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RATE AND EQUILIBRIUM CONSTANTS OF THE TETRAMERIZATION PROCESS OF PHOSPHORYLASE *b*

INFLUENCE OF AMP AND Mg^{2+}

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The association-dissociation equilibrium of phosphorylase *b* at different enzymatic concentrations has been studied at 25°C in this paper, pointing out how this equilibrium is affected by both AMP and Mg^{2+} concentrations. It has also been proved that association of phosphorylase *b* in the presence of AMP and Mg^{2+} follows second-order and first-order rate laws in the direction of tetramerization and dimerization, respectively. Rate constants have also been calculated and their dependence upon protein, AMP and Mg^{2+} concentrations studied thoroughly.

1. Introduction

Glycogen phosphorylase (EC 2.4.1.1, 1,4-α-D-glucan : orthophosphate α-D-glucosyltransferase) is a key enzyme in the regulation of carbohydrate metabolism [1].

Glycogen phosphorylase *b* from rabbit skeletal muscle may exist in an equilibrium between tetrameric and dimeric species. In the absence of its allosteric activator, dimer-dimer interaction of phosphorylase *b* is very weak so that it is essentially a dimer [2–4]. In the presence of its activator, AMP, the percentage of tetramer increases, the equilibrium being highly dependent on temperature [3–6]. The AMP-induced association may be enhanced principally by Mg^{2+} [7], glucose 1-phosphate and phosphate [5].

Wang et al. [8] showed by ultracentrifugation that the effect of AMP concentration on the association takes place in two stages. As AMP concentration is increased to 1 mM, enhanced enzyme tetramerization is observed. Further increase in AMP concentration results in partial reversal of enzyme association.

Weak nucleotide activators do not induce association of phosphorylase *b* [4] although an activator plus phosphate or glucose 1-phosphate yields a small increase in tetramer percentage [5].

Although the association-dissociation process seems to be related to the catalytic activity of phosphorylase [2,9], since the tetramer seems to possess a lower specific activity [8,10], the association equilibrium of phosphorylase has not been studied in depth. The few data available to date in the bibliography are discordant, at least from a quantitative point of view [11–13], and no systematic study has been carried out taking into account the different factors involved in this equilibrium. On the other hand, the determination of

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equilibrium constants is necessary for the understanding of the allosteric effector binding curve and therefore for the determination of thermodynamic parameters of this binding.

The tetramerization equilibrium of glycogen phosphorylase *b* at 25°C has been studied in this paper. The equilibrium constant has been determined at different enzyme, AMP and Mg^{2+} concentrations using light-scattering measurements.

The orders of both the dimerization and tetramerization reactions have been also determined as well as the rate constants of both processes at every concentration of AMP and Mg^{2+}.

2. Materials and methods

Phosphorylase *b* was obtained from rabbit skeletal muscle by the method of Krebs et al. [14]. The enzyme was recrystallized three times before use.

The enzyme concentration was measured spectrophotometrically using an extinction coefficient, $E^{1\%}_{1cm}$, of 13.2 at 280 nm [15]. The enzyme activity was assayed in the direction of glycogen breakdown following the procedure first described by Helmreich and Cori [16].

The buffer used in all experiments was 50 mM glycylglycine, 50 mM KCl and 0.2 mM EDTA, adjusted to pH 6.9 at 25°C. A molecular weight of 97 500 per monomer was used [17].

Light-scattering measurements were made using an FICA model 42000 photogoniodiffusometer provided with a xylene bath thermostated at 25 ± 0.1°C. Calibration was done using benzene, assuming that its Rayleigh ratio at 546 nm has a value of 6.3×10^{-6} cm^{-1} [18].

