (ppase-1), based on a classification of the mammalian protein phosphatases into two groups on the basis of substrate specificity and inhibitor-sensitivity [7]. ppase-1 has been isolated in which a catalytic subunit is associated with a glycogen-binding subunit [8,9]. The catalytic subunit of rabbit skeletal-muscle ppase-1 has been expressed in *Escherichia coli* [10]. Many investigations have described the kinetic and thermodynamic parameters of the individual enzymes or the interactions between adjacent pairs of the enzymes. Some effectors, such as AMP, caffeine, glucose, glucose 6-phosphate (Glc-6-*P*), glycogen and Glc-1-*P*, can change the rate of ppase-1 reaction [11–13], and these effects appear to be due to a change in the conformation of the substrate phosphorylase *a* [14,15].

The irreversible modification of enzymes has attracted particular attention because of its usefulness in elucidating the nature of the functional groups located at the active site, essential for enzyme activity, and likely to be involved in the catalytic process. Some years ago a systematic study of the kinetics of the irreversible modification of enzyme activity was presented [16]. It has been shown that not only can the apparent rate constant for the irreversible modification of enzyme activity be obtained in a single experiment, but that the effect of substrate on the modification can also be ascertained. This approach has been extended beyond the treatment of relatively simple cases of irreversible modification of an enzyme catalysing a single-intermediate, one-substrate reaction to include more complicated models [17].

Enzyme regulation processes in which one enzyme acts to modify the activity of another have the inherent ability to modify signal strength during transmission. In the present study, the progress-curve method is used to study the inactivation kinetics of phosphorylase *a* by ppase-1. On the basis of the kinetic equation of the substrate reaction in the presence of ppase-1, all microscopic kinetic constants for the free enzyme and enzyme–substrate(s) complexes have been determined. The results indicate that both the substrates, glycogen and Glc-1-*P*, strongly protect against dephosphorylation by ppase-1.

Abbreviations used: ppase-1, protein phosphatase-1; Glc-1-*P*, glucose 1-phosphate; Glc-6-*P*, glucose 6-phosphate; MTGuo, methyl-6-thioguanosine; DTT. dithiothreitol; MWC, Monod, Wyman and Changeux; KNF, Koshland, Némethy and Filmer.

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MATERIALS AND METHODS

Materials

Dithiothreitol (DTT), Hepes, EDTA, DMSO, methyl iodide, thiourea, 2-amino-6-chloropurine riboside and bacterial purine nucleoside phosphorylase were purchased from Sigma.

Rabbit skeletal-muscle glycogen phosphorylase *b* was isolated as described by Fischer and Krebs [18] and recrystallized three times. Phosphorylase *b* was converted into the *a* form as described by Krebs and Fischer [19] using rabbit skeletal-muscle phosphorylase kinase [20], then the enzyme was passed through a small Sephadex G-25/Norite column and crystallized [21]. After the fourth crystallization, the crystals were dissolved in 50 mM Hepes buffer, pH 7.0, containing 2mM DTT and 50 % glycerol and stored at −20 °C (final concentration, 29 mg of protein/ml), where it is stable for at least 3 years. Recombinant rabbit skeletal-muscle ppase-1 catalytic subunit [10] was a gift from Dr. E. Y. C. Lee, University of Miami School of Medicine, Coral Gables, FL, U.S.A. Conventional radiolabelling assays for ppase activity using ^{32}P-labelled phosphorylase *a* were carried out as described previously [22] with the exceptions that the temperature and assay buffer mixture used in the phosphorylase *a* continuous assay were used. A unit of phosphatase activity is defined as the amount of enzyme that releases 1 nmol of phosphate/min. Methyl-6-thioguanosine (MTGuo) was prepared by the procedure of Broom and Milne [23] as modified by Webb [24]. The yellow MTGuo, after precipitation with acetone, was washed with acetone, dried, and stored at −70 °C. Stock solution of MTGuo in DMSO were prepared daily. The concentration of MTGuo was determined at 331 nm, using a molar absorption coefficient of 32 000 $M^{-1} \cdot cm^{-1}$ [24]. Glycogen was purified with Norit A according to the procedure of Sutherland and Wosilait [25].

The protein concentrations of phosphorylase *a* was determined by measuring the absorbance at 280 nm and using an absorption-coefficient ($A_{280}^{1\%}$) value of 13.1 [26]. The Bradford method [27], using BSA as a standard, was used to determine the protein concentration of purine nucleoside phosphorylase. The subunit molecular mass of phosphorylase *a* was taken as 100 000 [26].

Enzyme assays

Enzymic activity was measured in the direction of glycogen synthesis according to the procedure of Sergienko and Srivastava [28], in which the P_i generated was coupled to the conversion of MTGuo into 7-methyl-6-thioguanine in the presence of purine nucleoside phosphorylase and the absorbance change at 360 nm was monitored. Quantification of phosphate release was determined using an absorption coefficient of 11 200 $M^{-1} \cdot cm^{-1}$ for the phosphate-dependent reaction at pH 7.0 [28]. Relative error in the measurement of the initial steady-state rate of the enzyme reaction was determined to be 5 %. Phosphorylase *a* was incubated with 2 % glycogen for at least 2 h at 25 °C prior to activity measurements. This prior incubation with glycogen is important with regard to phosphorylase *a*, in the light of the evidence presented concerning the stabilization of the enzyme into its more active dimeric form by incubation with glycogen. Under these conditions, linear progress curves were obtained, indicating (i) the coupling enzyme was in sufficient quantity to ensure actual measurement of the primary enzyme's behaviour, and (ii) the substrate consumption could be neglected and the initial-rate conditions was satisfied during activity measurement.

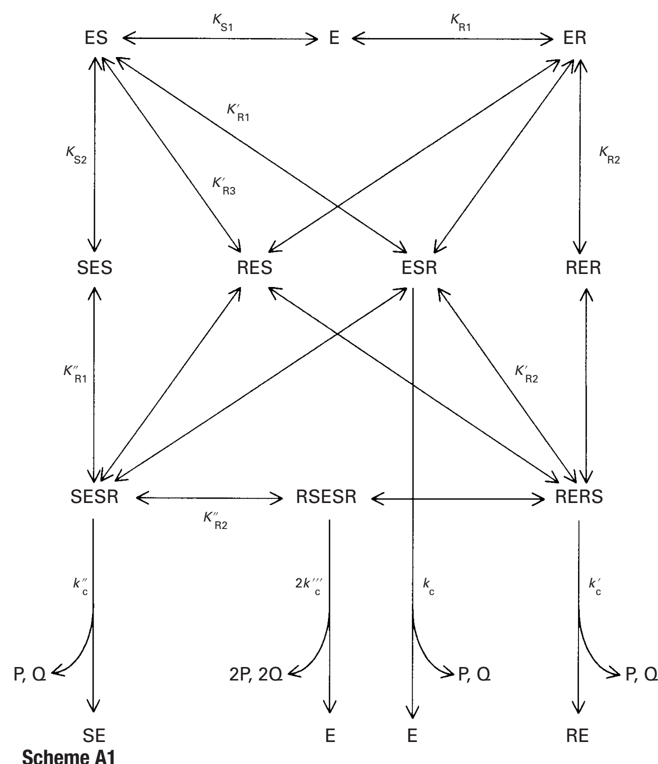
The kinetics of the inactivation reaction was followed by the substrate reaction in the presence of the ppase-1 as described previously [16]. In the experiments of inactivation kinetics, different concentrations of ppase-1 were included in the assay buffer. After 5 min equilibration at 25 °C, the reactions were initiated by adding a small volume of the preincubated enzyme/glycogen mixture, and the absorbance at 360 nm was recorded. In a theoretical study, Wang and Zhao [29] have shown that the substrate-reaction method using a coupled enzyme assay can be used for the study of enzyme inactivation kinetics. In control experiments, ppase-1 did not affect the activity of purine nucleoside phosphorylase under identical conditions.

