# A novel tau-tubulin kinase from bovine brain

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**Abstract During purification of tau protein kinase I and II from the bovine brain extract, a new tau protein kinase was detected and purified with phosphocellulose, gel filtration, S-Sepharose and AF-Heparin column chromatography. The molecular mass of the enzyme was determined to be 32 kDa by gel filtration and activity staining on SDS-PAGE. The enzyme is a Ser/Thr protein kinase phosphorylating tau, β-tubulin, MAP2 and α-casein. Employing many synthetic peptides, the recognition site of this enzyme appears to be –SR–. The enzyme requires no second messenger and is inhibited with high concentration of heparin, but not by inhibitors of CKI. These results indicate that this enzyme, tau-tubulin kinase is novel and distinct from TPKI, II and CKI, II.**

*Key words:* Protein kinase; Tau; Tubulin; Ser-Arg motif; Bovine brain

## 1. Introduction

We have previously reported that tau protein kinases I and II (TPKI, -II) from bovine brain extract phosphorylate tau and form paired helical filament (PHF) epitopes [1–3]. Although phosphorylation of tau by TPKI and -II was stimulated by tubulin under the conditions of microtuble formation, these enzymes have no ability to phosphorylate tubulin [1]. In the course of the purification of TPKI and -II, we found a novel tau protein kinase which phosphorylates both tubulin and tau. The enzyme, tau-tubulin kinase (TTK) was purified primarily as a microtuble associated protein (MAP). However, TTK was also found in the supernatant when separated from microtubles using temperature-dependent polymerization and depolymerization method.

It is known that cAMP-dependent protein kinase, calmodulin kinase and casein kinase (CK) II phosphorylate tubulin [4–6]. Recently, it has been reported that casein kinase-like kinases from a neuronal cell line PC12 and bovine brain phosphorylate β-tubulin, but not the CKII-specific synthetic peptide [7]. The molecular mass of these enzymes are different from TTK and there is no description on tau phosphorylation.

The characterization of TTK will clarify its role in the phosphorylation of tau and tubulin and may contribute to the elucidation of the possible roles of microtubles and PHF-tau formation. In this paper, the purification and the properties of TTK are described and the phosphorylation site sequence for TTK was determined.

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*Abbreviations:* PHF, paired helical filaments; TTK, tau-tubulin kinase; TPK, tau protein kinase; CK, casein kinase; MAP, microtuble-associated protein; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

## 2. Materials and methods

### 2.1. Materials

Adult bovine brains were obtained from the Nakashibetsu preparation center, Mitsubishi Kagaku Co. Tau, tubulin and MAP2 were prepared from bovine brain extracts [1,8]. Tau peptides K1, K2, K3 and 4 repeat, p25 protein peptides and their analogs, glycogen synthase peptide GS-1 were synthesized chemically with the solid phase method, on a Biosearch model 9500 or ABI model 431A peptide synthesizer [8–11]. In accordance with the amino acid numbering of human tau, the sequences of K1, K2, K3, and 4 repeat were found in the regions of 168–182, 133–166, 307–349 and 194–224, respectively. Peptides, 8655, 8656 and 8669 were obtained from Funakoshi. Most chemicals, except where noted, were obtained from Sigma.

### 2.2. Preparation of bovine brain extract

Bovine brains were homogenized in buffer A (100 mM MES–NaOH, pH 6.5, 0.5 mM Mg-acetate, 1 mM EGTA) and treated with assembly–disassembly cycles to prepare MAPs as described previously [1]. The MAP fractions were used for the preparation of TPKI and -II [1]. While, the supernatant eliminating MAP fractions was used to prepare TTK. During the purification, all buffer solutions contained 0.02% Tween 20, 10% glycerol, 5 mM 2-mercaptoethernol, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg /ml leupeptin, 1 μg /ml pepstatin, and 1 μg /ml antipain.

### 2.3. Kinase detection assays

Phosphorylation of tau and other substrates were measured by quantifying the radioactive phosphate incorporated from [γ-$^{32}$P]ATP (Amersham) into the substrates at 37°C as described previously [8]. The reaction mixture contained 600 μM [γ-$^{32}$P]ATP, substrate (0.3–0.6 mg/ml) in buffer B (100 mM MES–NaOH, pH 6.5, 5 mM Mg-acetate, 1 mM EGTA). In case of synthetic peptides, the reaction mixture contained 600 μM [γ-$^{32}$P]ATP, 0.66 mg peptide in buffer A. One unit of the kinase was defined as the amount of the enzyme required to incorporate 1 pmol of $^{32}$P into the substrate in 1 min at 37°C. The protein concentration was measured spectrophotometrically assuming that 1 absorbance unit at 280 nm is equal to 1 mg/ml.