Calculation of molecular weights was accomplished making use of Debye's equation [19]; modified by Townend and Timasheff [20], for systems undergoing an association-dissociation equilibrium:

$$\frac{Kc}{R_{90°}} = \frac{1}{\overline{M}_w} + Bc = \frac{1}{\overline{M}_{w,app}}$$

$$K = 2\pi^2 n^2 (dn/dc)^2 / N_A \gamma^4 \quad *$$

where $\overline{M}_w$ is the weight average molecular weight, B the second virial coefficient for the nonaggregated enzyme, c the protein concentration in g/ml, $R_{90°}$ the Rayleigh ratio of the solution at an angle of 90°, dn/dc the specific refractive index increment of the protein, n the refractive index of the solvent, γ the wavelength of primary light in vacuum (540 nm) and $\overline{M}_{w,app}$ the apparent molecular weight.

In our case, the second virial coefficient is negligible for phosphorylase *b*, due to working conditions [20].

The photogoniodiffusometer has been equipped with a graphic recorder to decrease as much as possible the error in the scattering measurements. In this way an average value was obtained, thus reducing the influence of mistakes that could be produced taking results at time intervals.

The dn/dc values for phosphorylase *b* were measured with a Carl Zeiss Jena LI 3 differential interferometer. Light-scattering and dn/dc measurements were carried out under the same experimental conditions.

The tetramerization kinetics of phosphorylase *b*, in the presence of AMP and magnesium acetate, were started by adding 1 M magnesium acetate to the solution of AMP and enzyme, previously thermostated at 25°C. The addition of magnesium acetate and subsequent stirring of the solution was carried out in a maximum time of 10–15 s in most cases, to allow a determination of $(dI/dt)_0$ as accurately as possible (see appendix A).

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* The right formula is:

$$\frac{K^*c}{R_\theta} = \frac{1}{\overline{M}_w} P^{-1}(\theta) + Bc$$

where R_θ is the measured excess scattering intensity of solution over that of pure solvent and $P^{-1}(\theta)$ the particle scattering function in terms of angle θ°. When scattering particles are isotropic and less than 1/20th of the wavelength of the light used (phosphorylase *b* fulfils these requirements, since the tetramer of phosphorylase *b* is a rectangular prism with dimensions 55, 109, 132 Å [21]). $P^{-1}(\theta)$ reduces to unity and light-scattering measurements to obtain molecular weights can be performed at any angle, usually at 90° [22]. The dissymmetry of scattered light (Z_d) has also been calculated and its value extrapolated to zero concentration (intrinsic dissymmetry). This extrapolation is lower than 1.05 in all cases which implies an error lower than 4% in light-scattering measurements [22], a conclusion already obtained by Steiner et al. [11].

3. Results

Values of dn/dc for the protein (table 1), obtained under the same experimental conditions used in light scattering, do not show significant differences when the protein is either alone or in the presence of changing concentrations of AMP and Mg^{2+}. Linearity of the results was perfect, irrespective of the conditions under which the measurements were carried out.

In the absence of ligands, an apparent molecular weight of 200 000 was obtained at any enzyme concentration (fig. 1A), in agreement with the bibliographic datum of 195 000 for the dimer of phosphorylase *b* [17].

From the slope of the line in fig. 1A, it can be inferred that the second virial coefficient is practically zero, therefore, little interaction among dimeric species exists. Since this coefficient does not change with aggregation [20] we can assume it to be negligible under our measuring conditions.

An increase in the molecular weight and a slightly negative slope can be observed in the presence of AMP (fig. 1B–F), which is the typical behavior of systems undergoing an association-dissociation equilibrium [20].

According to Wang et al. [8], association induced by AMP binding to the enzyme shows a biphasic behavior as a function of AMP concentration. As the nucleotide concentration is raised to 1 mM, enhancement of tetramerization is observed, while further increase in AMP concentration induces partial reversal of the equilibrium. Since there are two AMP-binding sites per enzyme monomer, this effect, as was suggested by Wang et al. [8], can be attributed to saturation of the second type of binding sites, possibly due to the presence of an AMP (second site)-pyridoxal 5′-phosphate interaction that weakens the AMP (first site)-enzyme interaction. The existence of these two sites was first considered by these authors and proved later by Buc et al. [23] through equilibrium dialysis and by Madsen et al. [24] from X-ray diffraction patterns.

