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ALDOLASE-TUBULIN INTERACTIONS: REMOVAL OF TUBULIN C-TERMINALS IMPAIRS INTERACTIONS

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SUMMARY: Aldolase copelleted with taxol stabilized microtubules with a B_{max} = 0.74 moles of aldolase per mole of tubulin dimer. Removal of the carboxy terminals from microtubules with limited subtilisin digestion, decreased binding to 0.16 moles of aldolase per mole of tubulin dimer. Aldolase inhibited subtilisin cleavage of the C-terminals while triose phosphate isomerase, an enzyme that does not interact with microtubules, did not affect subtilisin activity. These data indicate that the carboxy terminals are involved in tubulin-aldolase interactions.

Compartmentation of glycolytic enzymes has been demonstrated in many tissues including skeletal muscle [1,2], smooth muscle, erythrocytes [3], brain [4], and tissue culture cells [5]. The mechanisms involved in compartmentation are suggested to involve interactions with actin [5-8], membrane transporters including the anion transporter of human erythrocytes (also called band 3)[9-11], sodium potassium ATPase [12], and membrane receptors [13] for aldolase and glyceraldehyde 3-phosphate dehydrogenase; porin of the mitochondrial membrane for hexokinase [14], and the cytoskeleton for several glycolytic enzymes [5,15]. The binding of glycolytic enzymes to tubulin and to microtubules has been demonstrated by several techniques [16] and the relative affinity of the enzymes is of a magnitude that suggests interaction occur *in vivo* [17,18]. Several other proteins interact with tubulin and frequently these interactions occur at the carboxyl terminals [19-21], e.g., MAP-2 and tau [22]. Although motor proteins may bind elsewhere on tubulin [23], dynein is suggested to interact with the C terminal residues because removal of the C-terminals with subtilisin resulted in loss of interaction [24].

Abbreviations: PEG, poly(ethyleneglycol); MES, 2-(N-morpholino)-ethanesulfonic acid; EGTA, Ethylene glycol-bis(β-aminoethyl ether) N,N,N'N'-tetra acetic acid; PMSF, phenylmethylsulfonyl fluoride.

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The glycolytic enzymes are basic proteins, most having pI's around 8 [25]. Three enzymes (glyceraldehyde 3-phosphate dehydrogenase, aldolase, phosphofructokinase) have been shown to bind to the acidic amino terminal of human erythrocyte Band 3 through electrostatic interactions [26,27]. Tubulin, a heterodimer made up of two highly homologous subunits each having a molecular weight of about 50 kDa, binds these same enzymes [28]. The binding site on the tubulin dimer has not been identified, but both α and β subunits of tubulin contain acidic carboxyl terminals, which have similarities to the acidic sequence at the amino terminal of band 3. These similarities raise the possibility that the C terminals of tubulin bind glycolytic enzymes. The acidic terminals are exposed on the surface of microtubules when tubulin polymerizes [29]. At physiological pH these carboxyl terminals are highly charged, in an extended conformation [30] and can be selectively removed without further degradation of the tubulin monomers by digestion with subtilisin [31]. In this paper we examine the effects of removal of the carboxyl terminals from microtubules on binding of aldolase.

MATERIALS AND METHODS

Taxol was provided by Dr. Matthew Suffness, Natural Products Branch of the National Cancer Institute. Glycolytic enzymes and intermediates, poly(ethyleneglycol) with an approximate molecular weight of 8,000, MES, EGTA and all other reagents were purchased from Sigma Chemical Company, (St. Louis, MO). The glycolytic enzymes were from rabbit muscle and purchased as lyophilized powders.

Tubulin/Microtubule Preparation

Tubulin was purified by the method of Keates [32] from fresh bovine brains in the presence of glycerol through two depolymerization/polymerization cycles. After the second polymerization step the resuspended pellet, containing microtubules, was placed over 5 M glycerol in resuspension buffer (RB: 0.1 M MES, 1.0 mM EGTA, 1.25 mM magnesium acetate, pH 6.6) and sedimented at 150,000 x g for 40 min. The pellet was stored at -70°C.

