

Study of self-association of muscle glycogen phosphorylase *b* by sedimentation equilibrium methods

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Rabbit skeletal muscle glycogen phosphorylase *b* (EC 2.4.1.1) is an excellent subject for the study of the mechanisms of regulation of enzymic activity, including allosteric and dissociative mechanisms and regulation by covalent modification [1,2]. Phosphorylase *b* molecule is a dimer with molecular mass of 194.8 kDa capable of associating into tetramers in the presence of allosteric activator AMP. An increase in enzyme concentration, a decrease in temperature, or the presence of sulphate ions promotes the formation of the tetramer [2,3]. It has been shown that the conversion of dimers into the tetrameric form is accompanied by the loss of enzymic activity because of the steric screening of the active sites with respect to high-molecular-mass substrate glycogen [4,5]. The dimer-tetramer transition can be considered as a test of changing the conformation of the phosphorylase *b* molecule induced by the binding of the allosteric effectors [5-9]. At neutral pH and low protein concentrations the phosphorylase *b* molecule exists in the dimeric form.

The self-association of phosphorylase *b* has been well investigated by the sedimentation velocity method in an analytical ultracentrifuge [7-9]. In the present study we have studied the association behaviour of phosphorylase *b* by sedimentation equilibrium methods. A dimer-tetramer model provides an appropriate description of the reversible association of phosphorylase *b*.

Analytical ultracentrifugation was performed with a Beckman Model E analytical ultracentrifuge equipped with a scanning absorption optical system. The 'low-speed' and 'high-speed' meniscus-depletion sedimentation equilibrium experiments were run with a six-hole An-G Ti rotor with five double-sector, 12 mm cells and a counterbalance at rotor speeds of 7200 and 10000 r.p.m. respectively. The experimental time required for the attainment of equilibrium was established by running at a given rotor speed until the scans were invariant for 3 h.

To reduce the transient time to equilibrium at low-speed experiments we used solution column lengths of 1-1.5 mm and 2-2.5 mm. In

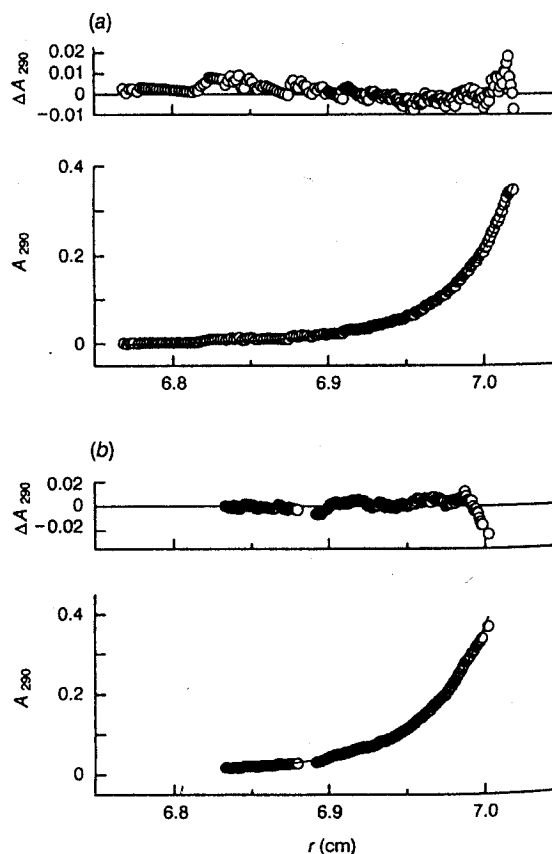
the latter case we used overspeeding (10000 r.p.m., 3 h) [10,11]. In low-speed experiments the equilibrium was achieved after 24 h.

High-speed experiments were run at 10000 rev./min. Overspeeding (15000 r.p.m., 3 h) was used to shorten the transient time to equilibrium [11]. Sedimentation equilibrium was attained after 24 h for a solution column length of 2.5-3.0 mm. FC-43 oil was placed on the bottom of

Figure 1

Concentration distribution and distribution of the residuals as the functions of radial position for phosphorylase *b* at sedimentation and chemical equilibrium after 26 h at 10000 r.p.m.

Overspeeding (15000 r.p.m., 3 h) was used. Sedimentation was at 17 °C in 50 mM glycylglycine buffer, pH 6.8, containing 0.2 mM EDTA, 0.125 M K₂SO₄, 1 mM AMP and 4 mM glucose 1-phosphate. FC-43 oil was placed at the bottom of the cells. The fitting line is calculated for a thermodynamically ideal homogeneous dimer-tetramer association of phosphorylase *b*. The loading concentration was 0.37 mg/ml (a) and 0.92 mg/ml (b)



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double-sector centrepieces to create a bottom meniscus.

