Steady-state kinetic mechanism of bovine brain tubulin:tyrosine ligase

Nancy L. DEANS,* R. Donald ALLISON and Daniel L. PURICH†

Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Health Science Center, Gainesville, FL 32610-0245, U.S.A.

The ATP-dependent resynthesis of tubulin from tyrosine and untyrosinated tubulin was examined to establish the most probable steady-state kinetic mechanism of the tubulin:tyrosine ligase (ADP-forming). Three pair-wise sets of initial rate experiments, involving variation of two substrates pair-wise with the third substrate held at a high (but non-saturating) level, yielded convergent-line data, a behaviour that is diagnostic for sequential mechanisms. Michaelis constants were 14 μM , 1.9 μM and 17 μM for ATP, untyrosinated tubulin and L-tyrosine respectively, and the maximal velocity was 0.2 $\mu\text{M}/\text{min}$. AMP was a competitive inhibitor with respect to ATP, and a non-competitive inhibitor versus either tubulin or tyrosine. Likewise, L-dihydroxyphenylalanine acted competitively relative to tyrosine and non-competitively with respect to either ATP or tubulin. These findings directly support a random sequential mechanism. Product inhibition patterns with ADP were also consistent with this assignment; however, inhibition studies were not practical with either orthophosphate or tyrosinated tubulin because both were very weak inhibitors. Substrate protection of the enzyme against alkylation by *N*-ethylmaleimide and thermal inactivation, along with evidence of enzyme binding to ATP-Sepharose and tubulin-Sepharose, also supports the idea that this three-substrate enzyme reaction exhibits a random substrate addition pathway.

INTRODUCTION

Tubulin:tyrosine ligase (ADP-forming) catalyses a unique ATP-dependent post-translational modification of tubulin in which a tyrosyl residue is specifically re-incorporated into the C-terminal of the α -subunit of the tubulin heterodimer. The physiological significance of the tyrosination of tubulin remains unclear, because the C-terminal tyrosyl residue is encoded by the mRNA for the α -tubulin subunit (Valenzuela *et al.*, 1981). However, some cells and tissues exhibit a high turnover of tyrosine at this position in tubulin (Barra *et al.*, 1974; Arce *et al.*, 1975; Raybin & Flavin, 1975, 1977). Furthermore, detyrosination is catalysed by an exoprotease known as tyrosine:tubulin carboxypeptidase (Hallak *et al.*, 1977; Argarana *et al.*, 1978, 1980). Tyrosinated tubulin is the major species in dynamic microtubules such as those found in the mitotic spindle, while untyrosinated tubulin is present in more stable tubules (Gundersen *et al.*, 1984, 1987; Gundersen & Bulinski, 1986*a,b*).

The ligase has been extensively purified (Murofushi, 1980; Flavin *et al.*, 1982; Flavin & Murofushi, 1984) and migrates on SDS/polyacrylamide gels as a single polypeptide corresponding to a molecular mass of 46000 Da (Murofushi, 1980). More recently, V8 proteinase digestion of the ligase indicates that the enzyme contains two domains, one of which interacts with the α -chain C-terminus and the other with the β -subunit (Wehland & Weber, 1987). To further characterize the ligase, we have carried out studies to elucidate the kinetic mechanism of this carboxyl-group-activating enzyme. Knowledge of the kinetic mechanism can sometimes provide valuable information on the occurrence of obligatory conformational changes implicit in ordered substrate binding as well as the occurrence of co-operative subunit interactions. As recently exemplified by studies of bacterial D-alanyl-D-alanine ligase (McDermott *et al.*, 1990), the design of specific mechanism-based enzyme inhibitors also can be facili-

tated by a clear understanding of the reaction kinetics. Accordingly, this paper describes the development of a reliable initial rate assay, the results of initial rate kinetic experiments, several competitive and product inhibitor studies and additional binding experiments, all of which were designed to probe the kinetic mechanism of the tubulin:tyrosine ligase reaction.

EXPERIMENTAL

Materials

The following chemicals were purchased from Sigma Chemical Co: Mes, Hepes, Tris, EGTA, EDTA, BSA, di-isopropyl-fluorophosphate-treated carboxypeptidase A, DL-dithiothreitol (DTT), cyanogen bromide-activated Sepharose 4B, *N*-ethylmaleimide (NEM), ATP, ADP, AMP, GTP and GDP. DEAE-cellulose 52 and 3MM filter paper discs (2.3 cm in diameter) were obtained from Whatman. Dextran-coated charcoal tablets were a WestChem product. All liquid scintillation cocktails were xylene-based and were purchased from Research Products, Inc. L-[U-¹⁴C]Tyrosine (specific radioactivity 350 mCi/mmol) was obtained from ICN Biomedicals. All other reagents were of the highest quality commercially available and were used without further purification.

Methods

Tubulin:tyrosine ligase was assayed by a modification of the method described by Flavin & Murofushi (1984). The standard reaction mixture (kept on ice) contained 50 mM-Hepes (pH 7.4), 12.5 mM-MgCl₂, 1.0 mM-DTT, 100 mM-KCl, 100 μM -tyrosine (containing [¹⁴C]tyrosine), 2.5 mM-ATP and 3 mg of microtubule protein/ml (30 μM) in a total volume of 50 μl . The reaction was initiated with the addition of 10 μl of ligase fraction. The mixture was quickly mixed with a vortex mixer and transferred to a warm water bath at 37 °C. An assay sample containing 10 μl of buffer

Abbreviations used: DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

* Present address: Department of Chemistry, Trenton State College, Trenton, NJ 08625, U.S.A.

† To whom correspondence should be addressed.

solution in place of the ligase was always included as a control. After 15 min at 37 °C, 50 μ l aliquots were removed and transferred to Whatman 3MM filter paper discs (2.3 cm in diameter). These filters had been previously soaked in 10 mM unlabelled L-tyrosine with 10% trichloroacetic acid and allowed to dry. The discs containing the sample were then placed in a beaker containing a cold solution of unlabelled L-tyrosine (10 mM) in 10% (v/v) trichloroacetic acid (approx. 2–4 ml per disc). After keeping the beaker on ice for 20 min, the discs were further washed by rotary agitation for 10 min in 10% (v/v) trichloroacetic acid (with five changes), 6 min in 5% (v/v) trichloroacetic acid (with three changes), 4 min in ethanol/diethyl ether (1:1, v/v) (with two changes), and 4 min in diethyl ether (with two changes). The filters were then dried with a hair dryer on a low setting, and radioactivity was counted in 5 ml of xylene-based scintillation cocktail. One unit of enzyme activity was defined as 1 nmol of L-tyrosine covalently incorporated into tubulin per min. The c.p.m./nmol values were determined by transferring a known volume of the stock radiolabelled L-tyrosine to a clean filter disc that was subsequently air-dried without washing and counted for radioactivity in the same scintillation mixture. Specific enzymic activity was defined as units/mg of protein.