Kinase activity staining on SDS-PAGE was done using the gel containing 0.4 mg/ml tau or 0.8 mg/ml tubulin by the method of Kameshita and Fujisawa [12]. The kinase was also detected by phosphorylation with [γ-$^{32}$P]ATP, followed by SDS-PAGE and autoradiography.

### 2.4. SDS-poly acrlyamide slab gel electrophoresis

SDS-PAGE was carried out with a 3% stacking gel and a 10% resolving gel. Gels were stained with Coomassie brilliant blue R250 or 2D-silver stain II (Daiichi Pure Chemicals). For autoradiography the gels were dried in vacuum and exposed to Kodak Omat RPI 6 film at −80°C.

### 2.5. Determination of phosphorylation sites in the synthetic peptide-F5b

For analysis to distinguish phospho-Ser from Ser, a modified sequencing protocol was used [13]. Phospho-Ser quantitatively forms dehydroalanine, followed by an addition reaction with DTT, yielding a DTT adduct of phenylthiohydantoin (PTH) dehydroalanine, while, Ser yields both PTH-serine and DTT adduct of PTH-dehydroalanine. The chemically synthesized peptide F5b was maximally phosphorylated with TTK. The phosphorylated peptide was sequenced and the result was compared with that of non-phosphorylated F5b which gave the ratio of PTH-serine and PTH-dehydroalanine 3:1 at normal serine site of cycle 4. While the phosphorylated peptide gave the ratio of those 1:1.8, indicating that the 4th serine is phosphorylated by TTK.


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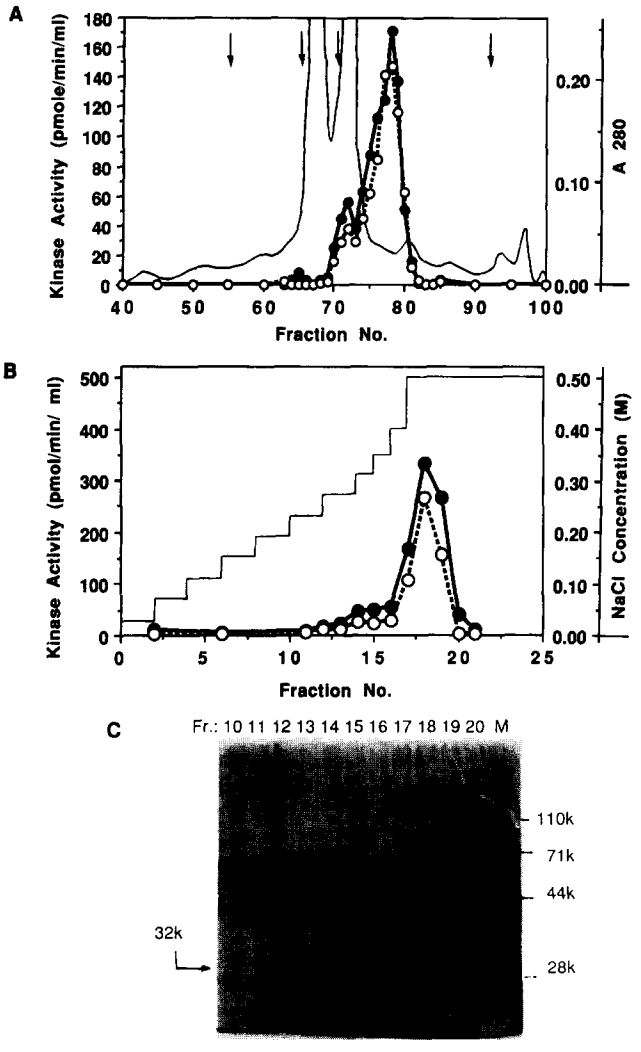


Fig. 1. (A) HPLC on G3000SW column of TTK. The arrows indicate the position of markers, lactate dehydrogenase (140 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa) and ATP, from left to right. $A_{280}$ (—). (B) AF-heparin column chromatography of TTK. The kinase activity was assayed with tau (●) and tubulin (○) as described in section 2. (C) SDS-PAGE of AF-heparin eluates. Aliquots (6 μl) were subjected to SDS-PAGE and the gel was stained with silver stain. Fraction numbers are indicated above each lane. M = pre-stained molecular weight markers (BRL).

