CYTOCHALASIN B REACTS WITH THIOLS

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1. Introduction

Cytochalasin B (fig.1) is a compound that is widely used in eukaryotic cell biology. Among other effects, it arrests membrane ruffling and cell locomotion, inhibits pinocytosis and cytokinesis, causes nuclear extrusion, and alters the rates of release of packaged secretions [1–3]. Some or all of these effects may result from the interaction of cytochalasin B with microfilaments, since the compound has been shown to cause limited fragmentation of actin filaments [4]. Cytochalasin B is also a potent inhibitor of the transport of monosaccharides, nucleosides, and nucleic acid bases into cells [5]. The inhibition of glucose transport in the human erythrocyte has been shown to be due to binding of the compound to specific sites on the cellular membrane [6,7].

In this paper we report that the α,β-unsaturated ester function of cytochalasin B (fig.1) reacts rather readily with thiols (Eqn.1)

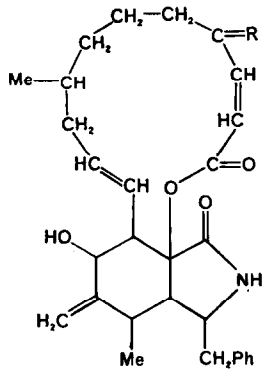
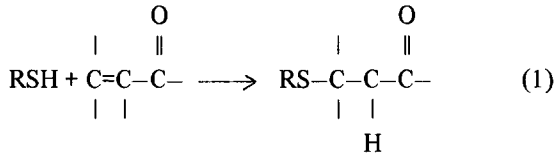


Fig.1. The structures of cytochalasins. B (R=H, OH) and A (R=O) [9].



(1)

We have examined the role of this reaction in the binding of cytochalasin B to erythrocyte membranes.

2. Materials and methods

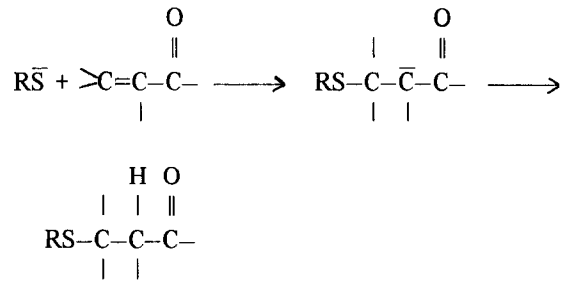
Cytochalasin B was purchased from Aldrich Chemical Company. Tritium-labeled cytochalasin B, which had been synthesized according to the procedure of Lin et al. [8], was purchased from New England Nuclear. Over 99% of the label in this preparation migrated in the same way as authentic cytochalasin B upon thin-layer chromatography (tlc). The concentration of [^{3}H]cytochalasin B, which had been purified by tlc, was determined spectrophotometrically [8]; this value in turn allowed calculation of the specific activity, which was 11 Ci/mole. Dihydrocytochalasin B, the derivative in which the double bond conjugated with the ester has been reduced [9], was the gift of Dr D. C. Aldridge. Tlc of cytochalasin B and its derivatives was performed on silica gel plates (E M Reagents 5538/0001) with ethyl acetate–chloroform (1:1 by volume); the compounds were located by staining with iodine vapor. Human erythrocyte membranes in 5 mM sodium phosphate buffer, pH 8, were prepared from blood bank cells that had been drawn 21 days earlier [10].

3. Results

3.1. *The reaction of cytochalasin B with propanethiol*

T.l.c. revealed that cytochalasin B (R_f, 0.33) rapidly formed a compound of higher mobility (R_f, 0.42) upon treatment with a mixture of 1-propanethiol and its sodium salt in ethanol at 25°C. Several milligrams of this compound were prepared through the reaction of 0.15 ml 55 mM cytochalasin B in ethanol with 0.05 ml 226 mM propanethiol–18 mM sodium propanethiolate in ethanol for 30 min, followed by t.l.c. of the reaction mixture and elution of the product with ethanol. High resolution mass spectrometry of the product, kindly carried out by Mr H. E. Ensley in the Department of Chemistry, Harvard University, gave a mass of 555.3026 for the parent peak. The calculated value for a one-to-one adduct of propanethiol with cytochalasin B is 555.3018. Since the α,β-unsaturated ester function in other molecules is known to undergo an addition reaction with thiols of the type shown in Eqn. 1 [11,12] and since there are no other groups in cytochalasin B that are likely to react readily with thiols, the structure of the adduct is almost certainly that given in Eqn. 1. This assignment is supported by a comparison of the ultraviolet spectra of cytochalasin B, dihydrocytochalasin B, and the adduct. The spectrum of cytochalasin B shows strong end absorption [8]; the value of the ratio of the absorbance at 230 nm to that at 258 nm, where the isolated phenyl group in the molecule absorbs maximally, is 17.8 with ethanol as the solvent. This end absorption must be due to the α,β-unsaturated ester, since the value for this ratio for dihydrocytochalasin B is only 2.8. The spectrum of the adduct in ethanol, after correction for the ultraviolet absorbance of material that eluted from the silica gel alone, gave a value of 4.5 for the 230/258 absorbance ratio.

