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Differential Scanning Calorimetry Study of Glycogen Phosphorylase *b*–Detergent Interactions

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The overall thermal denaturation of glycogen phosphorylase *b* is irreversible and our results conform to the theoretical prediction of a reversible process followed by a slower irreversible process. The basic thermodynamic parameters of glycogen phosphorylase *b* denaturation have been worked out and found to be: critical temperature 57.0 ± 0.5°C, transition half-width 8 ± 1°C, and calorimetric enthalpy change and Van't Hoff enthalpy change of the denaturation process 450 ± 50 and 105 ± 15 kcal/mol of enzyme monomer, respectively, at pH 7.4. These parameters have been found to be largely altered by the detergents octylglucoside, cholate, and deoxycholate at or below their critical micelle concentration, but not by Triton X-100 nor by lecithin liposomes. Organic solvents, such as dimethyl sulfoxide and methanol, and the presence of sarcoplasmic reticulum membranes produces an alteration of the denaturation thermogram of glycogen phosphorylase *b* similar to that produced by the above-mentioned detergents. These results allow us to hypothesize that hydrophobic domains of glycogen phosphorylase *b* are involved in its association to sarcoplasmic reticulum membranes in the sarcoplasmic reticulum/glycogenolytic complex of mammalian skeletal muscle.

KEY WORDS: Glycogen phosphorylase; detergents; bile salts; differential scanning calorimetry; sarcoplasmic reticulum.

INTRODUCTION

The compartmentation of glycogen phosphorylase in glycogen particles and in association with sarcoplasmic reticulum membranes has been established from the work carried out in several laboratories (Meyer *et al.*, 1970; Heilmayer *et al.*, 1970; Wanson and Drochmans, 1972; Entman *et al.*, 1980). However, the structural organization of these supramolecular particles has not been worked out in detail yet, and the basic bioenergetics of their formation has remained unknown. We have shown that glycogen phosphorylase *b* interacts with the sarcoplasmic reticulum membrane with partial loss of enzymatic activity (Gutierrez-Merio, 1983; Centeno and Gutierrez-Merino, 1986), and that this interaction is modulated by phosphorylation of glycogen phosphorylase (Cuenda *et al.*, 1991). In this regard, it is to be noted that this enzyme shows a polymorphic behavior with clear regulatory implications; see, for example, Fletterick and Madsen, 1980. In addition, a question that has been largely discussed is the physiological role of the conformational change produced by AMP on binding to phosphorylase *b* (Busby and Radda, 1976; Fletterick and Madsen, 1980; Davuluri *et al.*, 1981). So far, its physiological relevance appears to be secondary in normal skeletal muscle.

Several hydrophobic compounds structurally unrelated to AMP have been shown to activate glycogen phosphorylase *b* (Dreyfus *et al.*, 1978; Uhing *et al.*, 1979; Sotiroudis *et al.*, 1981, 1983), and in some instances up to levels of activity similar to those

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produced by this nucleotide. Some of these compounds are bile salts, widely used as detergents in membrane biochemistry, e.g., cholate and deoxycholate. This strongly suggests the presence of hydrophobic sites on this enzyme which have regulatory properties, but whose physiological relevance is poorly understood. In this regard, it is to be recalled that Madsen *et al.* (1983) have presented ample experimental evidence showing that aniline naphthalene sulfonate, a putative indicator of hydrophobic binding sites (Stryer, 1965), interacts with the activator nucleotide site in competition with AMP. In addition, the energetics of binding of compounds such as caffeine to the inhibitory site located about 10 Å from the catalytic site of glycogen phosphorylase has been shown to be dominated by the "hydrophobic contribution" (Sprang *et al.*, 1982). In accordance with this observation, Uhing *et al.* (1979) showed that several organic solvents alter the binding properties of AMP to the nucleotide activator site and of caffeine to the inhibitor site of glycogen phosphorylase, and stimulates the activity of the *b* form up to a level similar to that reached in the presence of 1 mM AMP. Nevertheless, Sotiroudis *et al.* (1983) have presented kinetic and spectroscopic evidence which strongly suggest that bile salts, such as cholate and hyocholate, bind to a hydrophobic site different from the regulatory binding sites in glycogen phosphorylase.

Because these hydrophobic domains of glycogen phosphorylase may be involved in the interaction of this enzyme with the sarcoplasmic reticulum membrane in the functional unit sarcoplasmic reticulum–glycogenolytic complex of the skeletal muscle, we have carried out DSC studies to further characterize the interaction of glycogen phosphorylase *b* with detergents and to explicitly consider the possibility of interaction with lipid bilayers.

MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles and purified glycogen phosphorylase *b* have been prepared as described elsewhere (Mata and Gutierrez-Merio, 1985; Gutierrez-Merio *et al.*, 1980). Glycogen phosphorylase *b* was found to be more than 98% pure on the basis of SDS[2] gel electrophoretic patterns. The protein concentration was determined following the method of Lowry using bovine serum albumin as standard (Lowry *et al.*, 1951), and also spectrophotometrically using an extinction coefficient at 280 nm for glycogen phosphorylase of $E^{1\%}_{1\,cm} = 13.2$ at pH 6.9 (Buc *et al.*, 1971). The activity of glycogen phosphorylase *b* has been measured as in Helmreich and Cori (1964), using the following reaction mixture: 12 mM KH_2PO_4 (pH 6.9)/10 mM magnesium acetate/0.63 mM $NADP^+$/50 mM imidazole/1 mM AMP/0.45 g/liter glycogen/1 IU phosphoglucomutase/1 IU glucose-6-phosphate dehydrogenase. A negligible activity was detected in the absence of AMP, amounting to less than 0.2% of the activity measured in the presence of 1 mM AMP, which was currently found to be about 20 IU at 20–22°C.

