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QUARTERLY

Halogenated benzimidazole inhibitors of phosphorylation, *in vitro* and *in vivo*, of the surface acidic proteins of the yeast ribosomal 60S subunit by endogenous protein kinases CK-II and PK60S*

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Several halogeno benzimidazoles and 2-azabenzimidazoles, previously shown to be relatively selective inhibitors of protein kinases CK-I and/or CK-II from various sources, including CK-II from yeast [Szyszka *et al.* (1995) *Biochem. Biophys. Res. Commun.* 208, 418–424] inhibit also the yeast ribosomal protein kinase PK60S. The most effective inhibitor of CK-II and PK60S was tetrabromo-2-azabenzimidazole (TetraBr-2-azaBz), which was competitive with respect to ATP (and GTP in the case of CK-II) with K_i values of 0.7 μM for CK-II, and 0.1 μM for PK60S.

PK60S phosphorylates only three (YP1β, YP1β′, YP2α) out of five polypeptides of pp13 kDa acidic proteins of 60S subunit phosphorylated by CK-II [Szyszka *et al.* (1995) *Acta Biochim. Polon.* 42, 357–362]. Accordingly, TetraBr-2-azaBz inhibits phosphorylation only of these polypeptides, catalysed by PK60S. Addition of TetraBr-2-azaBz to cultures of yeast cells, at concentrations which were without effect on cell growth, led to inhibition of intracellular phosphorylation of ribosomal acidic proteins, paralleling that observed *in vitro*. TetraBr-2-azaBz is shown to be a useful tool for studies on the intracellular regulation of phosphorylation of the ribosomal 60S acidic proteins, which are involved in formation of active ribosomes.

Bacterial ribosomes contain two acidic proteins, L7 and L12, associated with the large 60S subunit, which play a key role in interaction of ribosomes with translation factors during protein synthesis [1]. In eukaryotes the number of such ribosomal acidic proteins varies from two in mammals to eight in *Trypanosoma cruzi* [2, 3].

The ribosomes of the yeast *Saccharomyces cerevisiae* contain four acidic proteins with molecular masses of about 13 kDa [4, 5], coded by four genes [4, 6, 7], and now denoted YP1α, YP1β′, YP2α, YP2β [8]. Protein YP1β is also found in a form truncated by 8 amino acids at the N-terminus, denoted YP1β′, so that 5 such acidic

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Abbreviations: CK-II, protein kinase CK-II (hitherto known as casein kinase II, EC 2.7.1.37); PK60S, protein kinase 60S ribosomal subunits. For abbreviations of inhibitors, see Table 1 in the text.

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proteins of yeast ribosomes are revealed by isoelectric focusing [9]. They are located on the surface of the 60S subunit, and are referred to as "split proteins" because of their facile release in ethanolic NH_4Cl [10]. A marked proportion of these proteins is found in the cytoplasm, depending on the growth phase of the cells. Ribosomes from cells in the exponential growth phase contain twice as much acidic proteins as ribosomes from cells in the stationary growth phase [11], pointing to the involvement of these proteins in translation. The acidic proteins in the cytoplasm are non-phosphorylated [12, 13], whereas the phosphorylated forms are involved in assembly of active ribosomes [5, 14–16].

Yeast ribosomal acidic proteins are known to be substrates *in vitro* for the multifunctional protein kinase CK-II and the specific ribosomal protein kinase PK60S [18]. CK-II phosphorylates serine residues in all five polypeptides YP1α, YP1β, YP1β', YP2α and YP2β, whereas PK60S phosphorylates only YP1β, YP1β' and YP2α [19]. It is not known whether kinases analogous to yeast PK60S exist in other organisms. In contrast to rat liver CK-II, yeast PK60S exhibits only minimal phosphorylation, if at all, of the acidic proteins of rat liver ribosomes [18]. Attempts to detect a PK60S-like activity in rat liver extracts, and in the amoeba *Acantomoeba castellanii*, by biochemical and immunological procedures did not give positive results (unpublished). The yeast 60S ribosomal subunit contains one additional acidic protein, a 38 kDa unit, denoted AO or PO, which is a member of the ribosomal core proteins [20, 21], and is also an *in vitro* substrate for both CK-II and PK60S [18].