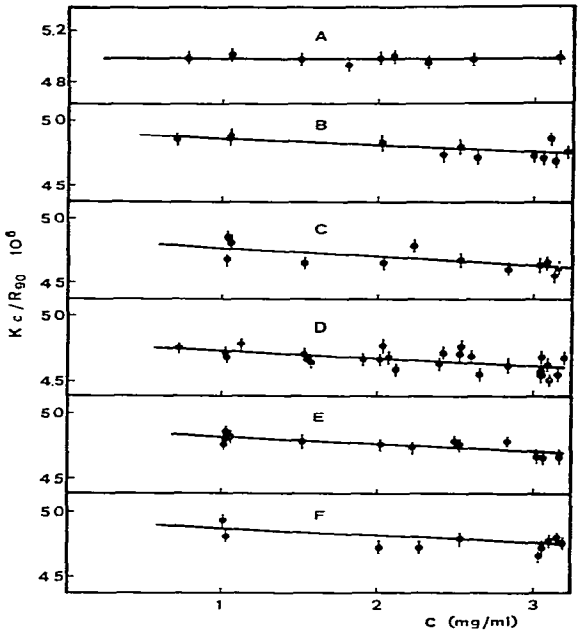

Fig. 1. Reciprocal weight average molecular weight of phosphorylase *b* at 25°C as a function of protein concentration. [AMP]: (A) 0, (B) 0.1, (C) 0.5 mM, (D) 1, (E) 5, (F) 10 mM.

Table 1
Specific refractive index increment

Temperature (°C)	Experimental conditions	dn/dc (ml g^{-1})
25	Phosphorylase *b*	0.185 (4)
25	Phosphorylase *b*	0.183 ± 0.002
25	Phosphorylase *b* + 10 mM AMP	0.187 ± 0.003
25	Phosphorylase *b* + 1 mM AMP + 55 mM Mg^{2+}	0.180 ± 0.003
25	Phosphorylase *b* + 1 mM AMP + 10 mM Mg^{2+}	0.182 ± 0.002
25	Phosphorylase *b* + 1 mM AMP + 30 mM Mg^{2+}	0.184 ± 0.002
25	Phosphorylase *b* + 5 mM AMP + 10 mM Mg^{2+}	0.183 ± 0.002
25	Phosphorylase *b* + 10 mM AMP + 10 mM Mg^{2+}	0.187 ± 0.003

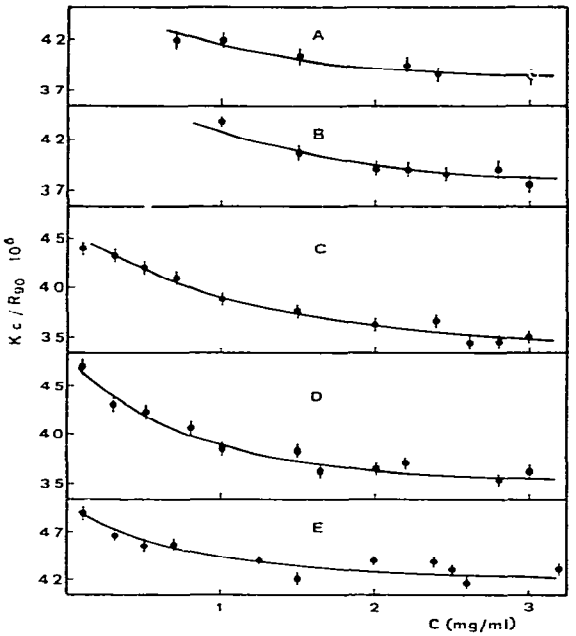

Fig. 2. Reciprocal weight average molecular weight of phosphorylase *b* at 25°C in the presence of 10 mM magnesium acetate as a function of protein concentration. [AMP]: (A) 10. (B) 5. (C) 1. (D) 0.5. (E) 0.1 mM.

