Incorporation of nitrotyrosine into α-tubulin by recombinant mammalian tubulin-tyrosine ligase

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Abstract

Tubulin-tyrosine ligase (TTL, EC 6.3.2.25) from porcine brain, which catalyses the readdition of tyrosine to the C-terminus of detyrosinated α-tubulin, was cloned and expressed in Escherichia coli as a glutathione S-transferase-fusion protein. Upon cleavage of the immobilised fusion protein, an electrophoretically homogeneous enzyme was obtained. Recombinant TTL, which exhibited similar catalytic properties as the mammalian enzyme purified from brain tissue, was capable of using nitrotyrosine as an alternative substrate in vitro. Incorporation of tyrosine into tubulin was competitively inhibited by nitrotyrosine with an apparent $K_i$ of 0.24 mM. The TTL-catalysed incorporation of nitrotyrosine as sole substrate into α-tubulin was clearly detectable at concentrations of 10 μM by immunological methods using nitrotyrosine-specific antibodies. However, in competition with tyrosine 20-fold higher concentrations of nitrotyrosine were necessary before its incorporation became evident. Analysis of the C-terminal peptides of in vitro modified α-tubulin by MALDI-MS confirmed the covalent incorporation of nitrotyrosine into tubulin by TTL. In contrast to the C-terminal tyrosine, pancreatic carboxypeptidase A was incapable of cleaving nitrotyrosine from the modified α-tubulin. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Microtubules are found in virtually all eukaryotic cells as a major component of the cytoskeleton. They are involved in numerous cellular processes, including cell division, directed intracellular transport, and movement of cilia and flagella, and in addition they influence the dynamic organisation of cellular morphology [1,2]. Microtubules are principally composed of the αβ-tubulin dimer and microtubule-associated proteins.

In higher eukaryotes, multigene tubulin families contribute to microtubule diversity [3]. In addition, tubulin undergoes several post-translational modifications, including tyrosination, acetylation, phosphorylation, polyglycylation and polyglutamylation [3,4]. These modifications generate further diversity within microtubule populations and are thought to influence their functions. Whereas acetylation and phosphorylation are more general post-translational
modifications, the other modifications, such as polyglutamylation, polyglycylcyclation and tyrosination, are tubulin-specific and occur at the highly variable C-termini of tubulin dimers (for references see [3,4]).

The de tyrosination/tyrosination cycle is entirely specific for α-tubulin [5–7] and is widespread in eukaryotes [4]. The C-terminal tyrosine of α-tubulin is usually encoded by the mRNA and tubulin is initially de tyrosinated at the C-terminus by a tubulin-specific carboxypeptidase [8,9]. De tyrosinated α-tubulin is then re tyrosinated by the action of a tubulin-tyrosine ligase (TTL) [10,11]. Stable microtubules are the preferred substrate for tubulin carboxypeptidase [9,12] and the αβ-tubulin dimers are preferably tyrosinated by TTL [13,14]. Consequently, stable microtubules are often highly de tyrosinated whereas dynamic microtubules contain high levels of tyrosinated tubulin [7,15,16]. Although the physiological relevance of the de tyrosination/tyrosination cycle is unknown, abrogation of TTL activity was recently suggested to be correlated with tumour growth [17].

The importance of the de tyrosination/tyrosination cycle was further demonstrated through the observation that in vivo incorporation of nitric oxide-derived nitrotyrosine into tubulin in mammalian cells leads to microtubule dysfunction [18]. The apparently irreversible nitrotyrosination of the C-terminal residue of α-tubulin was proposed to be catalysed by TTL and was suggested to alter microtubule function, leading to changes in cell morphology and loss of epithelial-barrier function [18]. These observations may provide an insight into the mechanism of inflammatory injury under pathological conditions entailing the formation of reactive nitrogen species [19–21]. Although TTL has been implicated in the in vivo nitrotyrosination of tubulin, its ability to covalently incorporate nitrotyrosine into tubulin has not been illustrated.