## 3. Results

### 3.1. Purification of TTK from bovine brain extract

Brain extract was prepared from 15 bovine brains. The supernatant of MAPs fractions was mixed with phosphocellulose (P11; Whatman) gel slurry previously equilibrated with PC buffer (20 mM MES–NaOH, pH 6.8, 5 mM Mg-acetate, and 1 mM EGTA). The gel was washed with PC buffer containing 0.1 M NaCl and packed on a column (3.6 × 30 cm) with the same buffer. Column chromatography was carried out using linear gradient with a 1000 ml of 0.1–1.0 M NaCl in PC buffer. Tubulin phosphorylating activity eluted between 0.3–0.5 M NaCl. This kinase fraction was loaded onto a S-Sepharose (Pharmacia LKB Biotechnology AB) column (1.7 × 9.0 cm) equilibrated with PC buffer. The column was washed with 100 ml of the same buffer and then with 100 ml of 20 mM HEPES–NaOH, pH 8.2, containing 5 mM Mg-acetate, 1 mM EGTA and 0.05 M NaCl. A 200 ml linear salt gradient of 0.05–0.3 M NaCl in the same buffer was used to fractionate proteins. The kinase eluted at 0.25 M NaCl. The active fractions were concentrated with YM 10 Diaflo membrane (Amicon) and applied on a G3000 SW column of HPLC (Tosoh; 7.5 × 600 mm), equilibrated with 0.3 M NaCl in buffer B. Fractions 2 ml were collected. The kinase eluted at the molecular mass of about 32 kDa (Fr.78) as shown in Fig. 1A. The active fractions (Fr.77–79) were loaded onto a AF-heparin Toyopeal 650 column (Tosoh; 0.7 × 1.5 cm) in sodium phosphate buffer (20 mM sodium phosphate, pH 7.6, 5 mM Mg-acetate, 1 mM EGTA). The chromatography was carried out with a series of step elution with 0.03 M, 0.07 M, 0.11 M, 0.15 M, 0.19 M, 0.23 M, 0.27 M, 0.31 M, 0.35 M (2 ml each) and 0.4 M, 0.45 M (1 ml each) and finally 0.5 M NaCl. Fractions of 1 ml were collected. Tau and tubulin phosphorylating activities were eluted as a single peak (Fr.17–19) at 0.5 M NaCl as shown in Fig. 1B. The silver staining patterns indicated that the protein band of TTK corresponds to one of two bands migrating slightly slower than the 28 kDa marker protein (Fig. 1C). To determine which protein is the enzyme, phosphorylation and activity staining on SDS-PAGE were carried out. Although further purification by 2nd HPLC with a G3000 SW column was tried on Fr.18 of AF-heparin, it was difficult to increase the specific activity. As shown in Fig. 2A and B, the band at 32 kDa and other bands were phosphorylated. However, the activity staining with tau or tubulin showed only a protein at 32 kDa phosphorylated the substrate (Fig. 2C,D). These results indicate that the band at 32 kDa is TTK. The purification procedure is summarized in Table 1. Significant separation of TTK activity from other tau phosphorylating kinases such as TPKI and -II occurred mainly with S-Sepharose column chromatography. Thereafter, the activity ratio on tau/tubulin became constant. The yield is calculated as based on tubulin phosphorylating activity. Unless otherwise noted, Fr.19 of AF-heparin column was used as the enzyme for the characterization experiments of TTK.

### 3.2. Catalytic properties of TTK

TTK phosphorylation activity had an optimum pH range of 5.5–6.0 in Tris-acetate buffer. The $K_m$ for phosphorylation of

Table 1
Purification of tau-tubulin kinase

| Step | Protein (mg) | Total activity tau/tubulin (pmol/min) | Activity ratio tau/tubulin | Specific activity tau/tubulin (pmol/min/mg) | Yield (%) |
|---|---|---|---|---|---|
| (1) Phosphocellulose | 70 | 30,415/15,435 | 1.9 | 289/147 | (100) |
| (2) S-Sepharose | 15 | 2,940/2,634 | 1.1 | 196/176 | (17.1) |
| (3) Gel Filtration | 0.48 | 2,244/1,596 | 1.4 | 3,740/2,660 | (10.3) |
| (4) Heparin | 0.02 | 765/528 | 1.3 | 16,650/13,250 | (3.4) |

