Kinetic mechanism of the glycogen-phosphorylase-catalysed reaction in the direction of glycogen synthesis: co-operative interactions of AMP and glucose 1-phosphate during catalysis

Eduard A. SERGIENKO and D. K. SRIVASTAVA¹

Biochemistry Department, North Dakota State University, Fargo, ND 58105, U.S.A.

We employed our newly developed, continuous, spectrophotometric method [Sergienko and Srivastava (1994) *Anal. Biochem.* **221**, 348–355] for measuring the glycogen-phosphorylase-catalysed reaction in the direction of glycogen synthesis, utilizing varied concentrations of AMP (2–400 μ M) and glucose 1-phosphate (G1P; 4 μ M to 41 mM). The experimental data revealed that the enzyme catalysis exhibits sigmoidal dependence on both AMP and G1P concentrations, with Hill coefficient and EC_{50} values (mutually) affected by the concentrations of the above substrates. A detailed kinetic analysis of the substrate-dependent activation, as well as glucose-inhibition data, lead us to propose the following mechanistic features of the glycogen-phosphorylase-catalysed reaction. (1) The enzyme exhibits cata-

lytic activity when two molecules of AMP and two molecules of G1P are bound to the dimeric unit. (2) The binding of one molecule of glucose (the competitive inhibitor of G1P) per dimeric unit results into a complete loss of the enzyme activity. (3) There is no restriction of binding of AMP or G1P when one of the dimeric subunits is already bound with the other ligand. For example, one or two G1P molecules can bind to the enzyme dimer when zero, one or two molecules of AMP are already bound. The magnitudes of rate and equilibrium constants for the glycogen-phosphorylase-catalysed reaction, derived from analyses of the experimental data in the light of a few selected minimal models, are presented.

INTRODUCTION

Rabbit-muscle glycogen phosphorylase is one of the most extensively studied enzymes from structural, mechanistic and regulatory points of view (for reviews see [1–4]). The dimeric enzyme molecule exists in phosphorylated (at Ser-14 residue) and non-phosphorylated forms, and these forms are conventionally referred to as phosphorylase *a* and phosphorylase *b* respectively [1,2]. Whereas phosphorylase *a* is catalytically active in the absence of any effector molecule, phosphorylase *b* requires AMP [2–4]. The active forms of the enzyme catalyse reversible phosphorolytic cleavage of glycogen to produce glucose-1 phosphate (G1P; eqn. 1).

The activity of glycogen phosphorylase is conventionally measured by monitoring the rate of formation of either G1P in the forward direction [5,6], or inorganic phosphate in the reverse direction [7,8]. Since the phosphate determination has been based on the sampling method until recently (see below), the kinetic mechanism of the enzyme in the direction of glycogen synthesis has not been elucidated in detail [1]. Although attempts have been made to measure the enzyme catalysis (in the direction of glycogen synthesis) by following the turbidity of glycogen solution spectrophotometrically at 310 nm [9], the sensitivity of the above assay procedure has been highly limited, and thus has led to a few unusual conclusions about the enzyme catalysis.

Realizing the limitations of the above assay procedures, we developed a continuous spectrophotometric method (based on the phosphate determination protocol of Webb [10]) for measuring the glycogen-phosphorylase-catalysed reaction in the direction of glycogen synthesis [11]. This method relies on the

utilization of inorganic phosphate (formed during the glycogen-phosphorylase-catalysed reaction) during the purine nucleoside phosphorylase-catalysed phosphorolytic cleavage of a chromophoric substrate, 7-methyl-6-thioguanosine (MTGuo) to yield 7-methyl-6-thioguanine (MTGua) and ribose 1-phosphate (eqn. 2):

Due to differences in the spectral properties of MTGuo and MTGua species, the glycogen-phosphorylase-catalysed reaction could be easily monitored at 360 nm ($\Delta\epsilon_{360} = 11\,200 \text{ M}^{-1} \cdot \text{cm}^{-1}$). By employing the above assay system, we observed that the enzyme-catalysed reaction exhibits sigmoidal dependencies on both AMP and G1P concentrations [11].

A casual perusal of the literature dealing with glycogen phosphorylase (for reviews see [1–4]) reveals that all the structural and kinetic data of the enzyme have been explained in the light of the concerted allosteric model of Monod, Wyman and Changeux (MWC) [12]. Several variations of the MWC model have been utilized by different laboratories to explain the enzyme kinetics data [9,13–15].

Our interest in the glycogen-phosphorylase-catalysed reaction emerged during our attempts to determine the minimal number of functional sites in enzymes that contain multiple binding sites for a ligand [16,17]. In this pursuit, while investigating

Abbreviations used: MTGuo, 7-methyl-6-thioguanosine; MTGua, 7-methyl-6-thioguanine; DTT, dithiothreitol; G1P, glucose 1-phosphate; MWC, Monod, Wyman and Changeux.

¹ To whom correspondence should be addressed.

the glycogen-phosphorylase-catalysed reaction in the direction of glycogen degradation, we discerned that of the two classes of AMP binding sites, the enzyme is functionally active even when only one site is occupied by AMP [17]. To confirm whether the above conclusion also holds when the reaction is performed in the direction of glycogen synthesis (i.e. under the conditions where the steady-state concentration of inorganic phosphate is negligible and the GIP concentration is considerably higher), as well as to ascertain the number of GIP binding sites required for the functional activity of the enzyme, we undertook a detailed kinetic investigation of the enzyme. As will be elaborated in subsequent sections, our approach towards deducing a specific kinetic model of the phosphorylase-catalysed reaction has been primarily based on the outcomes of the experimental data, rather than on fitting results of different preconceived models [9]. The experimental results and the underlying conclusions are contained in the subsequent sections.

EXPERIMENTAL

Materials

D-GIP (disodium salt), AMP (free acid), BSA, dithiothreitol (DTT), EDTA, magnesium acetate, Bistris, methyl iodide, thio-urea, 2-amino-6-chloropurine riboside and bacterial purine nucleoside phosphorylase (EC 2.4.2.1) were purchased from Sigma. All other reagents were analytical reagent grade.