Here we demonstrate that TTL is indeed capable of covalently incorporating nitrotyrosine into the C-terminus of α-tubulin in vitro using recombinant porcine brain TTL, which when recovered from transformed Escherichia coli exhibits similar catalytic properties as the mammalian enzyme purified from brain tissue. Furthermore, we show that, in contrast to in vivo findings [18], TTL has more than 35-fold lower affinity for the modified amino acid than for tyrosine.

2. Materials and methods

2.1. Cloning and expression of recombinant TTL

The full-length cDNA of TTL was subcloned from the embryonic porcine brain lgt10 cDNA library as described previously [22]. This 1137-bp cDNA was used as template for polymerase chain reaction to generate BamHI sites on the TTL cDNA. The pig TTL–BamHI fragment was inserted in frame into the pGex-6P-2 vector glutathione S-transferase (GST) gene fusion system (Amersham/Pharmacia Biotech, Freiburg, Germany) and expressed in E. coli strain TgII. TTL expression was induced either for 4 h or overnight with 0.1 mM isopropyl-β-D-thiogalactopyranoside at an OD of 0.6. All subsequent steps were performed at 4°C: the bacteria were harvested by centrifugation for 10 min at 6000×g. The pellet was washed twice with TTL stabilisation buffer (25 mM K+ morpholinoethanesulfonic acid (MES), pH 6.8, 150 mM KCl, 12.5 mM MgCl2, 2.5 mM ATP, 1 mM DTT) containing glycerol (15% v/v), the bacteria were repelled and taken up in an equal volume of the above buffer plus the mini Complete protease inhibitors (Roche Diagnostics, Mannheim, Germany) following the manufacturer’s instruction. Bacteria were lysed using the French press (SLM-Amicon). Lysed cells were centrifuged for 1 h at 30000×g and the supernatant was incubated for 1 h with glutathione–Sepharose 4B (Amersham/Pharmacia) pre-equilibrated with the TTL stabilisation buffer. The Sepharose matrix was then washed extensively with TTL stabilisation buffer and incubated overnight with PreScission Protease (Amersham/Pharmacia) as recommended by the manufacturer. Cleaved TTL was finally eluted with stabilisation buffer and stored at –70°C.

2.2. Characterisation of recombinant TTL

TTL activity was measured as described previously [10] in 50 μl stabilisation buffer containing 100 μg homogeneous bovine brain tubulin (purified by cycles of assembly and disassembly followed by chromatography on phosphocellulose [23]; PC-tubulin), 4 μM L-[3,5-3H]tyrosine (specific activity, 46 Ci/mmol, Amersham Pharmacia Biotech, UK) and 10 μl TTL containing maximally 0.05 U. Initial
rate assays were performed as above using 0.4–10 μM L-[3,5-3H]tyrosine. After incubation for 30 min at 37°C, the samples were transferred onto Whatman 3MM paper discs (1.3×1.3 cm), fixed for 10 min with 10% acetic acid, washed twice with ethanol, dried, then counted in 4 ml Quicksafe scintillation mixture (Zinsser Analytic, Frankfurt, Germany).

Inhibition of the uptake of radiolabelled tyrosine was measured as described above, but in the presence of either 0–1400 μM nitrotyrosine (Sigma, Germany) or 0–50 μM unlabelled tyrosine. For the determination of the K_i of nitrotyrosine initial rate assays were performed as above using 1, 4 and 10 μM L-[3,5-3H]tyrosine and either 0, 200 or 600 μM nitrotyrosine. These tests were performed using either PC-tubulin or carboxypeptidase A-treated PC-tubulin. In the latter case, PC-tubulin was incubated with 1.0 μg/ml pancreatic carboxypeptidase A (Sigma) for 15 min at 37°C (see reference [6]). All assays were carried out at least in triplicate. Kinetic parameters were analysed using the GraFit program [24] and represent the apparent rather than true parameters as the measurements were made at single constant values of tubulin and ATP.

