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Identification of an actin binding region in aldolase

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Fragmentation of the actin binding glycolytic enzyme, aldolase, with cyanogen bromide yields an 18K actin binding fragment which corresponds to residues 1–164 of the aldolase sequence. Within this fragment there is a region of sequence (residues 32–52) which is highly homologous to a region of sequence near the C-terminus of actin itself and which is also found in the actin binding domains of a number of other actin binding proteins. A synthetic peptide corresponding to the aldolase sequence 32 52 encompassing this region of homology binds to F-actin and specifically competes with native aldolase for binding to this cytoskeletal protein.

Aldolase, Actin; Actin binding peptide

1. INTRODUCTION

For many years now it has been known that the glycolytic enzyme aldolase can interact with F-actin. In fact, aldolase was one of the first actin binding proteins to be described when it was found to be localised within the I-band of skeletal muscle myofibrils and to associate with F-actin in vitro [1,2]. Further in vitro studies have revealed that aldolase is capable of interacting with F-actin under physiological conditions and that the aldolase/actin association affects not only the kinetics of the enzyme and its ability to interact with other enzymes but also it can influence the structural organisation of the actin filaments themselves [3–6]. Electron microscopy and other physical studies have shown that aldolase is capable of organising actin filaments into highly ordered three-dimensional structures indicating that the enzyme/actin interaction may have a functional duality, one catalytic and the other structural [7]. Recently, microinjection studies of fluorescent labelled aldolase into living cells have revealed that aldolase is associated with actin in vivo, attesting to the physiological relevance of this interaction [8,9]. However, no molecular details of the aldolase/actin interaction site(s) have yet been determined. There have been numerous reports [10–12] identifying the interaction sites between actin and a variety of other actin binding proteins and in many of these studies the basic experimental approach has involved chemical or proteolytic cleavage of the binding protein with subsequent screening of the resultant peptides for their ability to interact with F-actin. Using a similar approach, we have identified an actin binding region within the aldolase molecule which contains a section of sequence homologous with a region at the C-terminus of actin itself [13].

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2. MATERIALS AND METHODS

2.1. *Preparation of aldolase peptides*

Aldolase from rabbit skeletal muscle was purchased from Boehringer-Mannheim GmbH and it was reduced, carboxymethylated and fragmented with cyanogen bromide and the peptides were separated using gel permeation chromatography on Sephadex G-75 as described by Freemont et al [14]. The isolated peptides were designated 25K (1–232), 18K (1–164), 7K (165–232), 2K (233–250) and 13K (251–363). A synthetic peptide with the sequence ADESTGSIAKRLQSIGTENTE corresponding to the rabbit muscle aldolase sequence residues 32–52 was synthesised using an Applied Biosystems Peptide Synthesiser at the Queensland Protein and Nucleic Acid Research Centre. After cleavage from the resin the peptide was purified by reverse phase HPLC on a C18 column (Spherisorb ODS2; 250 × 10 mm) using a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid over 55 min. For use in the binding studies, peptide was lyophilised and resuspended in the appropriate buffer (see below). Peptides were quantified using a fluorescamine assay [15] with glycine as standard and by reference to the known lysine content of the peptides.

2.2. *Screening of peptide binding to F-actin*

To screen for binding to F-actin, samples of the cyanogen bromide peptides 25K, 18K, 13K, 7K and 2K were incubated in the absence or the presence of 110 μg of F-actin in a final volume of 110 μl of 10 mM PIPES, 40 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, pH 6.8, in Beckman TL 100 ultracentrifuge tubes. After incubating at room temperature for one hour, 10 μl of each mixture was removed and retained. Samples were centrifuged at 80,000 rpm for 10 min and the supernatants were collected. The pellets were gently washed in the above buffer and then resuspended in 100 μl of 0.1 M sodium phosphate buffer, pH 7.5, 1 mM EDTA, 1 mM DTT. Samples of the original mixture, supernatant and pellet were analysed by SDS-PAGE.