Methods

Glycogen phosphorylase *b* was purified and crystallized (4 times) from rabbit muscle according to Fischer and Krebs [18]. The crystallized enzyme thus obtained was judged to be homogeneous by SDS/PAGE. Before use, the crystallized enzyme was desalted on a Sephadex G-25 (fine grade) column that was equilibrated with a 20 mM Bistris buffer, pH 7.0, containing 0.4 mM EDTA and 2 mM DTT. The desalted enzyme was appropriately diluted in the above buffer containing 0.5 mg/ml BSA for the activity measurement. Under these conditions, the enzyme was stable for approx. 24 h at room temperature.

The glycogen-phosphorylase-catalysed reaction was routinely measured by the continuous spectrophotometric method developed in our laboratory [11]. The latter method involved the use of MTGuo as a chromogenic substrate and purine nucleoside phosphorylase as the coupling enzyme. MTGuo was synthesized and its concentration was determined as described earlier [10,11]. The enzyme-catalysed reaction progress was monitored at 360 nm ($\Delta\epsilon_{360} = 11200 \text{ M}^{-1} \cdot \text{cm}^{-1}$) either on a double-beam Perkin-Elmer (lambda 3-B) or a Diode Array (Beckman 7400) spectrophotometer. To ensure homogeneous mixing, the reaction mixture of the enzyme reaction was stirred continuously by magnetic stirrer, installed at the bottom of the thermostatically controlled cuvette holders of these spectrophotometers. This stratagem allowed us to acquire the data of the enzyme-catalysed reaction progress just after 5 s of addition of the reagents.

The protein concentration of glycogen phosphorylase *b* was determined by measuring the absorption at 280 nm ($A_{280}^{0.1\%} = 1.31$ [19]). The protein concentration of purine nucleoside phosphorylase was determined according to Bradford [20], utilizing BSA as a protein standard. The relative molecular mass of the dimeric glycogen phosphorylase was taken to be 200000 [19].

All the kinetic experiments were performed in a 50 mM Bistris buffer, pH 7.0 (at 25 °C), containing 50 mM KCl, 2 mM magnesium acetate, 0.1 mM EDTA and 1 mM DTT, unless stated otherwise. This buffer is referred to as the standard assay buffer for measurements of the glycogen-phosphorylase-catalysed reac-

tions. The reaction mixture, containing MTGuo (dissolved in DMSO to give a final concentration of the solvent in the cuvette of less than 1 %, v/v), glycogen and AMP, was added to purine nucleoside phosphorylase, followed by GIP. The addition of appropriately diluted glycogen phosphorylase at this stage led to a steady state increase in the absorption at 360 nm for several minutes. The initial rate of the glycogen-phosphorylase-catalysed reaction was determined from the slope of the time-dependent absorption changes (at 360 nm) in the linear region.

The experimental data were analysed by using a non-linear regression analysis program, Sigma-Plot 5.0, from Jandel Scientific. The program utilizes the Marquardt algorithm, and in addition to providing standard errors and coefficients of variation, it gives information about the dependencies of one parameter on another. The latter serves as an indicator of the redundancy of the associated parameters.

Theoretical considerations

For an enzyme that contains multiple binding sites for its ligand, the initial rate of enzyme catalysis can be expressed as a function of the ligand (L) concentration, by the ratio of two polynomials in L, as elaborated by Wong [21] and Bardsley and Childs [22]. For an enzyme containing two binding sites for its ligand, the above polynomial relationship can be given by eqn. (3):

$$v = \frac{A_0[L]^0 + A_1[L]^1 + A_2[L]^2}{B_0[L]^0 + B_1[L]^1 + B_2[L]^2} \quad (3)$$

The coefficients A_i and B_i attain constant values if the concentration of the other ligands remains practically unchanged (as is usually the case during the initial rate measurements) during the enzyme catalysis. Furthermore, if the enzyme-catalysed reaction conforms to a rapid equilibrium condition, the terms in the numerator and denominator refer to the enzyme species that are functionally active and the enzyme species that are bound with the ligand(s) respectively. Consistently, the constant terms A_i and B_i are related to 'activity' and 'binding' properties of the enzyme respectively.

If the ligand serves as a substrate (i.e. $L = S$), no enzyme catalysis would be expected in the absence of any bound substrate, and thus the first term (i.e. $A_0[L]^0$) in the numerator would be equal to zero, resulting in the simplification of eqn. (3) in the form of eqn. (4):

$$v = \frac{A_1[S] + A_2[S]^2}{B_0 + B_1[S] + B_2[S]^2} \quad (4)$$

Eqn. (4) implies that there are three enzyme species, i.e. E, ES and ES₂ (corresponding to the successive denominator terms), of which ES and ES₂ (corresponding to the successive numerator terms) are functionally active. If there is no distinction between the enzyme sites, i.e. both sites bind the substrate with equal affinities, and all enzyme-substrate complexes exhibit identical functional behaviour, eqn. (4) will be reduced to eqn. (5):

$$v = \frac{\text{Constant}_1[S]}{\text{Constant}_2 + \text{Constant}_3[S]} \quad (5)$$

Eqn. (5) takes the form of the Michaelis-Menten equation on division of numerator and denominator by Constant₃ (eqn. 6):

$$v = \frac{(\text{Constant}_1/\text{Constant}_3)[S]}{(\text{Constant}_2/\text{Constant}_3) + [S]} \quad (6)$$

In eqn. (6), Constant₁/Constant₃ and Constant₂/Constant₃ are the measures of $V_{\max}$ and K_m values respectively.

One of the interesting aspects of presenting the rate equation in the above format is that it does not require extensive model-based derivations of specific equations. The above format is particularly useful for enzymes that contain multiple binding sites for ligands and exhibit functional activities at specified stoichiometric combinations of such ligands. As will be elaborated below, such stoichiometry can be ascertained by analysing the experimental data by our graphical method [17].

A generalized equation for enzyme catalysis containing multiple binding sites for a ligand (L) can be given by eqn. (7):

$$v = \frac{A_k[L]^k + A_{k+1}[L]^{k+1} + \dots + A_{m-1}[L]^{m-1} + A_m[L]^m}{B_0 + B_1[L] + B_2[L]^2 + \dots + B_{n-1}[L]^{n-1} + B_n[L]^n} \quad (7)$$

where k and m in the numerator represent the minimal and maximal number of the ligand-bound sites, which are functionally active, and n in the denominator represents the total number of ligand binding sites per enzyme molecule.

