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Influences of Temperature Adaptation on the Concentration and Composition of Brain Microtubule Proteins in a Poikilothermic Animal

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The European pike is a poikilothermic animal living for long periods at temperatures of 0-5 °C. The fact that these animals live and actively swim at temperatures around 0 °C implies that the microtubules required for proper neuronal function are cold-stable, while those of warm blooded animals disassemble at temperatures between 5 and 25 °C. Therefore we addressed the question of cold-stable microtubules. Groups of pikes were reared for 14 days at either 18 °C or 4 °C, and the microtubule protein fractions from these two groups were compared. The microtubule protein preparations from pikes reared at the lower temperatures contained a considerably lower amount of high-molecular weight microtubule-associated proteins and have a lower ability to assemble compared to those reared at higher temperatures. We also offer evidence for the presence of a 130 kD cold-stabilizing factor. The results obtained from cold- and warm-reared pikes correlate very well with the results obtained from pikes kept in outdoor tanks and processed during the winter and summer months respectively.

Key Words: fish brain, temperature adaptation, cold-stable microtubules, MAPs,

Introduction

The nervous system of the European or Northern pike (*Esox lucius*), especially the olfactory system, has been exploited for a long time as a convenient model system for the biochemical and morphological characterization of fast axonal transport [7, 25, 27]. Axonal transport has long been suggested [for reviews see 24, 26] and is now proven to be microtubule-dependent [1, 2, 19]. Such investigations require detailed knowledge of the biochemical characteristics of the microtubules and their associated proteins and should enable us to better understand the molecular mechanisms and to identify the role of microtubules and their associated proteins.

Thus we isolated microtubule proteins (MTPs) from the central nervous system of pikes and investigated their protein composition and certain aspects of their assembly characteristics. Since the pike is a poikilothermic animal living for long periods of time at temperatures of 0-5 °C we addressed also the question of cold-stable microtu-

bules, which is in the present still incompletely understood. The fact that these animals live and actively swim at temperatures around 0 °C implies that the microtubules required for proper neuronal function [17, 24] are cold-stable while those of warm blooded animals disassemble at temperatures between 5 and 25 °C [6]. While in most other studies on this subject either warm-blooded or stenothermic animals were used [5, 9, 13], we took advantage of the fact that pikes are poikilothermic and live at temperatures between 0 and 19 °C. In the present study we compared the MTPs of groups of fish of the same species reared at the two extreme temperatures in order to describe their differences in composition, assembly characteristics and stability of the reassembled structures.

Material and Methods

Purification of microtubule proteins

MTPs were purified by taxol-induced polymerization [21] or by two or more cycles of temperature-dependent assembly/disassembly [10]. MTP fractions prepared in the presence of taxol are designated as "T"-fractions in the figures and tables. To inhibit proteolytic breakdown [see 12] and for preservation of MAPs we included the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) at 1mM in all buffers used. The purification schemes of both methods and the nomenclature of the individual fractions are shown in Fig. 1.