Relatively little is known about the mechanism by which phosphorylation of the acidic proteins of eukaryotic ribosomes is regulated, and effectors of the activities of protein kinases have been sought for with a view to resolving this problem. Halogenated benzimidazoles and 2-azabenzimidazoles [22] are reasonably specific inhibitors of CK-I and CK-II from mammalian and plant sources [23, 24], but in the yeast they are effective only with CK-II. We have found now that these benzimidazole analogues inhibit also the yeast PK60S enzyme. This observation has now been also applied to *in vivo* studies on intracellular regulation of phosphorylation of yeast ribosomal acidic proteins from the 60S subunit.

MATERIALS AND METHODS

Enzymes. Purified yeast protein kinases CK-II [25] and PK60S [18] were obtained as previously described. One unit of kinase activity is defined as the amount of enzyme required for incorporation of 1 pmol phosphate from [γ-^{32}P]ATP into substrate/min.

Phosphorylation of ribosomes in vitro. Highly purified yeast 80S ribosomes, devoid of endogenous kinase activity, were phosphorylated under standard conditions with CK-II [25] or PK60S [18], with [γ-^{32}P]ATP (Amersham, 500–2000 c.p.m./pmol) as a phosphate donor.

Intracellular phosphorylation of ribosomes. An 80-ml culture of yeast cells in the low-phosphate medium of Retel Planta [26] was divided into two portions, and to one of them TetraBr-2-azaBz was added to a concentration of 5 μM. Following one hour incubation at 30°C, 500 μCi of [^{32}P]H_3PO_4 was added to each culture, and incubation continued for 3–4 h to attain the logarithmic growth phase ($A_{600\ nm} \cong 1.2$). The cells were collected by centrifugation, washed with physiological fluid, and ribosomes isolated and purified as described earlier [18].

Electrophoretical techniques. Electrofocusing of ribosomal acidic proteins ("split proteins") was performed on 5% polyacrylamide gel slabs (210 × 100 × 0.8 mm) containing 2% Pharmacia ampholytes, pH range 2.5–5.0, as described elsewhere [19]. SDS/PAGE of ribosomal proteins followed the procedure of Laemmli [27].

Kinase inhibitors. Syntheses of the inhibitors employed in this study have been described elsewhere [22–24], and are listed, along with their abbreviations, as a footnote to Table 1.

Other procedures. Protein was measured by the Bradford procedure [28] using bovine serum albumin as a standard.

RESULTS

In vitro inhibition of phosphorylation

Several synthetic peptides containing target residues surrounded with clusters of glutamic and aspartic acids have been found to be good

Table 1

K_i values for inhibition of yeast CK-II and PK60S by several selected halogeno benzimidazoles and 2-azabenzimidazoles (benzotriazoles).

Each incubation medium included 5 units of protein kinase, 400 μg of 80S ribosomes, inhibitor in the range 0.2–500 μM, and ATP at concentrations of the K_m values for CK-II (7.5 μM) and PK60S (13.2 μM).

Inhibitor[a]	K_i (μM)	
	CK-II	PK60S
DRB	35	>200
DiBr-DRB	19	>200
α-AraDRB	93	>200
5(6)-Br-AraBz	116	53
TetraCl-2-azaBz	4.0	23
TetraBr-2-azaBz	0.7	0.1

[a]DRB, 5,6-Dichloro-1-(β-D-ribofuranosyl)benzimidazole; DiBr-DRB, 5,6-Dibromo-1-(β-D-ribofuranosyl)benzimidazole; αAraDRB, 5,6-Dichloro-1-(α-D-arabinofuranosyl)-benzimidazole; 5(6)-Br-AraBz, 5(6)-Bromo-1-(β-D-arabinofuranosyl)benzimidazole;TetraCl-2-azaBz, 4,5,6,7--Tetrachloro-2-azabenzimidazole;TetraBr-2-azaBz, 4,5,6,7--Tetrabromo-2-azabenzimidazole.

substrates of yeast CK-II [29]. The primary structures of these peptides resemble those of acidic regions at the C-termini of polypeptide chains of ribosomal substrates [9]. It is, however, not known whether both kinases share, to some extent, similar target amino acid(s) in the ribosomal substrate.

