The interaction of troponin C with phosphofructokinase

Comparison with calmodulin

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Phosphofructokinase (PFK) is a calmodulin (CaM)-binding protein [Mayr & Heilmeyer (1983) FEBS Lett. **195**, 51–57]. We found that troponin C (TnC), which is homologous to CaM, also binds PFK and affects PFK's catalytic activity, aggregation states and conformational changes as CaM does in most cases. PFK titration of *N*-acetylaminomethyl-5-naphthylamido-1-sulphonate ('AEDANS')-TnC showed that its apparent dissociation constant is comparable with that of PFK–CaM. Fluorescent labels were also used to probe contact regions on TnC and CaM. It is likely that the C-terminal end of the connecting strand of the TnC molecule is close to PFK in the binary complex. Hydrophobic regions of TnC and CaM also possibly play roles in the binding and polymerization of PFK. TnC and CaM deactivate PFK through accelerating PFK conformational change as well as through accelerating PFK tetramer dissociation, as implied in the results of activity, light-scattering, fluorescence and c.d. experiments. The intact molecule of CaM appears to be required to deactivate PFK, because neither half of the CaM molecule has an effect on PFK activity.

INTRODUCTION

Phosphofructokinase (PFK) is a key element in glycolysis. It is a tetrameric enzyme and is regulated by many low-molecular-mass effectors [1]. Calmodulin (CaM), which is ubiquitous in eukaryotic systems, combines with and regulates numerous enzymes [2–4]. Mayr and co-workers [5–8] found that rabbit muscle PFK is a CaM-binding protein and studied the influence of Ca²⁺-liganded CaM on the aggregation state, the conformation and the catalytic properties of PFK. Our interest arose in part from the fact that troponin C (TnC), which is abundant in muscle and resembles CaM in both amino acid sequence and three-dimensional structure [9–11], may also bind PFK and may also be a potential factor in any physiologically relevant interaction between Ca²⁺-binding proteins and PFK. Histochemical studies [1,12] have indicated that the glycolytic enzymes, including PFK, occur within the I-band of mammalian muscle fibrils, which corresponds to F-actin–troponin–tropomyosin filaments. Other studies have indicated that PFK interacts with the F-actin–troponin–tropomyosin system [13].

TnC has two reactive sites (Met-25 and Cys-98) for the specific attachment of a fluorescent label which can be used as a reporter group in the study of the binding. Wheat-germ CaM, which has a reactive Cys-27 residue, may also be used for this purpose.

Parallel experiments using different approaches may shed more light on the structural principles of Ca²⁺-binding-protein–target-protein interactions. In this work we have employed catalytic-activity assays (with native Ca²⁺-binding proteins and their fragments), light-scattering analyses, static and dynamic fluorescence and c.d. measurements to investigate the interactions of Ca²⁺-binding proteins with PFK.

A primary objective of this study was to identify the structural regions of CaM and TnC which are in contact with PFK and to determine whether there is any parallel with other systems. The principal experimental approach has involved the use of specifically located fluorescent probes.

MATERIALS AND METHODS

Proteins and their derivatives

PFK was purchased from Boehringer Mannheim Biochemicals and stored at 0–5 °C. Before use, an appropriate volume of the crystal suspension was centrifuged at 10000 *g* for 10 min, and the supernatant was discarded. After dissolving the crystals in a buffer containing 100 mM-phosphate, 0.5 mM-EDTA and 3 mM-dithioerythritol (pH 8.0), the solution was re-centrifuged to remove insoluble material and then dialysed against 500 vol. of the same buffer three times for 8 h each. The concentration of PFK was determined by using *A*₂₈₀ (1 mg/ml) = 1.07 [14].

