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3-Azido-L-tyrosine as a photoinhibitor of tubulin:tyrosine ligase

Role of thiol groups

Katleen Coudijzer and Marcel Joniau

Interdisciplinary Research Center, KU Leuven, Campus Kortrijk, B-8500 Kortrijk, Belgium

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We have synthesized the photoactivatable probes 3-azido-L-tyrosine and *p*-azido-L-phenylalanine and studied their capacity to inhibit the incorporation of [^{3}H]tyrosine into tubulin catalyzed by tubulin:tyrosine ligase. Without illumination, only 3-azido-L-tyrosine reversibly inhibits the enzyme. Upon illumination, both reagents irreversibly photoinactivate the enzyme in a similar way. The ligase can be protected against photoinactivation by reversibly blocking essential thiol groups with pCMB during illumination.

Tubulin; Tubulin:tyrosine ligase; Posttranslational modification; 3-Azido-L-tyrosine; Photoinactivation; Thiol group

1. INTRODUCTION

One of the best-documented posttranslational modifications of tubulin is tyrosination [1-4]. In this process the α-subunit of brain tubulin is reversibly tyrosinated at its carboxy-terminus, due to the presence of tubulin:tyrosine ligase (TTLase). At present, the physiological role of this modification remains unknown [5].

Recently we have reported on the specificity of TTLase for a series of tyrosine derivatives [6,7]. We also used this enzyme to label specifically the C-terminus of α-tubulin with [^{125}I]iodo-L-tyrosine [8]. Our studies have enabled us to delineate the minimal requirements, as well as the degrees of freedom, of Tyr-derivatives to act as good inhibitors of TTLase. They should all possess the L-configuration, but substitutions at ring position 3 are allowed.

In the present work we choose to investigate the azido-derivatives of Tyr and Phe with the aim of using them as photoaffinity labels of the tyrosine-binding site of TTLase. In the literature several reports exist about photolabeling with aryl azides [9]. More particularly the synthesis of N_3Phe has been described as well as its incorporation in oligopeptides to serve as affinity labels for chymotrypsin [10]. Hitherto, the synthesis and use of N_3Tyr has not been reported.

Correspondence address: M. Joniau, Interdisc. Res. Center, KU Leuven, Campus Kortrijk, B-8500 Kortrijk, Belgium

Abbreviations: TTLase, tubulin:tyrosine ligase; Tub, tubulin; NH_2Tyr, 3-amino-L-tyrosine; N_3Tyr, 3-azido-L-tyrosine; NH_2Phe, *p*-amino-L-phenylalanine; N_3Phe, *p*-azido-L-phenylalanine; pCMB, *p*-chloromercuribenzoic acid; DTE, dithioerythritol; DTNB (Ellman's reagent), 5,5'-dithiobis-(2-nitrobenzoic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; ME, β-mercaptoethanol

2. MATERIALS AND METHODS

Tyr and Phe were products of Sigma (USA) and Serva (FRG), respectively. NH_2Tyr and NH_2Phe were purchased from Janssen Chimica (Belgium). L-[3,5-^{3}H]Tyrosine (sp. act. 51 Ci/mmol) was obtained from Amersham (UK).

N_3Tyr and N_3Phe are not available commercially and as no synthesis has been described for the former, we have based our preparation procedure on that described by Fahrenholz and Schimmack [11] for the synthesis of 4-azido-2-nitrophenylalanine. In the case of N_3Tyr, 2 mmol NH_2Tyr is dissolved in 3.2 mmol HCl. While stirring in an ice-bath, 2.4 mmol $NaNO_2$ is added dropwise. After 10 min, a solution containing 5 mmol NaN_3 is added dropwise in the dark. After 1 h at 0°C the precipitate is recovered by filtration. After dissolving in 2 mmol HCl, N_3Tyr is reprecipitated as white crystals by addition of concentrated NaCl solution. The dried crystals are redissolved in water and purified over Sephadex G10 (yield about 40%).

The synthesis of N_3Phe has been described [12,13]. We have prepared N_3Phe following a procedure similar to that of N_3Tyr. However, the precipitate is dissolved in ethanol and recrystallized by adding diethylether (yield about 45%).

The synthesized products were analyzed by thin-layer chromatography on cellulose F_{254} plates in BuOH/AcOH/H_2O (5:2:3, v/v). After staining with ninhydrine the R_f-values for N_3Tyr and N_3Phe were 0.5 and 0.6, respectively.

The IR-spectrum of N_3Tyr showed the typical peak at 2140 cm^{-1} [11].

