Tubulin-tyrosine ligase catalyzes covalent binding of 3-fluoro-tyrosine to tubulin: kinetic and [^{19}F]NMR studies

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Abstract The use of 3-fluoro-tyrosine as an alternative substrate for the enzyme tubulin:tyrosine ligase which catalyzes the incorporation of tyrosine into the α-tubulin subunit was investigated. The incorporation of tyrosine into tubulin was inhibited competitively by 3-fluoro-tyrosine with an apparent K_i of ~25 μM. The affinity for this analog was similar to that of tyrosine, confirming that the hydrogen at position 3 of the aromatic ring is not essential for the reaction catalyzed by TTLase. The incorporation of 3-fluoro-tyrosine into the C-terminus of the α-tubulin subunit was demonstrated through [^{19}F]NMR spectroscopy. The 3-fluoro-tyrosine signal at −58.6 ppm (trifluoroacetic acid as external standard), with a bandwidth of 24.7 Hz presented a chemical shift of 0.75 ppm upfield and an enlargement in the bandwidth (30.5 Hz) when incorporated into tubulin. These results strongly suggest that this amino acid is exposed to the solvent in tubulin. Tubulin covalently labeled with 3-fluoro-tyrosine was competent to polymerize into microtubules. The use of fluorinated tubulin in [^{19}F]NMR spectroscopy for studying questions concerning protein conformation and interactions will be discussed.

Key words: Tubulin; Tyrosination; Tubulin:tyrosine ligase; 3-Fluoro-tyrosine; [^{19}F]NMR

1. Introduction

Tubulin is a heterodimer composed of two subunits, denominated α and β, that self-assembles to form microtubules [1]. The structure of tubulin remains essentially unknown due to the lack of appropriate crystals of this labile and microheterogeneous protein which has a tendency to aggregate [2]. In vivo, the α subunit of tubulin can be reversibly tyrosinated at the C-terminus. Two enzymes are involved in this reaction: tubulin:tyrosine ligase (TTLase) and tubulin carboxypeptidase, which participate in the incorporation and release of a tyrosine residue, respectively [3–5]. TTLase has been purified to homogeneity [6] and, through in vitro assays, it has been shown that is able to incorporate tyrosine, dihydroxyphenylalanine, phenylalanine [5] and monoiodotyrosine [7] into tubulin.

In this report, we introduce the use of 3-fluoro-tyrosine, a fluorinated derivative of tyrosine, as an alternative substrate of TTLase that is incorporated into tubulin. The advantage of using this compound is that it permits the fluorination of tubulin and its signal can be followed using [^{19}F]NMR spectroscopy for tubulin structural studies. The specificity of the attachment of this probe will permit NMR studies of the interactions that occur at the C-terminal region of α-tubulin.

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2. Materials and methods

2.1. Reagents

GTP (type III), ATP, tyrosine and 3-fluoro-DL-tyrosine were purchased from Sigma. L-U-[^{14}C]tyrosine (sp. act. 475 mCi/mmol) was obtained from Amersham.

2.2. Chicken brain tubulin purification

Chicken brains were dissected from freshly slaughtered chickens (donated by Pollos Ariztía), kept on ice and used within 2 h. Tubulin was purified by the method of Weisenberg et al. [8,9], as modified by Lee et al. [10]. The stock protein was stored at −70°C in the presence of 1 M sucrose [11]. The experimental samples were prepared by batch equilibration of the stock protein with 5–10 vol. of dry-packed Sephadex G-25 fine equilibrated in the experimental buffer (specified for each experiment) at 4°C, followed by a filtration at 4°C through a Sephadex G-25 column equilibrated with the experimental buffer [12,13]. Tubulin concentrations were determined measuring the absorbance at 275 nm of samples diluted 10-fold in 6 M guanidine hydrochloride, using an absorptivity value of 1.03 l g^{-1} cm^{-1} [14].