Hence, following the demonstration that a number of halogenated benzimidazoles and 2-azabenzimidazoles (benzotriazoles) are effective inhibitors of yeast CK-II, but not CK-I [22], it appeared logical to examine the effects of these compounds on yeast PK60S. Using highly purified yeast ribosomes as a substrate for purified yeast CK-II and PK60S, the effects of six previously characterized inhibitors of CK-II from various sources [23, 24], including yeast [22], were compared. The results are listed in Table 1, from which it is clear that several of the compounds are effective inhibitors of PK60S, and that the most effective inhibitor of both enzymes is TetraBr-2-azaBz, previously shown to be the most effective inhibitor of CK-II from yeast and other sources with casein as a protein substrate [22]. Furthermore, it will be noted that this analogue is 7 times more effective *vs* PK60S (K_i = 0.1 μM) than *vs.* CK-II (K_i = 0.7 μM) using ribosomes as an endogenous substrate for either enzyme.

As previously shown with CK-II from both yeast and mammalian sources [22], all the compounds tested were competitive inhibitors with respect to ATP for both CK-II and PK60S, as illustrated by means of Dixon plots with TetraBr-2azaBz (Fig. 1). It was also earlier shown that the same inhibitors are competitive with respect to GTP for CK-II [22].

The foregoing is further illustrated (Fig. 2A) by the decrease in phosphorylation of the ribosome substrate with increasing concentrations of TetraBr-2-azaBz (0.2–2.0 μM), and the subsequent reversal of inhibition (Fig. 2B) by addition of ATP at concentrations exceeding the K_m values for CK-II (7.5 μM) and PK60S (13.3 μM). Using the latter enzyme, Fig. 2C depicts the same results in the form of an autoradiograph of the electrophoretic pattern on SDS/PAGE of both the low-molecular mass surface proteins pp13 kDa (which are not resolved under these conditions) and the 60S pp38 kDa core protein.

The biological role of the family of "split proteins" (pp13 kDa) is much better understood than the function of the single core protein pp38 kDa [2]. This directed our subsequent attention to the low-molecular mass proteins which are differentially phosphorylated by CK-II and PK60S [19]. Therefore we have exam-

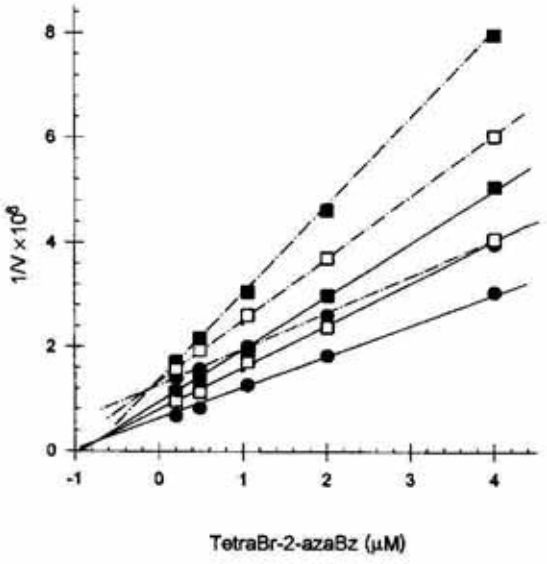

Fig. 1. Dixon plots for inhibition of CK-II (—) and PK60S (— · —) by TetraBr-2-azaBz, with ATP concentrations of 8 μM (■), 12 μM (❑) and 16 μM (●).

ined whether TetraBr-2-azaBz inhibits phosphorylation of all five of the pp13 kDa polypeptides which are substrates of CK-II, and all three of them which are substrates of PK60S. The low-molecular mass "split proteins" were extracted from the ribosomes phosphorylated by either kinase and resolved by isoelectric focusing (Fig. 3, insert), followed by counting of the ^{32}P incorporated by each protein. It will be seen from Fig. 3 that inhibition of phosphorylation involved all five substrates of CK-II (Fig. 3, upper panel) and all three substrates of PK60S (Fig. 3, lower panel).