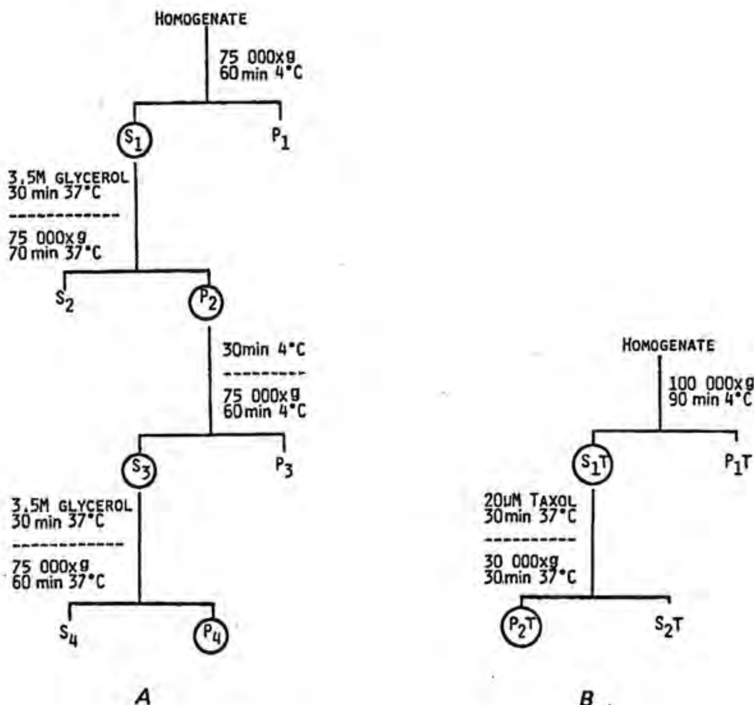

Fig. 1. Purification schemes and nomenclature of MTP-fractions
A — Preparation of MTP by cycles of temperature dependent polymerisation/depolymerisa-tion: S — supernatants; P - pellets. Encircled fractions are typically enriched in MTPs.B — Preparation of MTP by taxol-induced polymerization: T - MTP-fractions prepared in the presence of taxol.

Experimental animals

Pike-brains were dissected out and immediately homogenized in a buffer consisting of 0.32 M sucrose, 1mM EGTA, 1mM PMSF, pH 7.0. A total number of 52 pikes were used in these experiments.

Rearing of pikes under controlled temperature conditions

Groups of 7 pikes were transferred to indoor tanks (2.5 m^3). The temperature of the warm tank was maintained between 17.5 and 18.5 °C and in the cold tank - between 3.5 and 5.5 °C. In the following text warm- and cold-reared pikes are referred to as "warm"- or "cold"-pikes. Since most temperature adaptation processes are known to occur in the range of days all animals were kept at constant temperatures for at least 14 days prior to use.

Gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis (PAGE) was performed according to L a e m m l i [14] on 1mm-thick 7.5% polyacrylamide slab gels. 5-15 µg of protein were loaded per sample well.

Densitometry

Coomassie Brilliant Blue R_{250}-stained gels were quantified on a computer-driven ELSCRIPT densitometer at 570 nm. The values obtained for the same fraction run on different gels were within 5%.

Silver staining

Fixed and Coomassie R_{250}-stained were destained and carried through the silver staining procedure of M e r r i l a n d G o l d m a n [16].

Results

Preparation of tubulin from pike brains

During the initial attempts to isolate MTPs from the brains of pikes we encountered certain difficulties due to the limited amounts of brain tissue (1 pike brain from a 70 cm-long fish weighs on average 0.4 g), and the very low yield of MTP when the preparation was attempted by cycles of temperature-dependent assembly/disassembly [10]. Assuming that the assembly characteristics of pike tubulin might differ from those of the commonly used warm-blooded animals we used in addition a method where tubulin polymerization is aided by the microtubule-stabilizing agent taxol [21]. This method resulted in considerably higher yields of pike-MTP. Because of the low yields of pike tubulin we tried to disrupt the native microtubules by incubating pike brain homogenates for an additional 30 min on ice (longer periods were not found to be more effective). This doubled the yield of tubulin in the final P_2T pellet. The additional inclusion of Ca^{2+} during the incubation of pike homogenates on ice did not release more MTP from the homogenates (Table 1).

Purification of MTP from pikes reared under controlled temperature conditions

Groups of pikes were reared simultaneously for 14 days at 3.5-5.5 °C or at 17.5-18.5 °C. MTPs from pikes reared under controlled temperature conditions were purified by cycles of temperature-dependent polymerization, which allows a better assessment of MTP-assembly, than the method employing microtubule stabilization with taxol.

The yield of MTPs in all fractions compared from "warm"-pikes was significantly higher than from "cold"-pikes (Table 1). Already after the first polymerization step

T a b l e 1. Protein yields (mg MTP/kg brain tissue) in MTP-enriched fractions

Method of preparation	Treatment of homogenate	Fraction	Pike			
Vallee [21, taxol-induced assembly]	none	P_2T	403- 457 (5)*			
	30 min at 0°C	P_2T	983-1192 (4)*			
	30 min at 0°C $+5\times10^{-3}$M Ca^{2+}	P_2T	873- 940 (4)*			
			"warm"	summer	"cold"	winter
Karr et al. [10] (temperature-induced assembly)	none	P_2	475 (2)	410 (2)	135 (2)	237 (2)
	none	S_3	385 (2)	315 (2)	105 (2)	163 (2)
	none	P_4	290 (2)	223 (2)	i.m.	i.m.