2.3. Chicken brain tubulin: tyrosine ligase purification

The enzyme was extracted following the first step of the purification procedure of Murofushi [6]. After DEAE-cellulose chromatography, the fractions with TTLase activity were pooled and stored in aliquots at −70°C. The frozen enzyme was stable for at least 2 months.

2.4. Assay for the tubulin: tyrosine ligase activity

The activity was determined using the assay described by Raybin and Flavin [4]. The assay contained in 100 μl of final volume, 25 mM Mes, pH 6.8, 0.15 M KCl, 12.5 mM $MgCl_2$, 2.5 mM ATP, 1 mM DTT, 1 mM tyrosine, 20 mCi/mmol U-[^{14}C]tyrosine, 0.3 mg of tubulin and, 40 μl (~5 mU) of TTLase, and was incubated at 37°C during 30 min. The reaction was stopped adding 100 μl of 10% TCA and filtering through Whatman 3MM disc filter paper. The filters were washed with 5% TCA, absolute ethanol, dried and counted. The background was determined using a reaction mixture where TTLase was omitted. The specificity of the reaction was checked by electrophoresis in SDS-polyacrylamide of the labeled tubulin and subsequent autoradiography.

2.5. Inhibition of U-[^{14}C]tyrosine incorporation into α-tubulin by 3-fluoro-tyrosine

Inhibition of the incorporation of radioactive labeled tyrosine by 3-fluoro-tyrosine was measured using the conditions described in section 2.4, with a tyrosine concentration range between 5–100 μM, and different 3-fluoro-tyrosine concentrations.

2.6. Incorporation of 3-fluoro-tyrosine to tubulin

The reaction mixture contained 25 mM Mes, pH 6.8, 150 mM KCl, 12.5 mM $MgCl_2$, 2.5 mM ATP, 1 mM DTT, 0.2 mM 3-fluoro-tyrosine, 40 mg of tubulin, 8 U of TTLase, in a final volume of 160 ml. The mixture was incubated at 37°C during 1 h with gentle agitation. The reaction was stopped by the addition of 80 ml of cold deionized water (4°C) and kept at this temperature in all subsequent steps. The resultant solution was loaded in a DEAE-cellulose column (1 × 7 cm) equilibrated in 25 mM Mes, pH 6.8, 0.1 M NaCl, washed with 25 mM Mes, pH 6.8, 0.2 M NaCl. In this condition, only tubulin was retained in the DEAE-cellulose, and was eluted with 25 mM Mes, pH 6.8, and 0.8 M NaCl. The fractions with higher concentrations of tubulin were pooled and concentrated in a Centripep (Amicon) at 2000 × g, and the concentrated tubulin (1 ml) was diluted 10-fold to dilute NaCl, and concen-

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trated again in the same way to a final volume of 0.9 ml. This sample contained 30–40 mg of tubulin and 0.1 ml of D_2O was added for the NMR spectroscopy.

2.7. [^{19}F]NMR spectroscopy

The spectra were taken in a AMX 300 Bruker instrument (Centro de Equipo Mayor, Universidad de Chile), which operates with Fourier transform, using 5-mm precision tubes. The volume of the sample was 1 ml in 10% D_2O, used as internal lock. The frequency utilized for [^{19}F]NMR spectra was 282.35 MHz at 298k, with the following parameters: 6.6-μs pulse width, 62.5-kHz spectral width, 0.26-s data acquisition time, 0.75-s delay time. Trifluoroacetic acid (TFA) was used as external standard. For the reference spectra 512 scans were taken, and 52,000 for the fluorinated tubulin. The width of the signal peaks was determined at the half-height and expressed in Hz.

2.8 Polymerization of tubulin covalently labeled with 3-fluoro-tyrosine

Tubulin labeled with 3-fluoro-tyrosine (1 mg/ml) in polymerization buffer (25 mM Mes, 12.5 mM $MgCl_2$, 0.1 mM GTP, 0.15 M KCl, 25% glycerol, 10 μM taxol, pH 6.8) was induced to polymerize at 37°C, and the assembly was followed by turbidity measurements at 350 nm until the plateau was reached [12] using a Hewlett-Packard 8452A diode array spectrophotometer. Microtubule formation was assessed through electron microscopy using a Philips EM-300 microscope. Samples of the polymerization reaction were negatively stained with 1% uranyl acetate [12].