Prior to conducting binding experiments, tubulin was subjected to a third cycle of depolymerization and polymerization. Depolymerization was accomplished by suspending the microtubules in 3-4 volumes of ice cold RB for 1 hr followed by centrifugation (412,000 x g, 16 min, 4°C). The tubulin was then polymerized by the addition of GTP to 1 mM and taxol to 10 μM and incubating the mixture at 37°C for 15 min. The solution, containing newly formed microtubules, was then centrifuged (412,000 x g, 16 min, 32°C) and the pellet was resuspended to a concentration of 6-7.5 mg of protein/ml in one fifth strength RB (0.02 M MES, 0.2 mM EGTA, 0.25 mM magnesium acetate, pH 6.6). Taxol was used in all studies to stabilize microtubules and prevent breakdown into smaller, nonsedimentable fragments [33].

Saturation Studies with microtubules

Aliquants of resuspended microtubules containing 300μg of protein were pipetted into microcentrifuge tubes and increasing amounts of aldolase from a stock solution of 10 mg/ml (in 1/5 strength RB) were added. The total volume of the mixture was adjusted to 100 μl with the same buffer and mixed by light vortex action then incubated at 15°C for 15 min and centrifuged (85,400 x g, 11 min, 15°C). The supernatants and pellets were immediately separated, the pellets resuspended in 100μl RB and both fractions were assayed for enzymatic activity.

Saturation Studies with Subtilisin Digested Microtubules

Thrice-cycled tubulin (as described under Saturation Studies) was polymerized by warming to 37°C for 15 min in the presence of 1 mM GTP and 10 μM taxol. Protein concentration was determined and the microtubules were digested for 30 min with 1% (w/w) subtilisin [31,34].

Controls were treated identically except subtilisin was not added to the incubation. Proteolysis was stopped by addition of 2 mM PMSF [31,34] and the mixtures were centrifuged to pellet the microtubules. Pellets were resuspended to a final protein concentration of approximately 6 mg/ml in 1/5 strength RB. Aliquants containing 300 µg of microtubules or subtilisin treated microtubules were pipetted into microcentrifuge tubes and mixed with increasing amounts of aldolase. The mixtures were adjusted to 100µl with 1/5 strength RB, incubated for 15 min at 15°C, centrifuged and assayed as above.

Effect of aldolase on subtilisin digestion of microtubules

Taxol stabilized microtubules (30 µM) were incubated with triose phosphate isomerase (30 µM) or aldolase (30 µM), with and without 4% PEG. Subtilisin was added to 1% and the mixture was incubated at 18°C. Subtilisin was inactivated at 10 or 30 min by adding PMSF to a final concentration of 2 mM. Aliquots were alkylated in 25 mM Tris buffer containing 50 mM iodoacetate and 170 mM SDS at pH 9.0 for 2 h in the dark [35] followed by mixing with an equal volume of SDS sample buffer [36]. SDS-PAGE was conducted in 8% polyacrylamide gels [36].

Enzyme Assays and Protein Determination

Aldolase was assayed according to Bergmeyer et al. [37]. Protein concentration was determined by the method of Bradford [38] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Treatment of microtubules with subtilisin almost abolished aldolase binding to microtubules (Table 1). This effect suggests that the carboxy terminals are responsible for aldolase binding to microtubules. The B_{max} of 0.74 is approaching one mole of aldolase tetramer per mole of tubulin dimer is similar to that derived by Somers et al [18] who have evaluated binding of glyceraldehyde-3-phosphate dehydrogenase to microtubules. The slope of the Hill plot was 1.03 ± 0.10 for the untreated microtubules indicating a single binding site for aldolase on tubulin.

