Phosphofructokinase is a calmodulin binding protein

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A trial to purify myosin light chain kinase from crude myosin led to the isolation of a M_r 85000 calmodulin binding protein different from this enzyme. Because it showed inherent phosphofructokinase activity we investigated its relation to this enzyme. We demonstrated identity to phosphofructokinase by a close to identical amino acid composition, by antigenic identity and a set of completely identical peptide maps. The calmodulin binding property was also shown for a fraction of the enzyme prepared by standard methods. First experiments show that Ca^{2+}–calmodulin is a potent regulator of phosphofructokinase polymerization.

Ca^{2+} effect *Calmodulin* *Myosin light chain kinase* *Phosphofructokinase* *M_r 85000 calmodulin binding protein*

1. INTRODUCTION

Phosphofructokinase (EC 2.7.1.11, PFK), a key enzyme of glycolysis, has been shown to be regulated by a variety of allosteric effectors, substrates and products [1] as well as by fructose 2,6-bisphosphate [2]. Phosphorylation–dephosphorylation has been postulated to be involved in activation of the enzyme [3,4]; the dependence of enzymatic activity on the aggregation state has been established [5]. Besides these well documented regulatory properties of the isolated enzyme in the intact muscle a direct activation of this enzyme upon contraction has been postulated [6] which points towards an involvement of calcium. Similarly calcium has been suggested to mediate an α-adrenergic PFK activation in heart [7]. However, in vitro Ca^{2+} have no or rather an inhibitory effect on PFK activity [8].

Here we demonstrate a hitherto unknown property of PFK – calmodulin binding – possibly important for the postulated Ca^{2+} regulation of PFK in vivo. Upon our search for isolation of myosin light chain kinase (MLCK) from crude skeletal muscle myosin by calmodulin–Sepharose affinity chromatography a different M_r 85000 calmodulin binding protein was characterized and proofed to be identical to PFK. These findings led us to investigate conventionally prepared PFK and we could demonstrate that indeed a fraction of the enzyme also binds to calmodulin. First experiments show that calmodulin is a potent regulator of PFK–polymerization.

Abbreviations: SDS, sodium dodecyl sulfate; DTE, dithioerythriol

2. MATERIALS AND METHODS

2.1. *Materials*

ATP, fructose-6-P and fructose-1,6-P_2 in highest purity were from Boehringer (Mannheim), [^{32}P]phosphoric acid from NEN (Boston MA). Fluphenazine was a gift of Heyden GmbH (Munich). [γ-^{32}P]ATP was prepared as in [9].

2.2. *Purification procedures*

Calmodulin from bovine brain was essentially isolated as in [10]. A final fluphenazine–Sepharose affinity chromatography was done as in [11]. Calmodulin–Sepharose 4B (0.6–0.9 mg/g gel) was prepared as in [12] at pH 9.5 in presence of 50 μM

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$CaCl_2$. Isolation of dephosphorylated calmodulin free light chains 2 (LC2) from rabbit skeletal muscle as well as of highly active myosin light chain kinase (>30 U/mg) was performed as in [13]. Second crystals of PFK (>120 U/mg) were obtained as in [14], twice precipitated myosin as in [15].

2.3. *Removal of an* M_r *85000 calmodulin binding protein from crude myosin by calmodulin–Sepharose affinity chromatography*

About 15 g of twice precipitated myosin was dissolved in 500 ml buffer A: 0.5 M KCl, 40 mM triethanolamine–HCl (pH 7.5), 1 mM DTE, 0.2 mM benzamidine; containing 0.1 mM EDTA and dialysed against 2 × 10 l of buffer A (without EDTA) for 8 h. After dilution to ~7 mg/ml with buffer A, $CaCl_2$ was added to 0.1 mM and the solution applied to two columns of calmodulin–Sepharose 4B (5 × 10 cm, 150 ml/h each) in parallel which were pre-equilibrated against buffer A including 0.1 mM $CaCl_2$. After washing with buffer A containing 0.1 mM $CaCl_2$ until no more protein was eluted a step to buffer A containing 1 mM EGTA was applied. About 50 mg of protein was eluted.