Tubulin preparation. Bovine brain microtubule protein was isolated according to the hypo-osmotic purification procedure described by Shelanski *et al.* (1973). The protein was taken through two polymerization/depolymerization cycles and then stored as microtubule pellets [in 35% (v/v) glycerol] at –80 °C. Immediately prior to use, the microtubule protein was further purified by an additional cycle. Tubulin was purified from hypo-osmotic microtubule protein by the method of Hamel & Lin (1984), centrifuged again as described by them, and the final supernatant was placed on ice and used within 10 min. The method of Flavin & Murofushi (1984) was used to determine the amount of pre-existing C-terminal tyrosine on the α -subunit of tubulin purified from hypo-osmotic microtubule protein. Based on the differential incorporation of labelled L-tyrosine before and after treatment with carboxypeptidase A to remove C-terminal tyrosine, we obtained a value of 0.40–0.45 mol of tyrosine per mol of tubulin. This agrees quite well with the discovery of Paturle-Lafanechere *et al.* (1991) of a major tubulin variant that cannot undergo tyrosination as a result of post-translational glutamylation. Accordingly, all plotted values of tubulin concentration were corrected to reflect the untyrosinated tubulin concentration. Moreover, we found that tyrosinated tubulin had no inhibitory effect under any conditions utilized in our kinetic experiments.

Affinity columns. A modification of the procedure described by Murofushi (1980) was used to prepare the ATP affinity column. Sebacic acid dihydrazide (0.4 g) was dissolved into 8 ml of acetic acid and left in a fume hood at room temperature. CNBr-activated Sepharose 4B (1.2 g) was washed successively with 100 ml of cold 1 M-HCl and 50 ml of distilled water. The sebacic acid dihydrazide solution was added, and the gel was stirred gently overnight (again in a fume hood) using a small gradient mixer. The gel was then washed successively with 100 ml of 50% (v/v) acetic acid, 200 ml of distilled water and 100 ml of 1.0 M-sodium acetate (pH 5.0) at room temperature. The gel was suspended in 4 ml of sodium acetate buffer and cooled on ice. Next, ATP was oxidized by mixing 1.2 ml of 20 mM-ATP (pH 7.0) with 1.2 ml of 18 mM-sodium *m*-periodate (freshly prepared) in the dark for 1 h at 0 °C. The oxidized ATP was then added to the Sepharose/sebacic acid hydrazide gel with gentle stirring for 3 h in the dark at 4 °C. Finally, the gel was washed successively with 50 ml of cold 1.0 M-NaCl, 50 ml of 1.0 mM-EDTA and 50 ml of distilled water. The gel was suspended in 10 ml of distilled water and kept at 4 °C until used within 2 days of preparation. The

amount of coupled ATP was calculated as the difference between the amount of total ATP added and the unbound ATP recovered in the salt wash. We found that about 3 μ mol of ATP was bound per g of Sepharose gel. Immobilized tubulin affinity resin was prepared according to the method of Murofushi (1980), and approx. 20 mg of protein was coupled per g of Sepharose gel.

Purification of tubulin:tyrosine ligase. Bovine brain tubulin:tyrosine ligase was isolated by the method of Flavin & Murofushi (1984) with modifications. All the column chromatography steps were performed at 4 °C. The starting material was the first warm supernatant (500 ml) from a bovine brain microtubule protein preparation diluted with 250 ml of Mes buffer [containing 25 mM-Mes (pH 6.8), 5.0 mM-MgCl₂ and 0.5 mM-DTT]. The mixture was then added to 200 ml of DEAE-cellulose, previously equilibrated with the same buffer, and the slurry was slowly stirred with a propeller stirrer for 1 h at 4 °C. The resin was collected on a Büchner funnel and resuspended in 200 ml 25 mM-Mes (pH 6.8), 50 mM-KCl, 5.0 mM-MgCl₂, 0.5 mM-EDTA and 0.5 mM-DTT. The slurry was then transferred to a column (10 cm $\times$ 5.25 cm diam.) and washed with 70 ml of the same buffer. The protein was eluted with a 0.05 M- to 0.30 M-KCl linear salt gradient [total volume of 1 litre, also containing 25 mM-Mes (pH 6.8), 5.0 mM-MgCl₂, 0.5 mM-EDTA and 0.5 mM-DTT]. Fractions (15 ml) were collected at a flow rate of 100 ml/h. Tubulin:tyrosine ligase was eluted as a broad peak between 0.15 and 0.25 M-KCl (results not shown).

The active fractions were pooled and applied at a flow rate of 50 ml/h to a Sepharose-ATP column (1.5 cm $\times$ 2.3 cm) pre-equilibrated with 25 mM-Mes (pH 6.8), 5.0 mM-MgCl₂ and 0.5 mM-DTT. The column was washed with the above buffer containing 0.5 M-KCl to remove non-specifically bound proteins. After the u.v. absorbance at 280 nm had decreased to 0.05 absorbance units, the ionic strength was lowered by washing with the same buffer containing only 0.1 M-KCl. The column flow was stopped for 1 h after adding 4 ml of elution buffer [25 mM-Mes (pH 6.8), 25 mM-ATP, 0.1 M-KCl, 25 mM-MgCl₂ and 0.5 mM-DTT]. The enzyme was then eluted, and five 10 ml fractions were collected at a flow rate of 15 ml/h. Active fractions (usually the first three or four) were pooled and diluted with an equal volume of 25 mM-Mes (pH 6.8), 5.0 mM-MgCl₂ and 1.0 mM-DTT to lower the salt concentration. The mixture was then centrifuged at 15000 *g* for 20 min at 4 °C to remove any precipitate.