In the case where L serves as an inhibitor, eqn. (7) can be rearranged in the reciprocal form, followed by multiplication of denominator by $[L]^p$ (eqn. 8):

$$\frac{1}{v[L]^p} = \frac{B_0 + B_1[L] + B_2[L]^2 + \dots + B_n[L]^n}{A_k[L]^{k+p} + A_{k+1}[L]^{k+p+1} + \dots + A_m[L]^{p+m}} \quad (8)$$

As elaborated by Wang and Srivastava [17], the plot of $1/v[L]^p$ versus $[L]$ would yield a horizontal asymptote when $p = n - m$, and the magnitude of p would be a measure of the minimal number of catalytic sites (bound with substrates, but free from any inhibitor) for exhibiting the enzyme activity. In the case of oligomeric enzymes, if L serves as a competitive inhibitor, the magnitude of p represents the minimal number of interacting subunits that exhibit the catalytic activity.

In the case where L serves as an activator, a somewhat different stratagem is employed to ascertain the minimal number of functional sites of the enzyme molecule. In this case, eqn. (7) is rearranged in the following format (eqn. 9):

$$v = \frac{A_k/[L]^{n-k} + A_{k+1}/[L]^{n-k-1} + \dots + A_{m-1}/[L]^{n-m+1} + A_m/[L]^{n-m}}{B_0/[L]^n + B_1/[L]^{n-1} + \dots + B_{n-1}/[L] + B_n} \quad (9)$$

Eqn. (9) can be rearranged in the reciprocal form, followed by multiplication of both sides by $[L]^p$ to yield eqn. (10):

$$\frac{[L]^p}{v} = \frac{B_0/[L]^n + B_1/[L]^{n-1} + \dots + B_{n-1}/[L] + B_n}{A_k/[L]^{n-k+p} + \dots + A_{m-1}/[L]^{n-m+p+1} + A_m/[L]^{n-m+p}} \quad (10)$$

As discussed previously, the plot of $[L]^p/v$ versus $1/[L]$ would yield a horizontal asymptote when $p = k$. In this case the magnitude of p would be a measure of the minimal number of enzyme sites, which must be ligated by the activator molecule, to yield the catalytic activity.

Since G1P and AMP, in the present case, essentially serve as activators (during the enzyme catalysis), the experimental data involving these ligands have been analysed by eqn. (10). On the other hand, glucose-dependent inhibition of the enzyme has been analysed by eqn. (8).

RESULTS

The continuous spectrophotometric method, involving MTGuo and purine nucleoside phosphorylase, developed in our laboratory [11] was utilized to measure the initial rates of the glycogen-phosphorylase-catalysed reaction in the direction of glycogen synthesis. This method was found to be highly sensitive for measuring enzyme catalysis utilizing G1P in the range of

Figure 1 Effects of AMP (A) and G1P (B) on the glycogen-phosphorylase-catalysed reaction in the direction of glycogen synthesis in the standard assay buffer, at 25 °C

[Glycogen] = 0.1% (w/v), [MTGuo] = 100 μ M, [purine nucleoside phosphorylase] = 138 μ g/ml, [glycogen phosphorylase] = 0.453 μ g/ml. The changing fixed concentrations of G1P and AMP are noted for the individual curves on panels (A) and (B) respectively. For clarity, several data sets are not included. The smooth solid lines are the best fit of the experimental data according to the Hill equation. The magnitudes of Hill coefficient (h) and EC_{50} values for both AMP and G1P-dependent reactions are given in Table 1.

4 μ M to 41 mM, and AMP in the range of 2–400 μ M. To the best of our knowledge, such a sensitivity has not been achieved by any continuous spectrophotometric method developed so far, for measuring the glycogen-phosphorylase-catalysed reaction in the direction of glycogen synthesis.

Figure 1 shows the initial rates of the enzyme-catalysed reaction as a function of AMP concentration at different fixed concentrations of G1P (Figure 1A), and as a function of G1P concentration at different fixed concentrations of AMP (Figure 1B). As noted previously [11], these data do not conform to the Michaelis dependence of the AMP and G1P concentrations. A qualitatively similar conclusion was discerned by other investigators [9,23–25]. Smooth solid lines are the best fit of the experimental data according to the Hill equation. The magnitudes of the Hill coefficient (h) and EC_{50} (the concentration of ligand required to achieve half of the maximal velocity), derived from the above analysis, are summarized in Table 1. These parameters suggest that, in the case of the G1P-dependent reaction, h decreases from 1.6 to 1.4, and EC_{50} decreases from 5.4 to 1.5 mM on an increase in AMP concentration from 5 to 300 μ M. However, for the AMP-dependent reaction, only EC_{50} decreases

Table 1 Summary of the Hill coefficient (h) and EC_{50} values derived from the best fit of the experimental data of Figure 1

Substrates	[Fixed substrate]	Hill coefficient (h)	EC_{50}
Variable substrate = AMP, fixed substrate = G1P	0.099 mM	1.59 ± 0.28	$74.7 \pm 12.1 \mu\text{M}$
	0.501 mM	1.52 ± 0.06	$67.8 \pm 2.5 \mu\text{M}$
	0.99 mM	1.5 ± 0.03	$55.3 \pm 1.14 \mu\text{M}$
	2.14 mM	1.6 ± 0.06	$36.7 \pm 1.0 \mu\text{M}$
	5.01 mM	1.6 ± 0.04	$24.9 \pm 0.51 \mu\text{M}$
Variable substrate = G1P, fixed substrate = AMP	21.4 mM	1.5 ± 0.06	$18.7 \pm 0.5 \mu\text{M}$
	5 μM	1.6 ± 0.1	$5.4 \pm 0.25 \text{ mM}$
	10 μM	1.7 ± 0.07	$4.3 \pm 0.14 \text{ mM}$
	16 μM	1.7 ± 0.06	$3.5 \pm 0.09 \text{ mM}$
	40 μM	1.5 ± 0.02	$2.4 \pm 0.03 \text{ mM}$
	300 μM	1.4 ± 0.02	$1.5 \pm 0.02 \text{ mM}$

Figure 2 Graphical analysis of the AMP-dependent activation of glycogen phosphorylase

The minimum number of activation sites has been determined by plotting the experimental data as $[AMP]^p/v$ ($p = 0, 1, 2$ and 3) versus $1/[AMP]$. The enzyme-catalysed reaction was measured in the standard buffer, utilizing the presence of 0.031% (w/v) glycogen, 10 mM G1P, 65 μM MTGuo, 65.4 $\mu\text{g/ml}$ purine nucleoside phosphorylase and 0.34 $\mu\text{g/ml}$ glycogen phosphorylase b . Note a horizontal asymptote when $p = 2$, suggesting that the minimum number of AMP molecules required for the activation of the dimeric enzyme molecule is 2. Solid lines are the best fit of experimental data according to eqn. (10).

from 75 to 19 μM on an increase in G1P concentration from 0.1 mM to 21 mM; no discernible effect of G1P concentration on h (approx. 1.6) was observed. These data clearly suggest that both G1P and AMP interact co-operatively with the dimeric enzyme molecule. A quantitative relationship between the Hill coefficient and a variety of kinetic model for the glycogen-phosphorylase-catalysed reaction will be published elsewhere.