N o t e s. Numbers in brackets = number of experiments; i.m. - insufficient material; * MTPs from pike were prepared by the method of V a l l e e [21] during winter. Values are given as average, differences were in no case greater than 10%.

the yield of "warm"-pike-MTP was about twice the MTP-yield from "cold"-pikes (Table 1, pike-P_2). In the subsequent depolymerization step this difference became even more pronounced (Table 1, pike-S_3). Upon assembly hardly any protein could be sedimented from the "cold"-pike-S_3, whereas pellets sufficient for further analysis were obtained from "warm"-pike-S_3. In addition we found a very good correlation between the amounts of MTP obtained from the brains of pikes processed during the summer and winter months and the MTP yields from "warm"- and "cold"-pikes respectively.

PAGE of MTP fractions

Analyses of the protein patterns of the final MTP pellets prepared by taxol-induced assembly revealed the almost complete absence of high molecular weight (over 190 kD) MAPs in the pike fractions (only 2% of the total protein).

Analyses of the protein patterns of MTP-fractions prepared by cycles of temperature-dependent assembly show the lowest amounts of tubulin occur in the homogenates of "cold"-pike brains. This difference persists in the first high-speed supernatants (fractions S_1) and in the first microtubule pellets (P_2). Significant amounts of high molecular weight-MAPs (HMW-MAPs) were found in almost all fractions (Fig. 2). However, the lowest amounts of HMW-MAPs were found in "cold"-pike fractions. The HMW-MAPs of fractions obtained after the first polymerization cycle (S_3) in most cases became detectable only on heavily overloaded gels or after silver-staining (Fig. 3).

Yield and distribution of tubulin

On the basis of the relative concentrations of tubulin in the individual fractions we calculated the %-yields of tubulin in each fraction and its distribution between supernatants and pellets obtained on each centrifugation step. From this analysis (Table 2) we can derive conclusions about the apparent cold-stability and polymerizability of tubulin at different stages of purification. Among the differences found between the yields and distribution of tubulin from warm- and cold-reared pikes a higher yield of tubulin from "cold"-pikes on depolymerization of the native microtubules (fraction

S_1) is evident. However, in the following polymerization/depolymerization cycle the tubulin from "warm"-pikes exhibits better polymerizability (pike P_2 fractions) and lesser cold-stability (pike P_3 fractions) in comparison to "cold"-pike tubulin. In the final polymerization step only "warm"-pike tubulin yielded sizeable pellets, whereas no microtubules could be pelleted from "cold"-pikes.

Differences between the non-tubulin proteins from "warm"- and "cold"-pike

Comparing the protein patterns of "warm"- and "cold"-pike S_3 (the most purified MTP fraction which we were able to obtain from "cold"-pikes) the following qualitative and quantitative differences become apparent. "Warm"-pike S_3 contains one or two prominent and very high molecular weight bands of unknown nature, which remain in the stacking gel (bands 1, Fig. 2), completely absent in "cold"-pike S_3. "Warm"-pike S_3 contains considerably higher amounts of proteins comigrating with pig MAP 1 and MAP 2 as compared to "cold"-pike S_3. The densitometric tracings (Fig. 3) illustrate in a more detailed manner the qualitative and quantitative differences observed in the high molecular weight region of pike S_3 fractions. The identity of the bands tentatively identified on the basis of migration in polyacrylamide gels as MAP 1 and MAP 2 of pike-MTP was confirmed by the antibodies, specific for these peptides.