The active enzyme fraction was then loaded on to a Sepharose-tubulin column (1.5 cm $\times$ 2.3 cm) pre-equilibrated with a solution of 25 mM-Mes (pH 6.8), 5.0 mM-MgCl₂, 50 mM-KCl and 1.0 mM-DTT at a flow rate of 30 ml/h. Non-specifically bound proteins were removed with 25 mM-Mes (pH 6.8), 5.0 mM-MgCl₂, 0.4 M-KCl and 1.0 mM-DTT. After halting the flow for 1 h, the ligase was eluted with the same high-salt Mes buffer, collecting 3 ml fractions at a flow rate of 9.5 ml/h. The active fractions (typically the second to the fourth) were pooled and glycerol was added to a final concentration of 1 M. The sample was then frozen by dropwise addition to liquid nitrogen and stored at –80 °C. The final specific activity was 840 units/mg.

Tubulin:tyrosine ligase, purified as described above, was stable for 2 months at –80 °C with less than 10% loss of activity. In the absence of glycerol, however, the ligase was far more unstable.

Initial rate assays. Initial rate assays were performed at 37 °C in 50 mM-Hepes (pH 7.4), 12.5 mM-MgCl₂, 1.0 mM-DTT, 100 mM-KCl, 0.3 units of tubulin:tyrosine ligase and various concentrations of charcoal-treated untyrosinated tubulin, tyrosine and ATP. Because the ATP concentrations utilized in these experiments never exceeded 0.1 mM, no corrections were necessary for depletion of the 12.5 mM total magnesium ion concentration. The method involved holding the non-varied substrate at a constant concentration (approx. 3–5 times the apparent

K_m value) while varying the concentration of the other two substrates from about 0.5 to 5 times their apparent K_m values. A 95 μ l reaction mixture containing all the reaction components except tyrosine (including [14 C]tyrosine) was preincubated at room temperature for 5 min. After preincubation, 30 μ l of radiolabelled L-tyrosine was added to initiate the reaction. The mixture was immediately placed in a 37 °C water bath. At timed intervals, 45 μ l aliquots were removed and transferred to tubes containing 15 μ l of BSA (5.2 mg/ml) and 8 mM unlabelled tyrosine on ice. This procedure effectively quenched any further radiolabel incorporation. After about 5 min in the ice bath, 50 μ l aliquots were spotted on to Whatman 3MM filter paper discs (previously washed in 10 mM unlabelled tyrosine in 10% trichloroacetic acid). The discs were washed, dried and counted for radioactivity as described. The data reported here are completely representative of kinetic experiments, which were all carried out at least in triplicate and were analysed using Cleland's software (Cleland, 1979). The reported K_m values were obtained by this procedure, and because our measurements were made at a single constant value of the non-varied substrate, we should emphasize that these are apparent kinetic parameters rather than extrapolated or true K_m values. The best-fit lines largely matched those drawn by sight. We also observed substrate inhibition as well as non-ideality imposed by the range of tubulin concentrations over which no polymerization took place, and the precision of points of convergence of lines in $1/v$ versus $1/[S]$ plots were not consistently achieved. Nonetheless, when the initial rate data were fitted to various multi-substrate kinetic schemes using Cleland's software, we found that ping-pong mechanisms with their inherent parallel-line behaviour cannot adequately account for the convergence observed in each of the plots that we obtained. Indeed, the statistical variation in rate data was always lowest at substrate concentrations above the respective Michaelis constants, and sequential mechanisms characteristically display convergence. It should also be noted that no attempt was made to use these statistical routines to analyse curvilinear data.

Substrate-protection studies. A typical NEM inactivation experiment was performed as follows. The preincubation mixture (total volume of 50 μ l) included 0.2 unit of tubulin:tyrosine ligase, 0.4 M-NEM, 50 mM-Hepes (pH 7.4), 1.0 mM-DTT, 100 mM-KCl and one or more of the substrates (at concentrations near their K_m values). Whenever ATP was included in the mixture, 12.5 mM-MgCl₂ was added as well. This mixture was left at room temperature for 8 min. Following this room temperature preincubation, a second mixture (containing the remaining reaction components) was added to give a final volume of 100 μ l. This solution was then incubated at 37 °C. At timed intervals, 40 μ l aliquots were removed, quenched, and the amount of L-tyrosine incorporation was determined as described above.

Protein analysis. Protein concentration was determined by the method of Lowry *et al.* (1951), using BSA as a standard, or by a modified procedure (Bensadoun & Weinstein, 1976) whenever the concentration was below the sensitivity of the Lowry procedure.

RESULTS

Evaluation of the kinetic mechanism and, in particular, the kinetic parameters of an enzyme-catalysed reaction can often be best approached through the analysis of initial rate data. The experimental procedures required for such analyses of three substrate enzymic reactions, however, are more complicated than bisubstrate kinetic studies. Two methods are commonly used to evaluate such systems. One method involves varying one

substrate while holding the other two substrates in a fixed ratio at different degrees of saturation within the range of their respective Michaelis constants (Fromm, 1967). Similarly, each of the other two substrates is varied in separate experiments, to allow the differentiation of the kinetic mechanism into sequential or ping-pong type. An alternative procedure involves holding one substrate at high but non-saturating concentrations, while the other two substrate levels are varied (Frieden, 1959). Again, three experiments are required to distinguish between ping-pong and sequential-type mechanisms. In both methods, double-reciprocal plots of the initial rate data allows the differentiation of the kinetic mechanism: ping-pong mechanisms result in one or more parallel lines in double-reciprocal plots, whereas sequential mechanisms exhibit non-parallel lines. Secondary replots using slope and intercept data allow for the determination of certain kinetic parameters. The Frieden method proved to be more accessible in these experiments using a protein substrate, and this protocol was followed for all initial rate studies.

Development of initial rate kinetic assays

Preliminary rate studies of the tubulin:tyrosine ligase reaction indicated lag-phase kinetics in the progress curve. This is most evident during the first 3 or 4 min after initiating the reaction (Fig. 1a). This behaviour represented a potential obstacle to the application of the initial rate kinetic strategies noted above. Nonetheless, we found that preincubation of the enzyme with ATP and tubulin at room temperature (followed by the addition of L-tyrosine and L-[14 C]tyrosine to initiate the reaction) eliminated or markedly reduced the lag phase (Fig. 1b), yielding satisfactory initial rate behaviour. All enzyme assays were performed under these conditions, and the incorporation of radiolabelled L-tyrosine was linear with reaction time or enzyme concentration (see Fig. 1c).