Scanning calorimetry measurements were carried out using a differential scanning calorimeter Microcal MC-2 operating at a scanning rate of 10–60°C/h, as indicated in the text, under approximately 1.5–2.0 kg/cm^2 pressure of nitrogen during the scan. All the scans reported in this paper are representative of, at least, duplicate experiments. The buffer used in scanning calorimetry experiments consisted of: 50 mM Tes/0.25 M sucrose/2 mM β-mercaptoethanol (pH 7.4). The samples were carefully degassed before loading the calorimeter. Calorimetric enthalpy was estimated using the algorithm proposed by Freire in its registered "MC-2 program" (EMF software). The Van't Hoff enthalpy ΔH_{VH} was calculated from peak height, assuming a single transition, and using the equation (Biltonen and Freire, 1978)

$$\Delta H_{VH} = (\langle \Delta C_p \rangle_{max} \cdot 4RT_c^2)^{1/2}$$

where T_c is the critical denaturation temperature, e.g., the temperature at which $\langle \Delta C_p \rangle$ reaches its maximum value $(\langle \Delta C_p \rangle)_{max}$.

Chemicals

Bovine serum albumin, AMP, ATP, phosphoenolpyruvate, cholate, deoxycholate, DMPC, L-α-lecithin (from egg yolk), EGTA, phenylmethyl sulfonyl fluoride, β-mercaptoethanol, Sephadex G-50, sodium dodecyl sulfate, glycogen, NADH, $NADP^+$, Tris (TRIZMA base), and Tes were obtained from sigma. Phosphoglucomutase, glucose-6-phosphate dehydrogenase, and octylglucoside were obtained

[2]Abbreviations used: cmc, critical micelle concentration; DMPC, dimyristoylphosphatidylcholine; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; Tes, 2-{[2-hydroxyl-1,1-bis-(hydroxymethyl) ethyl]amino} ethanosulfonic acid; T_c, critical denaturation temperature; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

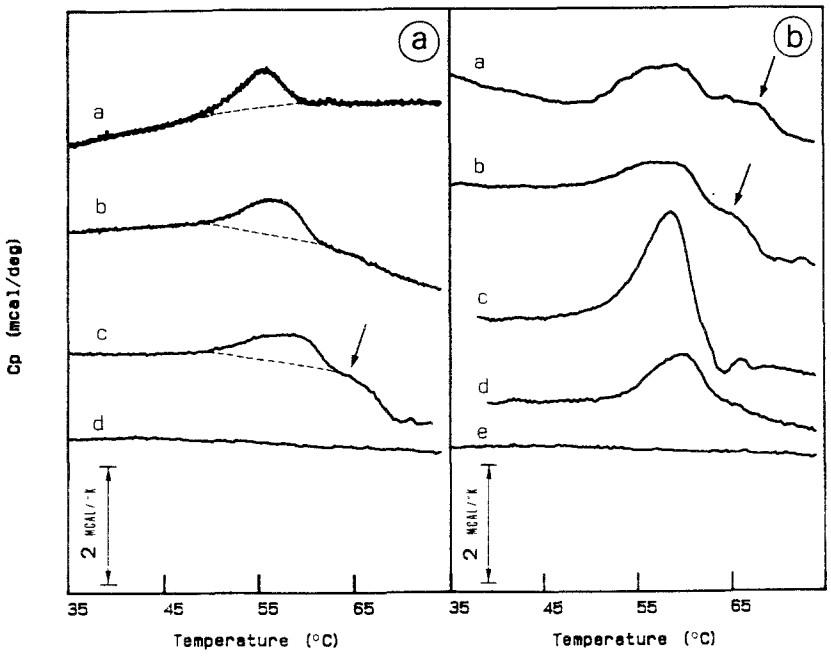

Fig. 1. DSC thermograms of glycogen phosphorylase *b*. Buffer as indicated in the Materials and Methods. The traces have been displaced in the *Y*-axis to avoid overlap of the thermograms. Panel A. Glycogen phosphorylase *b* in buffer at 1 mg/ml at 10 (a), 30 (b), and 60°C/h (c). A buffer baseline recorded at 60°C is included (d). Panel B. Glycogen phosphorylase *b* in buffer at 1 mg/ml plus 5 mM DMPC (a) or plus 0.45 g/liter glycogen (b). Traces c–e: glycogen phosphorylase *b* (3 mg/ml) in buffer (c); thermogram of a sample heated up to T_c, then cooled and rescanned (d); and rescan of a sample heated up to 75°C, then cooled up to 25°C (e). Scan rate (a–e): 60°C/h.

from Boehringer Mannheim. Triton X-100 is a trademark of Rohm & Hass, Dimethyl sulfoxide was from Carlo Erba, and methanol was of HPLC grade.