TnC, CaM, their labelled derivatives and CaM fragments were prepared as described in refs. [15–21]. [Dns-Tyr⁹⁹]CaM was prepared by selectively nitrating Tyr⁹⁹ of bovine CaM with tetranitromethane, by the procedure of Richman & Klee [18]. The NO₂-Tyr⁹⁹ derivative was then reduced to [NH₂-Tyr⁹⁹]CaM by treatment with Na₂S₂O₄ as described in refs. [16] and [19]. Substitution of the NH₂-Tyr⁹⁹ group with dansyl (Dns) chloride was carried out in a heterogeneous reaction at pH 5.0, as described in ref. [16]. However, the earlier procedure was modified in that, before reduction of the nitrotyrosine, the lysine groups were reversibly blocked by treatment with citraconic anhydride [17], which was subsequently removed after completion of the Dns chloride reaction by incubation at pH 3. This procedure ensures that dansylation is confined to the aminotyrosine group and that other amino groups are unaffected. A similar procedure was used to prepare [Dns-Tyr¹³⁹]CaM (wheat germ), except that Cys²⁷ was protected by reaction with *p*-chloromercuribenzoate, which was subsequently removed by treatment with dithiothreitol. The derivatives retained their native structure, as judged by their ability to activate myosin light-chain kinase. [AEDANS-Cys⁹⁸]TnC was prepared by treatment of TnC with IAEDANS, by the method of Cheung *et al.* [20]. [AEDANS-Cys²⁷]CaM was prepared by treatment of wheat-germ CaM with IAEDANS by the method of Strasburg *et al.* [21].

Abbreviations used: PFK, phosphofructokinase; TnC, troponin C; CaM, calmodulin; DANZ, *N*-dansylaziridine; IAEDANS, *N*-iodoacetylaminomethyl-5-naphthylamido-1-sulphonate; AEDANS, *N*-acetylaminomethyl-5-naphthylamido-1-sulphonate; TNS, 2-*p*-toluidinylnaphthalene-6-sulphonate; Dns, dansyl; Fru-6-*P*, fructose 6-phosphate; Fru-2,6-*P*₂, fructose 2,6-bisphosphate.

Fig. 1. PFK deactivation on dilution in the absence and presence of Ca^{2+} -binding proteins

PFK was diluted from a concentrated stock to $1.1 \mu\text{M}$ (protomer) into preincubation (see the Materials and methods section) without TnC or CaM (top $\bullet$ line) and with (from top to bottom) $0.4 \mu\text{M}$, $0.8 \mu\text{M}$, $1.2 \mu\text{M}$, $2.1 \mu\text{M}$ and $3.0 \mu\text{M}$ TnC ($\bullet$ lines as indicated in the Figure) and with $3.0 \mu\text{M}$ CaM ($\times$ line, as comparison), and PFK catalytic activity was analysed. At 151 min 2 mM-MgATP and $0.2 \text{ mM-Fru-2,6-P}_2$ were added. Temperature = 25°C . Inset: PFK activities remaining at 150 min plotted versus TnC concentration.

Chemicals and reagents

ATP (substantially vanadium-free), fructose 6-phosphate (98%), fructose 2,6-bisphosphate (95%), NADH (98%), aldolase (15 units/mg), glycerol-3-phosphate dehydrogenase (243 units/mg), triosephosphate isomerase (5500 units/mg), *N*-dansylaziridine (DANZ), Dns chloride and 2-*p*-toluidinyl-naphthalene-6-sulphonate (TNS) were purchased from Sigma. IAEDANS was purchased from Molecular Probes. All other chemicals were reagent grade or better.

PFK activity assay

PFK activity and its variation were determined by a coupled enzyme system basically in accordance with Mayr's procedure [6,22]. The enzyme was diluted to $0.2\text{--}1.0 \mu\text{g/ml}$ into a quartz cuvette containing 1 mM-ATP , 3 mM-MgCl_2 , $3 \text{ mM-dithioerythritol}$, $2 \text{ mM-fructose 6-phosphate (Fru-6-P)}$, 0.2 mM-NADH , 0.1 mM-EGTA , 50 mM-Tris/HCl (pH 8.0), $1 \text{ mM-(NH}_4)_2\text{SO}_4$, $0.05 \text{ unit of aldolase/ml}$, $3 \text{ units of glycerol-3-phosphate dehydrogenase/ml}$ and $12 \text{ units of triose-phosphate isomerase/ml}$. The decrease in A_{365} was recorded for 1 min on a Gilford Response Spectrophotometer 30 s after mixing PFK into the assay solution, and the rate was calculated by the manufacturer's program. Sometimes, to decrease the background kinase activity in the system, a smaller (50%) amount of aldolase-dehydrogenase-isomerase was used and appropriate calibrations were made. The preincubation buffer (or experimental buffer), containing 20 mM-Hepes , 1.5 mM-MgCl_2 , 200 mM-KCl , 0.1 mM-CaCl_2 , 0.05 mM-ATP and $5 \text{ mM-dithioerythritol}$ (pH 7.5), was also used in the other experiments in this work unless otherwise specified.

