SENSITIZATION OF COLCHICINE BINDING PROTEIN TO ULTRAVIOLET LIGHT BY BOUND COLCHICINE

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1. Introduction

The microtubulues of sea urchin sperm tails have been shown to be composed of colchicine binding protein [1, 2]. Other materials which are rich in microtubules also tend to be rich in colchicine binding protein, e.g. mitotic apparatus and brain [3]. Weisenberg et al. [4] developed a procedure for purifying colchicine binding protein from mammalian brain. They found that 0.1 mM GTP + 10 mM $MgCl_2$ could slow but not stop the decay of the very labile binding activity of the purified protein. Once formed, however, the protein–colchicine complex is quite stable, which indicates that the presence of colchicine in the binding site protects it against denaturation [3, 5]. Colchicine would therefore appear to be the ideal stabilizer. This introduces another problem, though: the unlabeled colchicine used to stabilize the protein during handling must be removed from the binding site before binding activity can be assayed with radioactive colchicine. Irradiation of free colchicine with long wavelength ultraviolet (UV) light converts it to a mixture of isomers (lumicolchicines) (see [6] for literature). Lumicolchicines do not bind to colchicine binding protein [5, 7]. Furthermore, Aronson and Inoue [8] found that dissolution of the mitotic spindle and inhibition of cleavage of sea urchin eggs caused by colcemid (*N*-desacetyl-*N*-methylcolchicine) could be reversed by irradiation with long wavelength UV, presumably through photoconversion of colchicine to lumicolchicines followed by release of the lumicolchicines thereby leaving the protein free to reform microtubules.

It seemed probable that the binding site of colchicine binding protein could be protected from denaturation by unlabeled bound colchicine, then freed of the unlabeled colchicine by irradiation with long wavelength UV, and finally assayed with radioactive colchicine.

2. Experimental

Colchicine binding protein was partially purified from bovine brain by the procedure of Weisenberg et al. [4], omitting the fractionation on DEAE-Sephadex. The preparation was lyophilized and stored at $-20°$ without loss of colchicine binding activity. Solutions of colchicine binding protein were made by dissolving the lyophilized powder in enough water to restore the original salt concentration. Insoluble material was removed by centrifugation at 48,000 *g* for 20 min at 0°.

In the standard assay procedure for colchicine binding activity, approx. 170 μg protein, assayed by the method of Lowry et al. [9] using bovine serum albumin as the standard, were incubated in 0.1 ml of 10 μM [^{3}H]colchicine (specific activity, 6.8×10^4 cpm/nmole) in 10 mM sodium phosphate pH 6.5, 10 mM $MgCl_2$, 0.1 mM GTP (P-Mg-GTP), for 90 min at 37°. The reaction was stopped by addition of 10 μl 1 mM unlabeled colchicine and 5 ml P-Mg-GTP. The amount of bound colchicine was determined by the DEAE-cellulose filter assay [4]. For further studies, the protein–colchicine complex was separated from free colchicine, lumicolchicines, or GTP by gel filtrations on Sephadex G-100 columns.

UV irradiations were carried out with a Blak Ray UVL 22 lamp (Ultraviolet Products Inc.), the emission

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spectrum of which had a broad maximum between 350 and 390 nm. The tip of the vial containing the test solution was placed directly on the lamp, covered with aluminum foil which was cooled with ice, and turned intermittently. The temperature of the test solution was thus maintained close to 0° during the irradation.

Chemicals: Colchicine and GTP (Type II-S) were obtained from Sigma Chemical Corp. Colchicine[^{3}H-methoxy] (ring C) was a product of NEN, and DEAE-Sephadex A-50 and Sephadex G-100 were from Pharmacia Co.

3. Results

The colchicine binding activity decayed with first order kinetics, with a half-life of 4 hr at 4° in P-Mg-GTP (fig. 1). This half life is much shorter than that reported for the protein purified from porcine brain [4], but it was an advantage in this work because it accentuated the stabilizing effect of colchicine. The protein–colchicine complex has a half life of about 170 hr at 4° in P-Mg-GTP after separation from free colchicine, but only if GTP continues to be present (fig. 2). This indicates that GTP is essential not only for stabilization of the binding site prior to complex formation, as shown by Weisenberg et al. [4], but also for stabilization of the binding site after complex formation.

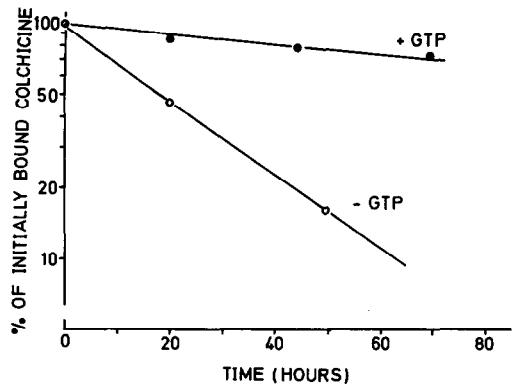

Fig. 2. Release of colchicine from protein–colchicine complex in presence and absence of GTP. Protein–colchicine complex (21,000 cpm) was subjected to gel filtration on Sephadex G-100, equilibrated with or without 0.1 mM GTP, to remove unbound colchicine and GTP. After the indicated times at 4° the amount of bound and unbound colchicine was determined after separation by gel filtration.