RESULTS

Denaturation of Glycogen Phosphorylase *b*

Glycogen phosphorylase *b* shows an endothermic peak in DSC scans, centered at 57.0 ± 0.5 or 58.5 ± 0.5°C when the scanning rate is 10° or 60°C/h, respectively. The overall endothermic transition is irreversible and corresponds to the denaturation of this enzyme; see Fig. 1. A change of the scan rate from 60 to 100°C/h produces a moderate change of the characteristic denaturation temperature (e.g., a shift of approximately 1–2°C), and of the calorimetric enthalpy of the overall process (an increase of 20% of the enthalpy value). In addition, at 60°C/h we have consistently observed a more asymmetric peak with a steeper decay of C_p of approximately 5°C above the T_c. As reviewed recently, this could be indicative of an overall denaturation process consisting of one or more reversible steps followed by an irreversible transition (Freire *et al.*, 1990), the latter transition lagging behind the reversible step. It has been shown that for these type of denaturation processes equilibrium thermodynamics can allow for an accurate estimation of the relevant thermodynamic parameters of the overall process (Sturtevant, 1987; Freire *et al.*, 1990). From the scans at 10 and 30°C/h we have obtained a denaturation enthalpy of 450 ± 50 kcal/mol of phosphorylase *b* monomer. In the case of the scans run at 60°C/h particular care need to be taken to correct for the baseline change accompanying the exothermic process lagging behind the endothermic transition. In most scans this is reflected as an abrupt decay of the C_p value above T_c, giving rise to a shoulder in the thermogram, as indicated by an arrow in trace c of Fig. 1 (Panel A). This feature is more clearly seen in the presence of DMPC or glycogen; see traces a and b of Fig. 1 (Panel B). When this baseline correction is done, the enthalpy value of the denaturation of glycogen phosphorylase determined at 60°C/h

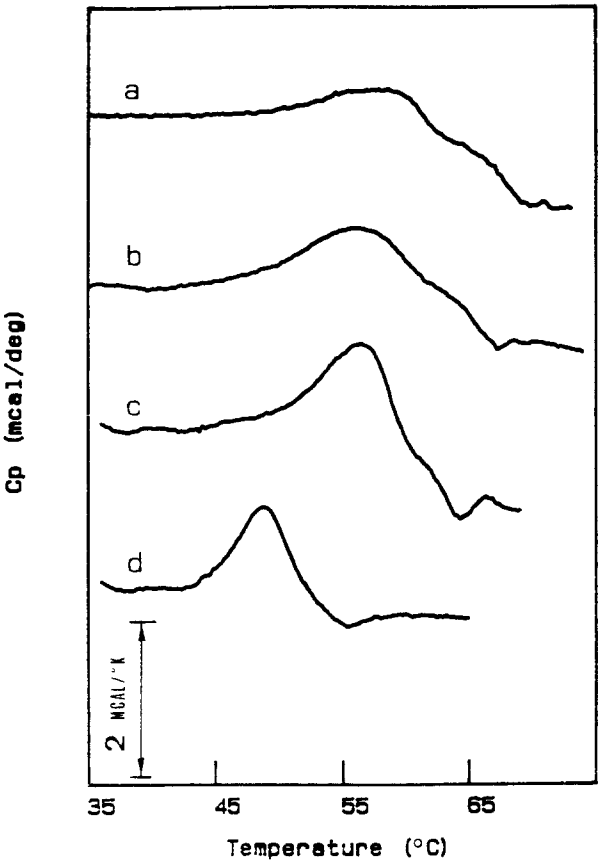

Fig. 2. Effect of Triton X-100 and octylglucoside on the DSC thermograms of glycogen phosphorylase *b*. Scanning rate: 60°C/h. Buffer as indicated in the Materials and Methods. The traces have been displaced in the *Y*-axis to avoid overlap of the denaturation thermograms. (a) Glycogen phosphorylase *b* (1 mg/ml) in buffer; (b) enzyme in the presence of Triton X-100 (1 mg detergent per ml); (c, and d) enzyme in the presence of octylglucoside, 2 and 8 mg detergent per ml, respectively.

approaches that determined from the scans performed at 30°C/h. A Van't Hoff enthalpy of 105 ± 15 kcal/mol of phosphorylase *b* monomer has been estimated, as indicated in Materials and Methods. We note that some atypical preparations showed values of enthalpy of denaturation and Van't Hoff enthalpy about 20–30% higher, e.g., 600 ± 50 and 150 ± 10 kcal/mol monomer, respectively. It follows that about 25% of the protein mass behaves as a dynamic structural unit in the maintenance of the native conformation of this enzyme.

Therefore, the overall denaturation process cannot be rationalized as a single two-state transition (Biltonen and Freire, 1978). In addition, at 3 mg/ml the shape is clearly asymmetric and only poorly fitted to a single gaussian. The steeper decay of C_p at temperatures higher than the critical temperature of maximum C_p is related to the contribution of an exothermic peak which overlaps the endothermic unfolding process, and is likely related to protein aggregation after unfolding, for at 1 or 0.5 mg/ml it is more neatly separated from the endothermic peak than at 3 mg/ml. Consistent with this observation the enthalpy of denaturation at 3 mg/ml is 390 ± 40 kcal/mol, i.e., about 10–20% lower than the value determined at 1 mg/ml (see above). Thus, the contribution of the exothermic process can be estimated as 50–100 kcal/mol monomer. In an attempt to separate reversible and irreversible transitions, samples were rescanned after cooling from T_c. Figure 1 (Panel B), trace d, shows a scan representative of the results obtained. It can be readily appreciated that this treatment eliminates the exothermic peak of the denaturation thermogram, but it also reduces the endothermic peak (to about half the value of untreated samples) and shifts the T_c about 1.5°C to higher temperatures. However, the Van't Hoff enthalpy is not significantly affected. Because of the large change of the enthalpy of denaturation, we have avoided this treatment in subsequent experiments.