Light-scattering measurement

Light-scattering was measured with a Brice Phoenix Universal Light-Scattering Photometer. Clear quartz cuvettes and centrifuged or filtered solutions were used. To follow the time course of the light-scattering of the sample, intensities at 90° (I_{90°) and at 436 nm were read at the desired time, and the light-

scattering of the buffer was subtracted. I_{90° is directly proportional to $\sum C_i M_i$, in which C_i is the weight concentration and M_i is the molecular mass of species i . This is one way to monitor the association states and interactions of macromolecules.

Fluorescence emission, polarization and intensity titration

Fluorescence measurements were carried out on an SLM 48000 spectrofluorimeter. To record emission spectra, excitation wavelengths were set at 330 nm for the conjugates and at 295 nm for tryptophan. Difference spectra were determined as the net difference in fluorescence emission between labelled TnC (or CaM) in the absence of PFK and in its presence. Blank and instrument response were corrected for. To monitor fluorescence polarization, emission wavelengths were set at the maximum of the difference spectra.

Fluorescence polarization (P) is defined as

$$P = (I_{vv} - gI_{vh}) / (I_{vv} + gI_{vh}) \quad (1)$$

where I_{vv} and I_{vh} are the intensities of the vertically and horizontally polarized components respectively, when exciting light is vertically polarized. The factor $g = I_{hv}/I_{hh}$ is to normalize instrument response, where I_{hv} and I_{hh} are as above, except that the exciting light is horizontally polarized.

In order to determine the dissociation constant K_d for TnC-PFK, AEDANS-TnC was selected for fluorescence-titration experiments. After adding PFK to labelled TnC, intensity was monitored as a function of time until equilibrium was considered to be reached when the reading reached a plateau. Usually this period was 10 min or more. Non-linear least-squares analysis was used to find the calculated curve which best fitted the observed titration values.

C.d. spectra and calculations

The c.d. measurements were carried out at room temperature on a Jasco 40A c.d. apparatus. Scans from 250 to 215 nm were made at the same time intervals after mixing PFK, PFK plus TnC, or PFK plus CaM into the experimental solution. Mean

molar residue ellipticity, $[\theta]$, is calculated from the observed ellipticity, θ , by using the equation

$$[\theta] = \theta M_r / 10lC \quad (2)$$

where M_r is the mean residue molecular mass computed from the PFK molecular mass (84000 Da), and the amino acid residue number, 668 [6,22], l is the cuvette path length (cm), and C is the concentration (g/ml). The α -helical fraction, f_H , is estimated by the empirical equation (Chang *et al.* [23]):

$$f_H = [\theta]_{223} / [\theta]_H (1 - k/n) \quad (3)$$

where $[\theta]_H$ is the mean molar residue ellipticity of an infinite helix ($= -37300^\circ$), k is the chain-length-dependence factor ($= 2.5$), and n is the average number of residues per helical segment ($= 9$).

Fig. 2. Light-scattering of PFK upon dilution and upon adding TnC or CaM

All solutions were in experimental buffer (see the Materials and methods section). The concentrations were: PFK 1.3 μ M, TnC or CaM 2.5 μ M. Light-scattering of 3 μ M-TnC alone was also measured. The experiment was at room temperature.

Fig. 3. Change of tryptophan-fluorescence intensity of 1 μ M-PFK with time upon dilution in the absence (●) or in the presence of 3 μ M-CaM (+) or in the presence of 3 μ M-TnC (x)

Emission wavelength was 335 nm; excitation wavelength was 295 nm. Temperature = 25 $^\circ$ C.