The standard assay for phosphorylase *a*, except where indicated, was carried out at 25 °C in 1.6 ml reaction mixtures containing 50 mM Hepes, pH 7.0, 50 μM EDTA, 2 mM DTT, 50 mM NaCl, 0.2 mM $MnCl_2$, 5 % glycerol, 1 % DMSO, 116 μM MTGuo and 150 μg/ml purine nucleoside phosphorylase. The steady-state and inactivation kinetic experiments were performed on a lambda 3-B Perkin–Elmer spectrophotometer equipped with a magnetic stirrer in the cuvette holder. The reaction mixture (1.6 ml) was continuously stirred using a magnetic stirrer. This stirring device allowed us to start the reaction and data collection (by computer) within 3 s after initiating the reaction. Initial rates were determined from the slopes of progress curves acquired using Perkin–Elmer software. Other data were analysed using a non-linear-regression-analysis program.

RESULTS

Kinetics of the glycogen phosphorylase *a*-catalysed reaction

In order to obtain the microscopic rate constants for the reaction of ppase-1, it was necessary first to determine the kinetic parameters of the phosphorylase *a*-catalysed reaction. If the enzyme and substrate(s) are in rapid equilibrium with the enzyme–substrate(s) complexes, the mechanism for a two-substrate dimeric enzyme can be represented by:



Scheme A1

where E, S and R represent the enzyme, Glc-1-*P* and glycogen, respectively. S or R on the left-hand side and the right-hand side of E denote the binding of these species on the different subunits

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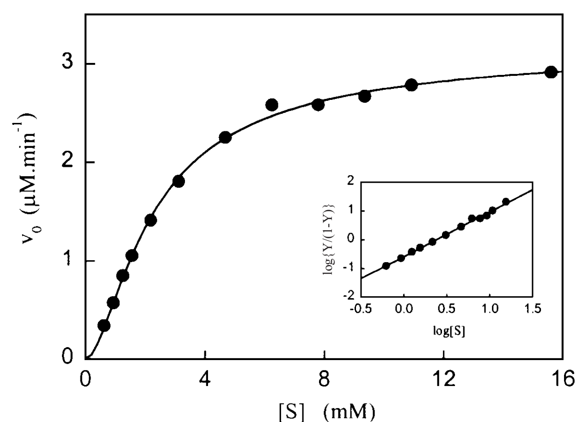

Figure 1 Initial velocity of glycogen phosphorylase *a*-catalysed reaction as a function of Glc-1-*P* concentrations at a fixed level of glycogen

Reaction mixture contained 50 mM Hepes, pH 7.0, 50 μM EDTA, 2 mM DTT, 50 mM NaCl, 0.2 mM $MnCl_2$, 5% glycerol, 1% DMSO, 116 μM MTGuo, 150 μg/ml purine nucleoside phosphorylase, 1.0 mg/ml glycogen, 0.58 nM phosphorylase *a* (dimer) at 25 °C. The continuous line is the best fitting result according to eqn.(2) with parameters $A_0 = 2.69$ mM^2, $A_1 = 1.47$ mM and $B_2 = 3.22$ μM·min^{-1} (see the text). The inset shows the Hill plot of the experimental data, where Y is the ratio of the initial rate to the maximum rate.

of phosphorylase *a*. The corresponding initial-rate equation can be written as

$$v_0 = \frac{B_1[\mathrm{S}] + B_2[\mathrm{S}]^2}{A_0 + A_1[\mathrm{S}] + [\mathrm{S}]^2} \tag{1}$$

where

$$A_0 = \frac{K_{\mathrm{S1}}K_{\mathrm{S2}}\left\{1 + \frac{2[\mathrm{R}]}{K_{\mathrm{R1}}} + \frac{[\mathrm{R}]^2}{K_{\mathrm{R1}}K_{\mathrm{R2}}}\right\}}{1 + \frac{2[\mathrm{R}]}{K''_{\mathrm{R1}}} + \frac{[\mathrm{R}]^2}{K''_{\mathrm{R1}}K''_{\mathrm{R2}}}}$$

$$A_1 = \frac{2K_{\mathrm{S2}}\left\{1 + \frac{[\mathrm{R}]}{K'_{\mathrm{R1}}} + \frac{[\mathrm{R}]}{K'_{\mathrm{R3}}} + \frac{[\mathrm{R}]}{K'_{\mathrm{R1}}K'_{\mathrm{R2}}}\right\}}{1 + \frac{2[\mathrm{R}]}{K''_{\mathrm{R1}}} + \frac{[\mathrm{R}]^2}{K''_{\mathrm{R1}}K''_{\mathrm{R2}}}}$$

$$B_1 = \frac{2[\mathrm{E}]_0K_{\mathrm{S2}}\left\{\frac{k_{\mathrm{c}}[\mathrm{R}]}{K'_{\mathrm{R1}}} + \frac{k'_{\mathrm{c}}[\mathrm{R}]^2}{K'_{\mathrm{R1}}K'_{\mathrm{R2}}}\right\}}{1 + \frac{2[\mathrm{R}]}{K''_{\mathrm{R1}}} + \frac{[\mathrm{R}]^2}{K''_{\mathrm{R1}}K''_{\mathrm{R2}}}}$$

$$B_2 = \frac{2[\mathrm{E}]_0\left\{\frac{k''_{\mathrm{c}}[\mathrm{R}]}{K''_{\mathrm{R1}}} + \frac{k'''_{\mathrm{c}}[\mathrm{R}]^2}{K''_{\mathrm{R1}}K''_{\mathrm{R2}}}\right\}}{1 + \frac{2[\mathrm{R}]}{K''_{\mathrm{R1}}} + \frac{[\mathrm{R}]^2}{K''_{\mathrm{R1}}K''_{\mathrm{R2}}}}$$

To study the effects of substrates on the kinetic behaviour of the phosphorylase *a*-catalysed reaction, two groups of experiments were carried out. The first group of experiments involved varying the concentration of Glc-1-*P* at a fixed concentration of glycogen. A sigmoidal dependence of the initial velocity on Glc-1-*P* concentration was found (Figure 1). The value of the Hill coefficient for Glc-1-*P* is 1.54 ± 0.06. By fitting eqn.(1) to the experimental data, the parameters were determined to be $A_0 = 5.01 \pm 2.43$ mM^2, $A_1 = 1.420 \pm 0.29$ mM, $B_1 = 1.58 \times 10^{-3} \pm 1.70 \times 10^{-3}$ mM^2·min^{-1} and $B_2 = 1.42 \times 10^{-3} \pm 0.29 \times 10^{-3}$ mM·min^{-1} respectively. It can be seen that the parameter B_1, as inferred from these data, had a very large standard deviation, such that its confidence interval would include the value zero. The large magnitude of the standard deviation in the estimate of B_1 could simply reflect the large scatter in the experimental data, or it could signify that the eqn. (1) does not provide a good description of the initial velocity in this case. To further clarify this problem, the graphical method of Wang and Srivastava [30] was used to analyse the experimental data. Figure 2 shows plots of $[\mathrm{S}]^p/v_0$ versus $1/[\mathrm{S}]$ at different values of p. It can be seen from this Figure that when $p = 2$, the plot approaches a horizontal asymptote, and when $p = 3$, a zero asymptote is observed. According to Wang and Srivastava [30], this result indicates that the coefficient B_1 in eqn.(1) is equal to zero, and therefore we have