Sedimentation analysis of association of phosphorylase *b* dimers (D) to tetramers (T) was performed in accordance with the thermodynamically ideal model. The concentration distribution of the enzyme as a function of radial position (*r*) in the cell at sedimentation and chemical equilibrium is described by [12,13]:

$$c_r = c_b \exp [AM(r^2 - r_b^2)] + K_{1-2} c_b^2 \exp [2AM(r^2 - r_b^2)] + A_0 \quad (1)$$

where *c_r* is the protein concentration at radius *r*, *c_b* is the concentration of the dimeric form at the radial position of the cell bottom (the concentra-

tions are expressed as absorbances), *r_b* is the radial position of the cell bottom, $A = (1 - \bar{v}\rho)\omega^2/2RT$, where \bar{v} is the partial specific volume, which is the same for dimer and tetramer (0.735 ml/g at 17 °C), ρ is the solution density (1.0146 g/ml at 17 °C), ω is the rotor angular velocity in radians per second, *R* is the gas constant, *T* is the absolute temperature, *M* is the dimer molecular mass (194.8 kDa), *K₁₋₂* is the association constant for the dimer-tetramer equilibrium and *A₀* is the baseline offset.

The molar association constant $K_{ass} = [T]/[D]^2$ may be calculated with the use of the parameter *K₁₋₂* as described in [12]: $K_{ass} = K_{1-2}\epsilon/l/2$, where ϵ is the molar absorp-

Figure 2

Concentration distribution and distribution of the residuals as the functions of radial position for phosphorylase *b* at sedimentation and chemical equilibrium after 24 h at 7200 r.p.m.

Sedimentation was at 17 °C in 50 mM glycylglycine buffer, pH 6.8, containing 0.2 mM EDTA, 0.125 M K₂SO₄, 1 mM AMP and 4 mM glucose 1-phosphate. The fitting line is calculated for a thermodynamically ideal homogeneous dimer-tetramer association of phosphorylase *b*. The loading concentration was 2 mg/ml (a) and 4 mg/ml (b)

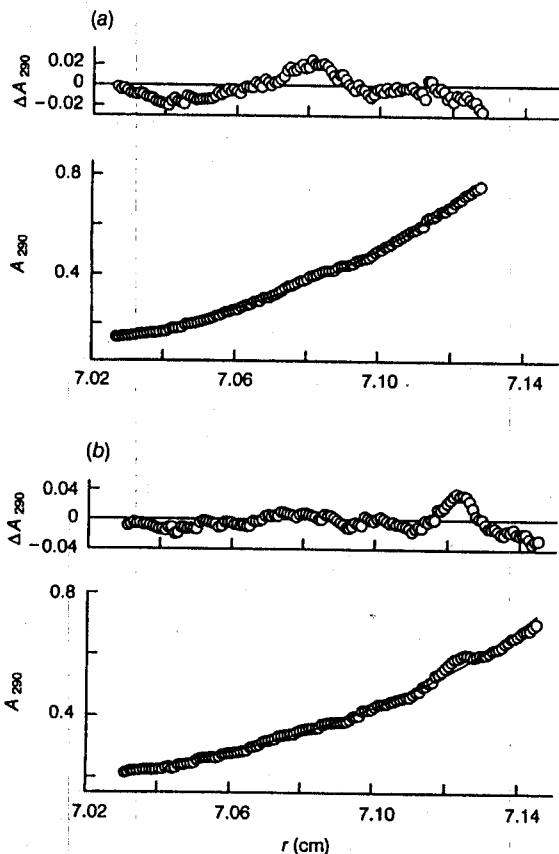
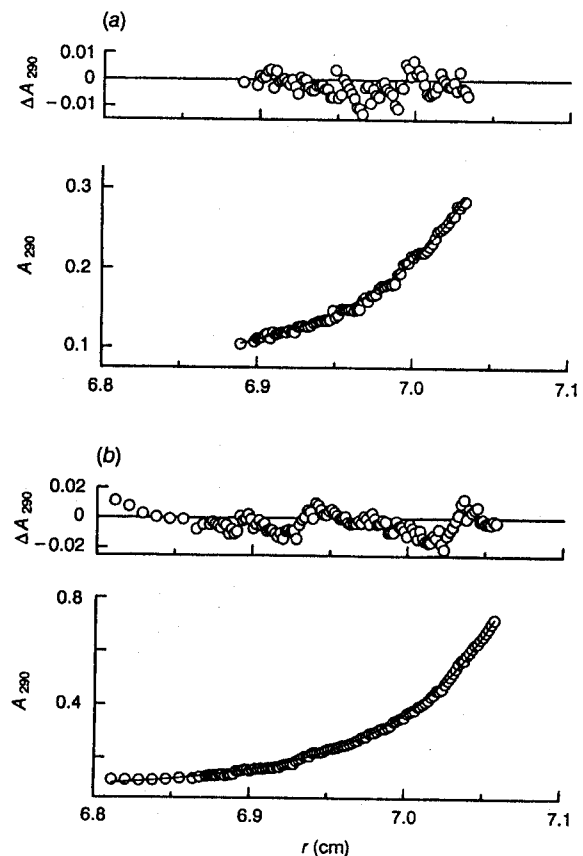


Figure 3

Concentration distribution and distribution of the residuals as the functions of radial position for phosphorylase *b* at sedimentation and chemical equilibrium after 24 h at 7200 r.p.m.

