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Biophysical Chemistry 113 (2005) 61–66

Biophysical Chemistry

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Effect of osmolytes on the interaction of flavin adenine dinucleotide with muscle glycogen phosphorylase *b*

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Received 3 June 2004; received in revised form 27 July 2004; accepted 27 July 2004
Available online 27 September 2004

Abstract

The effect of three osmolytes, trimethylamine *N*-oxide (TMAO), betaine and proline, on the interaction of muscle glycogen phosphorylase *b* with allosteric inhibitor FAD has been examined. In the absence of osmolyte, the interaction is described by a single intrinsic dissociation constant (17.8 μM) for two equivalent and independent binding sites on the dimeric enzyme. However, the addition of osmolytes gives rise to sigmoidal dependencies of fractional enzyme-site saturation upon free inhibitor concentration. The source of this cooperativity has been shown by difference sedimentation velocity to be an osmolyte-mediated isomerization of phosphorylase *b* to a smaller dimeric state with decreased affinity for FAD. These results thus have substantiated a previous inference that the tendency for osmolyte-enhanced self-association of dimeric glycogen phosphorylase *b* in the presence of AMP was being countered by the corresponding effect of molecular crowding on an isomerization of dimer to a smaller, nonassociating state.

Keywords: Glycogen phosphorylase *b*; Osmolyte effects; Difference sedimentation velocity; Enzyme isomerization

1. Introduction

At neutral pH, glycogen phosphorylase *b* is a dimer of two identical subunits, each containing an active site and a glycogen storage site as well as separate activatory and inhibitory sites [1]. The catalytic site is buried in the centre of the subunit at the junction of two domains. As well as a nucleoside-binding inhibitor site located at the entrance to a catalytic site channel, there is another allosteric inhibitory site that is a target for the design of new antidiabetic drugs [2,3]. Ability of the dimeric enzyme to adopt different conformational states is responsible for its allosteric behaviour. The binding of AMP to the activatory site of dimeric phosphorylase *b* favours the active isomeric state and also the formation of tetramers [4–6]. Indeed, advantage can be taken of the extent of self-association to monitor effector-induced transitions between the inactive (T) and active (R) states of the enzyme [7–9]. On the other hand, nucleosides (e.g., FAD) and the antidiabetic drugs under development favour the inactive dimeric state of phosphorylase *b*.

This propensity for glycogen phosphorylase *b* to undergo so many structural transitions makes the enzyme a logical candidate for studies designed to examine the effects of molecular crowding on protein interactions [10–12]. Whereas enhanced self-association is the outcome of such thermodynamic nonideality [13,14], the corresponding effect on an R ← T interconversion is displacement of the equilibrium towards the smaller isomeric state [15–17]. In a previous study [18], the marginal effect of molecular crowding by trimethylamine *N*-oxide (TMAO) on the self-association of dimeric glycogen phosphorylase *b* in the presence of AMP was taken to imply that the effects of thermodynamic nonideality on the dimer–tetramer equilibrium were being countered by those displacing the T ← R

Abbreviations: TMAO, trimethylamine *N*-oxide.

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doi:10.1016/j.bpc.2004.07.040

isomerization equilibrium towards a smaller, nonassociating T-state of the enzyme.

In the present investigation, we examine the effects of osmolytes on the interaction between FAD and glycogen phosphorylase *b*. Because the binding of flavins to the inhibitory nucleoside binding site of phosphorylase *b* favours the nonassociating T-state of the enzyme [19], the complications of interpretation arising from concomitant self-association are eliminated. This study should thus allow a more direct appraisal of the earlier inference [16] that the T-state is smaller (or more symmetrical) than the R-state of muscle glycogen phosphorylase *b*.

2. Materials and methods

2.1. Chemicals

FAD, TMAO, betaine and L-proline were commercial preparations obtained from Sigma. Concentrations of FAD were based on a molar absorptivity of 1.13×10^4 M^{-1} cm^{-1} at 450 nm for the flavin coenzyme [20].

2.2. Preparation of enzyme solutions

As in the previous investigation [18], phosphorylase *b* was isolated from rabbit skeletal muscle by a slight modification [21] of the method described by Fischer and Krebs [22]. After four crystallizations, the AMP-free phosphorylase *b* preparation was stored at −20 °C in 2-phosphoglycerate/NaOH (pH 6.8) supplemented with an equal volume [18].