The kinetics of reaction of cytochalasin B with excess propanethiol were determined (table 1). Within experimental error, the observed first order rate constants are directly proportional to the concentration of the thiol anion (last column, table 1). This result is consistent with a mechanism in which this anion attacks the double bond to form an enolate anion, which is then protonated:



Under the conditions described in table 1 the reaction must proceed to near completion since the total

Table 1
Rate constants for the reaction of propanethiol with cytochalasin B, at 25.0°C

Propanethiol–sodium propanethiolate, (mM)	k, (min^{-1})	k/[sodium propanethiolate], ($M^{-1}\,min^{-1}$)
3.0–2.0	0.033	16.5
8.0–2.0	0.042	21.0
6.0–4.0	0.071	17.8

The reaction was followed by the decrease in absorbance at 230 nm, in stoppered cuvettes of 1.0 mm path length. The initial concentration of cytochalasin B was 1.0 mM; the solvent was ethanol–water (1/1, by vol). The product was identified as the adduct by tlc of the reaction mixtures after acidification with acetic acid. A relatively small and slow decrease in absorbance, which is probably due to oxidation of the thiol, occurs in the absence of cytochalasin B. After the absorbance changes were corrected for this background, the plots of ln (absorbance at time t minus the absorbance at the end of the reaction) versus t were linear and gave the first-order rate constants.

decrease in absorbance was the same for all the reaction mixtures, even though the concentration of the thiol varied by a factor of two.

In order to determine the rate of reversal of adduct formation, we incubated 0.1 mM adduct with 0.2 mM of the colorimetric thiol reagent 5,5'-dithio-*bis*(2-nitrobenzoic acid) in 5 mM potassium phosphate buffer, pH 7.0, – 20% ethanol by volume at 23°C [13,14]. Comparison of the absorbance change at 412 nm with those in a control lacking the adduct and in a control containing 0.1 mM propanethiol and cytochalasin B in place of the adduct showed that there was less than 5% reversal in three days.

3.2. *Binding of cytochalasin B to the erythrocyte membrane*

The human erythrocyte membrane contains approx. 300 000 sites that bind cytochalasin B with high affinity [6]. The fact that this binding is prevented by the thiol reagent p-mercuribenzoate [7] suggests that an addition reaction of the type described above may be involved. The association of cytochalasin B with the erythrocyte membrane and the dissociation of bound cytochalasin B occur within minutes [6]. Consequently, if binding does involve the formation of an adduct with a protein cysteinyl residue, the rates in both directions must be accelerated relative to those of the reaction with propanethiol, presumably because of the unique conformation of the binding site. This reasoning led us to determine whether treatment of the cytochalasin B-membrane complex with protein-denaturing reagents resulted in stably bound cytochalasin B.

Table 2 summarizes the results with five different denaturing agents, several of which completely solubilize the membrane [15]. Other experiments, in which the ghosts were separated by centrifugation [6], showed that under the conditions described in table 2, in the absence of denaturing agents, 83% or more of the cytochalasin B (about 60 000 molecules per cell membrane) is bound reversibly. None of the denaturating treatments resulted in an amount of stably linked cytochalasin B that is greater than that in the corresponding control experiment. Moreover, except in the case of heat treatment, the amount stably bound is only 0.1–0.2% of the amount reversibly bound. It is likely that heating accelerates the nonspecific reaction of cytochalasin B with nucleophilic groups of the membrane.

4. Discussion

The fact that radioactively labeled cytochalasin B

Table 2
Extent of irreversible binding of [^{3}H]cytochalasin B to the erythrocyte membrane

Treatment	Molecules bound per ghost. cytochalasin B added. After treatment	Before treatment
(1) 100 mg/ml sodium dodecyl sulfate	140	140
(2) Heated at 100°C for 5 min	700	680
(3) 1.0 N perchloric acid for 5 min, then neutralized with K_2HPO_4	160	160
(4) 7 M urea–0.1 M acetic acid	41	50
(5) 5 M guanidine thiocyanate	36	41

About 2 × 10^9 ghosts were incubated with 190 pmol of [^{3}H]cytochalasin B in a total volume of 0.2 to 1.0 ml for two min at 22°C and then treated as described above. In a parallel set of reaction mixtures, the cytochalasin B was added immediately after the treatment (in the case of no. 2, the mixture was heated for 5 more min after this addition). The reaction mixtures were dialyzed three times against 80 vol of 1 mM potassium phosphate, pH 7.0,–20% ethanol (no. 1, 2, 3), 8 M urea–0.1 M acetic acid (no. 4), or 6 M guanidine HCl, pH 5.3 (no. 5), for 24 h periods each time. The molecules bound per ghost were calculated from the non-dialyzable radioactivity.

has recently become available [8] is likely to stimulate further investigation of its mode of action in a variety of systems. Investigators should be aware that the reaction of this compound with the thiol anion is sufficiently facile so that covalent attachment to proteins may be encountered. The halftime for reaction with 4 mM propanethiol anion is about 10 min at 25°C.

Our results with the erythrocyte membranes suggest that the thiol addition reaction is not involved in the binding of cytochalasin B to the high affinity sites. However, we cannot exclude the possibility that denaturation of the cytochalasin B–receptor complex proceeds via dissociation of a covalent adduct with cytochalasin B, followed by denaturation of the receptor. Whether the reaction has any role in the many other effects of cytochalasin B is not known.

Kuo and Lampen have recently reported that inhibition of the growth of yeast and of the uptake of sugar into yeast by cytochalasin A is completely prevented by 1 mM cysteine or dithiothreitol [16]. The reason for this protection by thiols is very likely to be that the thiol adds to the conjugated double bond of cytochalasin A (fig.1) to yield a derivative that is inactive. Because the double bond of cytochalasin A is conjugated with both the ester function and a ketone function (fig.1), it should be even more reactive than that of cytochalasin B, and also thiol addition can occur at either carbon atom of the double bond.

Acknowledgement

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