One unit of enzyme is defined as the amount of enzyme transferring 1 nmol of tyrosine to tubulin in 1 min under the conditions described above. The cpm/nmol were determined by determining 10 μl of L-[3,5-3H]tyrosine onto Whatmann 3MM filter paper, which was air dried without washing and counted in the scintillation mixture. Specific activity is defined as units/mg protein. Protein concentration was determined using the standard assay kit from Bio-Rad (Munich, Germany) with bovine serum albumin as standard.

N-terminal sequence analysis of purified recombinant TTL was performed by automated Edman degradation using an Applied Biosystems Model 494A Procise pulsed liquid-phase sequencer with an on-line PTH amino acid analyser (PE Biosystems, Weiterstadt, Germany).

2.3. Production of polyclonal antibodies against nitrotyrosine

Polyclonal antiserum against nitrotyrosine was prepared by immunisation of two rabbits with nitrotyrosine coupled to ovalbumin by glutaraldehyde crosslinking. Antiserum was used at a 1:500 dilution in the immunoblots.

The rat monoclonal antibody YL1/2 against tyrosinated tubulin and the mouse monoclonal antibody against TTL (LA/C4) were recently described [7,25]. Horseradish peroxidase conjugated goat anti-mouse, goat anti-rat and goat anti-rabbit antibodies were purchased from Dianova (Hamburg, Germany) and diluted 1:2000. Immunoblots were processed with the ECL kit (Amersham, Braunschweig, Germany).

2.4. In vitro incorporation of nitrotyrosine into tubulin

The in vitro incorporation of nitrotyrosine into tubulin was measured as described above using carboxypeptidase A-treated PC-tubulin and either 0.1–500 μM nitrotyrosine or 0.1–100 μM tyrosine as sole substrates, or 50–5000 μM nitrotyrosine in the presence of 50 μM tyrosine as cosubstrate. The reaction was stopped after 30 min by boiling the mixture in SDS buffer and the degree of modification of the α-tubulin was qualitatively assessed by immunoblotting as described below.

The ability of carboxypeptidase A to cleave C-terminal nitrotyrosine from α-tubulin was tested by incubating nitrotyrosinated tubulin with pancreatic carboxypeptidase A followed by immunoblotting with nitrotyrosine-specific antibodies.

2.5. SDS–PAGE and immunoblotting

Nitrotyrosinated tubulin (500 ng) was separated using modified 7.5% SDS–PAGE, with the SDS buffers for the stacking and separation gels containing 6 M urea (SDS-L-5057, Sigma, Germany), to enable the separation of α- and β-tubulin [26]. Separated proteins were transferred electrophoretically onto polyvinylidenedifluoride (PVDF) membranes (Immobilon-P, Millipore, Germany).

2.6. Isolation and analysis of the C-terminal fragments of tubulin

The α- and β-tubulins were separated by SDS–PAGE in the presence of 6 M urea as described above. C-terminal peptides of α-tubulin were obtained by digestion with endoproteinase Lys-C, purified by anion exchange chromatography on Mono Q
and by reverse phase HPLC as described [27]. All peptide peaks from the HPLC profile were characterised by mass spectrometry using a KRATOS MALDI 4 time of flight spectrometer (Shimadzu, Duisburg, Germany).

3. Results and discussion

3.1. Expression and purification of recombinant TTL

Purification of the mammalian TTL from brain tissues has been significantly simplified by the application of immunoaffinity chromatography using monoclonal antibody LA/C4 immobilised on Sepharose [25]. Nevertheless, purification of large amounts of mammalian TTL has proven to be a major problem due to the low expression levels of the enzyme in neuronal tissues (only 1 mg of pure TTL can be obtained from 1 kg of porcine brain). This labour-intensive purification has consequently hampered attempts to elucidate the structure–function relationship of mammalian TTL and to approach its structural analysis by crystallisation. In order to solve this problem, the cDNA of the porcine brain TTL, which codes for a protein of 43.4 kDa [22], was cloned and expressed as a GST-fusion protein in Escherichia coli.