Solutions of phosphorylase *b* in 0.08 M HEPES buffer (pH 6.8) containing 0.2 mM EDTA were prepared by exhaustive dialysis of the stored enzyme against the buffer. Protein concentrations in the dialyzed solutions were then determined spectrophotometrically based on an absorption coefficient $\left(A^{1\%}_{1cm}\right)$ of 13.2 at 280 nm [23]. When required, the corresponding coefficient at other wavelengths was deduced from the above value and the ratio of absorbances at the two wavelengths. In binding studies, the weight–concentrations were converted to a base–molar scale by dividing by 97400, the molecular weight of phosphorylase *b* monomer.

2.3. Binding studies

The binding of FAD to phosphorylase *b* was monitored spectrophotometrically by sedimentation velocity studies at 20 °C in a Beckman model E ultracentrifuge operated at $60\,000\times g$ [7,24]. The absorbance at 450 nm in the protein-free region of the sedimentation velocity pattern was considered to reflect the free FAD concentration, $[L]$, in the mixture with composition ($[L]_{tot}$, $[E]_{tot}$), where $[L]_{tot}$ and $[E]_{tot}$ denote the respective total concentrations of ligand and enzyme in the solution subjected to velocity sedimentation. The fractional saturation, Y, (mol FAD bound/base mol of phosphorylase *b*), was then determined as

$$Y = \left([L]_{tot} - [L]\right)/[E]_{tot} \quad (1)$$

For these experiments, the base-molar concentration of phosphorylase *b* ($[E]_{tot}$) ranged between 1.5 and 100 μM, whereas the concentration of FAD ($[L]_{tot}$) ranged between 15 and 25 μM.

2.4. Difference sedimentation velocity

The existence of FAD-induced conformational changes in dimeric phosphorylase *b* was detected by the difference sedimentation velocity method [25], modified to take into account the inability of the current Beckman instruments to record simultaneously the absorbance scans for enzyme in the presence and absence of ligand [26,27]. Solutions of phosphorylase *b* (0.4–0.7 mg/ml) in HEPES buffer (pH 6.8) and in buffer supplemented with 50 μM FAD were centrifuged at 20 °C and $50\,000\times g$ in a Beckman XL-I ultracentrifuge. Migration of the enzyme was followed by scans at 280 nm of the cell with no ligand (cell 1), whereas the corresponding migration of enzyme–FAD complex in the other (cell 2) was monitored at 450 nm. Results obtained at angular velocity ω (5236 radians per second) were then analyzed in terms of the expression [26]

$$\ln(r_1)_{t_1} - \ln(r_2)_{t_2} = (s_1 - s_2)\omega^2 t_1 + \left[\ln(r_m)_1 - \ln(r_m)_2 - s_2\omega^2(t_2 - t_1)\right] \quad (2)$$

where $(r_1)_{t_1}$ and $(r_2)_{t_2}$ denote the boundary positions (midpoints) in cells 1 and 2 at times t_1 and t_2, respectively: $(r_m)_1$ and $(r_m)_2$ are the respective positions of the air–liquid meniscus in the two cells. Based on the approximate constancy of the time interval between scans of the two cells ($t_1 - t_2 \approx 1.5$ min), the difference in sedimentation coefficients ($s_1 - s_2$) was inferred from the slope of the dependence of $[\ln(r_1)_{t_1} - (r_2)_{t_2}]$ upon $\omega^2 t_1$ [26].

3. Results

In the absence of osmolytes, the binding of FAD to dimeric glycogen phosphorylase *b* is well described by a rectangular hyperbolic dependence of fractional enzyme-site saturation, Y, upon free inhibitor concentration and an intrinsic dissociation constant (K_d) of 17.8 (±0.5) μM^{-1} (Fig. 1a). The number in parentheses denotes the uncertainty (±2 S.D.) of the estimate obtained by nonlinear least-squares curve-fitting of the (Y, $[L]$) data. This dissociation constant essentially duplicates a previously reported estimate [24] of 18.1 μM for the interaction of FAD with two equivalent and independent sites on phosphorylase *b* under similar conditions (0.05 M glycylglycine/0.1 M KCl/0.2 mM EDTA, pH 6.8).