RESULTS

Effect of TnC on PFK activity

The catalytic activity of PFK decreased with time after PFK had been diluted from a concentrated stock to about 0.1 mg/ml; TnC caused PFK activity to decrease further (Fig. 1), in parallel with the effect of CaM [5,6]. The effect was TnC-concentration-dependent. On plotting the remaining PFK activity at 150 min versus the TnC concentration, a plateau appeared around 1.1 μ M (Fig. 1, inset), which was the value of PFK protomer concentration. This suggests a 1:1 effective binding. The addition of the activators, MgATP and Fru-2,6- P_2 , restored PFK activity; the higher the TnC level, the less PFK activity was restored, again in parallel with CaM-PFK.

PFK dissociation and effect of TnC in light-scattering

On dilution of PFK, a decay with time of I_{90° was observed, as reported by Mayr [6], which was correlated with the decrease in PFK catalytic activity (Figs. 2 and 1). This may be interpreted as a partial dissociation of active tetramer of PFK to inactive dimer until equilibrium was reached [6]. When TnC was added to the above PFK solution, an increase which was much greater than I_{90° of TnC alone was first observed, followed then by a slow decrease in I_{90° (Fig. 2). It was the same as for CaM-PFK [6], and was further evidence of the interaction between TnC and PFK.

Table 1. Fluorescence of labelled TnC and CaM influenced by PFK

Concentrations of samples were all 1 μ M; PFK was 2 μ M. The excitation wavelength was 330 nm. Other experimental conditions are described in the Materials and methods section. Abbreviations: K_{S-V} , Stern-Volmer constant for quenching by acrylamide; $\lambda_{max}\Delta I$, wavelength of maximum difference in intensity.

Sample	Additive	I_{max}	λ_{max} (nm)	Polarization	K_{S-V} (M^{-1})	ΔI_{max}	$\lambda_{max}\Delta I$ (nm)
AEDANS-TnC	–	2.19	501	0.051	6.5		
	PFK	2.38	493	0.107	3.9	0.412	463
DANZ-TnC	–	1.21	536	0.112			
	PFK	1.28	524	0.140		0.107	500
[Dns-Tyr ⁹⁹]CaM	–	0.45	544	0.094			
	PFK	0.46	534	0.151		0.023	495
[Dns-Tyr ¹³⁹]CaM	–	0.72	527	0.108			
	PFK	0.75	519	0.156		0.071	483
AEDANS-CaM	–	2.23	507	0.054	7.1		
	PFK	2.24	502	0.090	5.3	0.189	458

Fig. 4. Fluorescence intensity titration of 1 μM -AEDANS-TnC by PFK

Points were fluorescence intensity readings at about 10 min after each addition of PFK and were stabilized for 2–3 min. The solid line is the calculated curve assuming 1:1 binding with an apparent dissociation constant of 0.29 μM . Resolution of emission spectra was 4 nm. For other conditions see the text.

When the incubations were prolonged, I_{90} for PFK alone rose substantially in 1–2 h, as reported by Mayr [6], whereas those for TnC-PFK or CaM-PFK did not (when the ratio was > 1) (Fig. 2). The former phenomenon was due to PFK polymerization after its dilution into experimental buffer [6]; the latter reflected the fact that TnC and CaM were able to prevent PFK polymerization, though there was some difference between the two, in that the effect of TnC lasted for no more than several hours, whereas for CaM the effect existed for a much longer time (results not shown). The increase in intensity is not obviously correlated with the slow decrease in catalytic activity, perhaps because the increase in scattering reflects the formation of a small amount of very large aggregates, which do not account for a major fraction of the PFK during the time observation.

Tryptophan fluorescence of PFK

When PFK alone was diluted into experimental buffer, its tryptophan fluorescence intensity decreased with time, whereas in the presence of TnC or CaM tryptophan fluorescence increased with time (Fig. 3). For the wavelengths of excitation (295 nm) and emission (335 nm) that were employed here, control measurements with turbid solutions indicated that scattering could not be detected. It is possible that one or more tryptophan residues are at the contact region between the PFK subunits and become exposed when the PFK tetramer dissociates; it could be protected from solvent by binding TnC or CaM. The fluorescence change may also possibly be the consequence of a more complicated conformational change in PFK upon dilution and upon binding.