The overall denaturation thermogram is independent of changes on the concentration of β-mercaptoethanol from 0 to 50 mM. Thus, it appears rather unlikely that crosslinking by disulfide bridge formation after denaturation significantly contributes to the overall enthaply change of the denaturation process. We have recently reported a similar pattern for the denaturation of sarcoplasmic reticulum membranes (Gutierrez-Merino *et al.*, 1989). However, in the case of glycogen phosphorylase the enthalpy change of this latter exothermic process is much lower than the endothermic peak, and at lower scan rates (e.g., 30°C/h) can be separated from the main endothermic peak, thus allowing for an accurate estimation of the overall enthalpy change of the unfolding process. AMP (1 mM), phosphate (10 mM), and glycogen (0.45 g/liter) do not largely alter (e.g., less than 20% change) the enthalpy change nor the peak temperature (< 2°C shift) of glycogen phosphorylase *b* denaturation (results not shown). Because AMP is known to produce large conformational changes in glycogen phosphorylase *b* (Johnson *et al.*, 1979; Fletterick And Madsen, 1980), this point was further assessed by extensive removal of this nucleotide from redissolved enzyme crystals using column chromatography plus

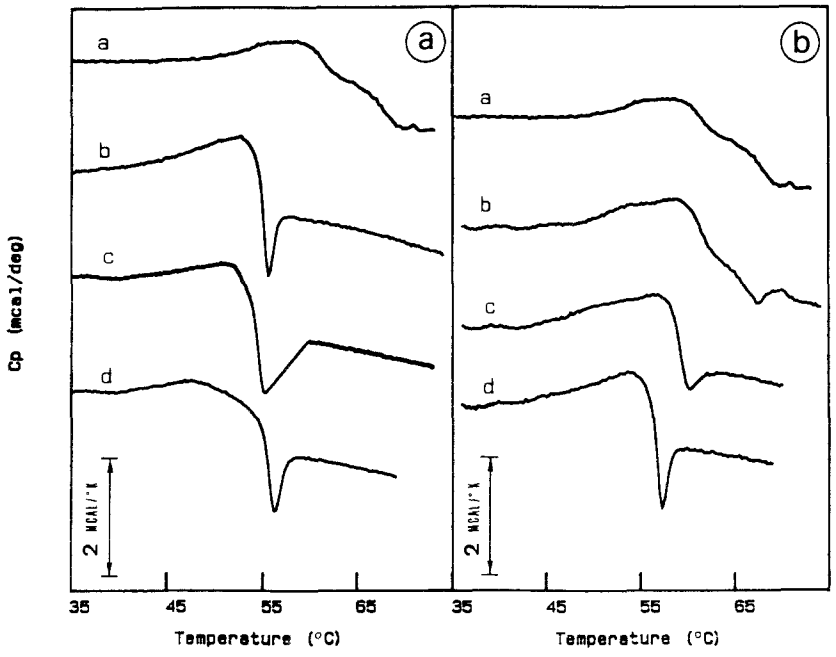

Fig. 3. Effect of deoxycholate (Panel A) and cholate (Panel B) on the DSC thermograms of glycogen phosphorylase *b*. Scanning rate: 60°C/h. Buffer as indicated in Materials and Methods. The traces have been displaced in the *Y*-axis to avoid overlap of the denaturation thermograms. Panel A: (a–d), glycogen phosphorylase *b* (1 mg/ml) in the presence of the following concentrations of deoxycholate: none, 0.25, 0.5, and 1 mg deoxycholate per ml. Panel B: (a–d), glycogen phosphorylase *b* (1 mg/ml) in the presence of the following concentrations of cholate: none, 0.25, 2, and 5 mg cholate per ml.

treatment with activated charcoal. The DSC thermograms obtained did not reveal any significant alteration upon this treatment, nor upon addition of AMP, up to 1 mM.

Effects of Detergents on the Denaturation Thermograms

The effects of several of the detergents more frequently used in membrane research on the DSC scans of glycogen phosphorylase *b* are presented in Figs. 2 and 3. The overall pattern of the denaturation of phosphorylase is drastically changed by most of these detergents. Because we did not find major differences in the DSC thermograms run at 30 and 60°C/h in the presence of cholate and deoxycholate, we have carried out the rest of this study at 60°C/h. It can be observed that in the presence of octylglucoside, deoxycholate, or cholate the endothermic peak of protein denaturation now clearly overlap a large exothermic peak and the denaturation temperature of the enzyme is shifted toward lower values in a concentration-dependent manner. From the analysis presented by Freire *et al.* (1990), it follows that these detergents produce a large increase on the rate of the irreversible step of the denaturation process, which as a consequence will take place under these experimental conditions in the same temperature range of the reversible step(s), and strongly distorts the denaturation thermogram.

Due to the large distortion of the endothermic peak by the exothermic process following after denaturation of glycogen phosphorylase, the effect of these detergents on the enthalpy of denaturation cannot be accurately determined from these data. Triton X-100, however, fails to produce large alterations of the DSC thermogram (see Fig. 2), and the only noticeable effect produced by Triton X-100 is a small shift of T_c to lower temperatures, ca. 1–2°C. The relative efficiency of cholate, deoxycholate, and octylglucoside to promote structural alterations on glycogen phosphorylase *b* correlates well with their relative cmc. These results clearly demonstrate the presence of binding domains in glycogen phosphorylase that interact with detergents.