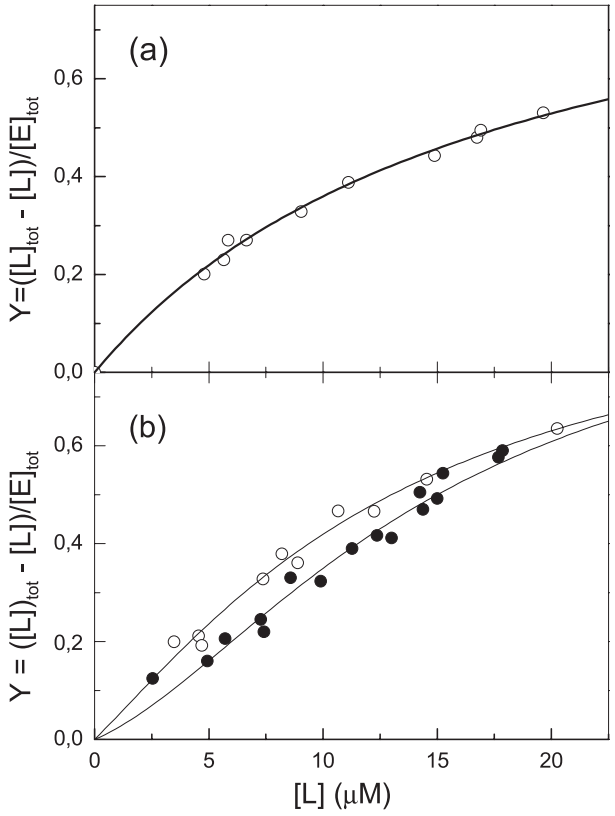
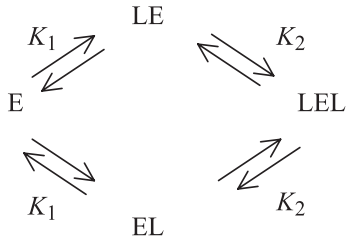


Fig. 1. Fractional saturation data for the interaction of FAD with glycogen phosphorylase *b*. (a) Results obtained by velocity sedimentation of mixtures in 0.08 M hepes–0.2 mM EDTA, pH 6.8, together with the best-fit description (——) in terms of a rectangular hyperbolic dependence of *Y* upon [*L*]. (b) Effect of 0.5 (○) and 1.0 M (●) TMAO on the interaction as well as the best-fit description (——) in terms of Eq. (3).

Markedly different binding behaviour is observed in the presence of osmolytes. In contrast with the rectangular hyperbolic dependence observed in Fig. 1a, the binding curve becomes progressively more sigmoidal with increasing osmolyte concentration—a factor evident from Fig. 1b which refers to the binding of FAD in the presence of 0.5 M (○) and 1 M (●) TMAO. The binding of ligand (L) by the two sites on the dimeric protein (E) may be represented by the reaction scheme



where LE and EL denote the two 1:1 complexes, and LEL, the fully saturated ternary complex. In that context, the lines drawn through the experimental data represent best-fit descriptions in terms of the relationship

$$Y = \frac{([L]/K_1)(1+[L]/K_2)}{1+2[L]/K_1+[L]^2/(K_1K_2)} \qquad (3)$$

Table 1
Apparent dissociation constants for the interaction of FAD with phosphorylase *b*

Osmolyte	Concentration (M)	K_1 (μM)[a]	K_2 (μM)[a]
–	–	17.8 (±0.3)[b]	17.8 (±0.3)[b]
TMAO	0.5	21 (±3)[c]	7.5 (±1.2)[c]
TMAO	1.0	50 (±12)[c]	5.5 (±3.0)[b]
Betaine	1.0	36 (±18)[c]	4.8 (±3.5)[c]
Proline	1.0	32 (±8)[c]	21 (±14)[c]

[a] Numbers in parentheses denotes uncertainties (±S.D.) of the estimates.
[b] Based on description of fractional saturation data (Fig. 1a) in terms of a rectangular hyperbolic dependence upon free ligand concentration ([*L*]).
[c] Best-fit description in terms of Eq. (3).

in which K_1 and K_2 denote the intrinsic dissociation constants describing the binding of FAD to E and EL (or LE), respectively: magnitudes of these parameters are given in Table 1. This minimal model of cooperative binding identifies the requirement for different values for K_1 and K_2, but does not specify a mechanism for the difference between their magnitudes. Consequently, variations in K_1 and K_2 merely signify a quantitative change in the form of the sigmoidal response.

On the grounds that the above binding behaviour would be consistent with the effect of molecular crowding by osmolyte on an enzyme conformational change, the effect of FAD on the shape of glycogen phosphorylase *b* has been examined by difference sedimentation velocity. Fig. 2 presents the results of those experiments in the manner suggested by Eq. (2). Whereas the binding of FAD has essentially no effect on the sedimentation coefficient of glycogen phosphorylase *b* in the absence of osmolyte (○), the negative slope of the plot obtained in the presence of 1 M TMAO (●) signifies a slightly larger sedimentation coefficient for the enzyme-FAD complex compared with that of glycogen phosphorylase *b*. The 3% change in sedimentation coefficient (−0.3 S in 8.2 S), and hence in Stokes radius, is of comparable magnitude to that observed