Influence of substrate concentration changes on the initial rate behaviour of the ligase

The effects of ATP and tubulin concentration changes on the reaction rates (at constant L-tyrosine) are presented in double-reciprocal form in Figs. 2(a) and 2(b). In both cases, the lines are not parallel, and converge on the corresponding $1/[S]$ axis. This behaviour is characteristic of a sequential mechanism for ATP and tubulin binding, as illustrated by the following rate law:

$$1/v = [\Phi + (\Phi_a/[ATP]) + (\Phi_t/[Tb]) + (\Phi_{at}/[ATP][Tb])]_{\text{const Tyr}}$$

where v is the initial velocity of the reaction, Φ is a collection of rate constants that also includes the constant level of tyrosine, Φ_a is a term akin to the Michaelis constant for ATP, Φ_t is a term containing the Michaelis constant for tubulin, and Φ_{at} is a complex combination of kinetic constants. The Φ_{at} term in the equation above is not a feature of ping-pong mechanisms, and the obvious convergence of the plotted data in Figs. 2(a) and 2(b) excludes the ping-pong mechanism for the ATP-tubulin substrate pair. Secondary plots of the rate data were obtained at constant L-tyrosine concentrations, and extrapolated values for V_{max} of $0.21 \pm 0.02 \mu\text{M}/\text{min}$, K_{Tb} (K_m for tubulin) of $1.3 \pm 0.4 \mu\text{M}$, and K_{ATP} of $20 \pm 3 \mu\text{M}$ were obtained at 100 μM -L-tyrosine.

The reaction velocity data as a function of tubulin and L-tyrosine concentrations at constant ATP levels are shown in Fig. 2(c) and Fig. 2(d). Again, the lines are non-parallel. By analogy with the above discussion, these data are consistent with a sequential mechanism for the addition of tubulin and L-tyrosine. The intercept and slope replots yielded the following kinetic parameters: V_{max} , $0.21 \pm 0.02 \mu\text{M}/\text{min}$, K_{Tb} , $2.4 \pm 0.5 \mu\text{M}$, K_{Tyr} , $18 \pm 4 \mu\text{M}$ at 50 μM -ATP.

The double-reciprocal plots of the initial rate data in which ATP and L-tyrosine were varied at a constant tubulin con-

Fig 1. Characteristics of the tubulin:tyrosine ligase-catalysed reaction

(a) Progress curve. The initial phase of the tubulin:tyrosine ligase-catalysed reaction was measured in the presence of 0.2 unit of enzyme/ml, 20 μM untyrosinated tubulin, 100 μM -L-tyrosine (including [^{14}C]tyrosine) and 1 mM-ATP in assay buffer. The reaction mixture was incubated at 37 $^{\circ}\text{C}$, and aliquots were removed at timed intervals from 0 to 20 min. The aliquots were assayed for tyrosine incorporation as described in the Experimental section. (b) Progress curve of the tubulin:tyrosine ligase-catalysed reaction after preincubation with tubulin and ATP. The enzyme-catalysed incorporation of radiolabelled tyrosine into untyrosinated tubulin was measured as a function of time. The reaction mixture was initiated with tyrosine (including [^{14}C]tyrosine) yielding final concentrations of 60 μM -tyrosine, 30 μM -ATP, 100 mM-KCl, 5 μM untyrosinated tubulin, and 0.4 unit of tubulin:tyrosine ligase. (c) Initial velocity versus tubulin:tyrosine ligase concentration. The effect of varying the enzyme concentration was examined by mixing 30 μM -ATP, 10 μM charcoal-treated tubulin and 100 μM -tyrosine (including [^{14}C]tyrosine) in 40 mM-Hepes (pH 7.4), 12.5 mM-MgCl₂, 1 mM-DTT and 100 mM-KCl with the indicated concentration of tubulin:tyrosine ligase. The reaction mixtures were incubated for 10 min at 37 $^{\circ}\text{C}$, and aliquots were then removed to determine the amount of radiotyrosine incorporation as described in the Experimental section.

centration are depicted in Figs. 2(e) and 2(f). As observed with the preceding data, these lines are non-parallel, again suggesting that a ping-pong mechanism is not operant for this pair-wise interaction in the tubulin:tyrosine ligase-catalysed reaction. The secondary plots yielded the following kinetic parameters: V_{max} , $0.18 \pm 0.01 \mu\text{M}/\text{min}$; K_{ATP} , $7.7 \pm 0.9 \mu\text{M}$; K_{Tyr} , $16.9 \pm 2.1 \mu\text{M}$ at 18 μM -tubulin.

Effects of inhibitors on the initial rate behaviour of the ligase

Taken together, the initial rate data cited above can be fitted to three-substrate sequential rate equations. To limit the number of kinetic mechanisms for this enzyme system, our initial rate data were supplemented with experiments involving competitive and product inhibitors for differentiating between random and ordered mechanisms (Fromm, 1979). Again, the most commonly used method involves holding two substrates at subsaturating levels in the range of their respective Michaelis constants, while the third substrate and inhibitor concentrations are varied. The initial rate data are then plotted in double-reciprocal form, and the inhibition patterns can be used to limit the possible kinetic pathways. Subsequent replots of the slopes or intercepts versus inhibitor concentration can also provide estimates of the inhibition constants.

AMP was found to act as competitive inhibitor of ATP (Fig. 3a) in the ligase reaction. Non-competitive inhibition was observed with respect to L-tyrosine (Fig. 3b), whereas non-linear inhibition was observed with respect to tubulin (Fig. 3c). The basis of this non-linearity, observed only in this set of experiments, remains unclear. The following inhibition constants (K_i) were obtained from replots: $0.27 \pm 0.04 \text{ mM}$ with respect to ATP and $1.3 \pm 0.6 \text{ mM}$ with respect to L-tyrosine.

Earlier studies showed that 3,4-dihydroxyphenylalanine inhibits tubulin:tyrosine ligase activity (Raybin & Flavin, 1977). By varying the concentrations of L-dihydroxyphenylalanine and L-tyrosine relative to each other, while holding ATP and tubulin levels constant, we found that L-dihydroxyphenylalanine exhibits a competitive inhibition pattern with respect to tyrosine (Fig.