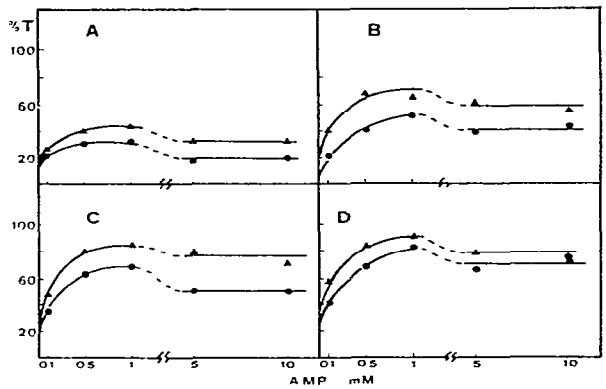

Fig. 3. Tetramer percentage of phosphorylase *b* at 25°C as a function of AMP concentration. [Mg^{2+}]: (A) 10. (B) 20. (C) 35. (D) 50 mM.

The presence of Mg^{2+} has a strong effect on AMP-induced enzyme aggregation, though the behavior is qualitatively similar to that observed in the absence of Mg^{2+} (fig. 2). At any given concentration of AMP and protein, the tetramer percentage increases with Mg^{2+} concentration (fig. 3). On the other hand, partial reversal of the equilibrium, related to saturation of the low-affinity AMP sites in the enzyme, becomes more important.

A previous knowledge of the orders of both the dimerization and tetramerization processes has been necessary to calculate rate constants. This task has been accomplished using Van't Hoff's differential method (appendix A).

Values for the order of the tetramerization reac-

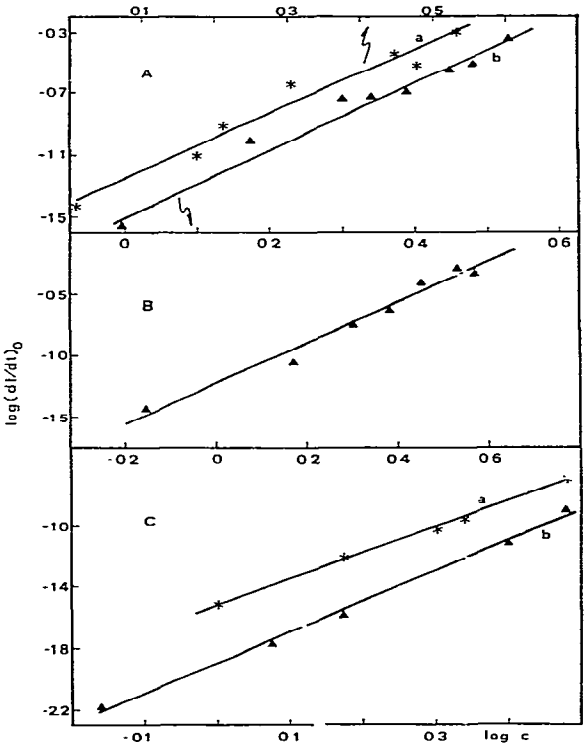

Fig. 4. Van't Hoff plot. It has been used to calculate the order of the tetramerization process at 25°C in the presence of 10 mM Mg^{2+}. (Aa) 0.5 mM AMP, slope 2.1; (Ab) 5 mM AMP, slope = 2.2; (B) 1 mM AMP, slope = 1.7; (Ca) 10 mM AMP, slope = 1.7; (Cb) 0.1 mM AMP, slope = 2.0.