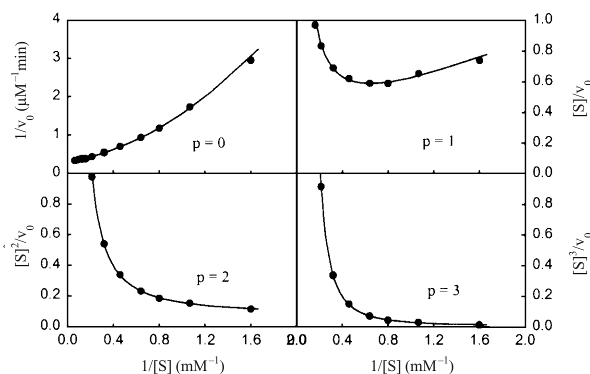

Figure 2 Replots of the experimental results of Figure 1 in the form of $1/(v_0[\mathrm{S}]^p)$ versus the reciprocal of the Glc-1-*P* concentration (1/[S])

The continuous lines represent the calculated lines according to eqn.(2) with parameters given in Figure 1. The *y*-axis values of these comparative plots are represented in arbitrary units.

$$v_0 = \frac{B_2[\mathrm{S}]^2}{A_0 + A_1[\mathrm{S}] + [\mathrm{S}]^2} \tag{2}$$

When the experimental data of Figure 1 were fitted by this equation, a remarkable correspondence was observed. The continuous lines in Figure 1 represent the best fit of the experimental results with parameters $A_0 = 2.69 \pm 0.3$ mM^2, $A_1 = 1.47 \pm 0.26$ mM and $B_2 = 3.22 \pm 0.076$ μM·min^{-1}. This result indicates that phosphorylase *a* reveals catalytic power exclusively upon the binding with two molecules of Glc-1-*P* ($B_1 = 0$ and hence $k_c = k'_c = 0$), which agrees with the experimental observation for the phosphorylase *b*-catalysed reaction [31].

In the second group of experiments, the concentration of glycogen was varied at a fixed concentration of Glc-1-*P*. Figure 3 shows the dependence of the initial velocity on the glycogen concentration. In contrast with the case of Glc-1-*P*, the initial velocity gives rise to the hyperbolic dependence on the glycogen concentration

$$v_0 = \frac{V_{\max}[\mathrm{R}]}{K_{\mathrm{m}} + [\mathrm{R}]} \tag{3}$$

where K_m is the apparent Michaelis constant for glycogen, and [R] is the concentration of glycogen. The experimental data fit the Michaelis–Menton equation with a K_m of 0.199 ± 0.011 mg/ml and a $V_{\max}$ of 4.3 ± 0.063 μM·min^{-1}. Similar experiments at another fixed concentration of Glc-1-*P* (3.08 mM) were also performed to illustrate the influence of the Glc-1-*P* concentration on the apparent Michaelis constant (results not

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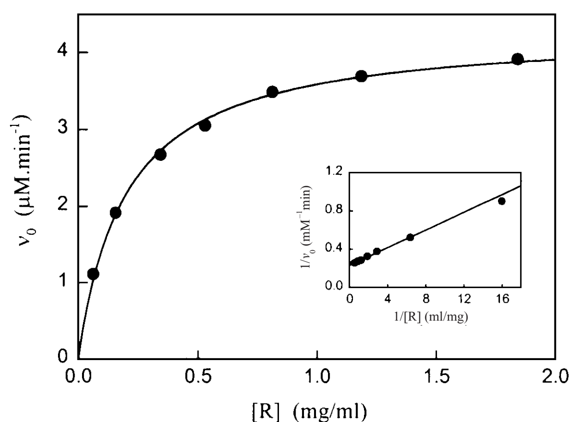

Figure 3 Initial velocity of glycogen phosphorylase *a*-catalysed reaction as a function of glycogen concentrations at a fixed level of Glc-1-*P*

Reaction mixtures contained standard assays similar to those described in Figure 1 with the exception that 15 mM Glc-1-*P* was used instead of 1.0 mg/ml glycogen. The continuous line is best fitting result according to the Michaelis–Menten equation with K_m = 0.199 mg/ml, V_{max} = 4.3 μM · min^{-1}. The inset shows the double-reciprocal plot of the experimental data..

shown). The K_m so determined is similar to that obtained previously, suggesting that (1) the binding of Glc-1-*P* at one active site does not influence either the binding of glycogen on the same subunit or the binding of glycogen on a different subunit, (2) the binding of first glycogen molecule at one subunit does not influence the binding of a second glycogen molecule on the adjacent subunit. In this case, we have, $K_{R1} = K_{R2} = K'_{R1} = K'_{R2} = K'_{R3} = K''_{R1} = K''_{R2} = K_R$, and $k'''_c = k''_c$. Therefore, the initial-velocity equation for the two-substrate dimeric enzyme can be simplified to

$$\nu_0 = \frac{\dfrac{2k''_c[S]^2[R][E]_0}{K_R K_{S1} K_{S2}}\left(1+\dfrac{[R]}{K_R}\right)}{\left(1+\dfrac{2[S]}{K_{S1}}+\dfrac{[S]^2}{K_{S1}K_{S2}}\right)\left(1+\dfrac{[R]^2}{K_R}\right)^2}$$

$$= \frac{2k''_c[S]^2[R][E]_0}{(K_{S1}K_{S2}+K_{2S}[S]+[S]^2)(K_R+[R])} \quad (4)$$

As expected, this mechanism will give rise to a second-degree steady-state equation in the concentration of S, but a hyperbolic dependence of velocity on the concentration of R. Comparing this equation with eqns.(2) and (3), the macroscopic terms in eqns.(2) and (3) exhibit the following relationship with the microscopic constants in eqn.(4):

$$A_0 = K_{S1}K_{S2}$$

$$A_1 = 2K_{S2}$$

$$B_2 = \frac{2k''_c[E]_0[R]}{K_R+[R]}$$

$$K_m = K_R$$

$$V_{max} = \frac{2k''_c[E]_0[S]^2}{K_{S1}K_{S2}+2K_{S2}[S]+[S]^2}$$

According to these relationships, all the kinetic parameters for the phosphorylase *a*-catalysed reaction were calculated and are listed in Table 1.

Let us discuss the possible conformational changes in phosphorylase *a* that are compatible with the observed experimental data. The fact that the binding of the first molecule of Glc-1-*P* to the enzyme changes the affinity for the binding of the second one is indicative of the conformational change in the dimeric enzyme molecule. Glc-1-*P* does not affect the binding of the glycogen molecule, suggesting that the conformational change in the enzyme induced by Glc-1-*P* does not extend on the binding site of glycogen. The dimeric enzyme molecule acquires catalytic activity exclusively upon the binding of two molecules of Glc-1-*P*. This fact indicates that the two subunits of the enzyme act concertedly for the enzyme activity, and therefore the dimer is the minimal function unit of phosphorylase *a*.