Since subtilisin cleaves only the carboxyl terminals when the polymerized form of tubulin is used as the substrate [39,40] and since aldolase binding is reduced by cleavage of the C-terminals (Fig. 1) it was postulated that aldolase may mask subtilisin cleavage sites on tubulin. In the absence of aldolase, subtilisin cleaved the bulk of the C-terminals within 30 min (Fig. 2).

Table 1: Binding of Aldolase to Microtubules and Subtilisin Treated Microtubules

	Untreated Microtubules	Subtilisin Treated Microtubules
B_{max}	0.74 ± 0.11	0.16 ± 0.06***
Slope of Hill Plot	1.03 ± 0.10	0.80 ± 0.23

Aldolase was incubated with 300 µg of either microtubules or subtilisin treated microtubules. After incubation for 15 min at 15°C the samples were centrifuged, the supernatants and pellets were separated and both fractions were assayed for enzyme activity. B_{max} is given as moles of aldolase bound per mole of tubulin in the dimer state. *** indicates significance at the 0.001 level, n = 6 ± SEM.

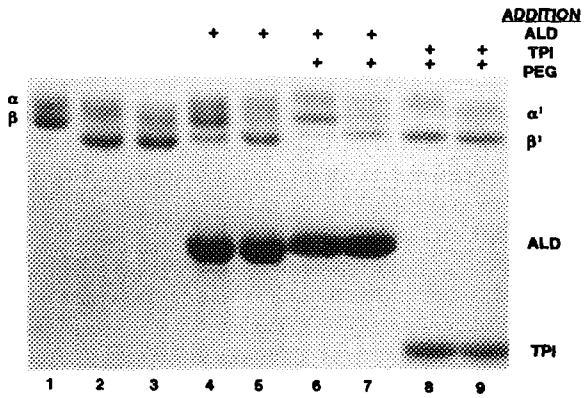

Figure 1. Microtubules were digested with subtilisin as described in the methods. Lane 1 is a sample of untreated microtubules, the upper band is α tubulin and the lower band is β tubulin. Lanes 2 and 3 are samples of microtubules digested with subtilisin for 10 and 30 min. The bands in lane 3 correspond to α' and β' tubulin. Lanes 4-7 represent incubations with added aldolase (ALD) and lanes 8 and 9 with added triose phosphate isomerase (TPI). PEG was included in incubations displayed in lanes 6-9. The additions are indicated by the plus signs above the gel. The migration of α and β are indicated on the left. Migration of α', β', aldolase, and triose phosphate isomerase are shown on the right. α' and β have virtually identical mobility.

When stoichiometric amounts of aldolase were included, the extent of cleavage was greatly reduced and when polyethylene glycol was included to enhance the interaction between aldolase and microtubules [17], the amount of cleavage was reduced further. Triose phosphate isomerase, an enzyme that does not bind to tubulin or microtubules [17], did not prevent the subtilisin cleavage of the C-terminals. N-terminal sequence analysis of both α and β tubulin and the subtilisin cleaved α and β tubulin revealed that this portion of the tubulin molecule was unaltered by the subtilisin treatment. The N-terminals of the untreated and the subtilisin cleaved fragments were sequenced. Both α and α' had the N-terminal sequence MRECISCHVG, and β and β' had the sequence MREIVHINAG indicating that the small segments released by subtilisin digestion were from the C-terminals as reported by others [30,41].

The glycolytic enzymes are basic proteins with pI's near 8 [25], while most proteins that bind glycolytic enzymes are acidic eg., actin [42], band 3 [43], and tubulin [44]. Steck has shown some glycolytic enzymes (aldolase, phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase) bind to the acidic carboxyl terminal of the erythrocyte Band 3 [9-11]. Like Band 3, both the α and β subunits of tubulin have highly charged acidic carboxyl terminals and removal of the carboxyl terminals reduced interaction with aldolase, consistent with the concept that carboxyl terminals are involved in aldolase binding. Experiments to identify the tubulin sequences involved in binding are now in progress.

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