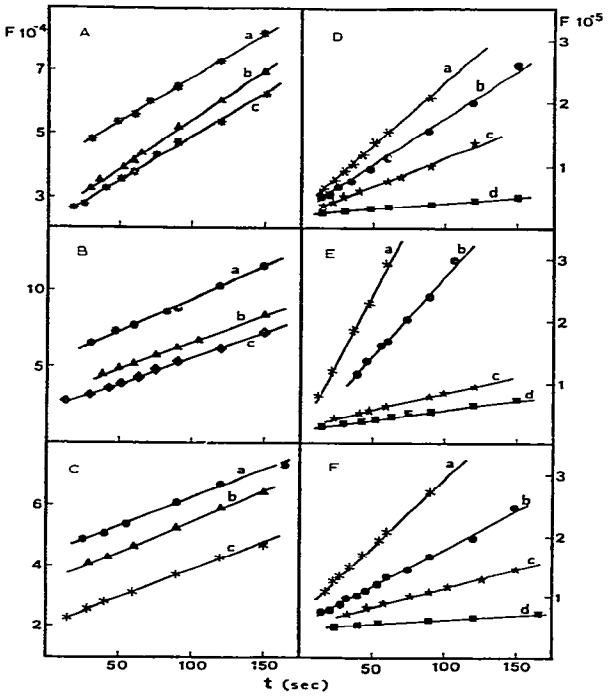

Fig. 5. Fitting of the association kinetics to an equilibrium which follows second-order and first-order reversible rate law in the direction of tetramerization and dimerization, respectively. (A) 10 mM Mg^{2+}, 0.5 mM AMP at different protein concentration (mg/ml): (a) 1.5, (b) 2.8, (c) 3.4; (B) 10 mM Mg^{2+}, 1 mM AMP at different protein concentrations (mg/ml): (a) 1, (b) 2, (c) 3.4; (C) 10 mM Mg^{2+}, 5 mM AMP at different protein concentrations (mg/ml): (a) 1, (b) 1.5, (c) 3; (D) 0.5 mM AMP, protein concentration 3 mg/ml; (E) 1 mM AMP, protein concentration 3 mg/ml; (F) 5 mM AMP, protein concentration 1 mg/ml (in D–F: (a) 10 mM Mg^{2+}, (b) 20 mM Mg^{2+}, (c) 35 mM Mg^{2+}, (D) 50 mM Mg^{2+}).

Table 2
Rate and equilibrium constants
The constants have been calculated at enzyme concentrations ranging from 0.6 to 3.7 mg/ml.

AMP (mM)	K_{eq} (M^{-1}) ($\times 10^{-4}$)	k_1 (M^{-1} min^{-1}) ($\times 10^{-4}$)	k_{-1} (min^{-1})
0.1	1.7±0.3	0.7±0.2	0.42±0.05
0.5	5.3±0.6	1.8±0.3	0.36±0.04
1	5.7±0.5	2.2±0.3	0.38±0.03
5	2.8±0.4	1.2±0.2	0.44±0.05
10	2.7±0.4	1.0±0.2	0.36±0.04

tion are in the range from 1.7 to 2.2 with no dependence on AMP and Mg^{2+} concentrations (fig. 4). Therefore, it can be concluded that, at 25°C and within the limits of experimental error, the tetramerization reaction follows a second-order rate law with respect to enzyme concentration.

Once the order of the tetramerization reaction has been determined, and assuming the dimerization reaction to be first order, the task of calculating the rate constants for the process has been accomplished (appendix B).

Fig. 5 shows the first expression in eq. B2 plotted versus time, at several concentrations of enzyme, AMP and Mg^{2+}. Fits are really good, with correlation coefficients higher than 0.996 in all cases. It can be concluded that the association-dissociation reaction for phosphorylase *b*, as induced by AMP and in the presence of Mg^{2+}, is a reversible process that follows second-order and first-order rate laws in the direction of tetramerization and dimerization, respectively, independently of AMP and Mg^{2+} concentrations. The

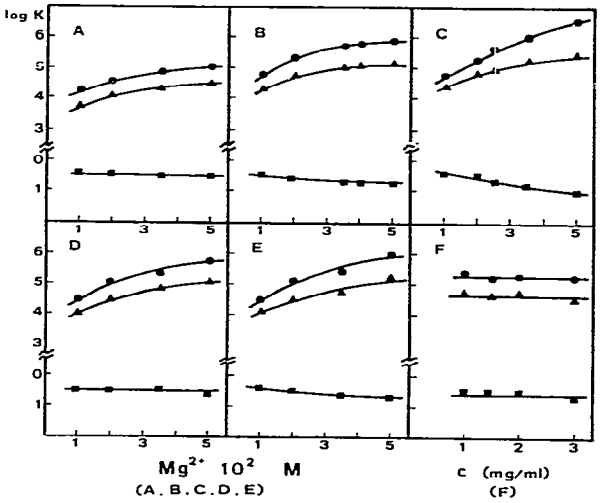

Fig. 6. Logarithm of the equilibrium constant (●, M^{-1}), rate constant of tetramerization (▲, M^{-1} min^{-1}) and rate constant of dissociation of tetramers into dimers (■, min^{-1}) plotted as a function of Mg^{2+} (M) ($\times 10^2$). [AMP]: (A) 0.1, (B) 0.5, (C) 1, (D) 5, (E) 10 mM; (E) logarithm of the equilibrium and rate constants plotted against enzyme concentration (mg/ml) in 1 mM AMP and 20 mM Mg^{2+}.