Overspeeding (10000 r.p.m., 3 h) was used. Sedimentation was at 17 °C in 50 mM glycylglycine buffer, pH 6.8, containing 0.2 mM EDTA, 0.125 M K₂SO₄, 1 mM AMP and 4 mM glucose 1-phosphate. FC-43 oil was placed at the bottom of the cells. The fitting line is calculated for a thermodynamically ideal homogeneous dimer-tetramer association of phosphorylase *b*. The loading concentration was 0.28 mg/ml (a) and 0.56 mg/ml (b)



tion coefficient calculated for the dimer ($\epsilon_{290} = 1.7 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and l is the optical path length (1.2 cm).

To find K_{1-2} we have developed software for an IBM-compatible computer. The software is based on a simultaneous approximation of the experimental curves obtained at different initial concentrations of phosphorylase b and different rotor speeds by eqn. (1). Optimization was verified by means of the sum of squares of the deviations of the calculated values of c_r from the experimental ones. The optimum K_{1-2} value, as the only globally varying parameter, was found by means of the one-dimensional optimization method at fixed values of A , M and r_b . At each specified value of K_{1-2} on the experimental curve, the optimum values of c_b were formed by the one-dimensional optimization method, and the A_0 values were determined by the least-squares method.

The value of the association constant K_{1-2} was determined simultaneously as the global parameter for all the experimental data obtained. The total number of sedimentation patterns used in our calculations was ten. In high-speed sedimentation experiments the phosphorylase b loading concentration was varied in the range 0.28–0.92 mg/ml (three patterns). Typical sedimentation patterns are presented in Figure 1.

In low-speed sedimentation experiments the phosphorylase b loading concentration was varied in the range 0.28–4.0 mg/ml (seven patterns). Typical sedimentation patterns are presented in Figures 2 and 3. The values of K_{1-2} and K_{ass} were 14.7 and $1.5 \times 10^6 \text{ M}^{-1}$ respectively. The theoretical curves obtained from eqn. (1) and the values of K_{1-2} , c_b and A_0 , which were the result of optimization, are shown in Figures 1–3 as the solid lines. Figures 1–3 also show the residual distribution (differences between the experimental and calculated values, ΔA_{290}). As can be seen from Figures 1–3, the model of self-association of phosphorylase b of the $2D \rightleftharpoons T$ type adequately describes the equilibrium sedimentation patterns.

To check the possible association of phosphorylase b into associates of higher order than a tetramer, we considered alternative dimer–tetramer–hexamer and dimer–tetramer–octamer models. These models are described by [12]:

$$c_r = \exp[\ln(b_b) + AM(r^2 - r_b^2)] + \exp\{2[\ln(c_b)] + \ln(K_{1-2}) + 2AM(r^2 - r_b^2)\} + \exp\{n[\ln(c_b)] + \ln(K_{1-n}) + nAM(r^2 - r_b^2)\} + A_0 \quad (2)$$

where c_r is the concentration at the radial position r , c_b is the concentration of the dimeric form of the enzyme at the radial position of the cell bottom, n is the stoichiometric factor ($n = 3$ or 4 for a hexamer or an octamer respectively), K_{1-2} is the association constant for the dimer–tetramer equilibrium and K_{1-n} is the association constant for the dimer– n -mer equilibrium (K_{1-3} and K_{1-4} are the association constant for the dimer–hexamer and dimer–octamer equilibria respectively). Note that eqn. (2) is presented in a form that excludes the appearance of negative value of the parameters K_{1-2} and K_{1-n} . To find the optimum values of two of the global parameters K_{1-2} , K_{1-3} or K_{1-4} , we used the Nelder–Mead minimization algorithm [14].

For the dimer–tetramer–hexamer equilibrium, the $\ln(K_{1-3})$ was (-9.45) and K_{1-3} was 7.8×10^{-5} . For the dimer–tetramer–octamer model, $\ln(K_{1-4})$ was (-10.9) and K_{1-4} was 1.8×10^{-5} , and the contribution of the term corresponding to a hexamer or an octamer in eqn. (2) can be neglected. The value of K_{1-2} calculated for both models was 14.5 and was practically identical with that for the dimer–tetramer model. Therefore we conclude that the $2D \rightleftharpoons T$ model is the best approximation to the sedimentation equilibrium patterns of muscle phosphorylase b and that associations of higher orders are absent.

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