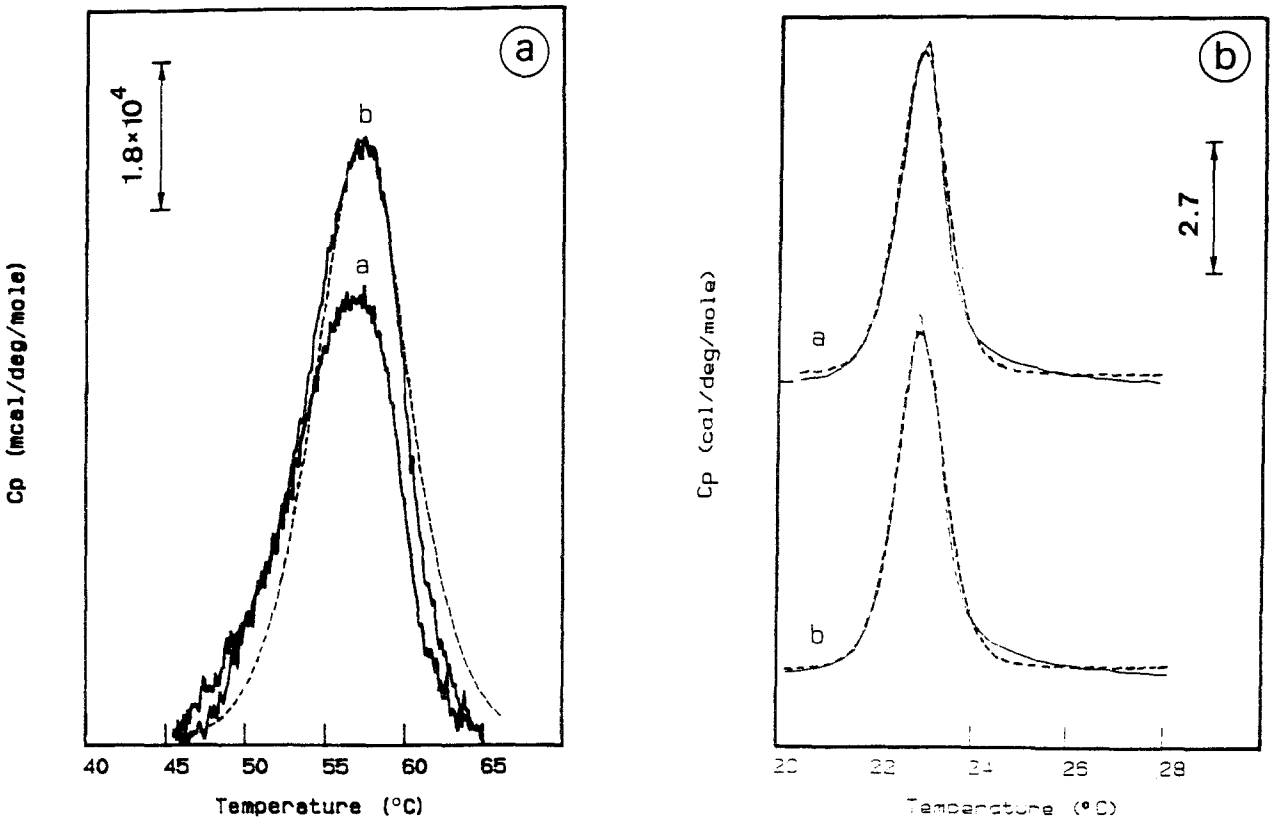

Fig. 4. DSC thermogram of glycogen phosphorylase *b* plus DMPC liposomes. Buffer as indicated in Materials and Methods. Panel A: (a) glycogen phosphorylase *b* (1 mg/ml) in buffer, scanning rate 30°C/h; (b) glycogen phosphorylase *b* (1 mg/ml) plus 1 mM DMPC liposomes, scanning rate 30°C/h. The dotted line illustrates that under the experimental conditions of (b) most of the denaturation endothermic peak can be fitted to a single gaussian describing a two-state transition, in contrast to what it is observed in the absence of lipid bilayers. Panel B: DSC thermograms of DMPC liposomes (1 mM) in buffer (a), and in the presence of glycogen phosphorylase *b* (5 mg/ml). Scanning rate 60°C/h.

Effect of Lecithin Liposomes and Sarcoplasmic Reticulum Membranes

As has been indicated in the introduction, glycogen phosphorylase *b* binds to sarcoplasmic reticulum membranes (Wanson and Drochmans, 1972; Entman *et al.*, 1980; Gutierrez-Merino, 1983; Cuenda *et al.*, 1991). Therefore, we have considered the possibility that this enzyme directly interacts with the lipid bilayer. To check this hypothesis, we have studied the effect of DMPC liposomes on the denaturation DSC pattern of phosphorylase. We have found an increase of approximately 20% of the enthalpy of denaturation, and a slight shift of T_c (about 1°C); see Fig. 4, Panel A. Similar results have been obtained in the presence of egg lecithin liposomes (not shown). Both effects can be due to a decreased rate of protein precipitation after unfolding. In fact, the exothermic process that follows the endothermic denaturation is largely reduced in the presence of lipid bilayers, and as a consequence the denaturation thermogram becomes more symmetrical, as expected on theoretical grounds if the rate of the irreversible process becomes much slower than that of the reversible step(s) during denaturation (Freire *et al.*, 1990).

We decided to test further our hypothesis that the interaction between folded glycogen phosphorylase *b* and DMPC is very weak. Consistent with this conclusion, glycogen phosphorylase *b* (up to 5 mg/ml) does not significantly alter the gel-to-liquid crystalline phase transition of DMPC centered at 24°C even after 1 h preincubation at the lipid melting temperature (Fig. 4, Panel B), and rescanning a DMPC/phosphorylase *b* mixture preheated up to 30°C shows a DMPC gel-to-liquid crystalline transition identical to that of DMPC in the absence of this enzyme.