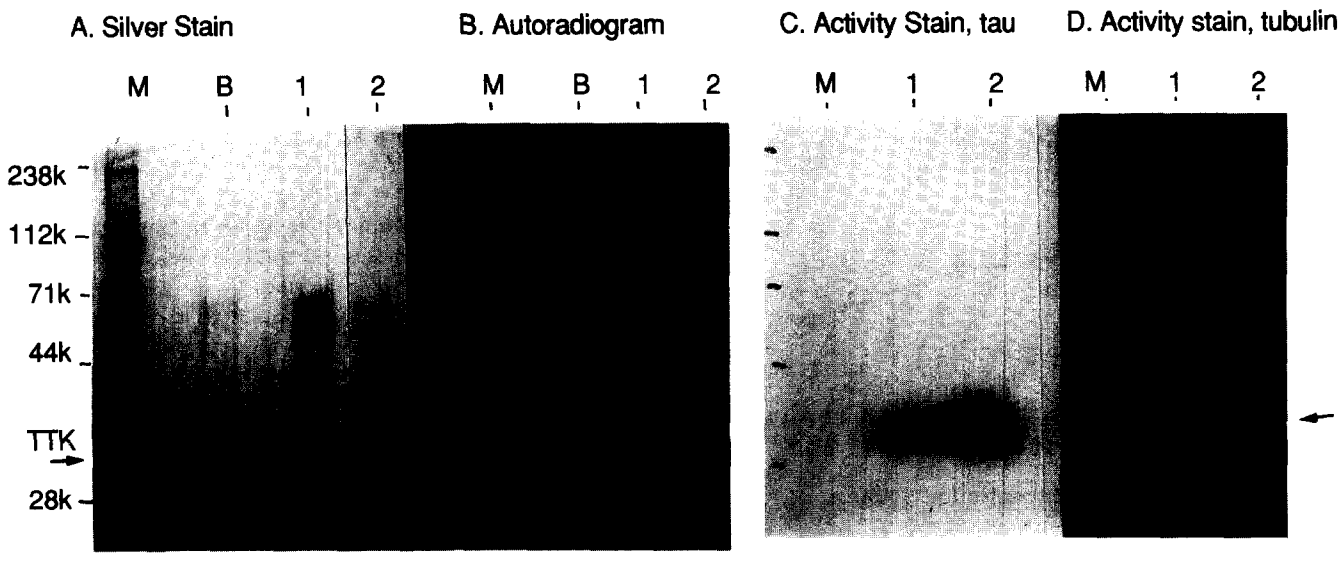


Fig. 2. Detection of TTK in SDS-PAGE. (A) Aliquots were labeled with [$\gamma$-$^{32}$P]ATP and gel was stained with silver stain. Lane 1 = second gel filtration fraction of AF-heparin Fr.18, 13 $\mu$l; lane 2, AF-heparin Fr.19, 6 $\mu$l; M, marker proteins; B, buffer blank. (B) Autoradiography of (A). (C,D) Phosphorylating activity in TTK fraction (15 $\mu$l) was detected by kinase activity staining of tau (C) or tubulin (D) included in the gel. Lane 1 = second gel filtration fraction of AF-heparin Fr.18; lane 2 = AF-heparin Fr.19; M, marker proteins.

tau is 12.5 $\mu$M and the $K_m$ for ATP was 27 $\mu$M. The optimum $Mg^{2+}$ concentration ranged from 1–20 mM toward general substrates, but a low level of 0.5 mM toward peptide substrates. Second messengers such as cAMP and calmodulin were determined to be unnecessary, as far as we examined (data not shown). As shown in Fig. 3A, the optimum concentration of $K^+$ is below 10 mM for tau, but 200 mM for tubulin. Inhibitors for casein kinase I, CKI-7, CKI-8 and hemin had no effect on TTK. However, heparin did show an inhibitory effect as shown in Fig. 3B. The $IC_{50}$ for heparin was 6 $\mu$M, about 600-fold high than the 9.3 nM for CKII inhibition [15]. At 6 $\mu$M heparin, TPKI activity was actually stimulated.

### 3.3. Substrate specificity of TTK

$P_i$ incorporation into tau and tubulin by TTK was estimated to be 6–7 and 1–2 mol/mol substrate, respectively (Fig. 4A). Phosphorylated amino acids were determined to be Ser and Thr (Fig. 4B), therefore the enzyme is a Ser/Thr protein kinase. Phosphorylated tubulin was confirmed to be $\beta$-tubulin by autoradiography on SDS-PAGE (Fig. 5).

As shown in Table 2, TTK phosphorylates proteins such as MAP2 and $\alpha$-casein as well as tau and tubulin, but not $\beta$-casein and histone H1, H2a, H2b which have been previously identified as substrates for TPKII [1].