Fluorescence of labelled TnC and CaM influenced by PFK

Five labelled Ca^{2+} -binding proteins were used in the study of binding with PFK: AEDANS-TnC labelled at residue Cys⁹⁸; DANZ-TnC, at Met²⁵; Dns-CaM (bovine), at Tyr⁹⁹; Dns-CaM (wheat germ), at Tyr¹³⁹; and AEDANS-CaM (wheat germ), at Cys²⁷. The changes in their fluorescence intensities and wavelengths of maximum intensity are summarized in Table 1. Of the five labels studied, the most pronounced change in properties upon complex-formation was shown by [AEDANS-Cys⁹⁸]TnC, which showed a substantial spectral shift to lower wavelengths, as well as a pronounced increase in protection from acrylamide quenching (Table 1). There is a definite indication

Fig. 5. TNS-PFK fluorescence

Emission resolution was 4 nm. All measurements were done in experimental buffer. Temperature = 25 °C. (a) TNS fluorescence emission spectra: (A) TNS (6 μM) in buffer; (B) 1 min after and (C) 1 h after adding 1 μM -PFK to (A); (D) difference spectrum from (C) and (B). (b) TNS fluorescence emission spectra: (A) TNS (6 μM) and CaM (3 μM) in buffer; (B) 1 min after and (C) 1 h after adding 1 μM -PFK to (A); (D) difference spectrum from (C) and (B). (c) Time course of TNS-PFK fluorescence intensity: TNS (6 μM) and PFK (1 μM) with (●) or without (○) 2 mM-MgATP and 0.2 mM-Fru-2,6- P_2 , or in the presence of 3 μM -TnC (×) or in the presence of 3 μM -CaM (+). (TNS-TnC or TNS-CaM fluorescence was subtracted.) (d) Time course of TNS-PFK fluorescence intensity net change: TNS-PFK (as above) with 3.0 μM -, 2.0 μM -, 1.0 μM -, 0.5 μM - and 0 μM -CaM (from the top). Prolonged incubation was carried out at room temperature.

that the Cys⁹⁸ region, which lies at the C-terminal end of the connecting strand, is involved in the zone of contact. [AEDANS-Cys²⁷]CaM, [DANZ-Met²⁵]TnC and [Dns-Tyr¹³⁹]CaM all show significant alterations in emission spectra upon complex-formation. In each case the interaction resulted in an increase in intensity and a shift of the emission maximum to lower wavelengths. For [AEDANS-Cys²⁷]CaM, complex-formation also results in partial shielding from acrylamide quenching. (The other derivatives were not quenched significantly, perhaps because of being embedded in the tertiary structure.) The changes in emission spectra are typical of those generally accompanying a transition to a more non-polar solvent-shielded micro-

Fig. 6. (a) C.d. spectra of PFK, TnC, CaM and their complexes, and (b) net PFK ellipticity versus time in the absence ($\circ$) and in the presence of TnC or CaM ($\bullet$)

(a) (A) 2 μ M-PFK (0.168 mg/ml) at 3 min (----), 37 min (---) and 71 min (—). (B) 4 μ M-CaM without PFK (upper —) and with 2 μ M-PFK at 3 min (----), 37 min (---) and 71 min (lower —). (C) 4 μ M-TnC without PFK (upper —) and with 2 μ M-PFK at 3 min (----), 37 min (---) and 71 min (lower —). Cuvette path length was 0.2 cm. The same buffer (see the Materials and methods section) was used; temperature = 25 °C.

environment. The simplest interpretation is that all four labels are involved to some degree in the zone of contact, which involves both the C-terminal region of the C-terminal lobe and the N-terminal lobe and includes the connecting strand. The effect of complex-formation on [Dns-Tyr⁹⁹]CaM is marginal, and may possibly be attributed to the indirect effect of an induced conformational change.

In principle, the above results could be explained in an alternative way, in terms of contact which is confined to the N- or C-terminal lobe and which results in a conformational change which is propagated to the balance of the molecule, causing the observed changes in the other labels. However, it is difficult to develop a plausible explicit form for this model, which would require that the structural transition be transmitted via the connecting strand; this seems unlikely, in view of the demonstrated independent thermal transitions of the N- and C-terminal half-molecules [24]. It is more likely that the observed microenvironmental changes reflect relatively short-range effects, including especially a direct contact or envelopment of the perturbed group by PFK.

Fluorescence-polarization results confirmed the interaction between the Ca²⁺-binding proteins and PFK, and also suggested that the label in [AEDANS-Cys⁹⁸]TnC was more immobilized in the binary complex, as expected (Table 1).