Well below their cmc concentrations, the detergents deoxycholate and cholate exert profound structural effects on glycogen phosphorylase *b*; see above. The overall pattern of the DSC thermograms of this enzyme in the presence of these detergents closely

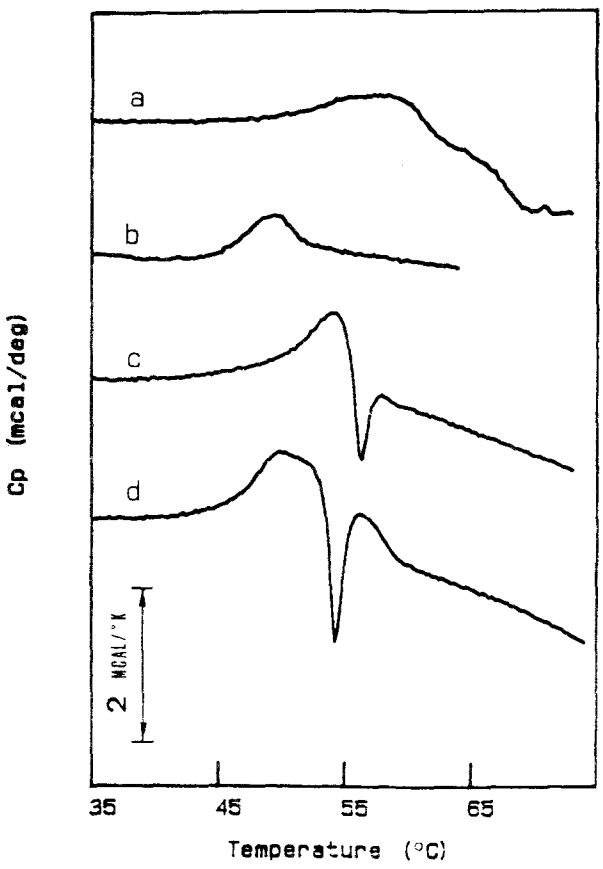

Fig. 5. DSC thermogram of glycogen phosphorylase/sarcoplasmic reticulum membranes mixture. Scanning rate 60°C/h. Buffer as indicated in Materials and Methods. (a) Glycogen phosphorylase *b* (1 mg/ml) in buffer; (b) sarcoplasmic reticulum membrane (1 mg protein per ml) in buffer; (c, d), glycogen phosphorylase *b* (1 mg/ml) plus sarcoplasmic reticulum membranes (1 mg protein per ml) in buffer plus and minus 1 M KCl, respectively.

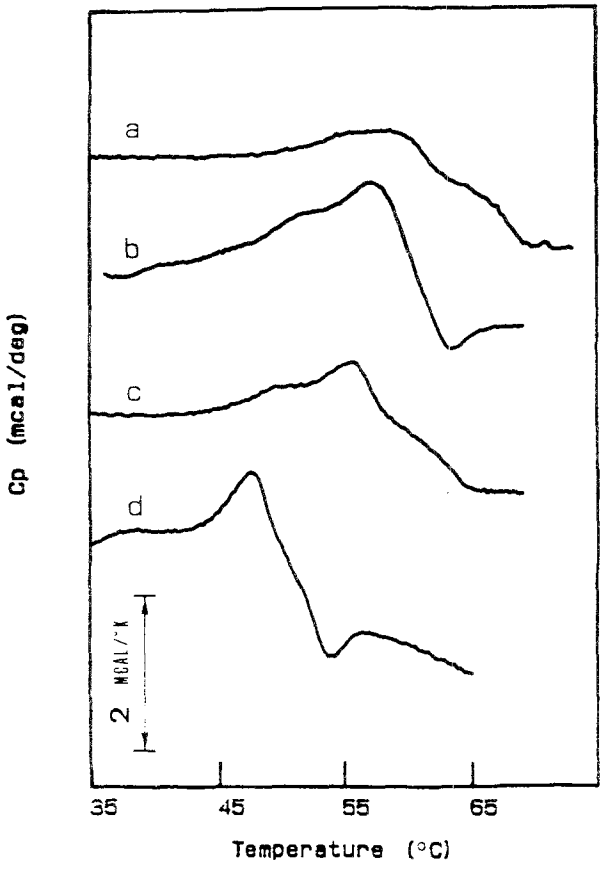

Fig. 6. Effects of organic solvents on DSC thermograms of glycogen phosphorylase *b*. Scanning rate 60°C/h. Buffer as indicated in Materials and Methods. Glycogen phosphorylase *b* (1 mg/ml) in buffer (a), plus 25% v/v dimethyl sulfoxide (b) or plus 10% and 25% v/v methanol (c and d), respectively.

mimics the denaturation pattern of glycogen phosphorylase in the presence of sarcoplasmic reticulum membranes; compare Figs. 3 and 5. Thus, these results strongly suggest that these detergents and the sarcoplasmic reticulum membrane induce similar structural alterations on glycogen phosphorylase, likely by interacting with a common protein domain of this enzyme. Because neither AMP (up to 1 mM) nor glycogen (up to 0.45 g/liter) largely alter the pattern of DSC thermograms of glycogen phosphorylase *b* in the presence of sarcoplasmic reticulum membranes, we conclude that this protein domain is not a nucleotide or polysaccharide-binding domain. In addition, high KCl concentrations (up to 1 M) only lower 2–4°C the critical denaturation temperature, without appreciably changing the overall shape of the thermogram (see Fig. 5). Therefore, it follows that these structural effects are due to hydrophobic interactions involving specific domains in phosphorylase.