3. Results and discussion

A tubulin saturation curve for the incorporation of U-[^{14}C]tyrosine into tubulin was performed to verify that high concentrations of tubulin did not inhibit its own tyrosination. The curve presented hyperbolic behavior with an apparent K_m of 10 μM (until concentrations 5-fold apparent K_m), using 0.1 mM tyrosine (1.5-fold apparent K_m) (not shown). The tyrosine saturation curve showed a hyperbolic behavior until 7.5 × the apparent K_m, whose value was 66.5 μM (not shown). In order to determine if 3-fluoro-tyrosine was an alternative substrate for

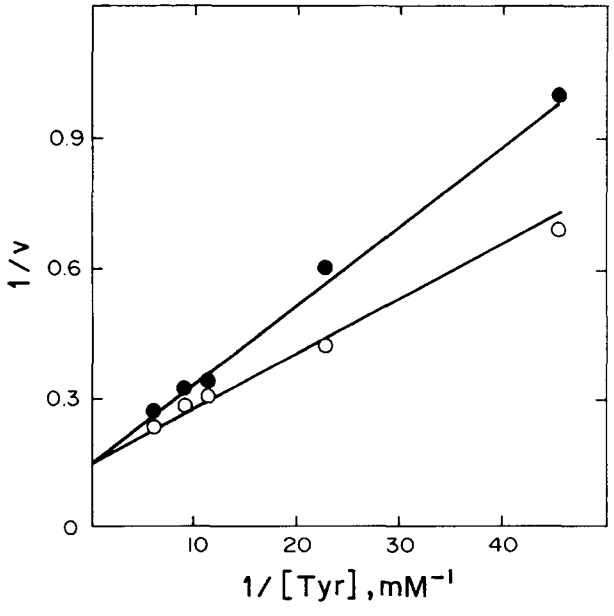

Fig. 1. Inhibition of [U-^{14}C]tyrosine incorporation into tubulin by 3-fluoro-tyrosine. The experiments were carried out as described in section 2. The saturation curves were performed in the absence (○), and in the presence (●) of 25 μM of 3-fluoro-tyrosine. The initial velocity is expressed in pmol of U-[^{14}C]tyrosine incorporated into tubulin per min.