To ascertain the minimum number of G1P and AMP binding sites for the functional (catalytic) activity of the dimeric enzyme molecule, we employed our previously developed graphical method ([17], see also the Theoretical considerations section). Since both these ligands essentially function as activators, we plotted $v/[AMP]^p$ versus $1/[AMP]$ and $v/[G1P]^p$ versus $1/[G1P]$ for different integral values of p (i.e. $p = 0, 1, 2, 3$). These plots are shown in Figures 2 and 3. Note that in the case of both AMP and G1P, the horizontal asymptotes are observed when $p = 2$. This is further confirmed by the fact that when $p = 1$, the plots attain oblique asymptotes, and when $p = 3$, they attain zero

Figure 3 Graphical analysis of the G1P-dependent reaction of glycogen phosphorylase

The minimum number of G1P binding sites has been determined by plotting the experimental data as $[G1P]^p/v$ ($p = 0, 1, 2$ and 3) versus $1/[G1P]$. The fixed concentration of AMP was 28 μM . Other conditions are the same as in Figure 2. Note a horizontal asymptote when $p = 2$, suggesting that the minimum number of G1P molecules required for the activation of the dimeric enzyme molecule is 2. Solid lines are the best fit of experimental data according to eqn. (10).

asymptotes. Since the horizontal asymptotes are obtained when $p = 2$, this implies that the minimum powers of AMP and G1P required for the functional activity of the enzyme are 2. Consistently, glycogen phosphorylase is expected to exhibit functional (catalytic) activity when two molecules of AMP and two molecules of G1P are bound to the dimeric enzyme molecule.

From the data of Figures 2 and 3, it is further evident that when $p = 0$, $1/v$ versus $1/[AMP]$ and $1/[G1P]$ plots exhibit non-linear dependence. Such profiles are characteristic of the sigmoidal dependence of the enzyme-catalysed reaction as functions of these ligands, as deduced from the analysis of the experimental data of Figure 1 by the Hill equation.

Inhibition of glycogen phosphorylase by glucose

It has been well documented that glucose serves as a competitive inhibitor against G1P during the glycogen-phosphorylase-catalysed reaction [26,27]. The latter is supported by the X-ray crystallographic data of the enzyme-ligand complexes [28,29]. We investigated the effects of glucose on the enzyme-catalysed reaction at selected concentrations of AMP and G1P. The data are shown in Figure 4. It should be pointed out that the inhibition data of glucose are best fitted by the Hill equation. The magnitudes of the Hill coefficient and EC_{50} are dependent on the concentrations of G1P and AMP. For example, at 2 mM G1P and 200 μM AMP, the glucose inhibition profile yields the Hill coefficient and EC_{50} values as 1.6 and 2.6 mM respectively. On increasing the G1P concentration to 20 mM (while maintaining the AMP concentration at 200 μM), whereas the Hill coefficient remains unchanged, EC_{50} increases to 18.8 mM. On the other hand, when the concentration of AMP is decreased from 200 μM to 20 μM (while maintaining the G1P concentration at 20 mM), the Hill coefficient and EC_{50} values are decreased to 1.2 and 2.8 mM respectively. These data, besides attesting to a competitive binding of glucose at the G1P binding site, also suggest that AMP and G1P interact co-operatively (heterotropic interaction). In other words, the binding of G1P influences the binding of AMP, and vice versa. Whether the above (hetero-

Figure 4 Inhibition of the glycogen-phosphorylase-catalysed reaction by glucose

Enzyme activity (% Activity) as a function of glucose concentration is plotted. The enzyme reaction was measured in the standard assay buffer, containing 0.031% glycogen, 50 μ M MTGuo, 71 μ g/ml purine nucleoside phosphorylase and 0.26 μ g/ml glycogen phosphorylase. The concentrations of G1P and AMP were 20 mM and 200 μ M (triangles), 2 mM and 200 μ M (squares), 20 mM and 20 μ M (circles) respectively. Solid lines are the best fit of the experimental data according to the Hill equation.

Figure 5 Graphical analysis of the inhibition of the glycogen-phosphorylase-catalysed reaction by glucose

The experimental data of Figure 4, obtained under the reaction conditions of 20 mM G1P and 200 μ M AMP, are plotted as $1/v[\text{glucose}]^p$ ($p = 0, 1, 2$ and 3) versus [Glucose]. The solid line is the best fit of the experimental data according to eqn. (8). Note a horizontal asymptote when $p = 2$, suggesting that both enzyme subunits must be free from any bound glucose molecules to exhibit the catalytic activity.

tropic) co-operative interaction is mediated within the same subunit or between two subunits is not clear at this time. These observations are qualitatively similar to those of Helmreich et al. [23].

We further analysed the glucose-inhibition data by our graphical method. To ascertain the minimal number of sites that must be free from glucose to exhibit the catalytic activity, we plotted $1/v[\text{glucose}]^p$ versus [glucose] at different values of p (i.e. $p = 0, 1, 2, 3 \dots$). These plots for the glucose-dependent inhibition of the enzyme (for one set of G1P and AMP concentrations) are shown in Figure 5. Note that the horizontal asymptote is attained when p becomes equal to 2. Hence the minimal number of enzyme sites

that must be free from glucose to exhibit the enzyme activity must be 2. In other words, the dimeric enzyme molecule, bound with one molecule of glucose and one molecule of G1P, would not exhibit the catalytic activity. Under the above conditions, the numerator terms $A_1[L]$ and $A_2[L]^2$ (where L is glucose) of eqn. (3) would be equal to zero. These data are further corroborated by the fact that the dimeric enzyme molecule is active only when two molecules of AMP and two molecules of G1P are bound (see above).