4a). However, non-competitive inhibition was observed with respect to ATP (Fig. 4b) and untyrosinated tubulin (Fig. 4c). Inhibition constants were calculated from the replots. K_i values of $0.45 \pm 0.06 \text{ mM}$ with respect to L-tyrosine, $1.9 \pm 0.9 \text{ mM}$ relative to ATP and $1.7 \pm 0.4 \text{ mM}$ with respect to tubulin were obtained from replots. It should be stressed that care was taken in choosing the range of inhibitor concentrations so that sufficient incorporation of radiolabelled L-tyrosine into tubulin occurred, and the fact that dihydroxyphenylalanine may be an alternative substrate does not affect the validity of its use as a competitive inhibitor of radiotyrosine uptake (Huang, 1979).

The high specificity of bovine brain tubulin:tyrosine ligase frustrated all efforts to identify a competitive inhibitor for tubulin. Even so, taken along with the results from the initial rate experiments, these data eliminate the possibility of an ordered quaternary substrate-addition mechanism, the first two substrates, ATP and L-tyrosine, followed then by tubulin binding; and the random BC mechanism wherein binding of L-tyrosine and tubulin occurs randomly after the initial compulsory binding of ATP. This leaves the fully random kinetic pathway as the most reasonable kinetic pathway.

Product inhibitor effects on ligase kinetics

Further support of a random substrate addition mechanism for tubulin:tyrosine ligase was adduced from product inhibition with ADP. We were forced to focus on ADP only because phosphate showed no inhibition, even at concentrations of 10 mM, and inhibition by tyrosinated tubulin appeared to be only slight. The effects of several constant concentrations of ADP on the rate behaviour relative to ATP are shown in double-reciprocal form in Fig. 5(a). Tubulin and L-tyrosine were held at levels approximately several times their respective K_m values. These data indicate that ADP behaved as a competitive inhibitor with respect to ATP, but as a non-competitive inhibitor relative to tubulin and L-tyrosine (Figs. 5b and 5c). The inhibition constants obtained from the secondary plots were: K_i of $32 \pm 5 \mu\text{M}$ with respect to ATP; K_i of $50 \pm 60 \mu\text{M}$ with respect to tubulin; and K_i

Fig 2. Ligase activity dependence on substrate concentration

(a) Reciprocal of the initial reaction velocity versus the reciprocal of untyrosinated tubulin concentration at various levels of ATP, and at a constant $100 \mu\text{M}$ concentration of L-tyrosine. The reaction components were mixed together with 0.36 unit of enzyme, incubated briefly at 37°C , and the reaction was initiated with L-tyrosine (including $[^{14}\text{C}]$ tyrosine). After 3 and 8 min incubation periods, aliquots were removed and the amount of tyrosine incorporated into tubulin was determined as described in the Experimental section. The reaction mixture contained a final untyrosinated tubulin (Tb) concentration (corrected) of $1.3 \mu\text{M}$, $1.8 \mu\text{M}$, $3.0 \mu\text{M}$ and $9.0 \mu\text{M}$. ATP concentrations were $35 \mu\text{M}$ (Δ), $11.7 \mu\text{M}$ ($\bullet$) and $7 \mu\text{M}$ ($\circ$). (b) Reciprocal of the initial rate velocity versus the reciprocal of the ATP concentration at various levels of untyrosinated tubulin, and at a constant $100 \mu\text{M}$ concentration of L-tyrosine. The reaction components were mixed together and the reaction was initiated with L-tyrosine (including $[^{14}\text{C}]$ tyrosine). The mixture was then incubated at 37°C with the ligase (0.36 unit). ATP concentrations were $7 \mu\text{M}$, $11.7 \mu\text{M}$ and $35 \mu\text{M}$. Untyrosinated tubulin concentrations (corrected) were $9 \mu\text{M}$ ($\blacktriangle$), $3 \mu\text{M}$ ($\triangle$), $1.8 \mu\text{M}$ ($\bullet$) and $1.3 \mu\text{M}$ ($\circ$). (c) Reciprocal of the initial reaction velocity versus the reciprocal of untyrosinated tubulin concentration at various levels of tyrosine and $50 \mu\text{M}$ -ATP. The reaction components were mixed together with 0.33 unit of enzyme, incubated briefly at 37°C , and the reaction was initiated with L-tyrosine. Untyrosinated tubulin (Tb) concentrations (corrected) were $1.3 \mu\text{M}$, $1.8 \mu\text{M}$ and $9 \mu\text{M}$. L-Tyrosine (including $[^{14}\text{C}]$ tyrosine) concentrations were $100 \mu\text{M}$ ($\blacktriangle$), $33 \mu\text{M}$ ($\triangle$), $20 \mu\text{M}$ ($\bullet$) and $14.3 \mu\text{M}$ ($\circ$). (d) Reciprocal of the initial reaction velocity versus the reciprocal of L-tyrosine concentration at various levels of untyrosinated tubulin, and $50 \mu\text{M}$ -ATP. The reaction components were mixed together with 0.33 unit of enzyme, incubated briefly at 37°C , and the reaction was initiated with L-tyrosine. Tubulin concentrations were $9 \mu\text{M}$ ($\blacktriangle$), $3 \mu\text{M}$ ($\triangle$), $1.8 \mu\text{M}$ ($\bullet$) and $1.3 \mu\text{M}$ ($\circ$). (e) Reciprocal of the initial reaction velocity versus the reciprocal of L-tyrosine concentration at various levels of ATP, and $10 \mu\text{M}$ untyrosinated tubulin (Tb). The reaction components were mixed together with 0.36 unit of enzyme, incubated briefly at 37°C , and the reaction was initiated with L-tyrosine. L-Tyrosine (including $[^{14}\text{C}]$ tyrosine) concentrations were $100 \mu\text{M}$, $33 \mu\text{M}$, $20 \mu\text{M}$ and $14.3 \mu\text{M}$. ATP concentrations were $35 \mu\text{M}$ ($\blacktriangle$), $11.7 \mu\text{M}$ ($\triangle$), $7 \mu\text{M}$ ($\bullet$) and $5 \mu\text{M}$ ($\circ$). (f) Reciprocal of the initial reaction velocity versus the reciprocal of ATP concentration at various levels of L-tyrosine, and a constant concentration of tubulin (Tb). The reaction components were mixed together with 0.36 unit of enzyme, incubated briefly at 37°C , and the reaction was initiated with L-tyrosine. ATP concentrations were $35 \mu\text{M}$, $11.7 \mu\text{M}$, $7 \mu\text{M}$ and $5 \mu\text{M}$. L-Tyrosine (including $[^{14}\text{C}]$ tyrosine) concentrations were $100 \mu\text{M}$ ($\blacktriangle$), $33 \mu\text{M}$ ($\triangle$), $20 \mu\text{M}$ ($\bullet$) and $14.3 \mu\text{M}$ ($\circ$).

of $65 \pm 12 \mu\text{M}$ with respect to L-tyrosine. The product inhibition data are in basic agreement with the conclusions reached from the competitive inhibition experiments.