2.4. *Electrophoretic techniques and protein determination*

Gel electrophoresis was carried out as in [16,17]. Peptide mapping in presence of SDS was performed as in [18]. Samples for proteolysis contained 10 mM DTE. CNBr digestion was performed as in [19]. Protein concentration was determined [20] using crystalline bovine serum albumin as a standard ($A_{280}^{1\%} = 6.6$).

2.5. *Assays of enzymatic activities*

All activities were assayed at 25°C. MLCK assays (0.05 ml) contained 0.1 M KCl, 40 mM triethanolamine–HCl (pH 8.0), 8 mM Mg-Ac_2, 0.1 mM $CaCl_2$, 1 mM DTE, 1 mM [γ-^{32}P]ATP (0.1 mCi/μmol), 1 μM calmodulin and 60 μM LC2. Aliquots were analysed as in [21]. For myosin light chain phosphatase [^{32}P]LC2 was prepared as in [22]. From assays (0.16 ml) of the same composition as for MLCK, except that ATP was omitted, aliquots were withdrawn, mixed with 0.15 ml cold 15% trichloroacetic acid and 0.05 ml bovine serum albumin (10 mg/ml), and 0.1 ml supernatant after 15 min centrifugation was counted for released $^{32}P_i$. ATPase was assayed at the conditions given below for the PFK assay without fructose-6-P and fructose-1,6-P_2 present. PFK was assayed at 5–20 μg protein/ml in 50 mM triethanolamine–HCl (pH 7.5), 50 mM KCl, 1 mM fructose-6-P, 1 mM [γ-^{32}P]ATP (1 μCi/μmol), 2 mM $MgCl_2$, 0.1 mM fructose-1,6-P_2, 0.5 mM EGTA (0.3 ml). After starting by ATP addition, withdrawn aliquots were mixed with a cold suspension of Norit A (5%, w/v) in 5% trichloroacetic acid. After 10 min standing on ice and 5 min centrifugation 0.05 ml of the supernatant were counted. Adsorption of ATP to Norit A was quantitative under these conditions, so that the supernatant radioactivity only resulted from [^{32}P]fructose-1,6-P_2 and $^{32}P_i$ generated. ATPase controls were routinely included for correction of PFK activity. Performing this assay and the ADP-coupled optical assay [1] with standard PFK under identical conditions led to equal activities.

2.6. *Immunological techniques*

Antibodies against purified M_r 85000 calmodulin binding protein were raised in two sheep as in [23]. Agar gel double diffusion [24] was performed in 0.4 M NaCl, 20 mM Na-phosphate (pH 7.1).

2.7. *Light scattering measurements*

All solutions were shortly centrifuged at 20000 × g prior to use. To 0.4 ml of PFK_{CM} (0.2 mg/ml) in buffer A containing either 1 mM EGTA or 1 mM EGTA, 1.05 mM $CaCl_2$ and 10 μM calmodulin, 1.6 ml of a solution containing 0.125 M KCl, 10 mM triethanolamine–HCl (pH 7.5), 1.25 mM $MgCl_2$, 0.05 mM $CaCl_2$, 1 mM DTE and 0.02% NaN_3 was quickly mixed in 4 ml cuvettes. Light scattering (90° angle, 530 nm) was measured repeatedly at 30°C.

3. RESULTS

3.1. *Purification of* M_r *85000 calmodulin binding protein*

With the intention to isolate MLCK directly from crude myosin and not from a cytosolic crude extract we performed a calmodulin–Sepharose chromatography (see section 2.3). The material eluted by EGTA already consisted by ~90% of an M_r 85000 protein which we first assumed to be