Induction of the recombinant E. coli TgII cells resulted in the accumulation of an additional protein with an apparent molecular mass of about 70 kDa in SDS–PAGE which was recognised by the anti-TTL monoclonal antibody LA/C4 (Fig. 1).

Expression of the recombinant TTL as a GST-fusion protein enabled its rapid purification by a single step on a glutathione-Sepharose affinity chromatography column. GST-TTL was extensively washed on the glutathione-Sepharose column to remove all unspecifically absorbed material and the enzyme was then separated from the fused GST by proteolytic cleavage with PreScission protease. Up to 50 mg of highly purified cleaved recombinant TTL, which was eluted from the column in stabilisation buffer, could be obtained from 1 l of E. coli culture. The recombinant TTL was active and stable for at least 4 months at −70°C in the stabilisation buffer.

3.2. Characterisation of recombinant TTL

Analysis of the cleaved recombinant TTL by SDS–PAGE demonstrated a single protein band of about 43 kDa which was recognised by the anti-TTL monoclonal antibody LA/C4 (Fig. 1). N-terminal sequencing revealed the following sequence: GPLGSMYTFVVRDEN. The first five amino acids corresponded to the C-terminal residues of the GST protein through which the fusion protein was attached to the recombinant TTL to enable cleavage. Residues 6–20 corresponded to the N-terminus of porcine TTL.

The initial rates of tyrosine incorporation into tubulin were determined at optimal concentrations of MgCl₂ and ATP [28] and varying concentrations of ³H-tyrosine. The incorporation of ³H-tyrosine into tubulin represented hyperbolic behaviour, demonstrating a linear relationship between rate and incubation time over the first 15 min, with the rates of tyrosine incorporation subsequently decreasing with additional incubation times and tailing off after 60 min (data not shown). The relationship between the time of incubation and rate of tyrosine incorporation was very similar to that previously observed with mammalian TTL [29,30]. Consequently, all kinetic assays were measured over a 15-min interval. From the double reciprocal plots of the initial rates of tyrosine incorporation into tubulin (see Fig. 2A) the following kinetic parameters were calculated: apparent Kₘ = 6.8 µM; Vₘₐₓ = 354 U/mg. These values compare favourably with those reported for the por-
merisation [7,32]. Thus, cocrystallisation of the recombinant enzyme with its substrate may enable elucidation of the crystal structure of the αβ-tubulin dimer and simultaneously provide important structural information about the interaction of TTL with tubulin.

3.3. In vitro incorporation of nitrotyrosine into tubulin

In cultured mammalian cells α-tubulin was recently shown to be post-translationally nitrotyrosinated by supplementing the medium with nitrotyrosine, which was presumably selectively incorporated into the C-terminal end of α-tubulin by the TTL [18].

In order to determine if nitrotyrosine is indeed an alternative substrate for TTL, an in vitro inhibition experiment of 3H-tyrosine incorporation into tubulin was performed. Although nitrotyrosine could inhibit the incorporation of radiolabelled tyrosine, 50% inhibition was achieved only at 50-fold higher concentrations of nitrotyrosine. In contrast, equimolar concentrations of unlabelled tyrosine effected 50% inhibition of radioactive tyrosine incorporation. The effect of nitrotyrosine on tyrosine incorporation was investigated further by determining the kinetic parameters of tyrosine incorporation. Fig. 2A shows the double reciprocal plot of 3H-tyrosine incorporation in the absence and presence of 0.2 and 0.6 mM nitrotyrosine. The curves present linear behaviour and converge at the same point on the ordinate axis, indicating that nitrotyrosine is a competitive inhibitor, or an alternative substrate, for TTL. Similar observations have been made with other tyrosine analogues, including phenylalanine [33], 3,4-dihydroxyphenylalanine [5], 3-iodotyrosine [34], 3-fluorotyrosine [35] and 3-azidotyrosine [36], indicating that modifications at position 3 of the tyrosine side chain do not sterically impair the acceptance of the modified substrates by the TTL. An apparent $K_i$ value of 0.24 mM was obtained for nitrotyrosine from a replot of the initial rates of tyrosine incorporation against nitrotyrosine concentration (Fig. 2B).