2.3 *Quantitation of peptide binding to F-actin*

Increasing amounts of 18K peptide were incubated with 100 μg of F-actin in 10 mM PIPES, 1 mM $MgCl_2$, 0.5 mM DTT, containing

either 40 mM or 80 mM KCl, at pH 6.8 in Beckman TL 100 ultracentrifuge tubes. Increasing amounts of synthetic peptide were incubated with 400 μg of F-actin in the same buffer as above containing 40 mM KCl. Following centrifugation as described previously, the resulting supernatants were collected and analysed by reverse phase HPLC to quantitate the amount of free peptide present. Samples containing 18K peptide were quantitated by analysis on a reverse phase C8 column (Aquapore RP 300; 250 × 4.6 mm, Brownlee Labs) developed with a linear gradient from 30% acetonitrile/propan-2-ol/methanol (1:1:1) to 50% acetonitrile/propan-2-ol/methanol (1:1:1) over 10 min. The column was monitored at 215 and 280 nm using a Linear 206 PHD detector. Quantitation of the 18K peptide in each sample was achieved by measuring the area under the peak using an on line integrator (ICI DP800 data interface) and comparison of this with a standard curve established for the 18K peptide. Samples containing the synthetic peptide (aldolase 32–52) were similarly quantified by analysis on reverse phase HPLC using a C18 column (Spherisorb 5S ODS-2; 50 × 4.6 mm) developed with a linear gradient from 10% acetonitrile to 90% acetonitrile containing 0.1% trifluoroacetic acid over 10 min

2.4. *Competition between aldolase or pyruvate kinase and the synthetic peptide for binding to F-actin*

100 μg of F-actin was incubated with a fixed amount of aldolase (1.8 μM) or pyruvate kinase (1.8 μM) in 10 mM PIPES, 40 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, pH 6.8. After a 1 h incubation at room temperature, all samples were centrifuged at 80,000 rpm for 10 min and the supernatants discarded. Pellets were resuspended in 0.1 M sodium phosphate buffer, pH 7.5, 1 mM EDTA, 1 mM DTT and the amount of aldolase or pyruvate kinase present in the pellet fractions was quantitated by measuring the enzymatic activity.

2.5. *Other methods*

Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt [16] except that a 0.8 M KCl step was used. Aldolase and pyruvate kinase were assayed according to the procedure of Scopes [17] and SDS-PAGE was performed using 16% gels using the buffer system of Laemmli [18] and stained with Coomassie blue.

3. RESULTS

To screen for the ability of the aldolase cyanogen bromide derived peptids to interact with F-actin, samples of each peptide were incubated in the presence and absence of actin, centrifuged and samples of the resultant supernatants and pellets, along with a sample of the original peptide/actin mixture were analysed on SDS-PAGE. Under the conditions studied the 13K, 7K and 2K peptides did not interact with the F-actin; however, as shown in Fig. 1, the 18K peptide corresponding to residues 1–164 of aldolase, clearly bound and sedimented with the F-actin. The 25K peptide was also capable of binding to F-actin but since this larger peptide contained the 18K peptide which was capable of binding F-actin, and the 7K peptide which was not, only the 18K peptide was investigated further. To further characterise this 18K peptide/actin association, a fixed amount of actin was incubated with increasing concentrations of 18K peptide at two different ionic strengths. The samples were centrifuged and the resultant supernatants were analysed by reverse phase HPLC and the amount of peptide bound at each experimental point was calculated. Fig. 2 shows the binding isotherms obtained for 18K peptide binding to F-actin at 40 mM KCl

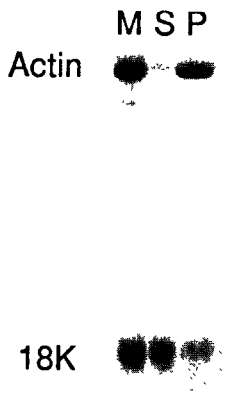

Fig. 1. Co-sedimentation of F-actin and the aldolase 18K peptide. F-Actin was incubated with the aldolase 18K peptide and following centrifugation samples of the original mixture (M), supernatant (S) and the F-actin pellet (P) were analysed by SDS-PAGE with Coomassie blue staining.

and 80 mM KCl. It can be seen that at 40 mM KCl the 18K peptide bound with a binding constant of approximately 1×10^5 M^{-1} and reached a final stoichiometric ratio with respect to actin monomers of approximately 1:2. At 80 mM KCl the apparent affinity of binding was decreased a little (binding constant of approximately 5×10^4 M^{-1}) but peptide binding still reached the same stoichiometric ratio of 18K peptide:actin.