slope of these straight lines is k_1 which is found to be independent of enzyme concentration (fig. 5A–C). This fact has been proved in all cases for, at least, six enzyme concentrations in the range from 0.6 to 3.7 mg.ml^{-1}.

Values of K, k_{-1}, k_1 are shown in table 2. These constants are independent of enzyme concentration in the studied range.

AMP influences both the equilibrium constant and the rate constant of tetramerization, while the rate constant of dimerization remains practically unchanged, within the limits of experimental error. Therefore, variations observed in the equilibrium constant with AMP concentration are due to the changes induced in the rate constant of the association process.

Fig. 6 shows how the rate and equilibrium constants depend upon the concentration of Mg^{2+} present in the solution. In a similar way to the results obtained as a function of AMP concentration, increasing concentrations of the divalent cation yield a great rise in both the aggregation levels and the rate constant of tetramerization, while the rate constant of dimerization diminishes only slightly as Mg^{2+} concentration is increased.

The strong rise produced in the aggregation levels of the enzyme by the presence of Mg^{2+}, in comparison with the values reached when solely in the presence of the allosteric activator, indicates clearly that Mg^{2+} has a specific effect on the aggregation process, possibly through formation of a ternary AMP-enzyme-Mg^{2+} complex.

4. Discussion

While it is widely accepted that, at high temperature and in the absence of effectors, phosphorylase *b* remains principally in the dimeric form, discrepancies appear when AMP is present in the solution.

Kastenschmidt et al. [25] and Buc and Buc [4] state that association in the presence of AMP is significant only at temperatures lower than 13°C. Appleman [26] and Wang et al. [8], however, point out that under the same conditions, association is appreciable even at high temperatures. Differences between these results may be due partly to the use of different techniques and buffer solutions, partly to the fact that measurements have been carried out in a rather incomplete way, working generally at only one concentration of enzyme and AMP.

Our results are halfway between those aforementioned, since we have observed systematically the existence of a certain amount of tetramer at 25°C, though its values were in all cases lower than those reported by Wang et al. [8].

On the other hand, Steiner et al. [11] point out that, when only in the presence of AMP, phosphorylase *b* is found totally in the dimeric form. Disagreement with out results can be accounted for by the fact that these authors have carried out their experiments at a pH of 8.6, while recent measurements made in our laboratory (unpublished data) show that pH has a great influence on the tetramer percentage.

Silonova and Kurganov [12] stated that the association constant varies appreciably from one enzyme preparation to another. Identical experiments have been carried out to verify this assertion, having arrived at the conclusion that for an enzyme with an activity of 100% according to the procedure described by Helmreich and Cori [16], the aggregation properties do not change from one preparation to another, within the limits of experimental error. Besides, it has been observed that 'ageing' of the enzyme produces a decrease in the enzyme activity, with simultaneous alterations in the aggregation state. Therefore, in all the experiments reported in the present paper, the enzyme used had an activity of 100%.

As it has been clearly shown in the results, the process of association-dissociation of phosphorylase *b*, induced by AMP in the presence of Mg^{2+} at 25°C, is an equilibrium that follows second-order and first-order reversible rate laws in the direction of tetramerization and dimerization, respectively.

Buc et al. [13] proved that, at low temperatures and in the presence of 0.8 mM AMP, the association-dissociation equilibrium obeys a first-order reversible rate law in both directions, the rate constant of dissociation of tetramer into dimers being equal to 0.05 min^{-1}. Nevertheless, the measurement were rather incomplete, since the authors generally worked at only one concentration of

enzyme and AMP, and without taking into account the existence of two binding sites for the allosteric effector. Moreover, the conformational transition reported at 18°C may affect the association equilibrium of phosphorylase *b* [7]. In addition to this, our results have been obtained in the presence of Mg^{2+} that could modify the mechanism of the association process.