Substrate binding studies

As a further probe of the substrate binding order of the tubulin:tyrosine ligase reaction, we investigated the ability of the various substrates to confer protection against inactivation. The tubulin:tyrosine ligase requires the presence of DTT for maximal activity, and either NEM or *p*-chloromercuriphenylsulphonic acid inhibits the enzymic activity (Murofushi, 1980). To determine if any of the substrates were capable of protecting the ligase from NEM-induced inactivation, this alkylating agent was

preincubated with the ligase in the presence of one or more of the substrates. The results of such experiments are shown in Table 1. In the absence of L-tyrosine, NEM caused 98% inhibition. However, L-tyrosine alone, L-tyrosine with ATP or L-tyrosine with tubulin decreased the NEM inactivation to approx. 50% under the conditions applied. On the other hand, tubulin alone and ATP alone did not protect against NEM-induced inactivation, perhaps suggesting that a reactive thiol is near the tyrosine-binding site.

Analogous experiments were performed to determine if any substrates or substrate combinations conferred some protection against heat inactivation of tubulin:tyrosine ligase (results not shown). Preincubation of the enzyme with tubulin alone or in

Fig 3. Inhibitory effects of AMP

(a) Reciprocal of the initial reaction velocity versus the reciprocal of ATP concentration at various concentrations of AMP. All reaction components (except tyrosine) were mixed together with 0.3 unit of enzyme, incubated briefly at 37 °C, and the reaction was initiated with L-[¹⁴C]tyrosine. The other substrates were 50 μ M-tyrosine (including [¹⁴C]tyrosine) and 6 μ M untyrosinated tubulin. The ATP concentrations were 30 μ M, 10 μ M, 6 μ M and 4 μ M. The AMP concentrations were 0.9 mM ($\circ$), 0.5 mM ($\bullet$) and 0.25 mM ($\triangle$); $\blacktriangle$ indicates the experimental velocity in the absence of inhibitor. (b) Reciprocal of the initial reaction velocity versus the reciprocal of L-tyrosine concentration at various concentrations of AMP. All reaction components (except tyrosine) were mixed together with 0.3 unit of enzyme, incubated briefly at 37 °C, and the reaction was initiated with L-tyrosine. The final concentration of other reaction components were 6 μ M-tubulin (corrected) and 20 μ M-ATP. L-Tyrosine (including [¹⁴C]tyrosine) concentrations were 70 μ M, 23 μ M, 14 μ M and 10 μ M; the AMP concentrations were 1.0 mM ($\circ$), 0.5 mM ($\bullet$) and 0.25 mM ($\triangle$); $\blacktriangle$ is the experimental velocity in the absence of inhibitor. (c) Reciprocal of the initial reaction velocity versus the reciprocal of tubulin (Tb) concentration at various concentrations of AMP. All reaction components (except tyrosine) were mixed together with 0.3 unit of enzyme, incubated briefly at 37 °C, and the reaction was initiated with tyrosine. The concentrations of the other components were 50 μ M-L-tyrosine and 20 μ M-ATP. The concentrations of untyrosinated tubulin were 9 μ M, 3 μ M, 1.8 μ M and 1.3 μ M. The AMP concentrations were 1.0 mM ($\circ$), 0.5 mM ($\bullet$) and 0.25 mM ($\triangle$); $\blacktriangle$ is the experimental velocity in the absence of inhibitor.

Fig 4. Inhibitory effects of L-dihydroxyphenylalanine

(a) Reciprocal of the initial reaction velocity versus the reciprocal of tyrosine concentration at various concentrations of L-dihydroxyphenylalanine. All reaction components (except tyrosine) were mixed together with 0.3 unit of enzyme, incubated briefly at 37 °C, and the reaction was initiated with L-tyrosine. The final concentrations of the other components were 30 μ M-ATP and 10 μ M untyrosinated tubulin. The L-tyrosine (including [¹⁴C]tyrosine) concentrations were 100 μ M, 33.3 μ M, 20 μ M and 14.3 μ M. The L-dihydroxyphenylalanine concentrations were 0.92 mM ($\circ$), 0.31 mM ($\bullet$), 0.18 mM ($\triangle$) and 0.13 mM ($\blacktriangle$); $\square$ is the experimental velocity in the absence of inhibitor. (b) Reciprocal of the initial reaction velocity versus the reciprocal of ATP concentration at various concentrations of L-dihydroxyphenylalanine. All reaction components (except tyrosine) were mixed together with 0.3 unit of enzyme, incubated briefly at 37 °C, and the reaction was initiated with tyrosine. The final concentrations of the reaction components were 50 μ M-L-tyrosine (including [¹⁴C]tyrosine) and 10 μ M untyrosinated tubulin. The ATP concentrations were 35 μ M, 11.7 μ M and 7 μ M. The L-dihydroxyphenylalanine concentrations were 0.92 mM ($\circ$), 0.31 mM ($\bullet$) and 0.13 mM ($\triangle$); $\square$ is the experimental velocity in the absence of inhibitor. (c) Reciprocal of the initial reaction velocity versus the reciprocal of untyrosinated tubulin (Tb) concentration at varying concentrations of L-dihydroxyphenylalanine. All reaction components except tyrosine were mixed together with enzyme (0.3 unit) at 37 °C, and the reaction mixture was initiated with L-tyrosine. The final concentrations of the reaction components were 50 μ M-L-tyrosine (including [¹⁴C]tyrosine) and 30 μ M-ATP. The untyrosinated tubulin concentrations (corrected) were 25 μ M, 8.4 μ M, 5 μ M and 3.6 μ M; the L-dihydroxyphenylalanine concentrations were 0.92 mM ($\circ$), 0.31 mM ($\bullet$) and 0.18 mM ($\triangle$); $\blacktriangle$ is the experimental velocity in the absence of inhibitor.