The preparation of pig brain Tub and TTLase was done as described in detail in our previous publication [8].

Ligase reaction and its inhibition by Tyr- and Phe-derivatives, in the absence of illumination, was carried out in ligase buffer: 50 mM K^+ Mes, pH 6.8, containing 50 mM KCl, 2.5 mM $MgSO_4$, 1 mM EGTA, 1 mM DTE and 10% glycerol (v/v) [8]. The reduced incorporation of [^{3}H]tyrosine, expressed as a percent of the incorporation in the absence of inhibitor, showed sigmoidal curves when plotted as a function of the log inhibitor concentration. The inhibitory capacity

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is expressed as the negative logarithm of the 50% inhibitory concentration (pIC_{50}).

Photoinactivation of TTLase was done by adding 0.05 E.U. of the enzyme to a logarithmic dilution series of N_3Tyr or N_3Phe, ranging from 10^{-3} to 10^{-6} M in ligase buffer. From a total of 800 μl for each incubation mixture, 400 μl were kept in the dark at 0°C. The rest was illuminated in a 2 mm path quartz cuvette for 10 min at 15°C in a nitrogen atmosphere at 350 nm (slit width 40 nm) in an Aminco SPF-500 spectrofluorometer with or without the presence of tyrosine. Of each sample, with or without illumination and added tyrosine, 350 μl were passed through a 2 ml gel filtration column (Sephadex G25-SF) in the dark. Protein-containing fractions were pooled and used to catalyse [^{3}H]tyrosine uptake into tubulin in the dark, as described [8], but with omission of the preincubation step.

Protection of the enzyme thiol groups was done by adding 10^{-2} M pCMB before illumination and 10^{-2} M DTE before gel filtration.

3. RESULTS

We first tested N_3Tyr and N_3Phe in the dark for their capacity to inhibit TTLase-catalyzed [^{3}H]tyrosine uptake into tubulin. Both azido-derivatives were compared with their respective parent molecules and the intermediate amino-derivatives (Fig. 1a and b).

The time course of photoinactivation of TTLase by N_3Tyr was investigated. Therefore, mixtures of TTLase with or without 1.6×10^{-3} M N_3Tyr were made. The mixtures were illuminated during time intervals going from 1 to 30 min. After illumination and gel filtration the protein fractions were tested for TTLase activity. Independent of illumination time, TTLase without N_3Tyr retains full activity. Upon illumination in the presence of N_3Tyr TTLase activity is gradually lost. Fifty percent inactivation occurs after 3.4 min. In further experiments, illumination was always done for 10 min, the minimum time required for complete inactivation.

Next, the effect of concentration of N_3Tyr and N_3Phe on the photoinactivation of TTLase was tested. Mixtures of TTLase and different amounts of N_3Tyr (or N_3Phe) were made up in duplicate and were illuminated or not. Both alternatives were then passed through a gel filtration column to remove excess N_3Tyr or N_3Phe and their photolysis products in order to prevent undesired dark reactions. The filtered samples were then analysed for TTLase activity (Fig. 2a and 2b: solid lines). From the mean of 4 similar experiments the pCI_{50}-values for N_3Tyr and N_3Phe were computed to be 4.6 and 3.3, respectively.

The effect of Tyr, the natural ligand of TTLase, as a potential protecting agent of the tyrosine-binding site

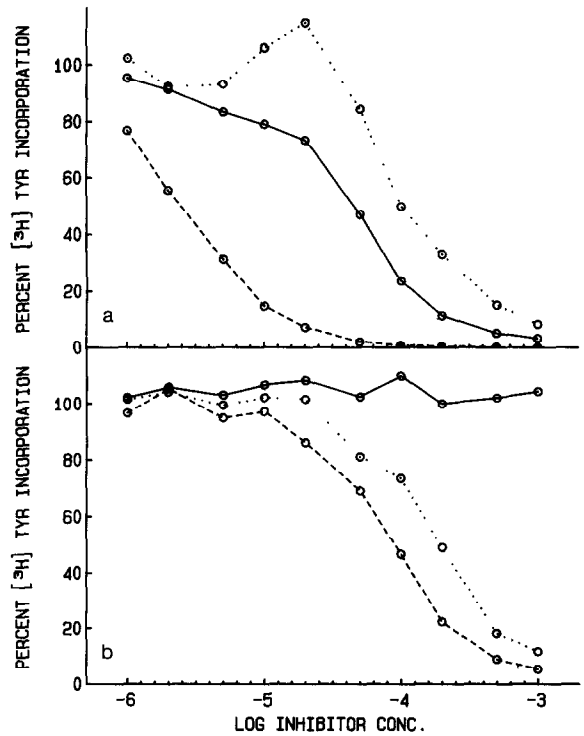

Fig. 1. Inhibition of TTLase-catalyzed [^{3}H]tyrosine uptake* into tubulin in the absence of illumination by (a) Tyr (– – –), NH_2Tyr (· · · ·), N_3Tyr (——); (b) Phe (– – –), NH_2Phe (· · · ·), N_3Phe (——).