Inracellular inhibition of phosphorylation

Attention was then directed to the feasibility of employing TetraBr-2-azaBz as an inhibitor of intracellular phosphorylation. The transport of halogeno benzimidazoles across cell membranes has not been hitherto investigated. But it was earlier shown that exposure of the giant salivary gland cells of *Chironomas tentans* to 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole and several of its analogues resulted in inhibition of mRNA transcription [30]. It was subsequently further demonstrated that DiBr-DRB also effectively inhibited mRNA transcription in intact HeLa cells [31].

It therefore appears reasonable to assume that TetraBr-2-azaBz can traverse the yeast cell membrane. Selection of the inhibitor concentration for the experiments *in vivo* was based on the ratio of K_m for ATP and K_i for TetraBr-2-azaBz. For PK60S this ratio (13.3 μM:0.1 μM) is 133. Since the intracellular ATP concentration in yeast cells is about 1 mM [32], or even lower [33], the inhibitor concentration of 5 μM was employed. Two cultures of yeast cells were prepared in liquid medium containing [^{32}P]orthophosphate. To one of these TetraBr-2-azaBz was added to a final concentration of 5 μM. Following attainment of the log growth phase (about 5 h at 30°C), the ribosomes from each culture were isolated, treated with pancreatic RNase, subjected to isoelectric focusing, and the individual low-molecular mass acidic proteins identified (Fig. 4). The individual proteins were excised from the gel, and levels of phosphorylation determined by scintillation counting (Table 2).

From panels A and B of Fig. 4 it will be noted that the level of phosphorylation of all five

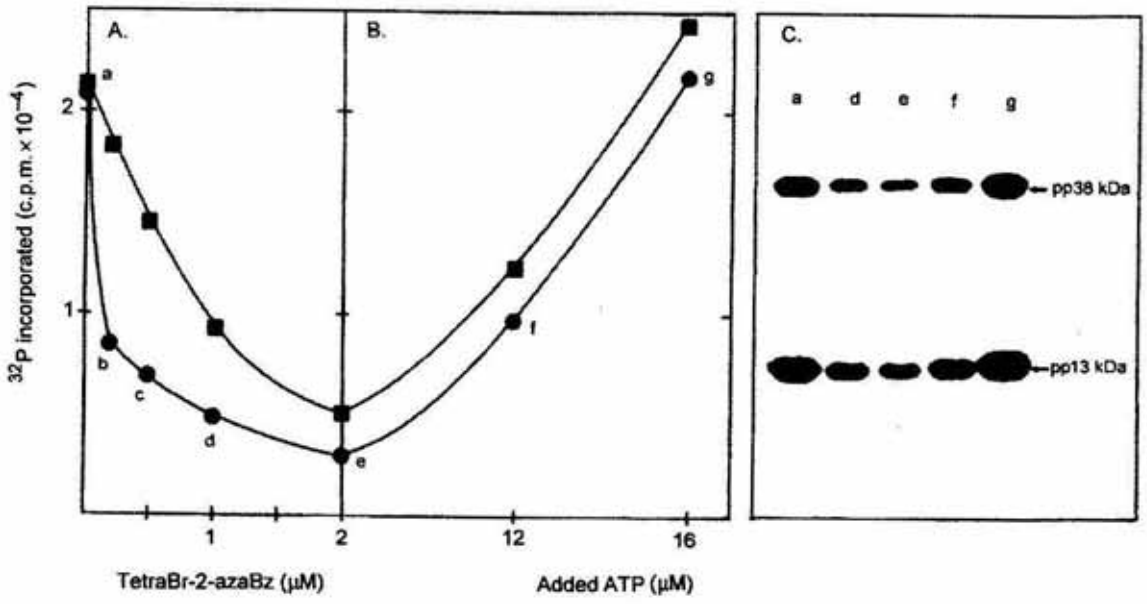

Fig. 2. Effect of TetraBr-2-azaBz on phosphorylation of ribosomal acidic proteins by CK-II (■) and PK60S (●).