Table 1 Kinetic constants of the phosphorylase *a*-catalysed reaction*

Constant	Value
K_{S1} (mM)	3.67 ± 1.04
K_{S2} (mM)	0.73 ± 0.13
K_R (mg/ml)	0.199 ± 0.011
k_c (s^{-1})	0
k'_c (s^{-1})	0
k''_c (s^{-1})	68.55 ± 1.01
k'''_c (s^{-1})	68.55 ± 1.01

*$K_{R1} = K_{R2} = K'_{R1} = K'_{R2} = K'_{R3} = K''_{R1} = K''_{R2} = K_R$.

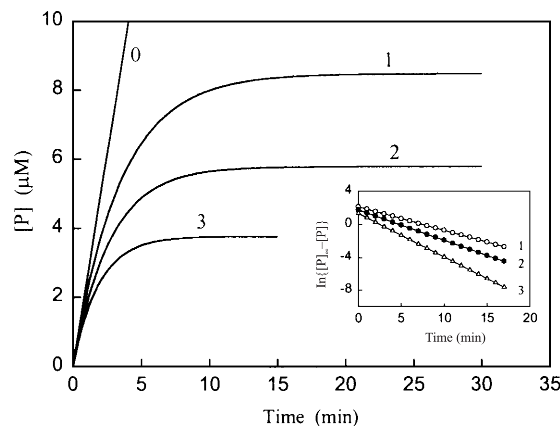

Figure 4 Time course of substrate reaction in the presence of different concentrations of ppase-1

Final concentrations were 0.625 mg/ml glycogen, 6.25 mM Glc-1-*P* and 0.58 nM phosphorylase *a* (dimer) in 50 mM Hepes buffer, pH 7.0, at 25 °C. Concentrations of ppase-1 were 0 units (control, curve 0), 3.89 units (curve 1), 5.0 units (curve 2) and 8.9 units (curve 3). Other conditions were the same as in Figure 1. The enzyme (5 μl) was added to the reaction mixture (1.6 ml) to start the reaction. The inset shows semi-logarithmic plots of curves 1–3 according to eqn.(5).

Inactivation kinetics of glycogen phosphorylase *a* by ppase-1

Time courses of the substrate reaction in the presence of different concentrations of ppase-1 are shown in Figure 4. It can be seen that [P] approaches constant final values, $[P]_\infty$, which decrease with increasing concentrations of ppase-1. Semi-logarithmic plots of the same data are given in the inset to Figure 4, and the apparent rate constant, k_{obs}, can then be calculated from the slopes of the straight lines obtained. Results presented in the inset of Figure 4 also show that the inactivation of phosphorylase *a* by ppase-1 is a monophasic pseudo-first-order reaction. There-

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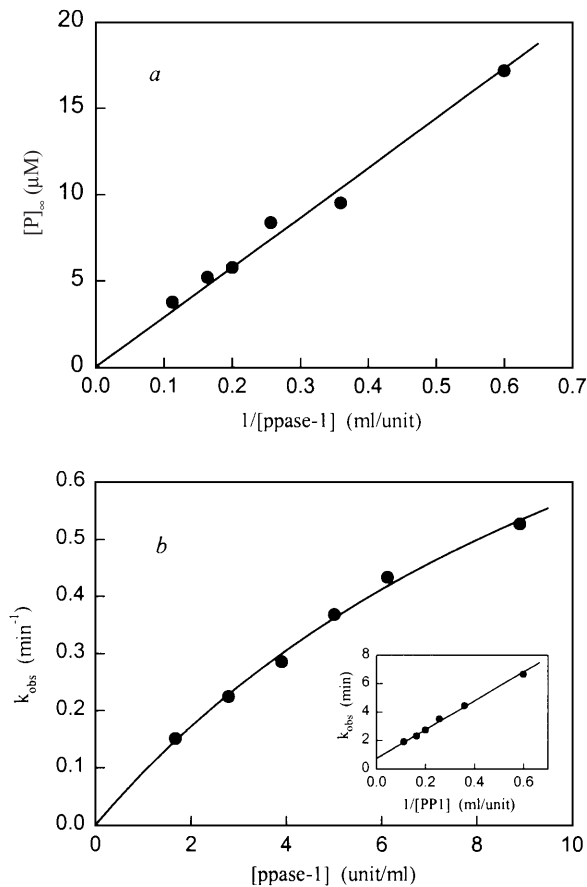


Figure 5 (a) Plot of $[P]_\infty$ versus the reciprocal of ppase-1 concentration for the inactivation of phosphorylase *a* by ppase-1 and (b) Plot of k_{obs} versus ppase-1 concentration for the inactivation of phosphorylase *a* by ppase-1

The values of the final product concentration, $[P]_\infty$, and the apparent rate constant, k_{obs}, were calculated from the time courses of substrate reaction given in Figure 4.

fore, the concentration of product formed at time t can be written as (see the Appendix):

$$[P] = [P]_\infty(1 - e^{-k_{obs}t}) \tag{5}$$

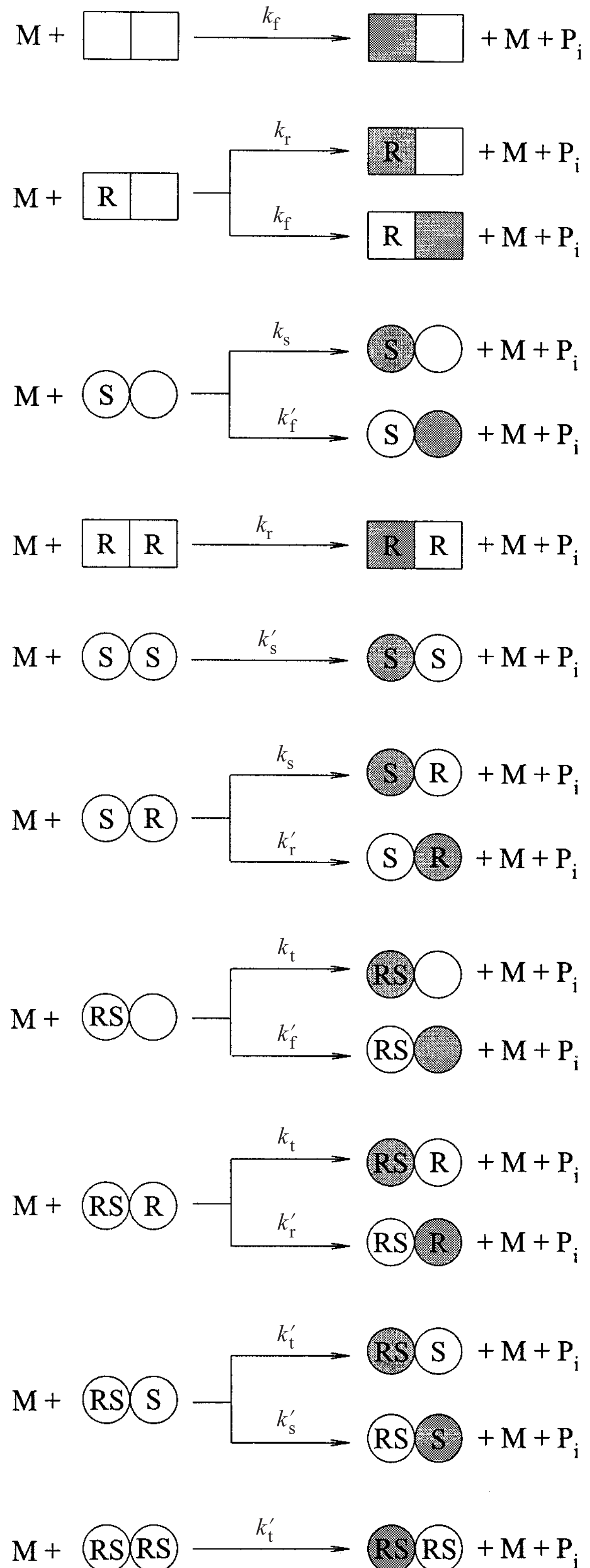
Both k_{obs} and $[P]_\infty$ are the functions of the concentrations of substrates and ppase-1. In the case of phosphorylase *a*, the expression of $[P]_\infty$ is given by [32]:

$$[P]_\infty = \frac{\dfrac{2k''_c[E]_0[S]^2[R]}{K_{S1}K_{S2}K_R}\left(1+\dfrac{[R]}{K_R}\right)}{[M]_0\Sigma} \tag{6}$$

where $[M]_0$ is the concentration of ppase-1, and

$$\Sigma = 2k_f + 2(k_r + k_f)\frac{[R]}{K_R} + 2k_r\frac{[R]^2}{K_R^2}$$
$$+ 2(k'_f + k_s)\frac{[S]}{K_{S1}} + 2k'_s\frac{[S]^2}{K_{S1}K_{S2}}$$
$$+ 2k'_t\frac{[S]^2[R]^2}{K_{S1}K_{S2}K_R^2} + 2(k_s + k'_r + k_t + k'_f)\frac{[R][S]}{K_RK_{S1}}$$
$$+ 2(k_t + k'_r)\frac{[R]^2[S]}{K_R^2K_{S1}}$$
$$+ 2(k'_t + k'_s)\frac{[S]^2[R]}{K_{S1}K_{S2}K_R}$$

k_f, k_r, k'_f, k'_r, k_s, k_t, k'_s and k'_t are the apparent second-order rate constants for the following modification reactions at low phosphorylase *a* concentrations [33]:



where □ and ○ represent different conformational states of a subunit in the dimeric phosphorylase, and the blank and cross-hatched ones represent phosphorylated and dephosphorylated

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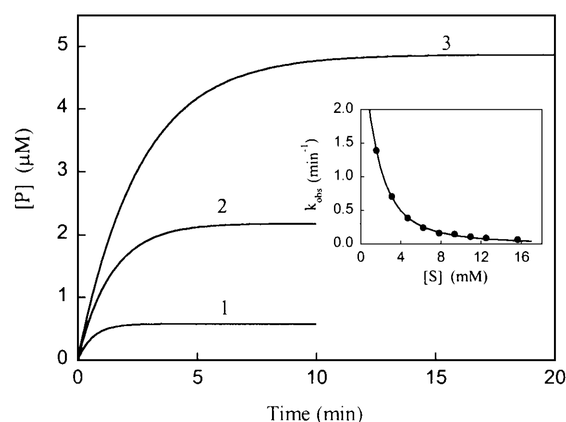

Figure 6 Time course of substrate reaction at different substrate concentrations in the presence of ppase-1

Final concentrations were 3.89 units of ppase-1, 1mg/ml glycogen and 0.58 nM phosphorylase *a* (dimer) in 50 mM Hepes buffer, pH 7.0, at 25 °C. Concentrations of Glc-1-*P* were 1.563 mM (curve 1), 3.125 mM (curve 2) and 4.688 mM (curve 3). Other conditions were the same as in Figure 1. The enzyme (5 μl) was added to the reaction mixture (1.6 ml) to start the reaction. The inset shows a plot of k_{obs} versus the concentration of Glc-1-*P*. The values of the apparent rate constant, k_{obs}, were calculated from the time courses of substrate reaction in the presence of ppase-1.

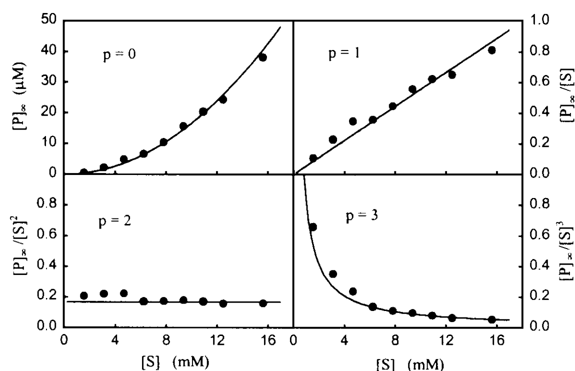

Figure 7 Plots of the experimental results of Figure 6 in the form of $[P]_\infty/[S]^p$ versus the concentration of Glc-1-*P* ([S])

The continuous lines represent the calculated lines according to eqn.(11) with parameters $A_0 = 2.69$ mM^2, $B_2 = 3.22$ μM·min^{-1} and $\lambda_0 = 7.19$ min^{-1}. The *y*-axis values of these comparative plots are represented in arbitrary units. The values of $[P]_\infty$, the final product concentration, were calculated from the time courses of substrate reaction given in Figure 6.

subunits respectively. Figure 5 shows plots of the final concentration of product formation, $[P]_\infty$, and the apparent inactivation rate constant, k_{obs}, against the concentration of ppase-1. As expected, $[P]_\infty$ is proportional to $1/[M]_0$, and k_{obs} gives rise to a hyperbolic dependence on $[M]_0$, indicating that the reaction obeys the complexing inhibition mechanism under the conditions of the present study [17].

Effect of substrates on the inactivation of phosphorylase *a* by ppase-1

Figure 6 shows the time course of enzyme inactivation using different Glc-1-*P* concentrations and a fixed concentration of glycogen. By fitting eqn.(5) to the experimental data, the values of k_{obs} and $[P]_\infty$ can be determined. A plot of the apparent inactivation rate constant, k_{obs}, versus the concentration of Glc-1-*P* is shown in the inset to Figure 6. On increasing the concentration of Glc-1-*P*, k_{obs} decreases and approaches zero, indicating that the substrate protects phosphorylase *a* against inactivation by ppase-1. Figure 7 shows a plot of $[P]_\infty$ against the concentration of Glc-1-*P*. It can be seen from eqn.(6) that, when keeping the concentration of glycogen constant, the general expression of $[P]_\infty$ for the phosphorylase *a*-catalysed reaction can be written as:

$$[P]_\infty = \frac{B_2[S]^2}{\lambda_0 A_0 + \lambda_1 A_1[S] + \lambda_2[S]^2} \tag{7}$$

where λ_0, λ_1 and λ_2 are the apparent inactivation rate constants

$$\lambda_0 = \frac{2[M]_0\left(k_f + k_r\dfrac{[R]}{K_R}\right)}{1+\dfrac{[R]}{K_R}} \tag{8}$$

$$\lambda_1 = \frac{[M]_0\left(k'_f + k'_r\dfrac{[R]}{K_R}\right) + [M]_0\left(k_s + k_t\dfrac{[R]}{K_R}\right)}{1+\dfrac{[R]}{K_R}} \tag{9}$$

$$\lambda_2 = \frac{2[M]_0\left(k'_s + k'_t\dfrac{[R]}{K_R}\right)}{1+\dfrac{[R]}{K_R}} \tag{10}$$