*Incorporation is expressed as a percent of the radioactivity uptake as observed in the absence of inhibitor.

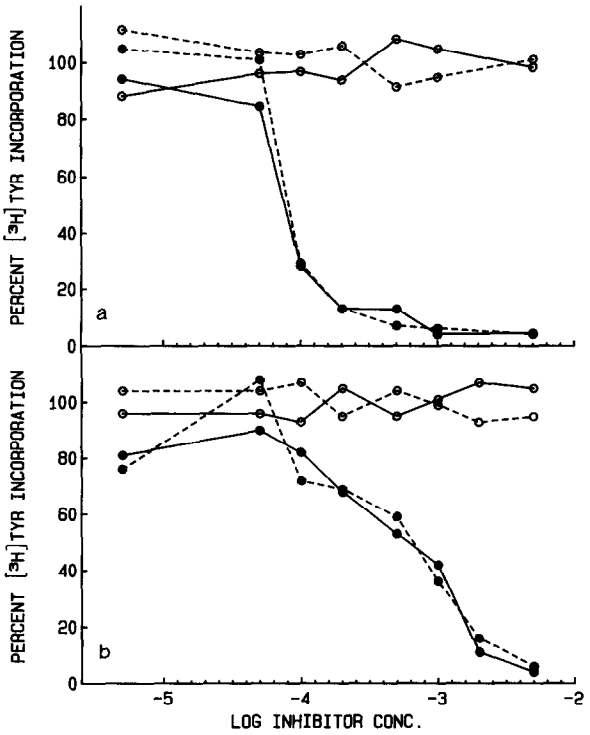

Fig. 2. Percent remaining TTLase activity, determined by [^{3}H]tyrosine uptake* into tubulin, incubated in the dark in the presence of (a) N_3Tyr (– ○ –), N_3Tyr plus 10^{-4} M Tyr (– – ○ – –); (b) N_3Phe (– ○ –), N_3Phe plus 10^{-4} M Tyr (– – ○ – –), and subsequently gel filtrated. Percent remaining TTLase activity after illumination in the presence of (a) N_3Tyr (– ● –), N_3Tyr plus 10^{-4} M Tyr (– – ● – –); (b) N_3Phe (– ● –), N_3Phe plus 10^{-4} M Tyr (– – ● – –), and subsequently gel filtrated.

*See Fig. 1.

Table I

Blocking of TTLase activity by increasing concentrations of pCMB correlated with loss of thiol groups

pCMB conc. [M]	% TTLase activity[a]	A_{412} nm (DTNB-derived)
10^{-2}	0	-
10^{-3}	33	0.099
10^{-4}	84	0.982
10^{-5}	90	0.975
10^{-6}	91	0.978
0	100	0.981

[a] Determined by [^{3}H]tyrosine uptake into tubulin.

against photoinactivation was studied. However, inactivation of TTLase caused by N_3Tyr or N_3Phe was not inhibited by the presence of 10^{-4} M (in some experiments 10^{-3} M) Tyr during illumination (Fig. 2a and b: dashed lines). Even 10^{-3} M Tyr, used in some experiments (not shown), yielded no protection.

In view of the possible reactions of thiol groups with aryl azides [14] the effect of protecting the thiol groups of TTLase by reversible mercaptide formation with pCMB was investigated. TTLase was transferred to ligase buffer (without DTE) by gel chromatography on a PD10 column (Pharmacia) and the enzyme-containing fractions were pooled. 300 μl mixtures were made up containing 3 mg/ml enzyme and different pCMB concentrations ranging from 10^{-6} to 10^{-2} M. After 30 min at room temperature, 80 μl samples were tested for TTLase activity (Table I). The rest of each mixture was incubated for another 30 min with 1 mM DTNB. Residual thiol groups, not blocked by pCMB, reduce the excess DTNB to form 2-nitro-5-benzoate anion, causing the development of a yellow colour (λ_{max} = 412 nm). From Table I it appears indeed that increasing pCMB concentration increasingly inhibits TTLase activity. This inactivation is accompanied by a loss of thiol groups. In our conditions at least 10^{-3} M pCMB is needed to block all thiol groups and most of the enzymatic activity.