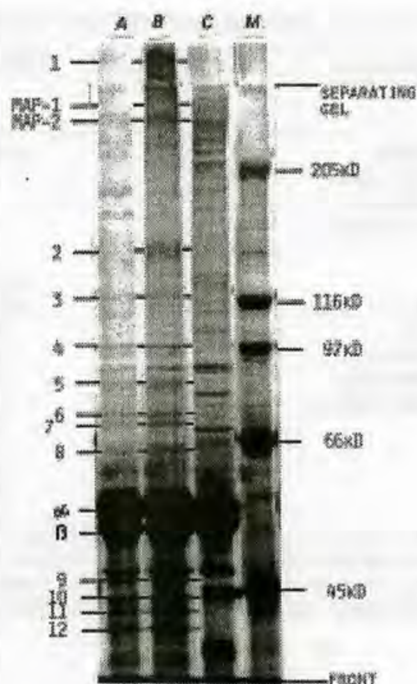

Fig. 2. Pike-specific protein bands
Silver-stained gels of S_3—fractions from: "cold"-pike (*A*), "warm"-pike (*B*), pig brain (*C*), marker proteins (*M*). Protein load - 7 µg/start

The "cold"-pike P_2- and P_3-fractions, which are enriched in cold-stable microtubules, contain a protein band of apparent molecular weight (MW) of 130kD. This protein is absent in the corresponding "warm"-pike fractions (Fig. 4). It should be noted that most of the bands shown here contain very little protein and are only detectable in silver-stained gels.

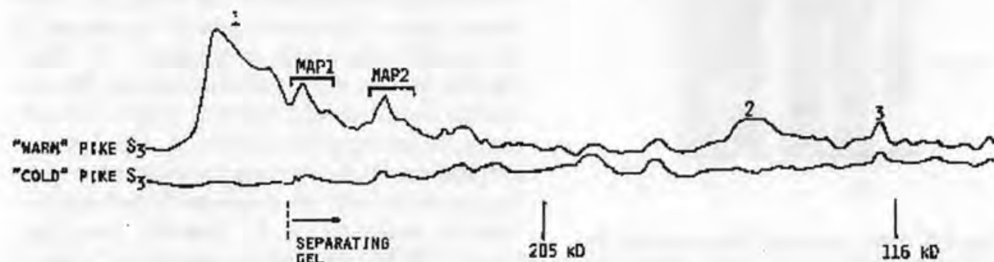

Fig. 3. Densitometric analysis of HMW proteins from "cold"- and "warm"-pikes
Densitograms of silver-stained gels showing the quantitative and qualitative differences between S_3-fractions obtained from "cold"- and "warm"-pikes. Protein load - 7 µg/start. Bands 1 to 3 are found only in pike MTP-preparations

T a b l e 2. Yield and distribution (%) of tubulin between fractions

Method of Preparation	Fraction	Yield of tubulin (%)* pike		Distribution of tubulin (recovered)* pike	
Vallee	HT	100.0		S + P = 100%	
[21], (taxol-	S_1T	31.2		32.4	
induced-	P_1T	65.1		67.6	
assembly)	P_2T	8.3		28.1	
	S_2T	21.1		71.9	
		"warm"	"cold"	"warm"	"cold"
K a r r et al.	H	100.0	100.0		
[10]	S_1	33.0	45.1	37.5	52.5
(cycles of	P_1	55.0	40.2	62.5	47.1
assembly/	P_2	7.8	5.8	30.0	15.7
disassembly)	S_2	19.1	31.1	70.0	84.3
	S_3	3.6	1.9	55.4	35.8
	P_3	2.9	3.5	44.5	64.2
	P_4	2.5	i.m.	73.5	i.m.
	S_4	0.9	i.m.	16.5	i.m.

N o t e s. i.m. - insufficient material;* yield of tubulin was calculated as % from the tubulin found in the homogenate (H). The amounts of tubulin in the individual fractions were estimated on the basis of the relative tubulin concentrations of these fractions by densitometry of polyacrylamide gels;** the sum of the tubulin amounts recovered in daughter fractions is taken as 100%.

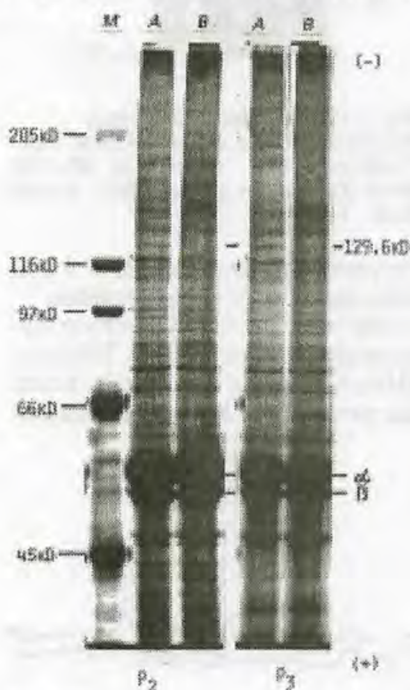

Fig. 4. Protein pattern of polymerizable (P_2) and cold-stable (P_3) fractions from pike brain