DISCUSSION

The continuous spectrophotometric assay system enabled us to measure the glycogen-phosphorylase-catalysed reaction at varying concentrations of G1P and AMP. This method further allowed us to investigate the effect of glucose (a competitive inhibitor of G1P) on the enzyme catalysis. Before this investigation, Klinov and Kurganov [9] attempted to deduce the kinetic mechanism of glycogen phosphorylase (in the direction of glycogen synthesis) via measuring the turbidity changes of glycogen solution (spectrophotometrically at 310 nm) during the enzyme catalysis. These authors investigated the enzyme catalysis, utilizing G1P and AMP concentrations lower and higher than their corresponding EC_{50} values respectively, presumably due to limitations in sensitivity of their assay system. We believe this has been the major cause of some of the discrepancies between our conclusions and those of Klinov and Kurganov (see below).

The experimental data presented in the previous section leads to the following conclusions. (1) Both G1P and AMP interact co-operatively (homotropic interaction) within the dimeric enzyme unit. (2) The enzyme exhibits catalytic activity, when and only when two molecules of G1P and two molecules of AMP are bound to the enzyme dimer. The enzyme does not exhibit catalytic activity at any other stoichiometric combinations of the above ligands. (3) The enzyme is catalytically inactive even when 1 molecule of glucose (competitive inhibitor of G1P) is bound to the dimeric enzyme unit. (4) The affinity of G1P is influenced by prior occupancy of the enzyme by AMP, and vice versa.

Based on the above experimental facts, we proceeded to analyse our experimental data on the basis of a general model such as that presented by eqn. (3). Since the enzyme catalysis involves one substrate (G1P) and one activator (AMP), the overall rate of catalysis can be expressed by eqn. (11):

$$v = \frac{(a_{00} + a_{01}[S] + a_{02}[S]^2) + (a_{10} + a_{11}[S] + a_{12}[S]^2)[A] + (a_{20} + a_{21}[S] + a_{22}[S]^2)[A]^2}{(b_{00} + b_{01}[S] + b_{02}[S]^2) + (b_{10} + b_{11}[S] + b_{12}[S]^2)[A] + (b_{20} + b_{21}[S] + b_{22}[S]^2)[A]^2} \quad (11)$$

In eqn. (11), A and S represent AMP and G1P; a_{ij} and b_{ij} are constants.

Eqn. (11) is essentially an expanded form of eqn. (3), with constant terms (A_i and B_i) expressed as a function of G1P (S) concentration, with new constants a_{ij} and b_{ij} . The statistical mechanics basis for deriving such an equation has been elaborated by Wang and Kihara [30]. Given that the enzyme is active only when two molecules each of G1P and AMP are bound to the dimeric unit, the first eight terms of the numerator, i.e. $a_{00}, \dots, a_{21}[S]$ can be taken to be equal to zero, leading to simplification of eqn. (11) to eqn. (12):

$$v = \frac{a_{22}[S]^2[A]^2}{(b_{00} + b_{01}[S] + b_{02}[S]^2) + (b_{10} + b_{11}[S] + b_{12}[S]^2)[A] + (b_{20} + b_{21}[S] + b_{22}[S]^2)[A]^2} \quad (12)$$

Figure 6 Effect of G1P on C_2 , D_0 and D_1

The experimental conditions are the same as in Figure 1. The smooth solid lines in the panels (A), (B) and (C) are the best fit of the experimental data by eqns. (16A), (16B) and (16C) respectively. The derived parameters for the stepwise fitting results are shown in Table 2, column 2.

In Eqn. (12), the activity parameter, a_{22} , can be taken as the product of the turnover rate of the enzyme (k) and the corresponding binding parameter (b_{22}), i.e. $a_{22} = k \cdot b_{22}$. Since the turnover rate (k) is expressed for each active site, it must be multiplied by a factor of 2 and the total enzyme concentration (E_t) to obtain the overall rate of the enzyme catalysis. These considerations yield eqn. (13):

$$v = \frac{2 * k * [E]_t * b_{22} [S]^2 [A]^2}{(b_{00} + b_{01}[S] + b_{02}[S]^2) + (b_{10} + b_{11}[S] + b_{12}[S]^2)[A] + (b_{20} + b_{21}[S] + b_{22}[S]^2)[A]^2} \quad (13)$$

Eqn. (13) can be further simplified by dividing the numerator and denominator by b_{20} (eqn. 14):

$$v = \frac{2 * k * [E]_t * c_{22} [S]^2 [A]^2}{(c_{00} + c_{01}[S] + c_{02}[S]^2) + (c_{10} + c_{11}[S] + c_{12}[S]^2)[A] + (1 + c_{21}[S] + c_{22}[S]^2)[A]^2} \quad (14)$$

where $c_{ij} = b_{ij}/b_{20}$ (with i or $j = 0, 1, 2$). Eqn. (14) can be presented in the form of eqn. (15):

$$v = \frac{C_2 [A]^2}{D_0 + D_1 [A] + [A]^2} \quad (15)$$

where C_2 , D_0 and D_1 are related by the constants of eqn. (14) as follows (eqn. 16):

$$C_2 = 2 * k * [E]_t * c_{22} [S]^2 / (1 + c_{21}[S] + c_{22}[S]^2) \quad (16A)$$

$$D_0 = (c_{00} + c_{01}[S] + c_{02}[S]^2) / (1 + c_{21}[S] + c_{22}[S]^2) \quad (16B)$$

$$D_1 = (c_{10} + c_{11}[S] + c_{12}[S]^2) / (1 + c_{21}[S] + c_{22}[S]^2) \quad (16C)$$

It should be emphasized that the rate of the enzyme catalysis as

Table 2 Summary of the parameters obtained from the stepwise concerted analysis of the AMP and G1P-dependent reaction of glycogen phosphorylase

The stepwise fitting results were obtained by the non-linear regression analysis of C_2 , D_0 and D_1 as a function of G1P concentration by eqns. (16A), (16B) and (16C) respectively. The cumulative fitting results were obtained by analysing the entire kinetic data (by using eqn. 14), using the stepwise fitting results as initial estimates.