Table 2
TTK activity toward various substrates

| Substrate | Activity (pmol/min/$\mu$g) |
|---|---|
| Tau | 20.0 |
| Tubulin | 14.5 |
| MAP2 | 22.2 |
| $\alpha$-Casein | 14.0 |
| $\beta$-Casein | N.G. |
| Neurofilament | N.G. |
| Histone H1 | 0.3 |
| Histone H2a | 0.15 |
| Histone H2b | 0.30 |

### 3.4. Phosphorylation site sequence of TTK

Several synthetic peptides, the designs of which were based on the sequence of tau, glycogen synthase and brain specific protein p25, were checked to identify the sequence required to be substrates for TTK (Table 3A). Synthetic peptides containing Ser–Arg sequences required to be substrates for TTK. Among these peptides shown in Table 3A, F5b, one of the peptides from p25 is a good substrate. However, a peptide (F5a) related to F5b except for the absence of 7 amino acids at its N-terminus was not phosphorylated. The result suggests that the 4th Ser of F5b peptide is phosphorylated. The phosphorylation site of F5b was identified as the 4th Ser by sequencing of phospho-serine (Fig. 6).

Furthermore, the phosphorylation site sequence of TTK was examined using synthetic peptides. A peptide composed of the N-terminal 7 amino acids of F5b was expected to be a good substrates for TTK. Therefore, the peptide F5n was synthesized and tested for its ability to act as a substrate for TTK. Surprisingly, as shown in Table 3B, the peptide F5n was not phosphorylated by TTK. Peptides containing either 3 (F5d) or 8 (F5f) additional amino acids were synthesized. Although peptide, F5d does not work as the substrate, F5f was phosphorylated at the one-third intensity of F5b. These results suggest that the whole sequence of F5b is necessary for the better substrate.

Two peptides 8655 and 8669, which are known to be substrates for cGMP-dependent protein kinase and have Ser–Arg sequence, were examined. Both peptides were for the substrates of TTK. The analog 8656 was used as a negative control. This result suggests that an –RSR– motif may be an effective substrate for TTK. Peptide F5h, identical to F5n except for replacement of D of position 3 with R, was found to be as good a substrate as F5b. But, when an Arg was substituted for Met (F5g) or a Glu (F5e) was added to the C-terminus, only inactive substrates were obtained. These results indicate –SR– sequence is necessary, but not sufficient for a substrate for TTK.

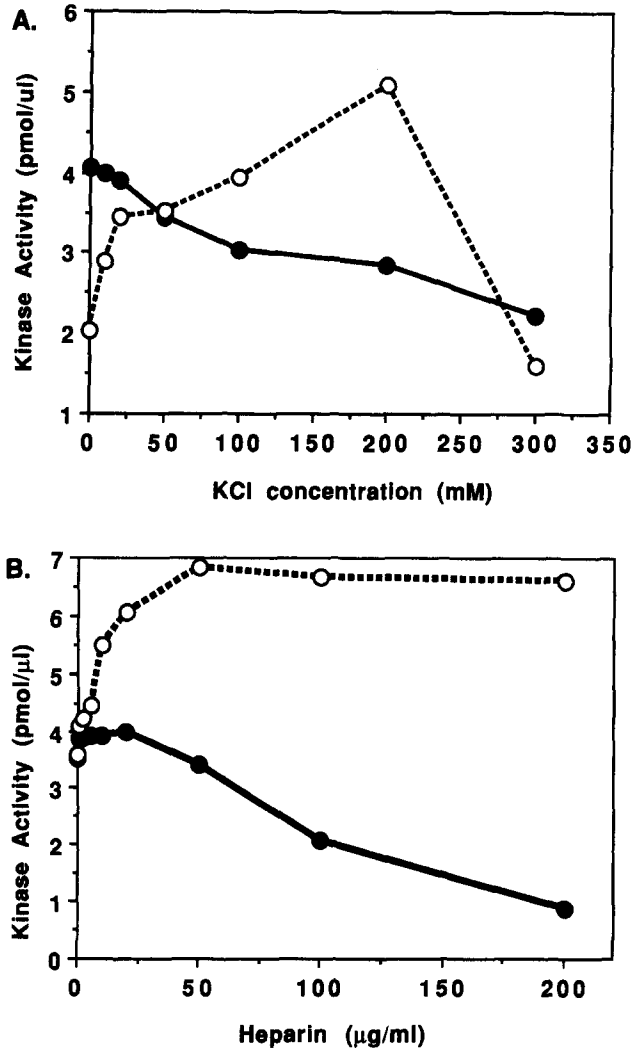


Fig. 3. Effects of KCl and heparin on TTK activity. (A) Effect of KCl concentrations. The activity toward tau (●) and tubulin (○) was assayed at various concentrations of KCl. (B) Effects of heparin at various concentrations were compared with TTK (●) and TPKI (○) activities on tau.