MLCK. Indeed a 60–100-fold enrichment in calmodulin dependent MLCK activity was found. However, the specific activity of 12–20 mU/mg still was <0.1% of that of homogeneous MLCK purified as in [13]. Following concentration by a 55% $(NH_4)_2SO_4$-precipitation, the material was redissolved in 10 ml of buffer A and directly applied to a calibrated column of Sephacryl S-300 (2.5 × 90 cm, 20 ml/h) which was equilibrated in buffer A containing 0.05 mM EGTA. The resulting elution profiles are shown in fig.1. PFK activity (and ATPase as control) were determined because it is known that crude myosin contains significant amounts of this enzyme [25]. Two main protein peaks were eluted and the gels in fig.1 showed that both consisted mainly of M_r 85000 polypeptide which obviously was in different aggregation states; in the first peak highly aggregated, in the second one oligomeric to monomeric. The first peak showed a very low MLCK activity, ATPase activity of ~30 mU/mg and ~40 mU/mg PFK activity. The second protein peak showed two distinct maxima of MLCK activity with the relative heights varying between different preparations. Both MLCK activities were >95% dependent of the presence of calmodulin; the gels in fig.1 allowed no attribution of certain polypeptide bands to these activity peaks. Gel filtration on the same column of 10 units of highly active MLCK [13] showing an M_r of 80000 in the same gel system (see fig.1,2A) resulted in an activity peak identical in elution volume with the first maximum (arrow in fig.1). Gel filtration of MLCK showing an additional M_r 55000 degradation band led to an additional activity peak close to the second maximum of fig.1 suggesting the presence of a similar degradation product. As only a very small amount of myosin light chain phosphatase was

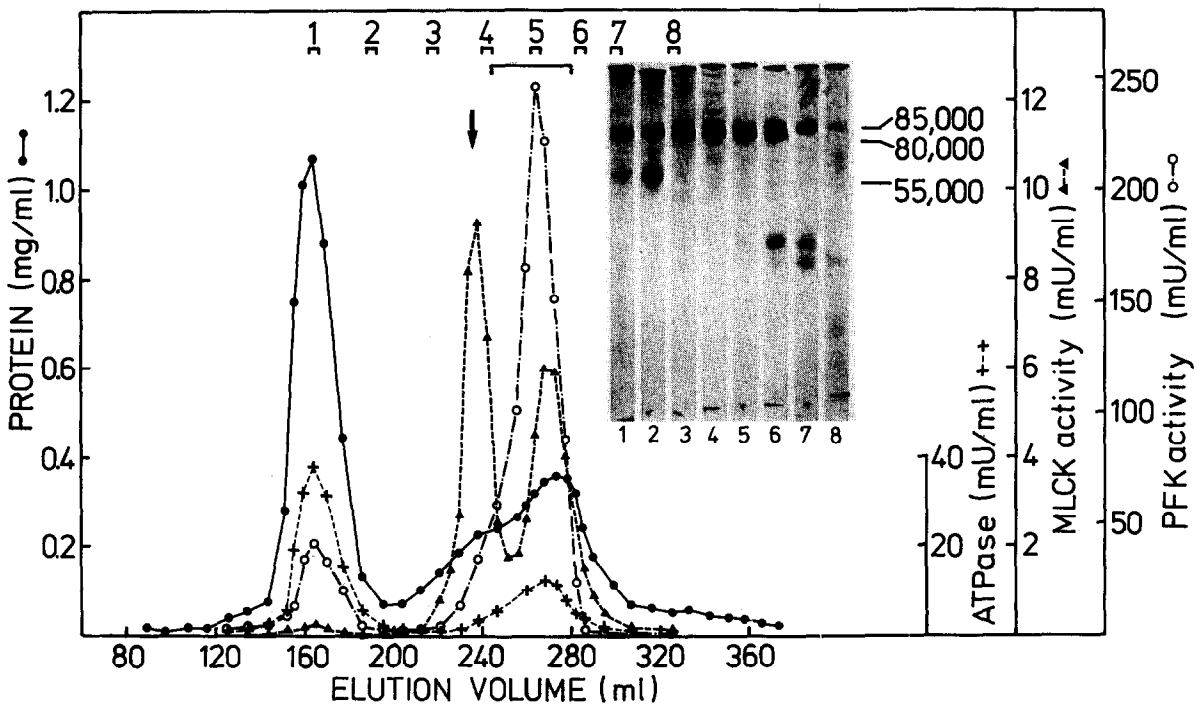

Fig.1. Chromatography of crude M_r 85000 calmodulin-binding protein on Sephacryl S300. Activities (symbols indicated at the corresponding scales) were measured as in section 2. A separate calibration was performed with proteins of known Stokes' radius (Pharmacia calibration kit). The arrow indicates the peak position of pure MLCK run separately. Numbers and small bars on top indicate the fractions from which gels, run as in [16], are shown in the inset on the right. Loadings were 10–15 μg for gels (1–6), 6 μg for (7), 4 μg for (8). The large bar on top indicates the pooled fractions further used. M_r values on the right of the inset indicate positions of pure PFK, MLCK and a M_r 55000 fragment of MLCK.