Apparent dissociation constant of TnC-PFK

Assuming 1:1 binding, an apparent dissociation constant of 0.3 μ M was obtained for AEDANS-TnC-PFK by fluorescence titration (Fig. 4). This value is similar to that reported for Dns-CaM-PFK from titration monitored by fluorescence anisotropy [25]. Although the binding stoichiometric ratios had different values ([6,8,25] and the present work), and the titration procedure had uncertain accuracy because of the presence of slow time effects, the order of magnitude of the dissociation constant could still be estimated under the same assumptions. The similar apparent dissociation constants suggest that TnC and CaM have comparable binding affinity to PFK.

TNS fluorescence

TNS in buffer has essentially no fluorescence (Fig. 5a, trace A). It gains a moderate fluorescence upon binding to Ca²⁺-CaM or PFK (Fig. 5b, trace A, and Fig. 5a, trace B); this behaviour is usually interpreted as arising from the binding of TNS to hydrophobic sites [26]. The initial acquired fluorescence intensities of TNS-CaM and TNS-PFK were additive (Fig. 5b, trace B). However, the TNS-CaM fluorescence was stable, whereas the TNS-PFK fluorescence kept on increasing, doubling the intensity in about 1 h (Fig. 5a, trace C).

It is noteworthy that the slow increase in TNS fluorescence is not correlated with the much more rapid dissociation into dimers (Figs. 2 and 5) and must reflect subsequent molecular events, which presumably involve a structural change resulting in the exposure of a hydrophobic zone. The change in TNS-PFK fluorescence was accelerated by the presence of CaM (Figs. 5b, 5c and 5d), and the net change in about 1 h in the presence of CaM (Fig. 5b, trace D) was more than twice as much as that of TNS-PFK without CaM (Fig. 5a, trace D). Prolonged incubation (46 h) showed that the system retained the increased TNS fluorescence if the CaM:PFK ratio was > 1, but the TNS fluorescence decreased from a maximum, or even returned to the original intensity, when there was less or no CaM with PFK (Fig. 5d). In this case the behaviour of TnC was different from that of CaM, and the effect on the change of TNS-PFK fluorescence was not remarkable (Fig. 5c). The fluorescence of TNS-PFK in the presence of activators was stable (Fig. 5c); at the beginning it was the same as that of TNS-PFK without activators.

C.d. spectra and calculations

C.d. spectra of TnC and CaM, and those of PFK or PFK plus CaM or PFK plus TnC at 3, 37 and 74 min after mixing into solution, are shown in Fig. 6. The c.d. spectrum of TnC or CaM was stable, but the other systems showed a decrease in ellipticity at 223 nm. Since the ellipticity at 223 nm arises largely from the α -helical structure, and the contribution of other secondary

Table 2. Change in ellipticity and helical content of PFK and the effect of Ca²⁺-binding proteins

Concentrations were: PFK 2 μ M (0.168 mg/ml), CaM or TnC 4 μ M. Abbreviation: f_H , fractional α -helical content, as estimated from eqn. (3).

Sample	<i>t</i> (min)	$-\theta_{223}$ (°)	$-\theta_{223}$ (°)*	$10^3 \times -[\theta]_{223}$ (°)†	f_H
PFK	3	0.0245	0.0245	9.44	0.350
	37	0.0230	0.0230	8.86	0.329
	71	0.0210	0.0210	8.09	0.300
CaM		0.0205			
PFK-CaM	3	0.0450	0.0245	9.44	0.350
	37	0.0425	0.0220	8.48	0.315
	71	0.0410	0.0205	7.90	0.293
TnC		0.0200			
PFK-TnC	3	0.0445	0.0245	9.44	0.350
	37	0.0420	0.0220	8.48	0.315
	71	0.0405	0.0205	7.90	0.293

* Assuming c.d. is additive, and Ca²⁺-CaM or Ca²⁺-TnC does not change conformation with PFK.

† Instrument correction factor 1.03 is included.

structure is less important and less certain [23], it is often used in the approximate estimation of the helical content. The results showed that PFK decreased its α -helical content upon dilution and that the presence of TnC or CaM accelerated or enhanced the change (Table 2 and Fig. 6b).