A, Inhibition of each enzyme by increasing concentrations of TetraBr-2-azaBz (b, c, d, e). The assays for CK-II and PK60S activity were carrried out at 8 μM and 16 μM [γ-^{32}P]ATP, respectively; B, Reversal of inhibition in the presence of 2 μM inhibitor incubated for an additional 15 min with ATP at increasing concentrations; C, Autoradiograph of the gel electrophoresis (12% SDS/PAGE) pattern of 60S ribosomal acidic proteins phosphorylated by PK60S in the presence of TetraBr-2-azaBz, and reversal of the inhibition by ATP. Each lane corresponds to 400 μg of 80S ribosomes labelled by [γ-^{32}P]ATP: (a) in the absence, and (d, e) in the presence of inhibitor, and (f, g) following addition of increasing concentrations of ATP. The letters a, d, e, f, g correspond to those in panels A and B.

Table 2

Intracellular incorporation of $^{32}P_i$ by the individual pp13 kDa acidic proteins of the yeast ribosomal 60S subunit in the presence and absence of the inhibitor TetraBr-2-azaBz.

The separated and identified phosphoproteins (see Fig. 4) were cut out from the gel and ^{32}P incorporated counted. Each value is the mean of three independent experiments, corrected for background.

Protein	^{32}P incorporated (c.p.m.)		Inhibition of phosphorylation (%)
	–Inhibitor (control)	+Inhibitor (5 μM TetraBr-2-azaBz)	
YP1α	1596	827	48.2
YP1β	476	122	74.0
YP1β′	1074	423	60.7
YP2α	1729	708	59.1
YP2β	1108	571	48.5

pp13 kDa acidic polypeptides decreased in the presence of TetraBr-2-azaBz. The extent of inhibition of phosphorylation was most pronounced for YP1β (and YP1β′) and YP2α (see Table 2), hence those phosphorylated preferentially by PK60S (see Fig. 3). Similar results were obtained with 1 μM inhibitor (not shown).