## 4. Discussion

TTK was found in bovine brain and phosphorylates tau, MAP2, tubulin and α-casein. TTK is a Ser/Thr protein kinase and distinct from TPKI and -II which are unable to phosphorylate tubulin. Although TTK was first found in MAPs from bovine brain together with TPKI and -II, the MAP-free supernatant was also useful as a source of TTK. Therefore, TTK does not necessarily bind specifically to microtubles.

TTK is also distinct from CKI and -II. TTK does not phosphorylate a CKII-specific synthetic peptide and is not inhibited by heparin at low concentration that do inhibit CKII. Inhibitors of CKI are also not effective in inhibiting the activity of TTK. It has been reported that a heparin-inhibited protein kinase from bovine kidney phosphorylates casein and has a molecular mass of 32 kDa on gel filtration. However, the substrate specificitey for tau and tubulin are unknown [16]. The 32 kDa molecular mass of TTK is distinct from the subunit molecular masses of CKII (38 kDa, 36 kDa and 25 kDa) [17]. It has been reported that CKII phosphorylates tubulin in addition to casein and phosphorylation is regulated by a neuronal protein of 185 kDa [18]. Recently, Crute and Buskirk have reported that several tubulin casein kinase activities copurified with tubulin from bovine brain [7]. These tubulin casein kinases phosphorylated both tubulin and casein, but none was able to phosphorylate the CKII-specific synthetic peptide RRREEETEEE. Although they did not report studies on phosphorylation of tau by the tubulin kinases, it would be interesting to know the recognition sites of these enzymes.

The results reported here indicate that TTK requires an Arg residue immediately C-terminal to the phosphorylatable residue, an –SR– motif (Table 3A,B, Fig. 6). TTK modifies about 6 residues of S/T in tau and 1–2 residues in β-tubulin (Fig. 4).

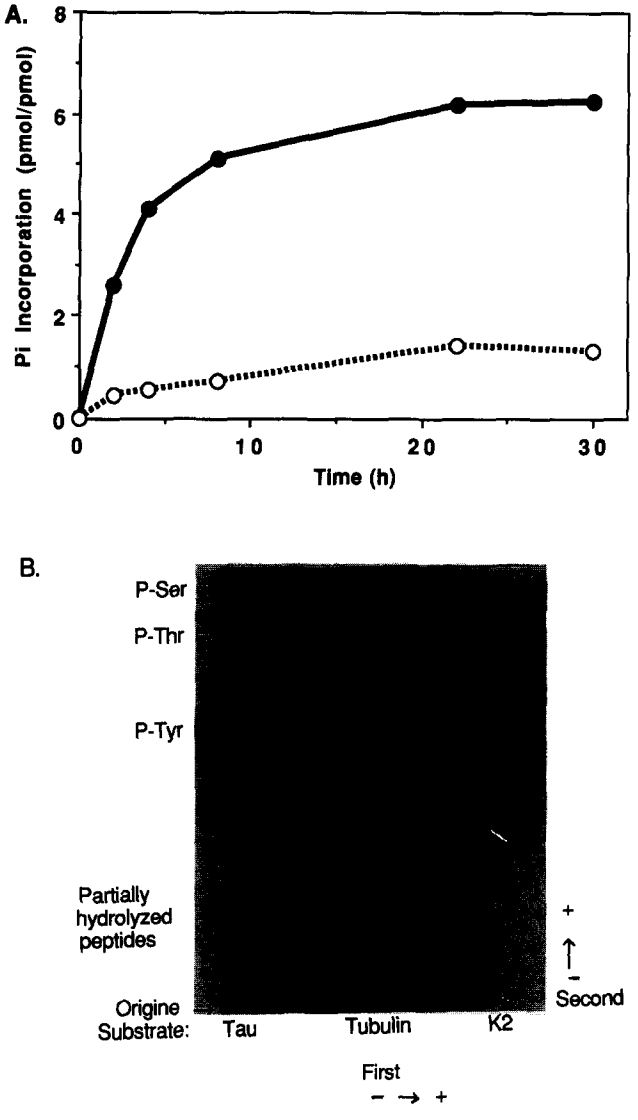


Fig. 4. Phosphorylation of tau and tubulin by TTK. (A) The phosphorylation of tau (●) and tubulin (○) was monitored as a function of time. (B) Phospho-amino acids of $^{32}P$-labeled tau, tubulin and K2 peptide were hydrolyzed and analyzed with two-dimensional electrophoresis. First dimension: acetic acid/formic acid/$H_2O$ (78:25:897), pH 2.0 at 450 V for 1 h; 2nd dimension, acetic acid/pyridine/$H_2O$ (50:5:945), pH 3.5, at 450 V for 3 h. The radio labeled amino acids were detected by autoradiography. The positions of the phosphorylated amino acid standards were visualized by ninhydrin staining as shown with dotted circles.