Effects of CaM fragments on PFK catalytic activity

The tryptic fragments of CaM, TR1C and TR2C, were used in the PFK activity assay. In the first step of preincubation, a 3-fold excess of TR1C or TR2C was added to PFK; no effect on PFK catalytic activity was observed in the second step of the activity assay. Neither were TR1C and TR2C antagonistic to CaM, because, in the presence of a 3-fold excess of TR1C or TR2C, CaM still deactivated PFK as before (results not shown).

DISCUSSION

The preceding results have demonstrated that TnC, as well as CaM, combines with PFK and affects its catalytic activity, conformation and state of association. In view of the pronounced structural homology of the two proteins, evidence obtained from both will be combined in order to present a model for the interaction. The properties of fluorescent probes located on the

N-terminal (Cys²⁷ of CaM and Met²⁵ of TnC) and *C*-terminal (Tyr¹³⁹ of CaM) lobes as well as on the connecting strand (Cys⁹⁸ of TnC), are substantially modified as a result of combination with PFK. Probes linked to Cys²⁷ of CaM and Cys⁹⁸ of TnC are shielded from an external quencher by complex-formation. These results are consistent with, and suggest, that the above labels are involved in the zone of contact, which includes portions of both the *N*- and *C*-terminal lobes and the connecting strand.

The transient increase and blue-shift of TNS fluorescence in the presence of PFK under dissociating conditions is suggestive of a time-dependent conformational change resulting in the exposure of hydrophobic binding sites for TNS after dissociation of the PFK tetramer. The slow subsequent loss of TNS fluorescence on standing is consistent with the competitive displacement of the bound TNS by involvement of the hydrophobic binding sites in polymer formation. The observation that combination with CaM stabilizes the TNS complex indicates that the TNS-binding sites of PFK are not blocked by CaM.

Binding studies with PFK fragments have shown that cationic segments with hydrophobic residues occur in the binding domains of PFK [22], in parallel with the case of other target proteins [27,28]. There is mounting evidence that the structural regions of CaM and TnC which contact the binding domains of the target proteins include elements of both the *N*- and *C*-terminal lobes, as well as the connecting strand and, in particular, involve the hydrophobic troughs present in the *N*- and *C*-terminal halves of the Ca²⁺-liganded forms of both molecules [15,29]. The results presented here for PFK fit into this pattern (Fig. 7), although the consequences of binding CaM or TnC differ somewhat from their effects on other systems. Although it is uncertain whether the *N*- and *C*-terminal half-molecule fragments of CaM are bound by PFK, their failure to inactivate the enzyme individually suggests that the intact molecule is required to achieve the structural effects described earlier. In combination with the results obtained with the fluorescent labels, these findings indicate that both *N*- and *C*-terminal lobes are involved.

The interaction of TnC with PFK may prove to be of physiological relevance, in view of the proximity of muscle PFK to the thin filaments of the myofibril, which contain troponin, and the reported ability of PFK to interact with these [12,13]. The activity of PFK in resting muscle is orders of magnitude lower than that observed under optimum conditions *in vitro*, suggesting that the greater part of PFK is inactive in the former state [1]. The concentration of PFK in muscle (> 0.1 mg/g) is too high to account for this decreased activity in terms of tetramer $\rightarrow$ dimer dissociation [1,6], suggesting that other inactivating mechanisms must be present. Perhaps combination with CaM or TnC is a factor in the decreased activity. Because of the proximity of PFK to the thin filaments, TnC may be a more likely candidate than CaM for this function. These studies should be extended to the more complex case where the other components of troponin are present.

Conclusions

(1) TnC interacts with PFK in the presence of Ca²⁺ in a manner parallel to CaM.

(2) For both proteins the interaction involves portions of the *N*- and *C*-terminal lobes, as well as the connecting strand.

(3) The products of the dissociation of the PFK tetramer undergo a time-dependent structural change which results in the exposure of hydrophobic surfaces, which bind TNS. This is promoted by interaction with CaM.

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Fig. 7. Proposed spatial relationship of PFK and CaM or TnC subunits in complex

In this model both the *N*- and *C*-terminal lobes of CaM make contact with PFK; the connecting strand of CaM is bent so as to make this possible.

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