To test the ability of TTL to incorporate nitrotyrosine into α-tubulin, TTL was incubated for 30 min with 100 μg detyrosinated PC-tubulin in a medium containing 0.1–5000 μM nitrotyrosine as sole substrate. The reaction was stopped by heating the mix-
In vitro incorporation of nitrotyrosine into the C-terminus of carboxypeptidase A-treated α-tubulin revealed by immunoblotting. Each lane contains 5 μg PC-tubulin. (A) Concentration-dependent incorporation of nitrotyrosine (probed with anti-NO₂-Y) and tyrosine (probed with YL1/2) as sole substrates into tubulin. (B) Competitive incorporation of nitrotyrosine into tubulin in the presence of 50 μM tyrosine and at initial nitrotyrosine concentrations of 500, 1000, 3000 and 5000 μM. The upper figure (anti-NO₂-Y) is probed for nitrotyrosine; the lower (YL1/2) for tyrosine. The same blot membrane was first probed for tyrosine incorporation, the membrane was then stripped, air dried and incubated with the anti-nitrotyrosine (anti-NO₂-Y) antibody. Stripping was performed by incubating the membrane three times for 10 min with each of the following: distilled water; solution A (8 M urea, 1% SDS, 0.5% β-mercaptoethanol) with sonication; solution B (50% ethanol and 10% glacial acetic acid); and 100% ethanol. No visible loss of protein occurred during the stripping procedure. (C) Treatment of C-terminally tyrosinated or nitrotyrosinated tubulin with carboxypeptidase A. Lanes 1 and 2, tyrosinated tubulin before (lane 1) and after (lane 2) carboxypeptidase A treatment (probed with YL1/2); lanes 3 and 4, nitrotyrosinated tubulin before (lane 3) and after (lane 4) carboxypeptidase A treatment (probed with anti-NO₂-Y).

Analysis of the C-terminal peptides of the in vitro modified α-tubulin by MALDI-MS confirmed the covalent incorporation of nitrotyrosine into tubulin by TTL (Fig. 4). MALDI-MS analysis revealed that TTL was also capable of incorporating a derivative of nitrotyrosine, nitrosotyrosine, a contaminant of the commercially used nitrotyrosine (data not shown). Furthermore, these experiments showed that the maximal nitrotyrosination level of α-tubulin in vitro was about 55% when using detyrosinated α-tubulin (data not shown). This is in accordance with the maximally achievable tyrosination levels of the α-subunit of neuronal tubulin [37], suggesting that the non-tyrosinatable tubulin also cannot be nitrotyrosinated by TTL.

In summary, we have demonstrated the ability of TTL to covalently incorporate nitrotyrosine in vitro into the C-terminus of α-tubulin, although the enzyme shows an approximately 35-fold lower affinity for the nitric oxide-modified derivative of tyrosine than for its natural substrate. In addition, we have confirmed previous observations that, in contrast to tyrosine, nitrotyrosine cannot be reversibly cleaved...
from the C-terminus of tubulin by pancreatic carboxypeptidase A. The potential inability of the presumptive endogenous tubulin-specific carboxypeptidase to cleave the C-terminal nitrotyrosine from α-tubulin should disrupt the tyrosination/detyrosination cycle in the cell, which could indeed detrimentally affect microtubule functions under pathological conditions, as recently implicated [18]. However, in contrast to the published in vivo results [18], significantly higher concentrations of nitrotyrosine were required for the in vitro nitrotyrosination of α-tubulin in competition with tyrosine. The large differences in the affinity of TTL for the two substrates implies that the discrepancies in the levels of in vivo and in vitro tubulin nitrotyrosination may be due to differences in the rates of uptake of the two tyrosine compounds by the cells [18]. Further investigations are required to determine the described preference for the incorporation of nitrotyrosine relative to tyrosine in the cell. Moreover, the impact of this modification on microtubule functions needs to be studied more thoroughly.

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**References**