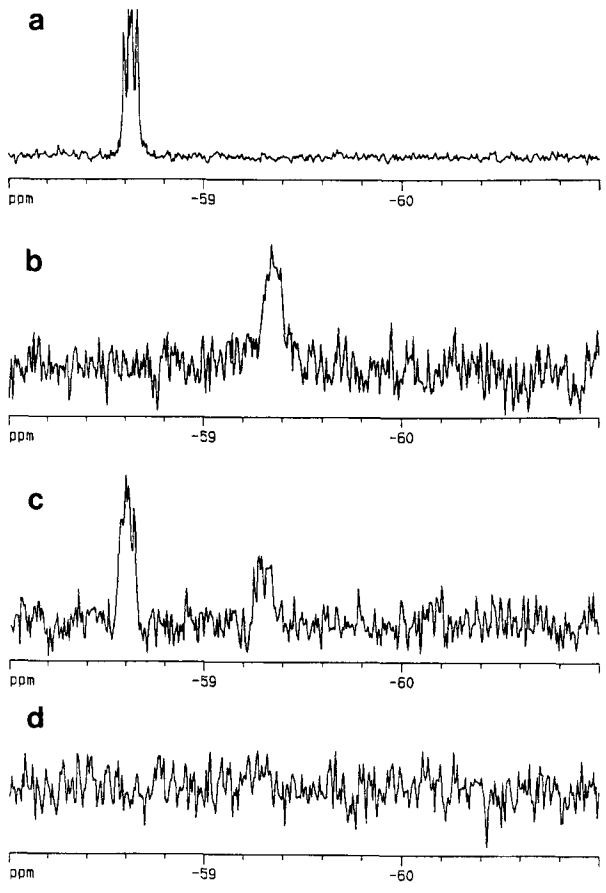

Fig. 2. [^{19}F]NMR spectra of 3-fluoro-tyrosine free and incorporated into tubulin. The experiments were performed as described in section 2. (a) 1 mM 3-fluoro-tyrosine in 25 mM Mes, pH 6.8. (b) 32 mg of tubulin tyrosinated with 3-fluoro-tyrosine in the same buffer. (c) The same as B plus 0.05 μmol of 3-fluoro-tyrosine. (d) 28 mg of tubulin that were treated using the 3-fluoro-tyrosination procedure in the absence of ATP.

TTLase an inhibition experiment of tyrosine incorporation into tubulin was performed. Fig. 1 shows the double reciprocal plot of tyrosine incorporation in the presence and absence of 3-fluoro-tyrosine. Both curves presented a linear behavior in this plot, with a higher slope when 3-fluoro-tyrosine was present, indicating that this compound inhibits tubulin tyrosination. Both curves converge in the same point in the ordinate axis, indicating that 3-fluoro-tyrosine is a competitive inhibitor, or an alternative substrate for TTLase. The apparent K_i value for 3-fluoro-tyrosine obtained through a non-linear computer program was 50 μM [15]. As 3-fluoro-tyrosine is a racemic mixture this value should be corrected by a factor of 2. Thus, the apparent K_i value for 3-fluoro-tyrosine is ~25 μM. This result confirms that the hydrogen at position 3 of the aromatic ring is not essential for the reaction catalyzed by TTLase as it was found using monoiodotyrosine substituted at position 3 of the ring [7] and dihydroxyphenylalanine [5].

Fig. 2 shows the [^{19}F]NMR spectra of free 3-fluoro-tyrosine (Fig. 2A) and 3-fluoro-tyrosine incorporated into tubulin. Free 3-fluoro-tyrosine displays a signal at −58.6 ppm with respect to the external standard TFA, with a bandwidth of 24.7 Hz. Fig. 2B presents the spectrum of tubulin treated with 3-fluoro-tyrosine as described in section 2. The fluorine signal of the

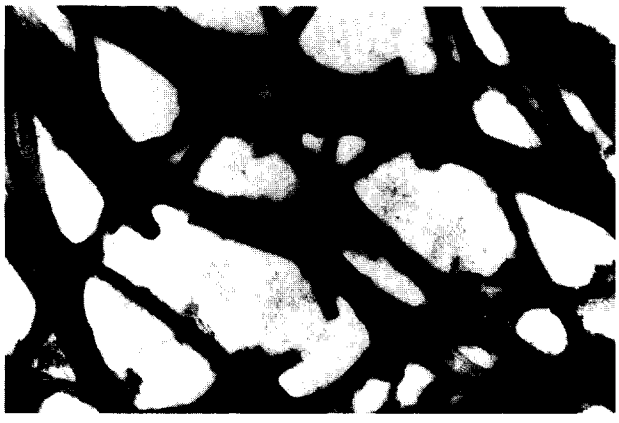

Fig. 3. Microtubules formed with tubulin labeled with 3-fluoro-tyrosine. Polymerization of fluorinated tubulin was carried out as described in section 2. The polymerized material was visualized by electron microscopy. × 38,000.