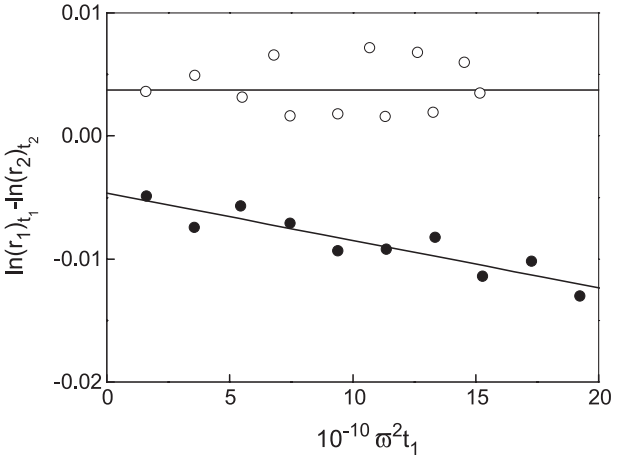


Fig. 2. Difference sedimentation velocity plots (50 000 rpm) of the effects of 50 μM FAD on the sedimentation coefficient of glycogen phosphorylase *b* in the absence (○) and presence (●) of TMAO (1 M).

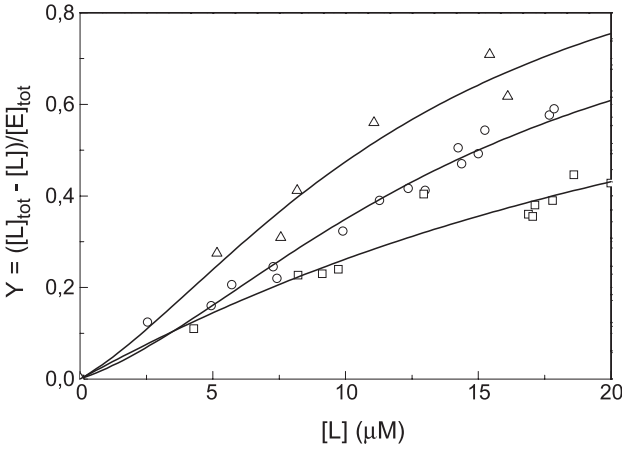


Fig. 3. Effect of osmolytes on the fractional saturation curve for the interaction of FAD with glycogen phosphorylase *b*. (——) Best-fit descriptions [Eq. (3)] of results obtained in the presence of 1 M concentrations of betaine (△), TMAO (○) and proline (□).

for the two isomeric states of rabbit muscle pyruvate kinase [15,26,27].

The ability of other osmolytes to influence the binding of FAD to glycogen phosphorylase *b* is demonstrated in Fig. 3 which compares the above saturation curve obtained in the presence of 1 M TMAO (○) with those determined in the presence of the same concentration (1 M) of betaine (△) and proline (□) as osmolyte. Although nonlinear regression analysis of the saturation data in terms of Eq. (3) returns different quantitative descriptions of the results obtained in the presence of 1 M TMAO and 1 M betaine (Table 1), the forms of the saturation curves are fairly similar. On the other hand, the form of the corresponding curve obtained in the presence of 1 M proline differs significantly from that of the other two. An important point to note in relation to Fig. 3 is its implication of a conformational change associated with the binding of FAD to glycogen phosphorylase *b* in the presence of osmolytes.

4. Discussion

The first observation requiring consideration is the rectangular hyperbolic dependence of FAD binding to glycogen phosphorylase *b* upon free allosteric inhibitor concentration (Fig. 1a). In view of the allosteric properties of glycogen phosphorylase *b* [28–33], this finding is amenable to two interpretations.

(i) Identical intrinsic dissociation constants describe the interaction of FAD with two equivalent and independent sites on both isomeric states of the enzyme.
(ii) FAD is binding to the isomeric state that already predominates in the absence of ligand.

Either interpretation of Fig. 1a would be consistent with the essentially horizontal nature of the difference sedimentation velocity plot (○, Fig. 2), which implies the identity of sedimentation coefficients for glycogen phosphorylase *b* in the absence (s_1) and presence (s_2) of FAD. However, the cooperative interaction of AMP with glycogen phosphorylase *b* [28,29] renders the latter as the more likely explanation because of the implication that AMP binds preferentially to the isomer (R-state) that is least abundant in its absence.