Silver-stained gels of fractions P_2 (containing microtubules) and P_3 (containing cold stable microtubules) obtained from the brains of "cold"-pikes (*A*), "warm"-pikes (*B*), marker proteins (*M*). Protein load - 15 μg/start

Discussion

Cold-stability of microtubules

The exact cause of the apparently greater cold-stability of "warm"-pike microtubules as compared to "cold"-pike microtubules which is found during the first cold treatment remains unclear. One possibility could be a different behavior of "stable tubules only"-protein (STOP) regarding its affinity to microtubules or its relative concentration.

The ensuing increase in the cold-stability of *in vitro* polymerized "cold"-pike microtubules in the course of the purification could be due to the presence of a soluble cold-stabilising factor in the brains of cold-reared pikes. This factor would be present in the crude high-speed supernatant - S_1. During the first in vitro polymerization the assumed factor would interact with microtubules, pelleting with them in P_2. On depolymerization such a protein should stay predominantly with the cold-stabilized microtubules pelleting in P_3. Indeed, an additional 130 kD protein is present in "cold"-pike P_2 and P_3 but not in the corresponding "warm"-pike fractions (see Fig. 4). The enhanced synthesis of such a soluble microtubule cold-stabilising factor can be one of a

number of mechanisms responsible for the processes of adaptation of the poikilothermic pikes to lowtemperature conditions.

A large spectrum of proteins of different MW have been suggested as cold-stability-conferring factors found in various species in the recent literature. Some of the 12 proteins which we found only in the MTP-preparations from pike (as compared to pig-brain derived MTPs, 11) can be correlated by comparison of MW with cold-stability-conferring proteins described in the literature. Pirollet et al. (18) describe a protein of an apparent MW of 33 kD responsible for cold-stabilization of sheep-brain microtubules. A protein of similar MW (35 kD) is found in our pike MTP-preparations (band 12, Fig.2).

Proteins from bovine brain in the MW regions of 64 and 70-80 kD have been described by J o b et al. [9]. Pike-specific protein bands are found in this MW region too (band 7, 8 and 9, Fig. 2). The major STOP has a MW of 145 kD (bovine) or 135 kD (rat) [4, 9, 15]. Further studies are clearly necessary before physiological functions can be assigned to these polypeptides.

Temperature adaptation

The differences between microtubule cold-stability, tubulin polymerizability and protein pattern of MTP-preparations obtained from "cold"- and "warm"-pikes is another example of the ability of living organisms to adapt to changing environmental conditions. One known mechanism for temperature adaptation is a change in the concentration of some proteins [e.g. enzymes, for review see 8]. Further possibilities involve changes of the catalytic activities of enzymes or modulation of enzyme-substrate and protein-protein interactions.

The differences observed between "cold"- and "warm"-pike-MTPs may be the manifestation of a complex interplay of the above-mentioned adaptation mechanisms, since we observe both changing amounts of tubulin and HMW-MAPs and the appearence of a 130 kD protein. It could also be suggested that temperature adaptation brings about a change between pike-MAPs and tubulin interactions [see also 3, 20]. It is also possible that an eurythermal adaptation process takes place through the presence of iso-forms with different thermal optima for a reaction, in this case assembly. This can be suggested on the assumption that the additional protein found in the tubulin region of pike-MTP [11, 23] is actually an iso-tubulin keeping the structural and dynamic characteristics [see e.g. 22] of pike-microtubules constant under different temperature conditions.

More detailed studies on the nature of proteins found in pike MTP-preparations tentatively identified as MAP1 and MAP2 by PAGE or as a putative tubulin iso-form are needed for a better interpretation of these results. Such knowledge may also prove of great importance for further work on the intracellular dynamics and turnover of cytoskeletal proteins, on their involvement in axoplasmic transport and microtubule-based organelle movement in general, as well as on cold-stabilization of microtubules.

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