3-fluoro-tyrosine incorporated into tubulin, taken at the center of the quartet appeared at −59.35 ppm with a bandwidth of 30.5 Hz. To demonstrate that the chemical shift was not due to non-covalent binding of 3-fluoro-tyrosine to tubulin or to the influence of the experimental conditions, 3-fluoro-tyrosine was added to the sample used in Fig. 2B (Fig. 2C). In this case, the spectrum presented two signals, at −58.6 ppm, with a bandwidth of 24.8 Hz corresponding to free 3-fluoro-tyrosine, and the other signal with the characteristics of 3-fluoro-tyrosine incorporated into tubulin (−59.35 ppm, bandwidth 28.6 Hz). Fig. 2D shows a control experiment in which the 3-fluoro-tyrosine incorporation into tubulin was carried out in the absence of ATP. No signal appeared in the spectrum indicating that the fluorinated amino acid 3-fluoro-tyrosine was not incorporated in the absence of ATP. This result confirms that in the presence of TTLase and ATP, 3-fluoro-tyrosine forms a covalent complex with tubulin. Additionally, no fluorine signal was detectable after removing 3-fluoro-tyrosine from tubulin through a treatment of microtubules with tubulin carboxypeptidase, confirming in this way that the fluorination occurred in the C-terminal position of tubulin.

To demonstrate that tubulin covalently labeled with 3-fluoro-tyrosine was able to assemble into microtubules, labeled tubulin was polymerized under the conditions described in section 2, and the polymerized material was visualized by electron microscopy. Fig. 3 shows that the polymerization products were microtubules. These microtubules were washed and resuspended in the polymerization buffer (without taxol) and incubated at 4°C to induce cold depolymerization. Depolymerized tubulin (29 mg/ml) was analysed through [^{19}F]NMR, and a fluorine signal appeared at the same position than the labeled tubulin showed in Fig. 2B.

The small chemical shift upfield from free 3-fluoro-tyrosine strongly suggest that 3-fluoro-tyrosine is rather exposed to the solvent as have indicated by Sykes and Hull [16], for the interpretation of [^{19}F]NMR spectra of proteins with this fluorinated amino acid. This is supported by the fact that the C-terminal region of α-tubulin must be exposed to solvent in order to be substrate of TTLase. After the covalent binding of the amino acid, this region should remain exposed to the solvent because this tyrosine is removed by tubulin carboxypeptidase in the tyrosination/detyrosination cycle [17–19]. The widening of the signal produced by 3-fluoro-tyrosine incorporated into tubulin would indicate a decrease in the relaxation time since the fluorinated compound is now bound to tubulin and it has a lower motional freedom. The data would be adequately described by isotropic motion of the C-terminal peptide of α-tubulin and by the tumbling of the protein molecule, although the contribution of dipolar relaxation should not be neglected [20]. The exposure of the labeled residue to the solvent could be also explained as the result of protein denaturation due to the long acquisition time needed for the spectra. However, the [^{19}F]NMR spectrum of fluorinated tubulin in the presence of 1 M guanidine hydrochloride presents a downfield shift of the fluorine signal, ruling out the possibility that tubulin is in a denatured state (A. López-Brauet and O. Monasterio, unpubl. data).

A fluorinated nucleotide GTP(γF) [21] has been used previously to study the distance between the E-site for GTP and the high-affinity Mg^{2+} site in tubulin through [^{19}F]NMR [22,23]. However, the possibility of studying tubulin structure using a fluoro-tyrosine protein derivative presents several advantages. The use of an ^{19}F label allows one to observe a specific amino acid residue as a well-resolved resonance, and is considerably more sensitive than ^{1}H to local magnetic environment. The well-resolved resonances will in turn provide a sensitive probe of the conformational state of the protein. Studies of the structure of bacterial alkaline phosphatase were carried out using 3-fluoro-tyrosine and [^{19}F]NMR [24]. In this case, the incorporation of 3-fluoro-tyrosine was achieved in vivo, by growing the cells in the presence of this compound.

The specific labeling of an amino acid residue of tubulin by TTLase, by what was called 'enzymatic protein engineering' [7], open a new way of studying tubulin structure. In this particular case, the changes in the environment of the C-terminal region of α-tubulin produced by the interaction with regulators of tubulin polymerization like MAPs (microtubule associate proteins) and calcium can be studied.

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