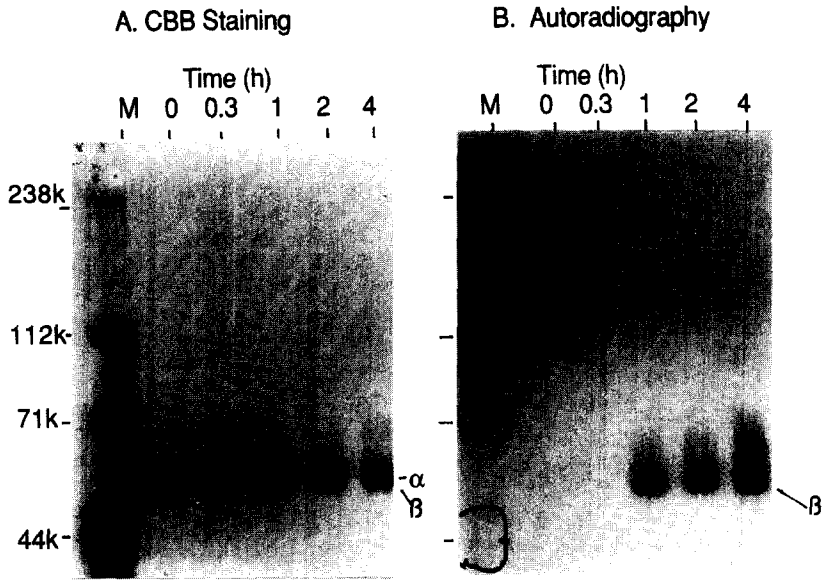


Fig. 5. Time course of phosphorylation of β-tubulin by TTK. Tubulin (α and β) was phosphorylated by TTK and the reaction products from various time were subjected on 7% SDS-PAGE according to the method of Serrano et al. [6]. The gel was stained with CBB (A) and the radio-labeled products were detected by autoradiography (B). M, standard markers.

Bovine tau contains 5 residues of –S/TR sequences [19], β-tubulin has only one –SR– sequence [20] and α-tubulin has none [21]. These data seem to agree with the number of phosphorylation sites by TTK. Although Ser-4 of F5b fragment of p25 protein was phosphorylated, F5n, F5e and F5g peptides, which contain the same Ser-4 residue, are poor substrates. These facts indicate a requirement for a larger structure for recognition and phosphorylation by TTK.

A list of phosphorylation site sequences for many protein kinases have been reported [22]. Protein kinases, which recognize the –S/TR-motif as a consensus for phosphorylation sites, is unknown. In this category there are enzymes which phosphorylate –S/TR-sequences such as CKI and GSK-3, cAMP-dependent protein kinase and cGMP-dependent protein kinase, hem-regulated eIF-2α-kinase, p68 kinase and protein kinase C.

PHF-tau accumulate in the brain of Alzheimer's disease patients and the main component is tau [23–25]. It has been reported that PHF-tau is highly phosphorylated in the tau 1 portion [26] and carboxy-terminal portion [27]. Recently 19 phosphorylation sites in PHF-tau have been identified, 10 of

Table 3
(A) TTK activity toward synthetic peptides

| Protein/fragment | Amino acid sequence | Activity (pmol/min/μg) |
|---|---|---|
| tau/K1 | VAVVRTPPKSPSSAK | 0.05 |
| tau/K2 | PKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPK | 0.60 |
| tau/K3 | PVVSSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASLAK | 0.09 |
| tau/4 repeat | DLKNVKSKIGSTENLKHQPGGGKVQIINKKL | 0.10 |
| p25/F3 | KAUSSPTVSRKTD | 0.70 |
| p25/F5a | PANKTPPKSPGEPAKDPAAK | 0.10 |
| p25/F5b | MADSRPKPANKTPPKSPGEPAKDPAAK | 7.80 |
| GS/GS-1 | YRRAAVPPSPSLSRHSSPHQSESEE | 0.40 |
| CKII substrate | RRREEETEEE | 0.10 |

Ser of –SR–motif is underlined.