Parameters	Stepwise fitting results	Cumulating fitting results
k (s^{-1})	79.9 ± 0.53	79.7 ± 0.45
c_{00} (mM^2)	$3.30 \times 10^{-3} \pm 1.45 \times 10^{-4}$	$6.66 \times 10^{-3} \pm 1.34 \times 10^{-3}$
c_{01} (mM)	$1.97 \times 10^{-3} \pm 2.17 \times 10^{-4}$	$4.98 \times 10^{-4} \pm 5.32 \times 10^{-5}$
c_{02}	$2.60 \times 10^{-4} \pm 2.75 \times 10^{-5}$	$3.00 \times 10^{-4} \pm 5.32 \times 10^{-5}$
c_{10} (mM)	$2.57 \times 10^{-2} \pm 9.99 \times 10^{-3}$	$8.70 \times 10^{-2} \pm 6.20 \times 10^{-2}$
c_{11}	$6.74 \times 10^{-2} \pm 1.49 \times 10^{-2}$	$2.75 \times 10^{-2} \pm 1.96 \times 10^{-2}$
c_{12} (mM)	$1.49 \times 10^{-2} \pm 1.89 \times 10^{-3}$	$1.60 \times 10^{-2} \pm 4.10 \times 10^{-3}$
c_{21} (mM^{-1})	1.63 ± 0.37	1.53 ± 0.43
c_{22} (mM^{-2})	1.92 ± 0.28	1.81 ± 0.36

a function of AMP concentration can be analysed by eqn. (15) to obtain the magnitudes of C_2 , D_0 and D_1 . Since the latter parameters are dependent on the concentration of G1P (eqn. 16), the analytical results yield different values of C_2 , D_0 and D_1 at different concentrations of G1P. The derived parameters as a function of G1P concentration are shown in Figure 6. Note that whereas C_2 increases with increasing G1P concentration, both D_0 and D_1 decrease.

To obtain all the intrinsic constants of eqn. (16), we performed stepwise analysis of the data of Figure 6. The dependence of C_2 on G1P concentration was first analysed according to eqn. (16A) (by a non-linear regression analysis method) to obtain the magnitudes of c_{21} , c_{22} and k (Table 2). By fixing the latter values, we analysed (by a non-linear regression method) the dependence of D_0 and D_1 on G1P concentration (Figures 6B and 6C) according to eqns. (16B) and (16C) respectively. The derived parameters from the above stepwise fitting results are summarized in Table 2 (column 2). The smooth solid lines in Figure 6 are the best fit of the experimental data.

It should be noted that in Figure 6, all the parameters (i.e. C_2 , D_0 and D_1) tend to acquire horizontal asymptotes at high concentrations of G1P. Of these, the horizontal asymptotes in D_0 and D_1 versus G1P plots are suggestive of the fact that the second G1P can be bound to the dimeric enzyme unit, irrespective of whether the enzyme is bound with 0 AMP (i.e. $c_{02} \neq 0$) or 1 AMP (i.e. $c_{12} \neq 0$). This is in contrast with the deduction of Klinov and Kurganov [9], who propose that the second G1P molecule can only bind to the dimeric enzyme when two molecules of AMP are already bound. This is presumably because the latter authors did not utilize a G1P concentration higher than its EC_{50} value (throughout their experiments), and thus they could not account for the terms associated with c_{02} , c_{12} and c_{22} of eqn. (14). Furthermore, since Klinov and Kurganov [9] utilized AMP concentrations higher than its EC_{50} value (once again due to the lack of sensitivity of their assay method), they could not account for the terms associated with c_{00} , c_{01} , c_{02} , c_{10} , c_{11} and c_{12} . As a result, the most pronounced effect of AMP and G1P concentrations was due to underestimation of c_{02} and c_{12} values by Klinov and Kurganov [9]. This is the reason why these authors did not observe any changes in the Hill coefficient for G1P as a function of AMP concentration.

Having determined the individual constants of eqn. (16) by stepwise fitting of the experimental data, we used these parameters as initial estimates for fitting the entire experimental data (referred

Figure 7 Three-dimensional plots of the glycogen-phosphorylase-catalysed reaction as a function of G1P and AMP concentrations

The dotted lines represent the calculated lines according to eqn. (14), utilizing the cumulative parameters of Table 2, column 3.

$$K_{a1} = 0.185 \pm 0.016 \text{ mM}; K_{a2} = 0.0235 \pm 0.0017 \text{ mM}$$

$$K_{s1} = 10.1 \pm 1.04 \text{ mM}; K_{s2} = 1.99 \pm 0.15 \text{ mM}$$

$$k = 79.4 \pm 0.34 \text{ s}^{-1}; a = 0.189 \pm 0.010$$

Scheme 1 Adair-type model

to herein as cumulative analysis) by non-linear regression analysis of eqn. (14). The resultant parameters obtained by the cumulative analysis (Table 2, column 3) are compared with those obtained by the stepwise analysis (Table 2, column 2). Note a marked similarity between these values.

Figure 7 shows a three-dimensional plot for the enzyme-catalysed reaction as a function of G1P and AMP concentrations. The dotted lines have been calculated according to eqn. (14), using the cumulative analysis parameters of Table 2. Note a remarkable correspondence between the experimental data and

the calculated lines, attesting to the internal consistency of our general model.

Specific models for the glycogen-phosphorylase-catalysed reactions

Given that all the experimental data for the glycogen-phosphorylase-catalysed reaction, presented herein, can be explained on the basis of the above general model, the question arises as to what is the relationship between the derived parameters of the general model and the putative equilibrium and rate constants of the overall catalysis. To formulate such a relationship, we must depict the enzyme catalysis by using specific models.

Adair-type model

Relying on the outcomes of our experimental data (presented in the previous section), we proceeded to develop a minimal model (Adair-type) for the glycogen-phosphorylase-catalysed reaction in the direction of glycogen synthesis. Scheme 1 represents such a model. In this scheme, S (G1P) and A (AMP) on the left and right hand side of E (enzyme) represent their binding at two different subunits of the dimeric enzyme molecule. Since AMP and G1P both interact co-operatively with the dimeric enzyme, their dissociation constants from the first and second subunits are represented by K_{a1}/K_{s1} and K_{a2}/K_{s2} respectively. The coefficient of interaction, 'a', is to denote the magnitude by which the binding affinity of one ligand (e.g. AMP) is altered when the other ligand (e.g. G1P) is already bound. Here, we have considered that the above co-operative interaction prevails only within the same subunit. Assuming that all the binding steps conform to the rapid-equilibrium condition, and the rate-limiting step is the break down of the ES_2A_2 complex (see above), the steady-state rate of the enzyme catalysis can be represented by eqn. (17):

$$v = \frac{2 * k_{cat} * E_t ([S]^2 [A]^2 / K_{a1} K_{a2} K_{s1} K_{s2} * a^2)}{1 + (2[S]/K_{s1}) + ([S]^2 K_{s1} K_{s2}) + (2[A]/K_{a1}) + ([A]^2 K_{a1} K_{a2}) + (2[S][A]/K_{a1} K_{s1}) + (2[S][A]/K_{s1} K_{s2} K_{a1} * a) + (2[S][A]^2 / K_{s1} K_{s2} K_{a1} K_{a2} * a^2)} \quad (17)$$