The rate constant of dissociation of tetramer into dimers remains constant as a function of AMP concentration. This fact seems to imply the existence of only one tetrameric species, independent of the amount of AMP present in the solution. The rate constant of dissociation of tetramer into dimers has a value of 0.4 ± 0.05, thus 1 mol of tetramer will be completely dissociated in 2.5 ± 0.3 min.

The presence of Mg^{2+} results in significant increase in both the equilibrium constant and the rate constant of tetramerization, this effect reaching its saturation point at a Mg^{2+} concentration of about 50 mM.

Finally, a conformational transition of the enzyme as a function of protein concentration in the range from 1.7 to 2.5 mg/ml has been recently described through different techniques [27,28]. Since the molecular weights and the rate equilibrium constants show no lack of continuity as a function of protein concentration, the above-mentioned transition must be supposed to happen between species of the same molecular weight.

Appendix A

The association-dissociation equilibrium for phosphorylase *b* can be expressed:

$$2D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} T \tag{A1}$$

$$K_{ass} = \frac{[T]}{[D]^2} \tag{A2}$$

where k_1 is the rate constant of tetramerization and k_{-1} the rate constant of dissociation of tetramer into dimers.

The rate of the association process is given by the expression:

$$v = k_1[D]^a - k_{-1}[T]^b \tag{A3}$$

where a and b are the orders of the forward and reverse reactions, respectively.

The initial concentration of tetramer (without Mg^{2+}) is very low [22], the initial rate can be expressed as:

$$v_0 = (d[T]/dt)_0 = k_1[D_0]^a \tag{A4}$$

an equation that can be rewritten in the following form:

$$v_0 = \text{constant}(dI/dt)_0 = k_1(c/M_d)^a \tag{A5}$$

where $(dI/dt)_0$ is the initial slope of the curve that shows the intensity of scattered light as a function of time, and c the dimer concentration.

If we convert eq. A5 to the logarithmic form:

$$\log\left(\frac{dI}{dt}\right)_0 = \text{constant} + a \log c \tag{A6}$$

Hence, $\log(dI/dt)_0$ is a linear function of $\log c$, its slope being the order of reaction in the direction of tetramerization.

Appendix B

If the tetramerization and dimerization reactions are of second and first order respectively, then the rate at which the concentration of tetramer changes will be given by:

$$v = k_1[D]^2 - k_{-1}[T] \tag{B1}$$

Taking into account that:

$$[T] = \frac{cw}{2M_d}; \qquad [D] = \frac{c(1-w)}{M_d}; \qquad w = \frac{M_w}{M_d} - 1$$

Integration of eq. B1 leads to the expression:

$$F = A + k_1 t \tag{B2}$$

$$F = \frac{M_d w_{eq}}{2c(1-w_{eq}^2)} \cdot \ln \frac{1 - w w_{eq}}{w_{eq} - w}$$

$$A = \frac{M_d w_{eq}}{2c(1-w_{eq}^2)} \cdot \ln \frac{1 - w_0 w_{eq}}{w_{eq} - w_0}$$

where M_d is the molecular weight of the dimer, c the protein concentration (in mg/ml) and w the weight fraction of tetramer (the subscripts 0 and eq denote initial and equilibrium conditions, respectively).

The plot of the first expression in eq. B2 versus time allows calculation of the rate constant of tetramerization (k_1). On the other hand, the equilibrium constant expressed as a function of tetramer percentage can be written as:

$$K_{ass} = \frac{[T]}{[D]^2} = \frac{w_{eq} M_d}{(1 - w_{eq}^2) 2c} \quad \text{(B3)}$$

Lastly, the constant of dissociation of tetramer into dimers (K_{-1}) can be determined making use of the relationship:

$$k_{-1} = k_1 / K_{ass} \quad \text{(B4)}$$

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