DISCUSSION

The experimental calorimetric data of the overall thermal denaturation of glycogen phosphorylase *b* closely conforms to the theoretical prediction of a reversible process involving more than two states ($\Delta H_{VH}/\Delta H_0 < 1$) followed by a slower irreversible process; see Freire *et al.* (1990). This is further supported by the thermograms of phosphorylase preheated up to the T_c and then cooled prior to the scan. The small effect of AMP on the basic thermodynamic

properties of the unfolding of phosphorylase *b* deserves to be emphasized, for it minimizes the direct contribution of the nucleotide-binding domain, a well-characterized part of the protein structure (Johnson *et al.*, 1979), to the thermal unfolding of the native (folded) structure of this enzyme. Because the enthalpy of denaturation of samples preheated up to the T_c is clearly lower than the enthalpy of denaturation of untreated samples, and there is a shift of T_c of approximately 2°C to higher temperatures in parallel to a shape change of the thermogram, it is likely that mixed aggregated forms of the type (folded monomer/unfolded or partially unfolded monomer) are generated during the denaturation process, and their relative concentrations must be dependent upon the rate of heating of the samples and of the particular kinetic constants of the intermediate states of the unfolding process. As indicated in the Introduction and by the results of this paper, glycogen phosphorylase has moderaly hydrophobic domains exposed to the solvent in the folded (active) state. It is likely that these domains can effectively interact with partially unfolded states, exposing to the solvent hydrophobic amino acid residues buried within the protein structure in the folded state. The study of the kinetics of the unfolding of phosphorylase, and characterization of the predominant intermediate states is out of the scope of this paper.

The overall process is thus irreversible, and under certain experimental conditions shows a complex pattern, with an exothermic peak 5–10°C above T_c. The fact that the overlapping of the endothermic peak and of the exothermic process is more evident at the highest protein concentration tested (e.g., 3 mg/ml), suggests that the rate of the irreversible process is higher at higher protein concentrations and, thus, further supports the notion that the exothermic process is likely to involve protein aggregation. It can be readily observed that glycogen phosphorylase *b* undergoes precipitation in largely aggregated form upon denaturation. Moreover, the exothermic process appears to be accompanied by an abrupt change of the DSC baseline, a characteristic feature of protein precipitation (Privalov and Khechinashvili, 1974). A minor distortion of a symmetrical gaussian peak is observed when scanning the temperature at 30°C/h, but distortion of this peak is readily evident at a scan rate of 60°C/h (see Fig. 1). From Eq. (10) of Freire *et al.* (1990) it can be estimated that the relaxation time of the exothermic irreversible process should be in the range of seconds between 61–62°C.

Our results show that the DSC thermogram of glycogen phosphorylase *b* is largley altered in the presence of several of the detergents currently used in biological research, namely cholate, deoxycholate, and octylglucoside. The difference in the relative potency of these detergents to solubilize lipid bilayers apparently correlates to the ratio between their cmc. However, at the concentrations of deoxycholate and cholate producing large structural changes in glycogen phosphorylase, they are well below their respective cmc, and thus it seems unlikely that a micelle/water interface could provide for the real active interacting specie. In addition, Triton X-100 fails to exert any appreciable effect on the denaturation of glycogen phosphorylase at total concentrations well above its cmc. Moreover, none of the relevant thermodynamic parameters of the denaturation process of glycogen phosphorylase *b*, i.e., critical temperature, calorimetric and Van't Hoff enthalpies, are largely altered by the presence of high concentrations of DMPC or lecithin liposomes, nor is the phase transition of DMPC liposomes significantly altered by glycogen phosphorylase *b*, nor is the activity of glycogen phosphorylase *b* modulated by the presence of liposomes of egg lecithin (Cuenda *et al.*, 1991). Therefore, these results strongly suggest that the effects of octylglucoside, deoxycholate, and cholate on the structure of glycogen phosphorylase *b* are not due to unspecific interfacial effects, but to specific interactions of these detergents with hydrophobic centers of this enzyme. In addition, the large increase of the contribution of the exothermic process to the total enthalpy of denaturation is one of the most striking features of the effects of these detergents, thereby confirming that this hydrophobic domain is involved in protein/protein interactions between folded and unfolded states, as suggested above.

The possibility that this hydrophobic domain, at least, partially overlapps the regulatory domain previously suggested to be involved in the estimation of glycogen phosphorylase *b* by organic solvents (Uhing *et al.*, 1979) has been considered. The effects of methanol and dimethyl sulfoxide in the thermograms of denaturation of glycogen phosphorylase *b* (Fig. 6) strongly support this hypothesis. It can be observed that methanol induces a perturbation of the denaturation thermogram of glycogen phosphorylase *b* larger than that produced by dimethylsulfoxide, in an inverse relationship with their relative values of dielectric constants. It is worth noting here that glycogen phosphorylase belongs to the group of enzymes that are stimulated by organic solvents (Klibanov, 1989).

From the data shown above, in the case of glycogen phosphorylase this effect is likely to be due, at least in part, to selective structural changes of protein domains.

In conclusion, our results suggest that the modulation of the activity of glycogen phosphorylase *b* by organic solvents, bile salts, and binding to the sarcoplasmic reticulum membrane involve a common regulatory domain of this enzyme. Because 1 M KCl does not significantly reverse the effect of bile salts or sarcoplasmic reticulum membranes on the DSC thermograms of glycogen phosphorylase *b*, we conclude that these effects are driven by hydrophobic interactions. This is an important point, for the physiological relevance of the association of glycolytic enzymes with the erythrocyte membrane (Maretzki *et al.*, 1989), on the basis of their reversion by physiological ionic strength, has been questioned recently, as they appear to be mostly dominated by the ionic interaction term. In addition, the kinetic results presented by Sotiroudis *et al.* (1983), and the lack of a large effect of AMP (up to 1 mM) and of glycogen (up to 0.45 g/liter) on the denaturation thermograms of glycogen phosphorylase *b* and of mixtures of glycogen phosphorylase *b* plus sarcoplasmic reticulum membranes (this paper and results not shown) strongly support the hypothesis of the existence of a regulatory hydrophobic binding site in phosphorylase *b* distinct to the activator and to the glycogen binding *loci*. It can be anticipated that X-ray diffraction should be very useful in defining precisely its location in the enzyme structure.