(B) Substrate specificity of TTK

| Peptide | Amino acid sequences | Activity (pmol/min/μg) | (%) |
|---|---|---|---|
| F5b | MADSRPKPANKTPPKSPGEPAKDPAAK | 8.2 | 100 |
| F5a | PANKTPPKSPGEPAKDPAAK | 0 | 0 |
| F5n | MADSRPK | 0 | 0 |
| F5d | MADSRPKPAN | 0 | 0 |
| F5f | MADSRPKPANKTPPK | 2.7 | 33 |
| F5e | MADSRPAE | 0 | 0 |
| F5g | RADSRPK | 0.7 | 9 |
| F5h | MARSRPK | 7.5 | 92 |
| 8655 | RKRSRKE | 2.8 | 34 |
| 8659 | RKRSRAE | 8.1 | 99 |
| 8656 | RKRARKE | 0 | 0 |

Ser of –SR–motif is underlined.

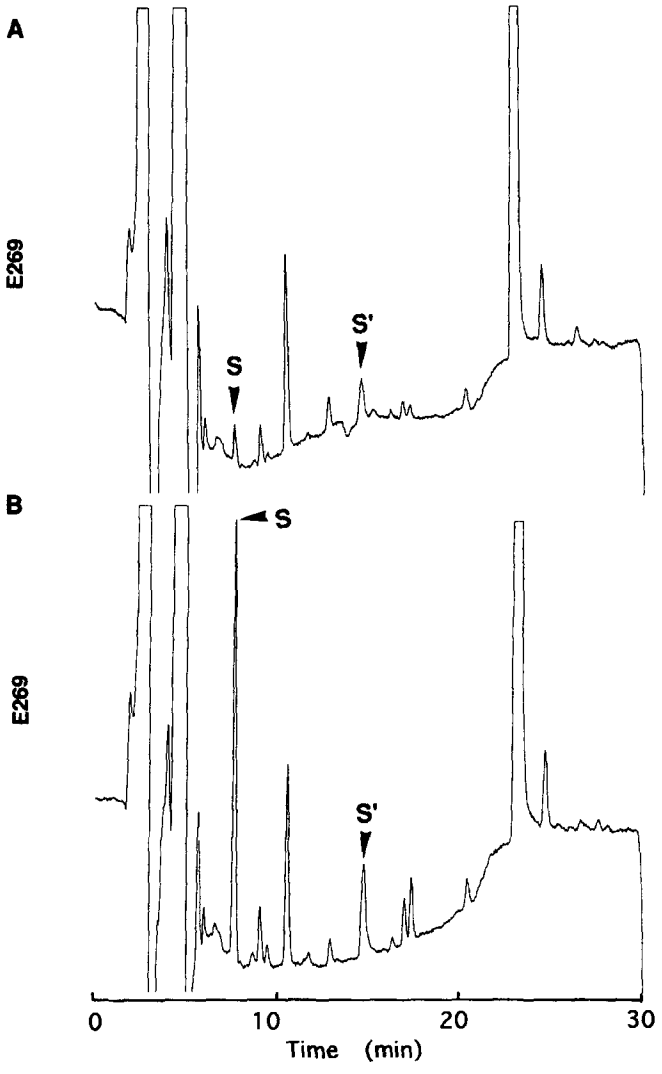


Fig. 6. Comparison of sequencing profiles of phospho-serine and the corresponding non-phospho-serine of F5b peptide in cycle 4. F5b peptide was phosphorylated by TTK (A) and the sequences were analyzed to compare with the non-phosphorylated peptide (B). S and S′ indicate elution position of PTH-serine and PTH-dehydroalanine in 4th cycle of the analysis, respectively.

them are proline-directed sites, the remainder are non-proline sites [28]. Among the non-proline-directed phosphorylation sites there are Ser-208 and Ser-210, which have –SR-motif and are located in K2 fragment/tau 1 portion. TTK phosphorylates K2 fragment as shown in Table 3A and Fig. 4B. The exact phosphorylation sites have not been determined, but it seems likely that TTK phosphorylates no other sequence than Ser-208 and Ser-210, –SR-motif of K2.

Initially, TPK was identified as a tubulin-dependent protein kinase [29] and it was confirmed that phosphorylation by TPKI and -II was stimulated in the presence of tubulin [1]. It is not known yet how tubulin stimulates TPKI and -II activities. It has been reported that the phosphorylation of MAP2 and tau results in the inhibition of microtuble assembly [30]. We are interested in the phosphorylation of tubulin and tau by TTK in the presence of TPKI and -II and the interactions related to microtuble assembly. This may aid in elucidating PHF formation in Alzheimer disease brain.

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