The constant terms of eqn. (17) can be arranged in the same format as that of eqn. (14). The specific relationships between the parameters of eqn. (14) and the aggregated constants of eqn. (17) are shown in Table 3 (column 2). By analysing the experimental data, we obtained the magnitudes of the microscopic constants of eqn. (17) (listed at the bottom of Scheme 1), and converted these constants to the macroscopic parameters of the general equation (Table 3, column 2). Note a remarkable correspondence between the macroscopic parameters (derived from the direct analysis of the experimental data by eqn. 14; Table 3, column 1), and those calculated from the microscopic parameters (Table 3, column 2). The above correspondence justifies the internal consistency of the Adair-type model of Scheme 1.

It should be noted (from the constants of Scheme 1) that the dissociation constants of AMP (K_{a1}) and G1P (K_{s1}) from the first enzyme site are severalfold higher than the corresponding constants (i.e. K_{a2} and K_{s2}) from the second site, attesting to the co-operative interactions of these ligands to the dimeric enzyme molecule. In addition, the magnitude of 'a' being equal to 0.2, suggests that the binding of AMP is approx. 5-fold tighter when a particular subunit is already occupied by G1P. A similar situation prevails for the binding of G1P when AMP is already bound to the enzyme site.

Macroscopic parameters are taken from Table 2. The microscopic constants for both Adair-type (Scheme 1) and MWC (Scheme 2) models were derived from the best fit of the experimental data by using eqns. (17) and (18) respectively.

General model	Adair-type model	MWC model
$k = 79.7 \text{ s}^{-1}$	$k = 79.4 \text{ s}^{-1}$	$k = 83.3 \text{ s}^{-1}$
$c_{00} = 3.30 \times 10^{-3} \text{ mM}^2$	$c_{00} = K_{a1}K_{a2}$	$c_{00} = (1 + L)K_a^2K_a'^2/(K_a'^2 + LK_a^2)$
$c_{01} = 4.98 \times 10^{-3} \text{ mM}$	$c_{00} = 4.23 \times 10^{-3} \text{ mM}^2$	$c_{00} = 1.00 \times 10^{-2} \text{ mM}^2$
$c_{02} = 3.00 \times 10^{-4}$	$c_{01} = 2K_{s1}K_{s2}/K_{s1}$	$c_{01} = 2K_a^2K_a'^2/(K_a'^2 + LK_a^2)$
	$c_{01} = 8.350 \times 10^{-4} \text{ mM}$	$c_{01} = 1.55 \times 10^{-4} \text{ mM}$
	$c_{02} = K_{s1}K_{a2}/(K_{s1}K_{s2})$	$c_{02} = K_a^2K_a'^2/K_s^2(K_a'^2 + LK_a^2)$
	$c_{02} = 2.10 \times 10^{-4}$	$c_{02} = 1.58 \times 10^{-4}$
	$c_{10} = 2K_{a1}$	$c_{10} = 2K_aK_a'(K_a' + LK_a)/(K_a'^2 + LK_a^2)$
$c_{10} = 8.70 \times 10^{-2} \text{ mM}$	$c_{10} = 4.59 \times 10^{-2} \text{ mM}$	$c_{10} = 1.40 \times 10^{-1}$
$c_{11} = 2.75 \times 10^{-2}$	$c_{11} = 2K_{a2}(1/K_{s1} + 1/aK_{s1})$	$c_{11} = 4K_aK_a'^2/K_s(K_a'^2 + LK_a^2)$
	$c_{11} = 2.85 \times 10^{-2}$	$c_{11} = 3.80 \times 10^{-2}$
$c_{12} = 1.60 \times 10^{-2} \text{ mM}^{-1}$	$c_{12} = 2K_{a2}/aK_{s1}K_{s2}$	$c_{12} = 2K_aK_a'^2/K_s^2(K_a'^2 + LK_a^2)$
	$c_{12} = 1.21 \times 10^{-2} \text{ mM}^{-1}$	$c_{12} = 3.88 \times 10^{-2} \text{ mM}^{-1}$
	$c_{21} = 2/aK_{s1}$	$c_{21} = 2K_aK_a'^2/K_s^2(K_a'^2 + LK_a^2)$
$c_{21} = 1.53 \text{ mM}^{-1}$	$c_{21} = 1.05 \text{ mM}^{-1}$	$c_{21} = 2.33 \text{ mM}^{-1}$
$c_{22} = 1.81 \text{ mM}^{-2}$	$c_{22} = 1/a^2K_{s1}K_{s2}^2$	$c_{22} = K_a^2/K_s^2(K_a'^2 + LK_a^2)$
	$c_{22} = 1.40 \text{ mM}^{-2}$	$c_{22} = 2.38 \text{ mM}^{-2}$

$$K_a = (8.16 \pm 0.16) \times 10^{-3} \text{ mM}; K_s = (4.90 \pm 0.42) \times 10^{-1} \text{ mM}$$

$$K_c = (1.52 \pm 0.45) \times 10^{-1} \text{ mM}; k = 83.3 \pm 0.57 \text{ s}^{-1}; L = 263 \pm 53$$

Scheme 2 MWC model

MWC model

Since most of the structural ligand binding and kinetic data on glycogen phosphorylase have been explained in the light of the MWC model ([1–4], and references therein), we considered whether or not our present kinetic data are consistent with the above model. In this endeavour, we realized that, unlike our Adair-type model, the MWC model cannot be formulated on the basis of the experimental evidence alone. Furthermore, as noted by other investigators [9,14,15], the glycogen-phosphorylase-catalysed reaction cannot be explained by the classic MWC model, i.e. the T-state can bind the ligand(s) but the catalysis

occurs only in the R-state. Our experimental data can be explained by the following variants of the MWC model: (1) T-state only binds G1P and is inactive, (2) T-state only binds AMP and is inactive, and (3) T-state is inactive and does not bind either AMP or G1P. However, based on the structural ligand-binding and kinetic data ([1], and references therein), we opted to analyse our experimental data on the basis of the MWC model in which the T-state binds only AMP (Scheme 2).