In view of the above considerations, the consequences of osmolyte inclusion on the saturation curves (Figs. 1b and 3) and difference sedimentation velocity results (Fig. 2) were surprising inasmuch as the T-state, to which FAD binds, is already the predominant isomer in the absence of osmolyte. It must therefore be concluded that osmolytes are influencing the equilibrium position of an additional structural transition involving a smaller (or more symmetrical) isomer than the T-state of glycogen phosphorylase *b*. The logic for that conclusion follows readily from the consideration of osmolyte effects on the statistical–mechanical basis of excluded volume. Specifically, the effect of thermodynamic nonideality on the enzyme isomerization equilibrium is described by the relationship

$$X_{\mathrm{M}} \approx X \exp\left\{\left(B_{\mathrm{T,M}} - B_{\mathrm{T^*,M}}\right)[M]\right\} \tag{4}$$

where X is the thermodynamic isomerization constant (ratio of thermodynamic activities) for the T ← T* conversion and X_{M}, the apparent value (ratio of concentrations) in the presence of a concentration $[M]$ of osmolyte M [14]. $B_{\mathrm{T,M}}$ and $B_{\mathrm{T^*,M}}$ are the second virial coefficients describing excluded volume interactions between cosolute and the respective enzyme isomer. Inasmuch as the magnitudes of these two coefficients are related to the sizes of the two isomeric states, the smaller size of the T* state is dictated by the requirement that X_{M} be greater than X in order to achieve osmolyte-mediated displacement of the isomerization equilibrium towards the T* state. Considerations of the same data in terms of protein solvation [32,33] or osmophobic effects [34,35] would also lead to the conclusion that the T* state is the favoured isomeric state in the presence of osmolyte because of the thermodynamic equivalence of all three treatments [36,37].

At first sight, the difference sedimentation velocity result obtained in the presence of 1 M TMAO (●, Fig. 2) seems to provide direct evidence for existence of the smaller (T*) state. However, a corollary of such interpretation of Fig. 2 in terms of preferential FAD interaction with the smaller (T*) state is that such binding should be enhanced by adding osmolyte—an inference that is negated by the experimental evidence (Fig. 1b) that increasing the concentration of TMAO has an inhibitory effect on the interaction between FAD and phosphorylase *b*: in other words, a decreased affinity for inhibitor is the outcome of the osmolyte-mediated enzyme transition. The FAD-mediated increase in sedimentation coefficient that is detected in Fig. 2 must therefore reflect a conformational change associated with inhibitor binding rather than displacement of an isomer-

ization equilibrium in favour of T* as the preferred binding state. The fact that the FAD-dependent conformational change occurs only in the presence of TMAO (Fig. 2) attests to the existence of an osmolyte-driven structural change within the enzyme—the putative T ← T* transition.

Thermodynamic nonideality certainly provides a rational explanation of the present findings in terms of the displacement of an isomerization equilibrium by molecular crowding/solvation effects. However, the requirement for high osmolyte concentrations invariably raises the possibility that the displacement of the isomerization may be reflecting weak preferential binding as well as thermodynamic nonideality effects. Indeed, the quantitative disparity between the effect of 1 M proline and that of the same concentration of either TMAO or betaine (Fig. 3) could well signify preferential binding of osmolyte to the T* isomer as a contributing factor in displacement of the structural transition towards the smaller T* state. In that regard, the inhibitory effects of high concentrations of glycerol, ethylene glycol, glucose and sucrose on glycogen phosphorylase activity [38–40] have been interpreted in terms of their preferential interaction with the T-state of the enzyme; but in the light of the present findings, those inhibitory effects could well be reflecting conversion of the enzyme to the T* state. Although the present interpretation of the effects of TMAO, betaine and proline on phosphorylase *b* isomerization in terms of thermodynamic nonideality is an oversimplification because of its failure to take into account any consequences of preferential binding, it draws attention to the corresponding shortcoming of the interpretation placed on similar results in terms of preferential chemical interaction [38,40]—failure to consider the consequences of molecular crowding by the high concentrations of glycerol, ethylene glycol and sucrose.

It remains to rationalize the present findings in the context of our earlier observation that high concentrations of TMAO had minimal effect on the self-association of glycogen phosphorylase *b* in the presence of AMP [18]. Because effects of thermodynamic nonideality should have favoured tetramer at the expense of dimeric glycogen phosphorylase *b*, it was proposed that that effect was being countered by the corresponding effect of thermodynamic nonideality on isomerization of the R-state to a smaller isomeric form. The basic correctness of that assertion remains unaltered despite the earlier identification of the smaller isomer as T rather than T* as the source of the osmolyte effect on the isomeric transition that is countering the corresponding effect on the dimer–tetramer equilibrium.

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (grant 02-04-49099), the Program "Molecular and Cellular Biology" of the Russian Academy of Sciences and the Program for the Support of the Leading Schools in Russia (grant 813.2003.4). The financial support of the Biotechnology and Biological Science Research Council (UK) is also gratefully acknowledged.

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