Fig 5. Inhibitory effects of ADP

(a) Reciprocal of the initial reaction velocity versus the reciprocal of ATP concentration at various concentrations of ADP. All reaction components (except tyrosine) were mixed together with 0.3 unit of enzyme, incubated briefly at 37 °C, and the reaction was initiated with tyrosine. The final concentrations of the other reaction components were 50 μM -tyrosine (including [^{14}C]tyrosine) and 8 μM untyrosinated tubulin. ATP concentrations were 31.5 μM , 10.5 μM , 6.3 μM and 4.5 μM ; ADP concentrations were 80 μM (○), 26.7 μM (●) and 16 μM (△); ▲ is the experimental velocity in the absence of inhibitor. (b) Reciprocal of the initial reaction velocity versus the reciprocal of tubulin (Tb) concentration at various concentrations of ADP. All reaction components (except tyrosine) were mixed together with 0.3 unit of enzyme, incubated briefly at 37 °C, and the reaction was initiated with tyrosine. The final concentrations of the other reaction components were 50 μM -tyrosine (including [^{14}C]tyrosine) and 20 μM -ATP. The untyrosinated tubulin concentrations (corrected) were 14 μM , 4.6 μM , 2.8 μM and 2.0 μM ; ADP concentrations were 190 μM (○), 63.3 μM (●) and 38 μM (△); ▲ is the experimental velocity in the absence of inhibitor. (c) Reciprocal of the initial reaction velocity versus the reciprocal of tyrosine concentration at various concentrations of ADP. All reaction components (except tyrosine) were mixed together with 0.3 unit of enzyme, incubated briefly at 37 °C, and the reaction was initiated with tyrosine. The final concentrations of the other reaction components were 20 μM -ATP and 8 μM -tubulin (corrected). The tyrosine (including [^{14}C]tyrosine) concentrations were 90 μM , 30 μM , 18 μM and 12.8 μM ; ADP concentrations were 190 μM (○), 63.3 μM (●) and 38 μM (△); ▲ is the experimental velocity in the absence of inhibitor.

Table 1. Substrate protection against NEM inactivation

All reaction mixtures contained 0.4 M-NEM in 50 mM-Hepes buffer (pH 7.4) containing 12.5 mM-MgCl₂ and 100 mM-KCl. When present, the substrate concentrations were 5 μM -tubulin, 10 μM -ATP and 20 μM -L-tyrosine. After 30 min in the presence of the alkylating agent, ligase activity was then measured with the standard enzyme assay containing 1 mM-DTT.

Conditions	Enzyme activity (pmol of L-tyrosine/min)
Control (preincubation in buffer only)	4.3
NEM	0.1
NEM + tubulin	0.1
NEM + L-tyrosine	2.2
NEM + tubulin + L-tyrosine	3.3
NEM + ATP	0.1
NEM + ATP + tubulin	0.1
NEM + ATP + L-tyrosine	2.2

combination with ATP or tyrosine stabilized tubulin against the heat inactivation of enzymic activity. BSA, on the other hand, had no such stabilizing effect, indicating that the stabilization by tubulin was not merely due to a non-specific protein-protein interaction, and that tubulin could bind to the free enzyme in the absence of other substrates. One should note, however, that such observations of binding and protection, while consistent with the kinetic data presented in this report, do not necessarily indicate that the protecting substrate is binding to the active site in a manner akin to that observed in the kinetic experiments.

DISCUSSION

The combined results of the experiments described in this report indicate that the tubulin:tyrosine ligase most probably operates by a random sequential pathway. All of the double-reciprocal plots, with substrates treated pair-wise, exhibit non-parallel lines converging to the left of the $1/v$ axis, thus excluding most of the so-called ping-pong pathways. While non-parallel-line data can also be observed in ping-pong mechanisms exhibiting substrate inhibition, no evidence of substrate inhibition was observed in the ligase-catalysed reaction. Our data also indicate that two other cases can be eliminated, i.e. the random AB three-substrate mechanism (in which the binding of the first two substrates, A and B, is random) and a rapid equilibrium ordered mechanism (in which the addition of substrates is completely ordered). Both of these mechanisms yield double-reciprocal data which converge on the $1/v$ axis, and such behaviour was not observed with any of the data that we obtained. One caveat concerning our rate data is that enzymes displaying sequential mechanisms, when kinetically analysed, as we did here, in terms of the method of Frieden (1959), should produce double-reciprocal plots in which the family of lines converge at a common point. Our results are at odds with this requirement, and we attribute this to statistical variation in the rate data.

Our kinetic experiments also attest to the value of competitive and product inhibitors in the elucidation of the kinetic mechanism of a three-substrate enzymic reaction. When combined with the initial rate data, these studies distinguish between ping-pong and sequential mechanisms. In addition, once the ping-pong type mechanism is eliminated, these experiments can be used to determine the most likely pathway of substrate addition to the

enzyme. The data presented are consistent with only two reaction pathways: a fully random mechanism or a partially ordered mechanism in which the addition of the second substrate, in this case L-tyrosine, is ordered. In attempts to distinguish ping-pong mechanisms from sequential mechanisms, the real problem arises when parallel-line behaviour is observed (Ning *et al.*, 1969). However, no parallel-line behaviour was observed in any of the double-reciprocal plots, indicating that no segment of the tubulin:tyrosine ligase kinetic reaction scheme exhibits either ping-pong behaviour or unusual sequential enzyme behaviour.