In Scheme 2, the dissociation constants of AMP in T- and R-states are taken as K_a' and K_a respectively. The dissociation constants of AMP and G1P are taken to be independent of the state of ligation of the enzyme molecule. For example, the affinity of AMP to a particular state is the same irrespective of whether the dimeric enzyme is free from any ligand, bound with one molecule of AMP, and/or with one or two molecules of G1P. The allosteric constant (L) is taken as the ratio of the enzyme population in T- to R-states in the absence of any bound ligand. Based on a rapid equilibrium assumption, the initial rate of the enzyme-catalysed reaction for the model of Scheme 2 can be given by eqn. (18):

$$v = \frac{2 * k * E_t * ([S]^2[A]^2 / K_a^2 K_{s^2})}{(1 + [A] / K_a)^2 (1 + [S] / K_s)^2 + L(1 + [A] / K_a)^2} \quad (18)$$

Table 3 summarizes the specific relationships between the parameters of the general model (eqn. 14) and the microscopic constants of the MWC model (eqn. 18). From the best fit of the experimental data by eqn. (18), we obtained the individual constants (shown at the bottom of Scheme 2) of eqn. (18), and from these constants, we calculated the parameters of the general model (listed in Table 3, column 3). Note a remarkable correspondence between these values, once again attesting to the internal consistency of the model of Scheme 2.

It should be noted that the magnitude of L (i.e. T_0/R_0), derived from the best fit of the experimental data, is 260 at 25 °C (Scheme 2). The latter falls in the range of the L value of between 6100 at 29 °C and 11 at 4 °C [13]. In the presence of a saturating concentration of AMP, the above allosteric constant shifts to 0.75, i.e. 43 % of the total enzyme predominates in the R-state. Since G1P does not bind to the T-state, in the presence of a saturating concentration of the latter, the total enzyme is expected to populate in the R-state. We are currently investigating the sensitivity of the Hill coefficient and EC_{50} values for different variants of contemporary models for the glycogen-phosphorylase-catalysed reaction, and we will report these findings subsequently.

We thank Dr. Z. X. Wang for his help in data analysis and stimulating discussions. This work was supported by a National Science Foundation (NSF)-EPSCoR grant (9452892).

REFERENCES

- 1 Oikonomakos, N. G., Acharya, K. R. and Johnson, L. N. (1992) Posttranslational Modification of Proteins (Harding, J. J. and Crabbe, M.J.C., eds.), pp. 81–151, CRC Press, Boca Raton, FL
- 2 Madsen, N. B. (1986) *Enzymes* 3rd Ed. **17**, 365–394
- 3 Graves, D. J. and Wang, J. H. (1972) *Enzymes*, 3rd Ed. **7**, pp. 435–482
- 4 Babsy, S. J. W. and Radda, G. K. (1976) *Curr. Top. Cell. Regul.* **10**, 89–160
- 5 Childress, C. C. and Sacktor, B. (1970) *J. Biol. Chem.* **245**, 2927–2936
- 6 Helmreich, E. and Cori, C. F. (1964) *Proc. Natl. Acad. Sci. U.S.A.* **51**, 131–138
- 7 Hedrick, J. L. and Fischer, E. H. (1965) *Biochemistry* **4**, 1337–1343
- 8 Engers, H. D., Shechosky, S. and Madsen, N. B. (1970) *Can. J. Biochem.* **48**, 746–754
- 9 Klinov, S. V. and Kurganov, B. L. (1994) *Arch. Biochem. Biophys.* **312**, 14–21
- 10 Webb, M. R. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4884–4887
- 11 Sergienko, E. A. and Srivastava, D. K. (1994) *Anal. Biochem.* **221**, 348–355
- 12 Monod, J., Wyman, J. and Changeux, J. P. (1965) *J. Mol. Biol.* **12**, 88–118

-
- 13 Kastenschmidt, L. L., Kastenschmidt, J. and Helmreich, E. (1968) *Biochemistry* **7**, 4543–4556
 - 14 Bresler, S. and Frisov, L. (1971) *Biopolymers* **10**, 1187–1205
 - 15 Madsen, N. B. and Shechosky, S. (1967) *J. Biol. Chem.* **242**, 3301–3307
 - 16 Wang, Z. X., Killilea, S. D. and Srivastava, D. K. (1993) *Biochemistry* **32**, 1500–1509
 - 17 Wang, Z. X. and Srivastava, D. K. (1994) *Anal. Biochem.* **216**, 15–26
 - 18 Fischer, E. H. and Krebs, E. G. (1962) *Methods. Enzymol.* **5**, 369–373
 - 19 Cohen, P., Duewar, T. and Fischer, E. H. (1971) *Biochemistry* **10**, 2683–2694
 - 20 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
 - 21 Wong, J. T. F. (1975) *Kinetics of Enzyme Mechanisms*, pp. 21–38, Academic Press, London
 - 22 Bardsley, W. G. and Childs, R. E. (1975) *Biochem. J.* **149**, 313–328
 - 23 Helmreich, E., Michaelides, M. C. and Cori, C. F. (1967) *Biochemistry* **6**, 3695–3710
 - 24 Reich, J. G., Wangermann, G., Falck, M. and Rohde, K. (1972) *Eur. J. Biochem.* **26**, 368–379
 - 25 Lowry, O. H., Schulz, D. W. and Passonneau, J. V. (1967) *J. Biol. Chem.* **242**, 271–280
 - 26 Cori, G. T. and Cori, C. F. (1940) *J. Biol. Chem.* **135**, 733–756
 - 27 Kasvinsky, P. J., Shechosky, S. and Fletterick, R. J. (1978) *J. Biol. Chem.*, **253**, 9102–9106
 - 28 Martin, J. I., Johnson, L. N. and Withers, S. G. (1990) *Biochemistry* **29**, 10745–10757
 - 29 Sprang, S. R. and Fletterick, R. J. (1979) *J. Mol. Biol.* **131**, 523–551
 - 30 Wang, Z. X. and Kihara, H. (1990) *J. Theor. Biol.* **143**, 455–464
-

Received 8 May 1997/27 June 1997; accepted 9 July 1997