Test mixtures made up of TTLase (3 mg/ml) and incubated with 10^{-3} M pCMB for 30 min at room temperature were subsequently treated with 10^{-2} M DTE or 10^{-1} M ME for another 30 min. After gel filtration (Sephadex G25-SF, 2 ml bed vol.) both samples recovered full activity.

Finally, we tested whether pCMB could protect TTLase against photoinactivation caused by N_3Tyr. Therefore we compared the enzymatic activity of two 800 μl mixtures made up of TTLase (3 mg/ml), incubated with excess pCMB (10^{-2} M) for 30 min before adding 10^{-3} M N_3Tyr. As a reference, we used TTLase not treated with pCMB. 400 μl of each mixture was illuminated for 10 min, while the rest was kept in the dark. Before gel filtration both fractions were treated with 10^{-2} M DTE as described. After gel filtration all samples were tested for TTLase activity (Table II).

4. DISCUSSION

The dark inhibition curve for N_3Tyr (Fig. 1a) shows that it is a rather good inhibitor (pIC_{50} = 4.4) with respect to Tyr (pIC_{50} = 5.6) and NH_2Tyr (pIC_{50} = 4.0). These data suggest that N_3Tyr is a candidate photoaffinity label of the tyrosine-binding site of TTLase. On the other hand N_3Phe (Fig. 1b) shows no measurable affinity (pIC_{50} <3), while Phe (pIC_{50} = 4.1) and NH_2Phe (pIC_{50} = 3.7) are moderate inhibitors of TTLase.

If, after incubation in the dark a gel filtration step is included, TTLase maintains its full activity over the whole concentration range (Fig. 2a and b). This means that neither N_3Tyr nor N_3Phe react with TTLase in the dark. In the case of illumination in the presence of N_3Tyr and subsequent gel filtration a sigmoidal curve (Fig. 2a) is obtained similar to the dark inhibition curve (cf. Fig. 1a). Photoinactivation of TTLase caused by N_3Phe also results in a sigmoidal curve (Fig. 2b), although no measurable affinity of this reagent for TTLase was observed in the dark. However, the curve is shifted one log unit to the right with respect to N_3Tyr which is a better photoinactivator. This is in agreement with the inhibitory capacity of the parent molecules (Tyr and Phe) for TTLase. Tyrosine, the natural ligand of TTLase, failed to protect the tyrosine-binding site of

Table II

Protection of TTLase activity by blocking thiol groups with pCMB during illumination in the presence of N_3Tyr

TTLase (3 mg/ml) treated with, in sequence:				% TTLase activity[c]
10^{-2} M pCMB	10^{-3} M N_3Tyr	Illuminated[a]	10^{-2} M DTE[b]	
-	-	-	-	100
-	-	+	-	100
+	+	-	-	17
+	+	+	-	18
+	+	-	+	103
+	+	+	+	85

[a] As described in section 2
[b] Added before gel filtration
[c] See footnote of Table I

TTLase against photoinactivation. The inhibition caused by N_3Phe, and the incapacity of Tyr to protect TTLase, point to the fact that photoinactivation is not due to reaction at the tyrosine-binding site.

In the literature [14] we found indications for dark reactions of aryl azides with thiols. Photolysis products of these azides could be even better reagents for thiols. Thus reactions at the thiol groups of TTLase could be the cause of the observed photoinactivation. This is confirmed by the use of a reversible thiol-blocking agent, pCMB, during illumination in the presence of N_3Tyr. Indeed TTLase can be protected for 85% against photoinactivation (Table II). These data prove, as presumed, that the photoinactivation by N_3Tyr is mainly due to a reaction of photolysis products (nitrenes) with essential thiol groups on the enzyme. It also explains why N_3Phe is a rather good photoinactivator of TTLase although it shows no measurable affinity for the enzyme in the dark. The fact that the photoinhibitor activity of N_3Phe does not equal that of N_3Tyr is probably not due to a difference in affinity, but rather to a difference in reactivity of the photointermediates.

We can conclude that both N_3Tyr and N_3Phe have not fulfilled their original promise to act as photoaffinity labels of TTLase. Nevertheless, they have demonstrated the existence of essential thiol groups present on TTLase, but outside the tyrosine-binding site. Our present work is focused on the identification of these essential side chains, through their photoreaction with azides.

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