detected (maximal activity 0.02 mU/mg, not shown in fig.1) the low MLCK activities most probably may be explained by only small amounts of the above mentioned MLCK species present not detectable on gels (see also section 3.2).

The major PFK activity (max. spec. act. ~0.75 U/mg) eluted in the range of the second protein peak where protein concentration was highest and fractions showed the highest purity of the M_r 85000 band on gels (see fig.1). Stokes' radii from 37–45 Å could be estimated for this material. A second ATPase activity peak (~35 mU/mg) closely paralleled the PFK activity. The most active fractions (8–12 mg) were pooled and after the addition of $CaCl_2$ to 0.1 mM the material was reapplied to a calmodulin–Sepharose column (2.5 × 15 cm, 40 ml/h) which was equilibrated against buffer A containing 0.1 mM $CaCl_2$. All the protein was adsorbed and ~90% could be eluted again by including 1 mM EGTA into buffer A. It exhibited ~1 U/mg of PFK activity, 30 mU/mg ATPase activity and still ~20–40 mU/mg of calmodulin-dependent MLCK activity. Electrophoretically the M_r 85000 protein now was ~98% homogeneous.

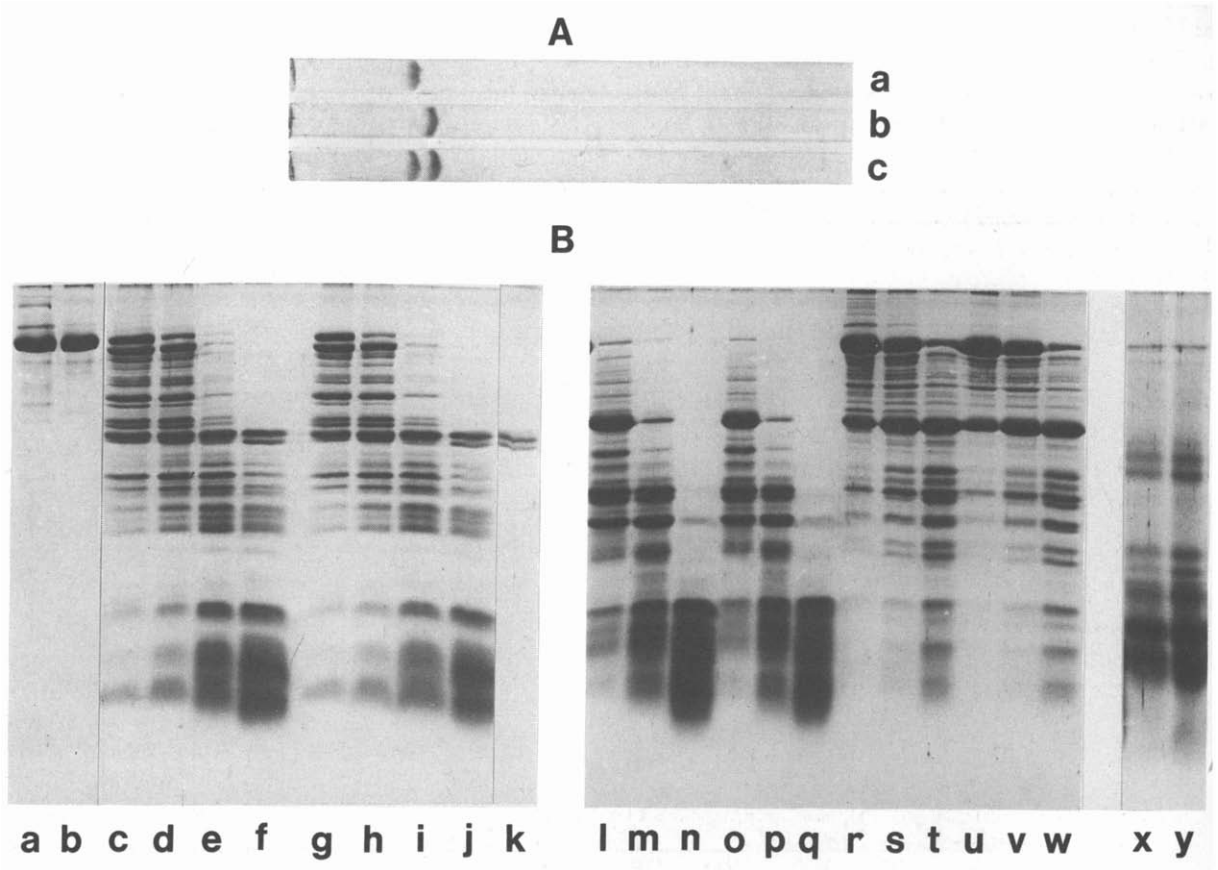

Fig.2. (A) Differentiation of MLCK and M_r 85000 protein on 7.5% gels according to [16]: (a) MLCK, 3 μg; (b) M_r 85000 protein, 3 μg; (c) 3 μg each of both proteins. (B) Peptide mapping of twice crystallized PFK and M_r 85000 protein. For separation of peptides generated as in [18] (a–w) 15% gels, for CNBr peptides (*x*, *y*) 18% gels were employed. Digestions by proteases were performed with 30 μg substrate for 30 min each: (a) PFK (10 μg); (b) M_r 85000 protein (10 μg); (c–j) *Staphylococcus* V8-protease digests of PFK (c–f) and M_r 85000 protein (g–j) with 0.08, 0.2, 0.6 and 2 μg protease each (from left to right); (k) 2 μg V8 protease incubated alone; (l–q) digests of PFK (l–n) and M_r 85000 protein (o–p) with 0.004, 0.02 and 0.1 μg papain each; (v–w) digests of PFK (v–t) and M_r 85000 protein (v–w) with 0.03, 0.1 and 0.4 μg chymotrypsin each; no band was detected after incubating 0.1 μg papain and 0.4 μg chymotrypsin; (x) CNBr peptides of M_r 85000 protein (20 μg); (y) CNBr peptides of PFK (30 μg).