Similarly, the difference between the maximum power of the substrate concentration in the numerator and denominator can be determined using the procedure of Wang and Srivastava [30]. Figure 7 shows plots of $[P]_\infty/[S]^p$ versus [S] at different values of *p*. It can be seen from this Figure that, when $p = 2$, the plot approaches a horizontal asymptote. Therefore, the difference between the numerator and the denominator is equal to 2. This result suggests that both the apparent inactivation rate constants, λ_1 and λ_2, are equal to zero. According to the definitions of λ_1 and λ_2, it can be concluded that $k'_f = k'_r = k_s = k_t = 0$ and $k'_s = k'_t = 0$. Therefore, eqn.(7) can be written as:

$$[P]_\infty = \frac{B_2}{\lambda_0 A_0}[S]^2 \tag{11}$$

This equation predicts that the $[P]_\infty$ will approach infinity when $[S] = \infty$. As both A_0 and B_2 are known, by fitting eqn.(11) to the experimental data the value of λ_0 was determined to be 7.19 ± 0.22 min^{-1}.

Figure 8 shows the time course of enzyme inactivation using different glycogen concentrations and a fixed concentration of Glc-1-*P*. A plot of the apparent inactivation rate constant, k_{obs}, against the concentration of glycogen is shown in the inset to Figure 8. On increasing the concentration of Glc-1-*P*, k_{obs} decreases and $[P]_\infty$ approaches a non-zero constant. This indicates that glycogen partially protects phosphorylase *a* against inactivation by ppase-1. Keeping the concentration of Glc-1-*P* constant, the relationship between $[P]_\infty$ and glycogen concentration is given by:

$$[P]_\infty = \frac{V_{max}[R]}{\beta_0 K_R + \beta_1[R]} \tag{12}$$

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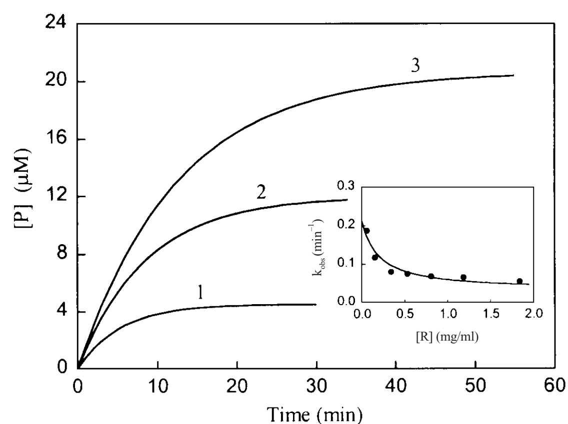


Figure 8 Time course of substrate reaction at different substrate concentrations in the presence of ppase-1

Final concentrations were 15 mM Glc-1-*P*, 3.89 units ppase-1 and 0.58 nM phosphorylase *a* (dimer) in 50 mM Hepes buffer, pH 7.0, at 25 °C. Concentrations of glycogen were 0.0625 mg/ml (curve 1), 0.1563 mg/ml (curve 2) and 0.3438 mg/ml (curve 3). Other conditions were the same as in Figure 1. The enzyme (5 μl) was added to the reaction mixture (1.6 ml) to start the reaction. The inset shows a plot of k_{obs} versus the concentration of glycogen. The values of the apparent rate constant, k_{obs}, were calculated from the time courses of substrate reaction in the presence of ppase-1.

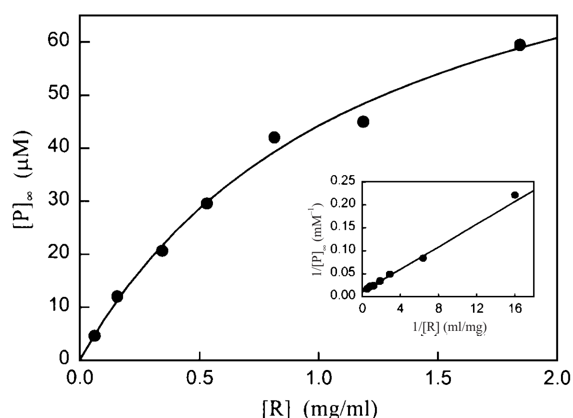


Figure 9 Plot of $[P]_{\infty}$ versus the concentration of glycogen

The continuous line is best fitting result according to eqn.(12) with $K_m = 0.199$ mg/ml, $V_{max} = 4.3\ \mu M \cdot min^{-1}$, $\beta_0 = 0.266\ min^{-1}$ and $\beta_1 = 0.045\ min^{-1}$. The inset shows the double-reciprocal plot of the experimental data. The values of the final product concentration, $[P]_{\infty}$, were calculated from the time courses of substrate reaction given in Figure 8.

where

$$\beta_0 = \frac{2k_f[M]_0}{1+\frac{2[S]}{K_{S1}}+\frac{[S]^2}{K_{S1}K_{S2}}}$$

$$\beta_1 = \frac{2k_r[M]_0}{1+\frac{2[S]}{K_{S1}}+\frac{[S]^2}{K_{S1}K_{S2}}}$$

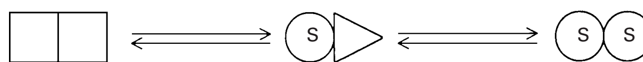
A plot of $[P]_{\infty}$ against the concentration of glycogen is shown in Figure 9. Similarly, as K_R is known, by fitting eqn.(12) to the experimental data, the values of β_0 and β_1 were determined to be $0.266 \pm 0.023\ min^{-1}$ and $0.045 \pm 0.004\ min^{-1}$. Therefore, from the expressions of β_0 and β_1, $k_f[M]_0$ and $k_r[M]_0$ can be calculated as 12.36 and 2.07 min^{-1} respectively. This agrees with the observation mentioned above that glycogen protects partially against the dephosphorylation of phosphorylase *a* by ppase-1. All the microscopic inactivation rate constants are summarized in Table 2.

Table 2 Inactivation rate constants of phosphorylase *a* by ppase-1

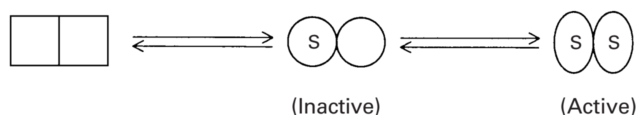
The constants $k_s = (k_{cat}/K_m)_s$, $k_t = (k_{cat}/K_m)_t$, $k_1' = (k_{cat}/K_m)_f'$, $k_r' = (k_{cat}/K_m)_r'$, $k_s' = (k_{cat}/K_m)_s'$ and $k_t' = (k_{cat}/K_m)_t'$ have a value of zero.

