INHIBITION OF PROTEIN SYNTHESIS BY CIBACRON BLUE F3GA IN EUKARYOTIC CELL-FREE EXTRACTS

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1. Introduction

NAD^+ (0.05–0.5 mM) added to rabbit reticulocyte lysates effectively replaces the creatine phosphate/creatine phosphokinase energy regenerating system and stimulates the initiation step of the protein synthetic process [1–4]. The effect of NAD^+ is correlated with the maintenance of a high adenylate energy charge and is most likely attributed to the activation of glycolysis by NAD^+ [1,2]. At NAD^+ >0.5 mM, protein synthesis is inhibited [4]. This inhibition has been suggested to result from an interaction between NAD^+ and some protein factor(s) essential for peptide initiation [3,4]. Reports have shown that blue dextran covalently linked to Sepharose has a specific affinity for proteins which interact with NAD^+ and other nucleotides. The affinity is apparently related to the structural similarity between the blue chromophore (cibacron blue F3GA) and NAD^+ [5–8]. Accordingly, we have tested the possibility of using cibacron blue F3GA to inhibit protein synthesis. We find that this dye is a potent inhibitor of protein synthesis in several mammalian cell extracts and in an in vitro translating system derived from wheat germ.

2. Materials and methods

Rabbit reticulocyte lysates and protein synthesis assays were with creatine phosphate/creatine phosphokinase as the energy regenerating system as in [1–4]. [^{3}H]Leucine was added to 40 μM final conc. (spec. act. 1.8 Ci/mmol). The hemin was 15 μM. Aliquots (10 μl) were removed at indicated times and processed for incorporation of leucine into polypeptides [2]. The initial dipeptide, methionylvaline, from rabbit reticulocyte lysates was isolated as in [9] by incubating the protein synthesizing system with [^{35}S]Met-$tRNA_f^{Met}$ (108 000 cpm/pmol, 26 pmol) at 30°C, 5 min (total vol. 0.32 ml). Ternary complex was assayed by Millipore filtration as in [4] except $MgCl_2$ (1 mM) was included in the incubation mixture. Initiation factor eIF-2 was partially purified by phosphocellulose column chromatography [10].

Mouse reticulocyte lysates were prepared as in [11]. Conditions for protein synthesis assays were identical to those of rabbit reticulocyte lysates except that the optimal hemin was 50 μM and the leucine was 10 μM.

Preparations of extracts and protein synthesis assays in HeLa cell lysates were as in [12].

Commercial wheat germ was obtained from General Mills and cell-free extracts prepared and assayed as in [13]. Wheat tRNA was omitted and globin mRNA (Miles Biochem.) was added to a 5 μg/ml assay.

Cibacron blue F3GA was purchased from Polysciences and purified as in [8]. The concentration of the dye was determined spectrophotometrically at 610 nm (ϵ = 13.6 $mM^{-1}\,cm^{-1}$) [6].

3. Results

In the absence of any added inhibitor, protein synthesis in rabbit reticulocyte lysates proceeds at a linear rate for 60 min (fig.1, left panel, ●). Addition of cibacron blue F3GA results in a marked, concentration-dependent, inhibition of polypeptide synthesis (fig.1, right panel). At 84 μM cibacron blue, there is a delay of 5–20 min (dependent on lysate) before the onset of inhibition is observed (fig.1, left panel, ○).

To test whether this dye can function as a general

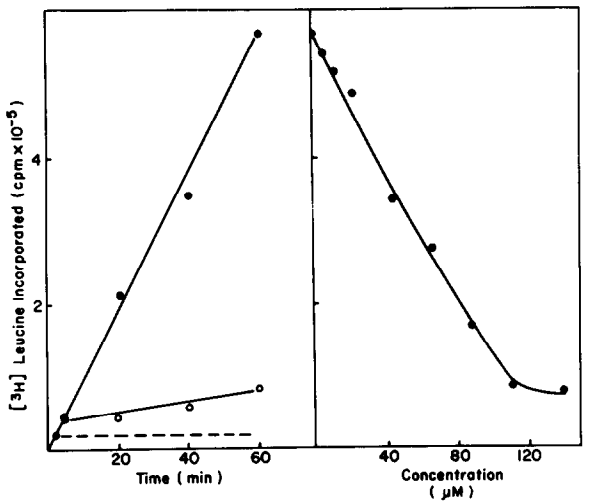

Fig.1. Effect of cibacron blue F3GA on polypeptide synthesis in rabbit reticulocyte lysates. Left panel, time course of polypeptide synthesis. The incorporation of [^{3}H]leucine into polypeptide was measured by removing 10 μl aliquots at the indicated times. (●–●) control; (○–○) control plus 84 μM cibacron blue; (– – –) polypeptide synthesis in the presence of pactamycin (2 μM). Right panel, effect of varying concentrations of cibacron blue. Inhibition was measured after a 60 min incubation, 30°C.