3.2. *Identification of the* M_r *85000 calmodulin binding polypeptide*

On SDS gels run according to Laemmli [17] a 1:1 (w/w) mixture of pure MLCK (see section 2.2) and the isolated M_r 85000 protein could not be separated. However, on gels run according to Weber and Osborn [16], pure MLCK migrated significantly faster (M_r 80000) than the M_r 85000 protein; two distinct bands were detectable for a 1:1 mixture (fig.2A), showing that the isolated protein indeed was different from MLCK which only was still present as a contamination not detectable on gels (see above, ~0.1% of protein as calculated from a specific activity of 30 mU/mg *vs* 30 U/mg for pure MLCK).

The significant PFK activity associated with the M_r 85000 calmodulin binding protein led us to assume that the isolated protein might be indeed similar or identical to PFK, the subunit M_r of which is in the same range [1]. The ATPase activity shown above to closely correlate with PFK activity then might represent the inherent ATPase of PFK [1]. Indeed by 3 protein chemical criteria the identity of the isolated M_r 85000 protein with PFK could be demonstrated: The amino acid composition (table 1) of the isolated protein showed a significant degree of similarity with that of crystallized PFK. Proteolytic peptide maps of both the M_r 85000 protein and crystallized PFK were generated in SDS using *Staphylococcus* V8 protease, papain, chymotrypsin, and in addition cyanogen bromide digests were analysed on SDS gels. Fig.2B clearly demonstrates a complete identity in all separated peptides. Antibodies raised in two sheep against purified M_r 85000 protein in both cases showed antigenic identity of the immunogen with crystallized PFK in the double diffusion test (fig.3).