Constant	Value ($min^{-1} \cdot units^{-1}$)
$k_f = (k_{cat}/K_m)_f$	3.18 ± 0.28
$k_r = (k_{cat}/K_m)_r$	0.53 ± 0.05

Positive co-operativity can be accounted for either by the concerted model of Monod, Wyman and Changeux (MWC) [34] or by the ligand-induced sequential model of Koshland, Némethy and Filmer (KNF) [35]. Many attempts have been made to distinguish between these two possible mechanisms for positive co-operativity. The present study provides a novel approach to distinguish whether the sigmoidal behaviour of phosphorylase *a* can be explained by the MWC concerted model or by the KNF sequential model. As mentioned above, in the case of this simple dimeric system, the general binding model can be written as:



The values of the inactivation rate constants for the different enzyme species by ppase-1 will depend on the properties of these species. If the conformation of intermediate species (△) is very close to that of the liganded subunit (○), the value of k_f' will be similar to that of k_s and the enzyme will behave as the simple concerted mechanism, whereas at the other extreme if the conformation of the intermediate species is similar to the free subunit (□), the value of k_f' will be close to that of k_f, and hence the behaviour of the enzyme can be explained on the basis of the simple sequential model. In the case of phosphorylase *a*, $k_f = 11.88\ min^{-1}$, and $k_f' = k_s = 0$, suggesting that the mechanism of the enzyme-catalysed reaction is consistent with the MWC concerted model. On the basis of all the above experimental observations, a minimal model for the binding of Glc-1-*P* to dimeric phosphorylase *a* can be represented as follows:



DISCUSSION

Glycogen phosphorylase was the first allosteric enzyme to be isolated and analysed in detail. Allosteric regulation of this enzyme has traditionally been described in terms of the concerted model of Monod et al. [34] or the sequential model of Koshland et al. [35]. It has been shown that the binding of AMP to phosphorylase *a* follows the concerted transition model [36]. On the other hand, the binding of most ligands to phosphorylase *b* follows the sequential model of Koshland et al. rather than the concerted model [4,37]. Therefore the phosphorylase system represents an example of how a simple chemical modification provides an enhanced subunit interaction sufficient to transform sequential allosteric transitions to the concerted mode. The kinetic mechanism of glycogen phosphorylase has been investigated by initial-velocity experiments, inhibition studies, and

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isotopic-exchange reaction at equilibrium [38–40]. The general conclusion is that the reaction proceeds though a rapid-equilibrium random Bi Bi mechanism which is not altered by interconversion between the *a* and *b* forms. This type of kinetic mechanism is particularly suitable for studying an allosteric enzyme because the apparent Michaelis constants are equivalent to dissociation constants. Klinov and Kurganov [31] used the non-linear regression method to analyse the application of 12 different variants of the MWC model for fitting the steady-state kinetics of the glycogen phosphorylase *b*-catalysed reaction. They suggested that the enzyme reveals catalytic power exclusively upon the binding with two molecules of AMP and two molecules of Glc-1-*P* under the conditions of saturation by glycogen. In the present study we use the kinetic method of the irreversible modification of enzyme activity to distinguish between the two models for positive co-operativity. The results suggest that the simple two-state concerted model of allosteric response in phosphorylase *a* is an over-simplification, and the allosteric behaviour of the enzyme should be interpreted in terms of a modified concerted model in which the binding of first Glc-1-*P* molecule at one subunit induces a concerted conformational change in both subunits to form an inactive intermediate state, and the binding of the second Glc-1-*P* molecule to the enzyme will result in a further conformational transition from the inactive state to active state.

It is known that AMP and Glc-1-*P* inhibit the reaction of phosphorylase phosphatase, whereas glucose, Glc-6-*P*, glycogen and caffeine stimulate this reaction. The effects of all the ligands on the phosphorylase phosphatase reaction were shown to be substrate-directed, since no compounds had any effect on the dephosphorylation of a small phosphopeptide derived from phosphorylase *a* [11–13]. The substrate-directed activations and inhibitions of phosphorylase phosphatase have been interpreted at the molecular level from consideration of the X-ray-derived crystal structure of phosphorylase and the effects on that structure of allosteric effectors. The dimeric phosphorylase *a* is composed of two identical subunits, each of which contains an active site and five effector sites [41–43]. The monomer can be subdivided into N- and C-terminal domains. The phosphorylation sites are located at the dimer interface within 1 nm (10 Å) of each other in the N-terminal domain. A distinct glycogen activation site also lies within the N-terminal domain. The active site resides within the subunit in a crevice between the N- and C-terminal domains. The binding of Glc-1-*P* at the active site could induce a conformation in which the serine-bound phosphate becomes unavailable to the phosphatase, whereas the binding of glucose, glycogen, Glc-6-*P* or caffeine to dimeric phosphorylase *a* appears to favour a conformation that renders phosphorylase *a* susceptible to phosphatase attack. It can be seen from Figures 6 and 8 that, under the conditions used in the present study, both Glc-1-*P* and glycogen inhibit the dephosphorylation reaction of phosphorylase *a* by ppase-1. The inactivation rate constant for the free enzyme is about 6-fold greater than that for the enzyme–glycogen complex, suggesting that glycogen provides protection against the dephosphorylation of phosphorylase *a* by ppase-1. This finding disagrees with previous studies that have suggested a stimulatory effect of glycogen on the phosphatase reaction [44]. One possible explanation for this difference is that glycogen has a biphasic effect on the dephosphorylation reaction of phosphorylase *a* by ppase-1. On the one hand, glycogen can shift the tetramer ↔ dimer equilibrium of phosphorylase *a* toward the dimeric form and stimulate the reaction of ppase-1, since the tetrameric form of phosphorylase *a* cannot be dephosphorylated by ppase-1. On the other hand, the binding of glycogen to dimeric phosphorylase *a* can decrease the reaction rate of ppase-1 probably as a result of steric hindrance at the binding site. Therefore, the net effect of glycogen on the dephosphorylation reaction of phosphorylase *a* will depend of the balance between these two opposing roles. When using the conventional method for ppase-1 activity assay, a higher concentration of phosphorylase *a* was incubated with ppase-1, and phosphorylase *a* existed mainly as a tetrameric form in the absence of other ligands. In this case, the dominant action of glycogen is to promote the dissociation of tetrameric phosphorylase *a*, and therefore its net effect shows a stimulation. In the present study, only the catalytic amount of phosphorylase *a* was contained in the assay system. Since the total enzyme concentration (5×10^{-10} M) in the assay system is far smaller than the apparent dissociation constant for the dimer ↔ tetramer equilibrium (6.7×10^{-7} M) [45], the rate of tetramer formation for the process $2D_T \rightarrow T_T$ is negligible. Therefore, all the enzyme molecules will be in the dimeric form and only inhibitory effect of glycogen can be observed. It can be concluded from the above discussion that the apparent stimulatory effect of glycogen observed before is directly related to the dissociation of tetrameric phosphorylase *a*, rather than a favourable conformational change in the dimeric phosphorylase *a*.