Table 1
Effect of cibacron blue F3GA on protein synthesis in eukaryotic cell-free systems

Translating system	Cibacron blue (μM)	Polypeptide synthesis (cpm)	Inhib. (%)
HeLa cells[a]	0.0	18 620	–
	3.6	18 110	3
	9.0	14 430	23
	13.4	12 540	33
	26.8	8330	55
Mouse reticulocyte lysate	0.0	428 620	–
	44.0	302 110	30
	66.0	236 810	45
	88.0	183 370	57
	110.0	137 670	68
Wheat germ lysate	0.0	265 080	–
	1.8	226 930	14
	3.6	186 660	30
	6.3	156 250	41
	13.4	90 350	66

[a] Conditions for incubations were as follows: HeLa cell extracts, 30 min, 30°C; mouse reticulocyte lysates, 60 min, 30°C; wheat germ extracts, 60 min, 25°C. In the wheat germ lysate, an incorporation of 6820 cpm, for assays done without added globin mRNA, has been subtracted

translational inhibitor, its effect on protein synthesis was examined in cell extracts derived from HeLa cells, mouse reticulocytes, and wheat germ (table 1). The results show that protein synthesis is effectively inhibited in all 3 systems.

In order to gain more information on the nature of translational inhibition by this dye, the effect of cibacron blue was studied in detail using the rabbit reticulocyte lysates. Results in table 2 show the effect of cibacron blue on the formation of the initial dipeptide, methionylvaline, as measured by the addition of valine to [^{35}S]Met-tRNA$_f^{met}$. At 21 μM and 63 μM of the dye, the initial dipeptide formation is inhibited by 16% and 43%, respectively. However, at these concentrations, the rate of completion of nascent chains (as indicated by leucine incorporation assayed in the presence of the specific initiation inhibitor, pactamycin, 2 μM) was not inhibited (data not shown), suggesting that, under these conditions, inhibition of initiation appears to be the primary effect of the dye.

Table 2
Effect of cibacron blue on the formation of the initial dipeptide, methionylvaline, in rabbit reticulocyte lysates

Addition	Total radioactivity/strip (cpm)	[^{35}S]Met–Val (cpm)	Met–Val (%)	Inhib. (%)
Control	67 330	4230	6.3	–
Plus 21.0 μM cibacron blue	66 580	3520	5.3	16
Plus 63.0 μM cibacron blue	42 540	1540	3.6	43

Incubation conditions and isolation of methionylvaline were as in section 2

Table 3
Effect of cibacron blue on ternary complex formation by eIF-2 from rabbit reticulocyte lysates

Addition	[^{35}S]Met-tRNA$_f^{met}$ bound (pmol)	Inhib. (%)
Control	0.26	–
Plus 27.0 μM cibacron blue	0.16	38
Plus 54.0 μM cibacron blue	0.06	77

GTP-dependent binding of [^{35}S]Met-tRNA$_f^{Met}$ (1.2 pmol, 28 560 cpm, 90% 5% cold trichloroacetic acid-precipitable) was assayed in a 50 μl reaction mixture containing 1.4 μg eIF-2 (2 μl) and 1 mM $MgCl_2$ as in section 2. Background without eIF-2 (0.005–0.01 pmol) was not subtracted

Because it is generally believed that a key step in the regulation of eukaryotic peptide chain initiation is the formation of a ternary complex between GTP, the initiator tRNA, and initiation factor eIF-2 [14–17], the effect of cibacron blue on ternary complex formation was also studied using a partially purified eIF-2 from reticulocytes. Addition of 27 μM and 54 μM of the dye resulted in a 38% and 77% inhibition of ternary complex formation (table 3), suggesting that translational inhibition may be related to the interaction between the dye and eIF-2, such that eIF-2 becomes inactive in protein synthesis. In support of this possibility, the inhibition of translation by cibacron blue in rabbit reticulocyte lysates is largely reversed by addition of partially purified eIF-2 (table 4).

Table 4
Addition of partially purified eIF-2 on inhibition of protein synthesis by cibacron blue in rabbit reticulocyte lysates

Addition	Polypeptide synthesis (cpm)		
	Minus eIF-2	Plus eIF-2 (μg/30 μl assay)	
		1.4 μg	4.2 μg
Control	62 760	61 600	65 640
Plus 10.0 μM cibacron blue	51 600	60 120	–
Plus 30.0 μM cibacron blue	33 920	48 000	58 880

Incubations were at 30°C, 60 min. Incorporation of [^{3}H]-leucine was measured by removing 10 μl aliquots

4. Discussion

Two sets of observations become evident from the present series of experiments.

1. Cibacron blue F3GA inhibits polypeptide synthesis in several commonly used eukaroytic in vitro translating systems (fig.1, table 1). The extent of inhibition, however, varies with the systems studied (table 1).
2. In rabbit reticulocyte lysates, the inhibitory effect of the dye (at the concentrations studied) appears to be primarily restricted to the initiation step of the protein synthetic process. This tentative conclusion is based on the following observations:

(i) There is a lag period of several minutes before the onset of inhibition (fig.1);
(ii) The formation of the initial dipeptide, methionyl-valine, is inhibited by the dye (table 2);
(iii) The formation of ternary complex between GTP, eIF-2, and initiator tRNA, is inhibited by the dye (table 3).

The ability of cibacron blue F3GA to inhibit ternary complex formation implies that there is selective interaction of eIF-2 with the dye. This, in turn, raises the possibility that blue dextran covalently linked to Sepharose may be useful as an affinity column for the specific purification of eIF-2. These studies are currently under investigation in this laboratory.

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