Interestingly, as shown in Fig. 3, this region of the molecule (residues 1–164) contains a section of sequence (residues 33–42) which is homologous not only to a region near the C-terminus of actin but also to sections of sequence found within the actin binding domains of a variety of other known actin binding proteins [13]. Consequently a synthetic peptide based on this region of homology and corresponding to residues 32–52 of the aldolase sequence was synthesised, purified and screened for its ability to interact with F-actin. Fig. 4 illustrates the binding isotherm obtained when increasing concentrations of the synthetic peptide were incu-

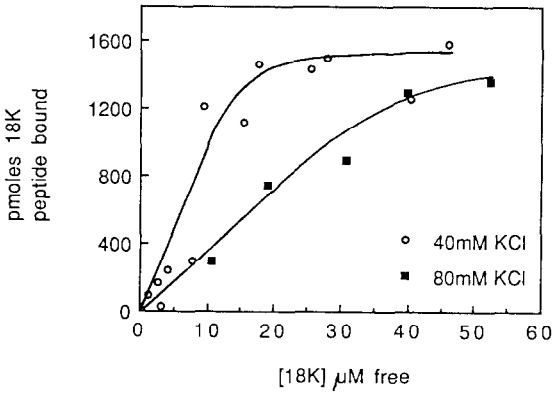

Fig. 2. Binding of the aldolase 18K peptide to 100 μg of F-actin in 100 μl of 10 mM PIPES, 1 mM $MgCl_2$, 0.5 mM DTT, pH 6.8, containing either 40 mM KCl or 80 mM KCl.

β-Actin	363	D	E	S	G	P	S	I	V	H	R	K	C	F	375	β-Actin
Aldolase Dm	33	D	E	S	G	P	T	M	G	K	R	L	Q	D	45	Aldolase Dm
Aldolase Rab.A	33	D	E	S	T	G	S	I	A	K	R	L	Q	S	45	Aldolase Rab.A
Aldolase Rat B	33	D	E	S	V	G	T	M	G	N	R	L	Q	R	45	Aldolase Rat B
Gelsolin	95	D	E	S	G	A	A	A	I	F	T	V	Q	L	108	Gelsolin
Severin	98	D	E	A	G	T	A	A	Y	K	T	V	E	L	111	Severin
α-Actin	363	D	E	A	G	P	S	I	V	H	R	K	C	F	375	α-Actin

Fig. 3 Amino acid sequence homology between aldolase and other actin binding proteins and the C-terminal end of actin. The boxes enclose regions of amino acid sequence homology with actin according to the following groups: S,T,G,A; I,V,M,P,L; H,R,K; D,E,Q,N; Y,W,F; C; Dm, *Drosophila melanogaster*; Rab. A, rabbit muscle aldolase A; Rat B, rat liver aldolase B.

bated with F-actin. While the apparent affinity of binding was much lower than that of the aldolase cyanogen bromide 18K peptide, it is clear that this synthetic peptide interacted with F-actin approaching an ultimate binding stoichiometric ratio of 1 peptide per actin monomer. Moreover, not only did this synthetic peptide bind to actin, it also specifically competed with native aldolase. When F-actin was incubated with a fixed amount of aldolase in the presence of increasing concentrations of synthetic peptide and centrifuged, it was observed that with increasing synthetic peptide concentration, there was a decrease in the amount of aldolase associated with the actin pellet (Fig. 5). However, the synthetic peptide was incapable of inhibiting the binding of another glycolytic enzyme, pyruvate kinase, to F-actin (Fig. 5) indicating that the synthetic peptide corresponding to aldolase residues 32–52 specifically competed with native aldolase for binding to F-actin.