ppase-1 activities are usually carried out using ^{32}P-labelled phosphoprotein substrates. After the reaction the released [^{32}P]P_i is separated from the remaining [^{32}P]phosphoprotein by precipitation with trichloroacetic acid [46]. Non-radioactive procedures have also been used. The conventional non-radioactive methods for determining the phosphatase activity have included taking aliquots from a phosphoprotein phosphatase incubation mixture at definite time intervals and monitoring changes in the enzymic activity of the protein substrates on dephosphorylation [46,47]. These methods are laborious, and not easily applied to fast reactions with a half-life of less than 1 min. In comparison with the conventional methods, the advantage of the progress-curve method is not only its usefulness in the study of fast modification reactions, but its convenience in the study of substrate effects on the interaction between the modifier and enzyme. Recently, a continuous spectrophotometric assay for protein phosphatases was developed [48]. The assay incorporates a coupled enzyme system, which used purine nucleoside phosphorylase and the chromophoric substrate MTGuo for the quantification of P_i. The drawback of this method is the higher amount of protein substrate required. This is not a problem when using phosphorylase *a*, since the rabbit skeletal-muscle enzyme can be readily prepared and is also available commercially. There may, however, be a problem in obtaining convenient amounts of other protein substrates. In these cases, the progress-curve method may be particularly appropriate, since only the catalytic amount of protein substrates is required in the assay system. Note that, according to our definition, k_f is equal to k_{cat}/K_m for the ppase-1-catalysed reaction [48]. Therefore it is interesting to compare the present result with that obtained by other methods. Using ppase-1 level of 2.22 units/ml, the kinetics of the ppase-1 reaction were determined by the continuous spectrophotometric assay [49]. The K_m of ppase-1 for phosphorylase *a* was determined to be 23.60 ± 2.78 μM and the V_{max} to be 15.0 ± 1.11 μM·min^{-1}. The corresponding value of k_{cat}/K_m is calculated to be $15/(23.6\times2.22) = 0.286$ min^{-1}·units^{-1}. It can be seen from Table 2 that the values of k_f determined in the present study is about an order of magnitude higher than the value of k_{cat}/K_m obtained by the continuous assay method. This difference between the values of k_{cat}/K_m is probably due to the fact that, in the continuous assay system, high concentration of salt (0.16 M NaCl) and caffeine (5 mM) have to be used in order to convert the tetrameric phosphorylase *a* into the dimeric form,

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and the presence of these may decrease the protein–protein interaction and increase the value of K_m for the ppase-1-catalysed reaction.

The rate of metabolic processes depends on both the amount and the catalytic efficiency of the enzymes concerned. Enzyme-catalysed chemical modification of enzymes is an important mechanism for the regulation of enzyme activity. The phosphorylation and dephosphorylation of proteins catalysed by protein kinases and protein phosphatases respectively is a well-known example of this type of mechanism. The kinetics of these reactions are ideally amenable to analysis by the present method. Particularly, the presence of substrate during the determination of the rate constant for the modification would be most desirable as, during the *in vivo* action of these modifying enzymes, the presence of substrate is inevitable. In this respect, the present study illustrates a good example. All information is obtained on how substrates can affect the action of an enzyme that utilizes another enzyme as a substrate.

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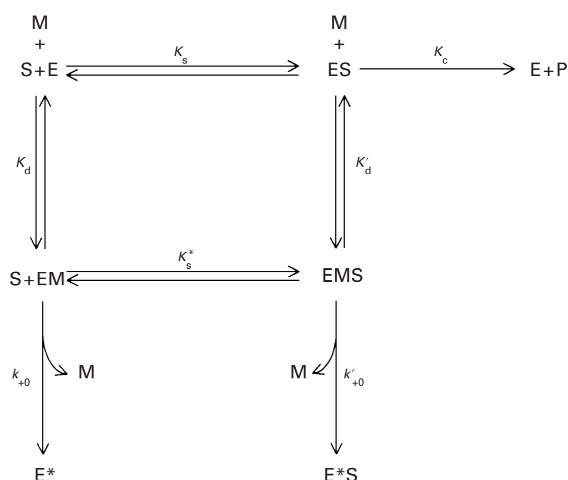
APPENDIX

The simplest single-substrate case will be considered first. In the presence of substrate, the enzyme inactivation mechanism can be described as follows:



Scheme A2

where E and E* represent active and inactive enzymes, S and M are substrate and modifier respectively. On the basis of the Scheme given above, we have:

$$[E_T] = [E]+[ES]+[EM]+[EMS] \quad (A1)$$

$$[E^*_T] = [E^*]+[E^*S] \quad (A2)$$

$$[E]_0 = [E_T]+[E^*_T] \quad (A3)$$

As before, it is assumed that the steady-state of the substrate reaction is rapidly established and that both [S] and [M] are much greater than [E]. In addition, it is also assumed that the formation of the EM and EMS complexes are fast reactions relative to the inactivation step. Therefore, the concentration of product formed at time t is given by:

$$[P] = [P]_\infty (1-e^{-k_{obs}t}) \quad (A4)$$

where k_{obs} is the apparent inactivation rate constant, and $[P]_\infty$ the product concentration at time infinity,

$$k_{obs} = \frac{(k_f K_S + k_s[S])[M]_0}{\left(1+\frac{[M]_0}{K_d}\right)K_S + \left(1+\frac{[M]_0}{K'_d}\right)[S]}$$

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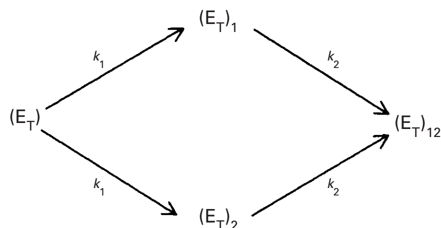
$$[P]_{\infty} = \frac{k_c[E]_0[S]}{(k_f K_S + k_s[S])\,[M]_0}$$

where [S] and $[M]_0$ are the concentrations of substrate and modifier, and $k_f = k_{+0}/K_d$ and $k_s = k'_{+0}/K'_d$ respectively [1]. For the dephosphorylation reaction, E and E* represent phosphorylated and dephosphorylated protein substrate, and M is a protein phosphatase. Therefore, k_{+0} and K_d are the catalytic-centre activity ('turnover number') and dissociation constant for the protein phosphatase-catalysed reaction respectively.

Similarly, in the presence of the substrates and a modifier, the modification reaction for a dimeric enzyme can be written as:



where (E_T) is the unmodified enzyme, $(E_T)_1$ denotes the enzyme molecule in which subunit 1 has been modified, $(E_T)_2$ in which subunit 2 has been modified, and $(E_T)_{12}$ the enzyme in which both subunits have been modified. k_1 and k_2 are the apparent modification rate constants. Both of them are the functions of concentrations of substrate(s) and the modifier. The concentration of unmodified subunit in solution at time t is given by [2]:

$$x = \frac{2[E]_0}{2k_1 - k_2}[(k_1 - k_2)e^{-2k_1 t} + k_1 e^{-k_2 t}] \tag{A5}$$

where $[E]_0$is the total enzyme concentration. The rate of product formation while the enzyme is being modified is:

$$\frac{d[P]}{dt} = \frac{v_0}{2k_1 - k_2}[(k_1 - k_2)e^{-2k_1 t} + k_1 e^{-k_2 t}] \tag{A6}$$

where v_0 is the initial velocity of the enzyme-catalysed reaction in the presence of the modifier. The concentration of product formed at time t is given by:

$$[P] = v_0\left\{\frac{3k_1 - k_2}{2k_1(2k_1 - k_2)} - \frac{k_1 - k_2}{2k_1(2k_1 - k_2)}e^{-2k_1 t} - \frac{k_1}{k_2(2k_1 - k_2)}e^{-k_2 t}\right\} \tag{A7}$$

When $k_1 = k_2$, eqn. (7A) becomes:

$$[P] = \frac{v_0}{k_1}\left(1 - e^{-k_1 t}\right) \tag{A8}$$

which is identical in form with eqn. (A4). This result indicates that, for a dimeric enzyme, if the modification of the first subunit has no affect on the modification of the second one, the dependence of product concentration on the reaction time will obey monophasic first-order kinetics.

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