The molecular basis of the exothermic transition lagging behind the endothermic transition is unclear at present. Although intramolecular and intermolecular disulfide bridge formation could contribute to the enthalpy change observed, the lack of effect of up to 50 mM β-mercaptoethanol seems to argue against this hypothesis as the dominant contribution to the exothermic enthalpy change observed. Alternatively, partial refolding of the denatured protein is likely to be taking place, since this process is favored when the protein is adsorbed onto micellar surfaces or in organic solvents. This has already been demonstrated to be the case for the sarcoplasmic reticulum Ca^{2+}, Mg^{2+}-ATPase, which has been shown to be largely enriched in β-sheet structures likely interacting with the lipid surface in the denatured state, whereas α-helical structures predominate in the native state (Jaworsky *et al.*, 1986; Jaworsky and Mendelsohn, 1987). Although out of the scope of this paper, regarding glycogen phosphorylase this point deserves to be further studied.

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REFERENCES

Biltonen, R. L., and Freire, E. (1978). *CRC Crit. Rev. Biochem.* **5,** 85–124.

Buc, M. H., Ullman, A., Goldberg, M., and Buc, H. (1971). *Biochimie* **53,** 283–289.

Busby, S. J. W., and Radda, G. K. (1976). *Curr. Top. Cell. Regul.* **10,** 89–161.

Centeno, F., and Gutierrez-Merino, C. (1986). Proceedings of the 13th Spanish Biochemical Society Meeting, August 2–15, Zaragoza.

Cuenda, A., Centeno, F., and Gutiérrez-Merino, C. (1991). *FEBS Lett.* **283,** 273–276.

Davuluri, S. P., Hird, F. J. R., and Stanley, I. J. (1981). *Comp. Biochem. Physiol.* **68B,** 369–375.

Dreyfus, M., Vandenbunder, B., and Buc, H. (1978). *FEBS Lett.* **95,** 185–189.

Entman, M. L., Keslensky, S. S., Chu, A., and Van Winkle, W. B. (1980). *Biol. Chem.* **255,** 6245–6252.

Fletterick, R. J., and Madsen, N. B. (1980). *Annu. Rev. Biochem.* **49,** 31–61.

Freire, E., van Osdol, W. W., Mayorga, O. L., and Sanchez-Ruiz, J. M. (1990). *Annu. Rev. Biophys. Biophys. Chem.* **19,** 159–188.

Gutierrez-Merino, C. (1983). *Trends Biochem. Sci.* **8,** 8.

Gutierrez-Merino, C., Garcia-Blanco, F., Pocovi, M., Menéndez, M., and Laynez, J. L. (1980). *J. Biochem.* **87,** 1483–1490.

Gutierrez-Merino, C., Molina, A., Escudero, B., Diez, A., and Laynez, J. L. (1989). *Biochemistry* **28,** 3398–3406.

Heilmeyer, L. M. G., Jr., Meyer, F., Haschke, R. H., and Fischer, E. H. (1970). *J. Biol. Chem.* **245,** 6649–6656.

Helmreich, E., and Cori, C. F. (1964). *Proc. Natl. Acad. Sci. USA* **51,** 131–138.

Jaworsky, M., and Mendelsohn, R. (1987). *Biophys. J.* **52,** 241–248.

Jaworsky, M., Brauner, J. W., and Mendelsohn, R. (1986). *Spectrochim. Acta Part A Mol. Spectrosc.* **42A,** 191–198.

Johnson, L. N., Stura, E. A., Wilson, K. S., Sansom, M. S. P., and Weber, I. T. (1979). *J. Mol. Biol.* **134,** 639–653.

Klibanov, A. M. (1989). *Trends Biochem. Sci.* **14,** 141–144.

Lowry, O. H., Rosebrough, H. J., Farr, A. L., and Randall, R. L. (1951) *J. Biol. Chem. 193,* **265–275.**

Madsen, N. B., Schechosky, S., and Fletterick, R. J. (1983). *Biochemistry* **22,** 4460–4465.

Maretzki, D., Reimann, B., and Rapoport, S. M. (1989). *Trends Biochem. Sci.* **14,** 93–96.

Mata, A. M., and Gutierrez-Merino, C., (1985). *Biochim. Biophys. Res. Commun.* **13,** 176–182.

Meyer, F., Heilmeyer, L. M. G., Jr., Haschke, R. H., and Fischer, E. H. (1970). *J. Biol. Chem.* **245,** 6642–6648.

Privalov, P. L., and Khechtnashvili, N. N. (1974). *J. Mol. Biol.* **86,** 665–684.

Sotiroudis, T. G., Oikonomakos, N. G., and Evangelopoulos, A. E. (1981). *Biochim. Biophys. Acta* **658,** 270–279.

Sotiroudis, T. G., Cazianis, C. T., Oikonomakos, N. G., and Evangelopoulos, A. E. (1983). *Eur. J. Biochem.* **131,** 625–631.
Sprang, S. R., Fletterick, R. J., Stern, M., Yang, D., Madsen, N. B., and Sturtevant, J. M. (1982). *Biochemistry* **21,** 2036–2048.
Stryer, L. (1965). *J. Mol. Biol.* **13,** 482–495.
Sturtevant, J. M. (1987). *Annu. Rev. Phys. Chem.* **38,** 463–488.
Uhing, R. J., Janski, A. M., and Graves, D. J. (1979). *J. Biol. Chem.* **254,** 3166–3169.
Wanson, J. C., and Drochmans, P. (1972). *J. Cell. Biol.* **54,** 206–224.