Table 1

Amino acid composition given in mol% of the M_r 85000 calmodulin binding protein and of phosphofructokinase (PFK) as reported in [30]

	Protein[a]	PFK[b]		Protein	PFK
Asx	8.9	8.9	Met	2.2[f]	2.8
Thr	6.4[c]	7.0	Ile	5.7[e]	5.8
Ser	5.4[c]	4.6	Leu	7.5	8.2
Glx	10.2	9.2	Tyr	2.3[g]	2.0
Pro	3.7	3.2	Phe	3.8	3.7
Gly	10.2	10.4	Trp	1.6[h]	1.6
Ala	7.9	8.6	Lys	5.6	5.2
Cys	2.0[d]	2.0	His	2.6	2.5
Val	7.8[e]	7.5	Arg	6.2	6.8

[a] Analysis was done from two different preparations of M_r 85000 protein after 24, 48 and 72 h hydrolysis in triplicate each in 6 N HCl at 110°C; a Biotronik-6000 analyser with a one column system (0.6 × 25 cm Durrum DC 6A resin), and lithium citrate buffers was employed

[b] The amino acid composition given in [30] has been converted into mol% values

[c] Obtained by extrapolation to zero hydrolysis time

[d] Determined as cysteic acid after performic acid oxidation according to [31]

[e] After 72 h hydrolysis

[f] Obtained from samples containing thioglycolic acid

[g] Mean of the values obtained spectrophotometrically as in [32] and those from amino acid analysis extrapolated to zero hydrolysis

[h] Determined as in [32] and colorimetrically as in [33]

3.3. *Calmodulin binding properties of crystallized PFK*

To determine whether conventionally prepared PFK also binds to calmodulin–Sepharose, 20 mg crystallized PFK were dissolved in buffer A at ~0.4 mg/ml, dialysed against 2 × 100 vol. buffer A and applied to a calmodulin–Sepharose 4B column (2.5 × 15 cm, 40 ml) as in section 3.1: 70–85% of the enzyme passed through unretarded under these conditions; the residual 15–30% could be eluted as above by inclusion of 1 mM EGTA into buffer A. Although the reason for only partial binding is not yet understood completely, this experiment showed, that conventionally prepared PFK at least in part possesses calmodulin binding potency. Repeating the affinity chromatography with the desorbed material again resulted in quantitative adsorption and specific desorption as above. Upon gel filtration as in section 2.1 most of the material eluted with a Stokes' radius of 37–50 Å and a protein profile similar to that of the second peak in fig.1. The specific activity of this material was in the range as observed in section 3.1 (up to 2 U/mg) in contrast to the high activity of the starting material (see section 2.2) before dialysis.

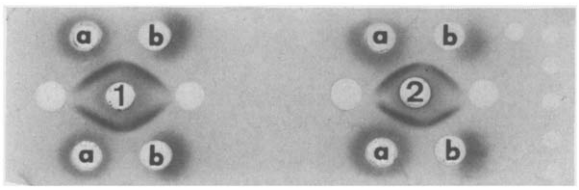

Fig.3. Agar gel double diffusion test performed with antiserum from sheep 742 (left) and sheep 744 (right): (1,2) 10 μl of antiserum each; (a) 10 μg M_r 85000 protein each; (b) 10 μg twice crystallized PFK each. Appropriate sample dilutions were made in the buffer (section 2), diffusion was allowed for 24 h and after 24 h washing in several changes of the same buffer the gels were stained with Coomassie blue.

3.4. *Effect of calmodulin on PFK desorbed from calmodulin–Sepharose (PFK_{CM})*

The specific activities of PFK_{CM} were low as compared with the ones of the conventionally prepared enzyme. A slow continuous increase in activity under assay conditions made it difficult to decide which maximum activity could be reached. Experiments which will clarify this reactivation are in progress. Preliminary data indicate a complex biphasic concentration-dependent activating and inhibiting effect of calmodulin on the activity of PFK_{CM} not simply correlating with the following polymerization phenomena. Whereas the standard enzyme preparation depolymerizes into dimers upon dilution below 150 μg/ml into buffers containing intermediate salt concentrations [5,26], PFK_{CM} polymerized upon dilution into buffers of <0.25 M KCl content at ≥ 10 μg/ml, especially at >20°C. The light scattering experiment in fig.4 showed, that polymerization of PFK_{CM} in the absence of calmodulin started after a short lag phase, reaching a constant plateau value after $\sim 10^4$ s. In the presence of μM levels of Ca^{2+}–calmodulin, however, polymerization was completely inhibited. Reducing $[Ca^{2+}]$ to $<10^{-7}$ M instantaneously released this inhibition and polymerization proceeded even faster.