Irradiation of the complex with long wavelength UV results in the release of the radioactivity (presumably lumicolchicines) with first order kinetics (fig. 3). After 20 min of irradiation 91 to 93% of radioactivity was released (table 1, expt. 2). When available binding

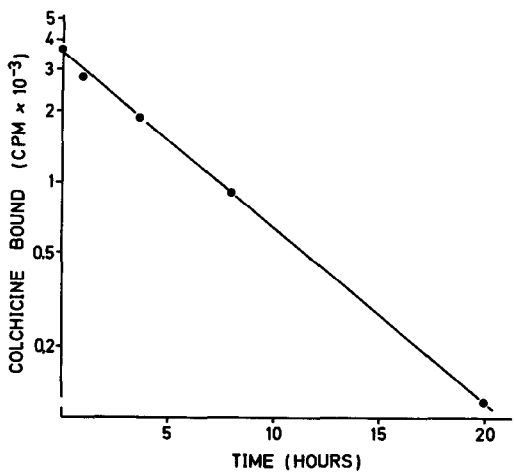

Fig. 1. Decay of colchicine binding activity during storage of protein at 4°. After the indicated times colchicine binding activity was determined at 37° by the filter assay.

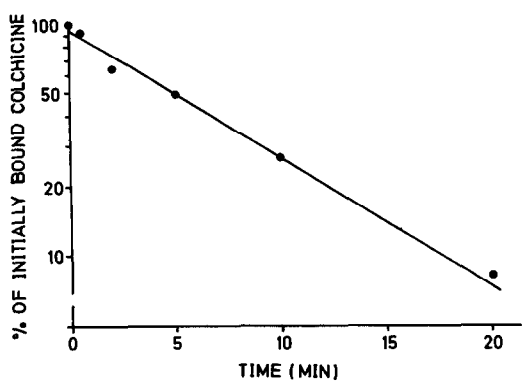

Fig. 3. Time course of colchicine release from protein–colchicine complex during irradiation with UV. After protein–colchicine complex (8250 cpm) had been formed under standard assay conditions, the reaction mixture was irradiated for the indicated times, and the amount of bound radioactivity was determined by the filter assay.

Table 1
Experiments on the mode of action of UV on colchicine binding activity.

Expt.	Pretreatment				Posttreatment 1				Posttreatment 2				[^{3}H]Colchicine bound as
	Additions	Time (min)	Temp.	UV	Additions	Time (min)	Temp.	UV	Additions	Time (min)	Temp.	UV	% of control
1	CBP+[^{3}H]CLC	20	0°	no	–	90	37°	no	–	–	–	–	100%
2	CBP+[^{3}H]CLC	90	37°	no	–	20	0°	yes	–	–	–	–	8
3	CBP+CLC	90	37°	no	–	20	0°	yes	[^{3}H]CLC	90	37°	no	33*
4	[^{3}H]CLC	20	0°	yes	CBP	90	37°	no	–	–	–	–	1.5
5	CLC	20	0°	yes	CBP+[^{3}H]CLC	90	37°	no	–	–	–	–	100
6	CBP	20	0°	yes	[^{3}H]CLC	90	37°	no	–	–	–	–	100
7	CBP+[^{3}H]CLC	20	0°	no	–	–	–	–	–	–	–	–	2
8	CBP+[^{3}H]CLC	20	0°	yes	–	–	–	–	–	–	–	–	4
9	CBP+CLC	20	0°	yes	[^{3}H]CLC	90	37°	–	–	–	–	–	92

Standard conditions for incubations and irradiations were employed. Bound colchicine was determined by the filter assay.

Abbreviations: CBP = colchicine binding protein. CLC = colchicine. UV = ultraviolet light.

* During 90 min at 37° without colchicine, about 33% of the binding activity was lost due to thermal inactivation. Since colchicine is present during the rebinding incubation after irradiation, and the protein–colchicine complex is thermostable, thermal inactivation can only account for an amount appreciably less than 33% out of the 60–64% of binding sites which are lost during irradiation and rebinding.

sites were assayed after such irradiation of the protei colchicine complex only about 36% (33 out of 92; se table 1, expts. 2, 3) of the sites expected could be detected. A series of experiments were designed to eliminate some possible mechanisms of loss of colchicine binding activity (see table 1). The loss of binc ing activity was not due to binding of lumicolchicine: (table 1, expt. 4) nor to inhibition by lumicolchicine: of colchicine binding (table 1, expt. 5). Furthermore, the loss was not due to direct damage to the protein by UV (table 1, expt. 6) nor to photosensitization of the protein by unbound colchicine (table 1, expts. 7–9). The latter experiment was done by irradiating a mixture of the protein and colchicine at 0°, before binding could occur.

4. Discussion

Our experiments have shown that bound colchicir can be destroyed photochemically while in the bindi site of colchicine binding protein and the photoprod is released, but about 2 times out of 3, the binding si itself is also destroyed by the photochemical reactior at least under the conditions we have employed.

This finding is not in conflict with that of Aronso and Inoue [8] because they did their experiments wi eggs undergoing the first divisions, and the sea urchin egg must have a pool of microtubule proteins sufficie for the formation of many mitotic spindles. Even if only 1/3 of the proteins survived the irradiation and no synthesis of microtubule protein were occurring, some spindle reformation would still be possible. Furthermore, destruction of the colchicine binding site need not affect the ability of the protein to assemble into microtubules.

Colchicine can inhibit an aggregation reaction of purified colchicine binding protein, and the aggregati reaction resumes following irradiation with long wav length UV [10, 11]. Borisy [10] stated that "there is no detectable effect on tubulin" from irradiation of the protein–colchicine complex, and after irradiatio "the protein is competent to rebind colchicine". Fro our results, we would infer that this is true for only about 1/3 of the colchicine binding protein after irra diation of the complex.

Hammond and Bryan [12] recently reported that irradiation of bound cyanoethyl colchicine results in

affinity labeling of a polypeptide. Bryan has also measured the recovery of colchicine binding activity after irradiation of the protein–colchicine complex and has obtained results in agreement with ours (J. Bryan, personal communication).

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