4. DISCUSSION

We have identified that one actin binding site on the aldolase molecule is located within residues 1–164. Contained within residues 1–164 of the aldolase polypeptide there exists a section of sequence between residues 33–42 which is homologous not only to sections of sequence within the actin binding domains of some other actin binding proteins such as gelsolin and severin (Fig. 3), but also to a region of sequence near the C-terminus of actin itself. The region of homology is based around the tetrapeptide sequence of 'DESG' or 'DEAG' to be found at positions 363–366 of β- or α-actin, respectively. Within actin itself this 'DE(S/A)G' motif is believed to be located near an actin–actin interaction site involved in polymer formation as it has been shown that cysteine 374 on one actin monomer can be crosslinked to lysine-191 on another actin monomer within the F-actin filament [19,20]. The recent model of the F-actin filament proposed by Holmes et al. [21] indicates that these two residues are spatially close and in close proximity to an actin–actin interface. It has previously been proposed by Tellam et al. [13] that the actin binding properties of proteins such as gelsolin, severin, villin, etc., which possess the 'DE(S/A)G' motif may in part be achieved by mimicry of actin–actin interaction sites. The present results indicate that aldolase may also fall into this category.

The synthetic peptide corresponding to the region of aldolase which contains this sequence motif (aldolase 32–52) was not only capable of binding to actin filaments, albeit with a lesser affinity than the native enzyme, but also it was capable of specifically competing with aldolase for binding to actin filaments. Such observations indicate that this smaller region within the aldolase 18K peptide constitutes, or is at least part of, an actin binding site on this enzyme. This region is located on a small loop on the exterior surface of the aldolase subunit and it has been shown that the conformation of this region of the molecule is altered by the binding of substrate [22]. It has also been reported that fructose 1,6-bisphosphate modifies aldolase binding to F-actin in vitro [7]. While not precluding the presence of other interaction sites elsewhere within the aldolase molecule, the present results would indicate the region identified above is involved in the interaction of this glycolytic enzyme with cytoskeletal actin.

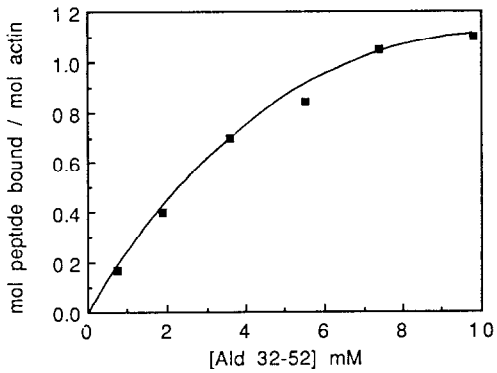

Fig. 4. Binding of aldolase synthetic peptide 32–52 to 100 μg of F-actin in 100 μl of 10 mM PIPES, 40 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, pH 6.8.

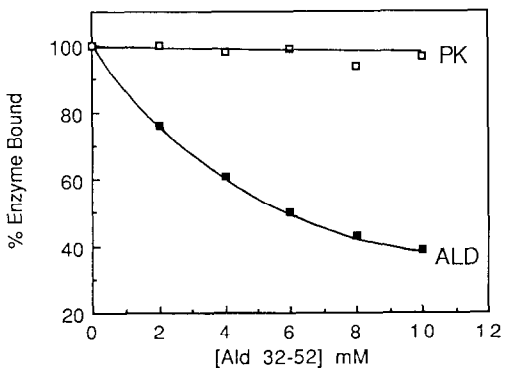

Fig. 5. Competition between aldolase synthetic peptide 32–52 and aldolase or pyruvate kinase for binding to F-actin. The binding of aldolase (1.8 μM) and pyruvate kinase (1.8 μM) to 100 μg of F-actin was determined in the absence or presence of increasing concentrations of aldolase synthetic peptide 32–52.

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