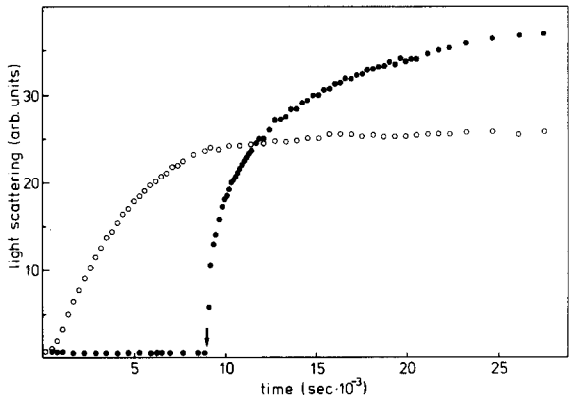

Fig.4. Effect of calmodulin on the polymerization of PFK_{CM}: (○) 40 μg/ml PFK_{CM}, 50 μM excess of $CaCl_2$ over EGTA; (●) 40 μg/ml PFK_{CM}, 2 μM calmodulin, 50 μM excess of $CaCl_2$ over EGTA. At the time indicated by an arrow, 20 μl of 20 mM EGTA were added to the latter solution. Buffer composition of both solutions: 0.2 M KCl, 1 mM $MgCl_2$, 10 mM triethanolamine–HCl (pH 7.5), 1 mM DTE, 0.016% NaN_3, 0.2 mM EGTA.

4. DISCUSSION

Our data clearly show that the low specific activity of MLCK purified from crude skeletal muscle myosin, also reported in [27] for a similar fractionation technique, is not mainly the consequence of denaturation of MLCK but results from the presence of an excess of an M_r 85000 calmodulin binding protein which we showed to be identical to PFK.

The much lower specific activities of this enzyme preparation as compared with a standard preparation of PFK may be explained by the lower polymerization state and a different inactive conformation. Tetrameric PFK (M_r ~350000, Stokes' radius 67 Å) is assumed to be the smallest *N*-mer still highly active [5]. As could be deduced from the Stokes' radius of PFK_{CM}, this species preferentially consists of the monomeric and dimeric form [5] explaining the low specific activities. A different conformation may be essential for the characteristic polymerization behavior of PFK_{CM} in the buffer system used and perhaps also for the calmodulin binding property. The slow reactivation mentioned in section 3.4 then would represent a reassociation following some conformational change of the monomers and/or dimers. Fig.1 showed that the higher polymers of PFK_{CM} in the first protein peak and in the ascending part of the second protein peak (Stokes' radius >50 Å) had only very low specific activity. A stabilization of such an inactive conformation of PFK_{CM} in those higher polymers would explain this finding.

Calmodulin binding to PFK_{CM} appears to be strong and specific:

(i) Binding to calmodulin Sepharose 4B is quantitative under the high salt conditions employed;
(ii) Desorption can be achieved by only chelating Ca^{2+} in the high salt buffer;
(iii) Complete inhibition of PFK_{CM} polymerization by calmodulin is achieved at the μM level and can only be relieved by reducing the $[Ca^{2+}]$.

A number of allosteric phenomena of PFK are reported to be correlated with changes of the polymerization state [26,28]. In analogy we propose that calmodulin-induced polymerization changes of PFK may play a role in enzymatic regulation. This hypothesis is attractive since it indeed would assign a regulatory role to Ca^{2+}.

Interestingly, the calmodulin effects on polymerization of PFK_{CM} are similar to the effects of calmodulin on tubulin polymerization [29] suggesting a more general modulatory function of calmodulin on a number of polymerizing systems.

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