Several other observations indicate that the tubulin:tyrosine ligase reaction is governed by a completely random mechanism. The second step of the purification protocol takes advantage of enzyme binding to an ATP affinity column in the absence of L-tyrosine. Likewise, the final chromatographic step involves enzyme binding to a tubulin affinity column in the absence of L-tyrosine. Although ATP is also present, clearly tubulin binding need not be preceded by tyrosine, suggesting that the ligase can bind tubulin or ATP before tyrosine addition. Moreover, the protection experiments described in this paper suggest that tubulin or L-tyrosine can bind to tubulin:tyrosine ligase in the absence of ATP. In the absence of tubulin and ATP, L-tyrosine stabilized the ligase against inhibition by *N*-ethylmaleimide, suggesting that L-tyrosine is capable of binding to the free enzyme. Preincubation of ligase with tubulin stabilized the enzyme against heat inactivation in the absence of L-tyrosine and ATP. Accordingly, the binding of L-tyrosine to free ligase eliminates the random AC mechanism (ordered binding of B, i.e. L-tyrosine), suggesting that the tubulin:tyrosine ligase reaction occurs by a fully random addition of substrates.

The kinetic constants were determined from the secondary plots of the initial rate data. These plots yielded similar values for all the kinetic parameters. The mean values for the Michaelis constants were: $K_{m,ATP}$, 14 μM ; $K_{m,Tb}$, 1.9 μM ; $K_{m,Tyr}$, 17 μM , and the mean maximum velocity was 0.2 $\mu\text{M}/\text{min}$. Except for the case of ATP, the values appear to be near the reported physiological concentrations of the substrates. The intracellular concentration of ATP in cerebral cortex is approx. 3 mM (Bachelard, 1971), suggesting that the ligase is always saturated with ATP. The concentration of L-tyrosine in brain tissue approaches 40 μM (Blosser & Wells, 1972), a value in good agreement with our K_m estimate for tyrosine. To determine the concentration of free tubulin, a plot of free tubulin versus total polymer concentration is extrapolated to the horizontal axis to obtain the critical tubulin concentration. This is the tubulin concentration in equilibrium with the polymer form. A typical *in vivo* tubulin critical concentration is around 1 mg/ml or 10 μM (Soltys & Borisy, 1985), suggesting that the physiological concentration of free tubulin is also in the range of the K_m value that we obtained.

Although the data in Fig. 3(c) are noticeably curvilinear with respect to the inverse of tubulin concentration, careful inspection of all kinetic data involving variation of tubulin concentration reveals evidence of some substrate inhibition by tubulin. Such non-linear behaviour can arise from the formation of so-called dead-end, or abortive, inhibitory complexes involving one or more substrates in multi-substrate enzyme reactions, and one must exercise care in choosing substrate concentrations that lie below the corresponding dissociation constant(s) for such abortive complexes (Rudolph, 1979). Another possible explanation for the occurrence of substrate inhibition in the case of tubulin:tyrosine ligase reaction is suggested by the very interesting proposal of Wehland & Weber (1987) that the ligase binds to a recognition site on the β -subunit, with a subsequent interaction of the ligase active site with the C-terminus of the α -subunit. Such a two-site interaction might be disturbed at high tubulin concentrations with the formation of inactive

enzyme·(tubulin)₂ complex rather than the catalytically productive enzyme·(tubulin)₁ complex. There are indications that the ligase is more efficient with tubulin as the substrate than with polymerized microtubule protein (W. C. Thompson, L. Wilson & D. L. Purich, unpublished work), and a non-linear tubulin concentration-dependence occurs even with colchicine or podophyllotoxin present at sufficient concentrations to totally block any assembly. Thus we do not believe that the non-linearity arises from tubulin polymerization, and we also know that our charcoal-treated tubulin cannot assemble, even in the presence of ATP.

There is no obvious allosteric regulation or co-operative substrate binding behaviour exhibited by the tubulin:tyrosine ligase-catalysed reaction, but the lag phase shown in Fig. 1(a) suggests some lag phase or hysteretic behaviour. Hysteretic enzymes are distinguished by slow, thermodynamically reversible transition(s) from an initial state of the enzyme to other state(s) having distinct physical and/or conformational properties manifested as different kinetic properties (Neet & Ainslie, 1980). Such slow transitions may occur on a time scale of minutes to hours, often allowing easy detection in kinetic studies. Alternatively, they may occur at slower rates than the other microscopic rate constants, and thus may be too slow to be seen in the time scale of typical kinetic assays. The observable lag phase can be significantly decreased by preincubating the enzyme with the protein and nucleotide substrates at room temperature. If this hysteretic behaviour occurs under physiological conditions, then this slow transition may be the result of a conformational change of the enzyme to a more active form. On the other hand, the apparent lag phase may be a result of the assay procedure itself, and may not be physiologically relevant. One should recall that, in the tubulin:tyrosine ligase purification protocol, the ligase is found to be associated with tubulin. This suggests that, under physiological conditions, this hysteretic phenomenon is probably physiologically insignificant. All available kinetic and binding evidence leads to the conclusion that the tubulin:tyrosine ligase operates by a random kinetic pathway:

where A, T and Y are ATP, tubulin and L-tyrosine respectively. At this point, however, we cannot evaluate the relative flux along each edge of the cube-like pathway that interconnects enzyme (E) with the various binary, ternary and quaternary complexes. In particular, methods such as isotopic trapping (Rose *et al.*, 1974) and equilibrium exchange (Purich & Allison, 1980) are frequently applied to analyse branched kinetic pathway mechanisms. In cases where a macromolecular substrate, such as tubulin, is involved, issues of protein solubility, possible polymerization and solution non-ideality are likely to frustrate the use of these valuable diagnostic kinetic approaches. Furthermore, the fact that this enzyme appears to operate by a random sequence says nothing about the chemistry of peptide synthesis by the ligase. A frequently drawn but mistaken impression is that the occurrence of covalent intermediates demands enzyme action by way of a ping-pong pathway. For example, the adenylosuccinate synthetase reaction may involve a 6-phosphoryl-IMP intermediate, but Rudolph & Fromm (1970) found that this enzyme exhibits rapid equilibrium random reaction kinetics. Likewise, in the case of glutamine synthetase, the kinetic reaction mechanism is random (Allison *et al.*, 1977), yet the participation of a glutamyl-

phosphate intermediate is widely accepted. The results presented here suggest that the three-substrate, three-product reaction catalysed by tubulin:tyrosine ligase also occurs via a random mechanism; however, any involvement of an analogous covalent activated α -glutamate carboxyl intermediate during catalysis requires further study.

We thank Dr. James Angelastro for helping with aspects of data reduction and plotting, and Dr. Herbert J. Fromm for providing the statistical software package used in our initial velocity analysis.

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Received 27 January 1992; accepted 6 March 1992