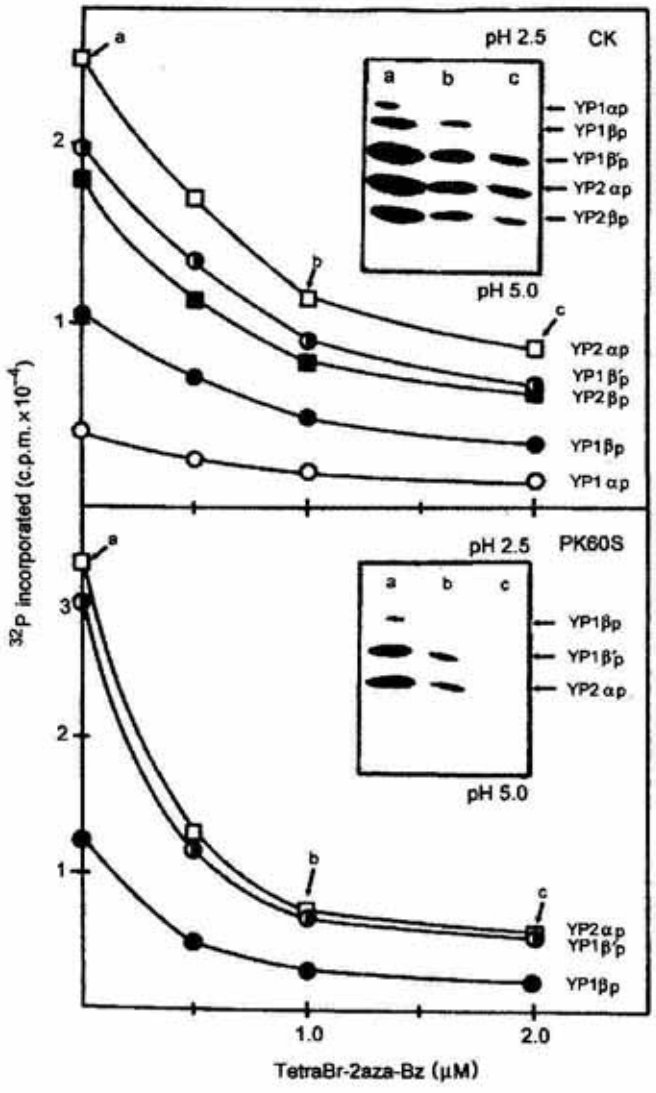

Fig. 3. Effect of TetraBr-2-azaBz on phosphorylation of the individual members of the pp13 kDa acidic proteins in intact 80S ribosomes by CK-II or PK60S.

Phosphorylation of 1 mg of 80S ribosomes by CK-II or PK60S (10 units) was carried out under standard conditions in the presence of [γ-^{32}P]ATP (2000 c.p.m./pmol) and the indicated concentrations of the inhibitor. The reaction was terminated after 15 min by addition of 0.5 M NH_4Cl in 50% ethanol, leading to liberation of the pp13 kDa acidic proteins [10]. An aliquot of each sample containing 6 μg of "split proteins" was subjected to isoelectric focusing on polyacrylamide gel. Following silver staining, the gel was dried and autoradiographed at –70°C (insert in each panel). The individual bands were cut out and ^{32}P incorporated counted. The dependence of the level of ^{32}P incorporated, by each enzyme, on the inhibitor concentration (a, b, c) is shown graphically and on the gels.

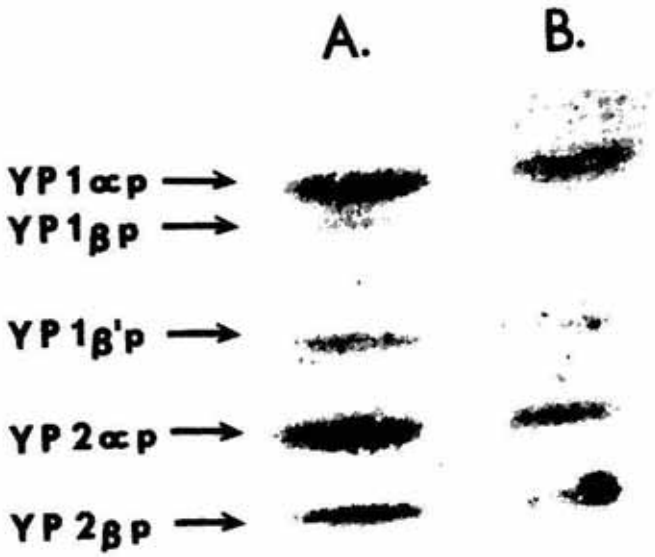

Fig. 4. Inhibition of intracellular phosphorylation of acidic proteins of the ribosomal 60S subunit by TetraBr-2-azaBz.
Samples of 0.5 mg of ^{32}P-labelled 80S ribosomes from yeast cells cultivated in the absence of the inhibitor (A), and in the presence of 5 μM inhibitor (B), were treated with 1 μg of pancreatic RNAse for 15 min at room temperature and the total ribosomal proteins directly subjected to isoelectric focusing on polyacrylamide gel. The separated acidic proteins were silver stained and autoradiographed.

DISCUSSION

Phosphorylation of yeast ribosomes *in vitro* is known to be due to at least two protein kinases with different properties, CK-II and PK60S [18, 19]. The finding that several halogeno benzimidazoles and 2-azabenzimidazoles (benzotriazoles), previously described as relatively specific inhibitors of CK-I and/or CK-II [22–24] are also inhibitors of PK60S is not entirely unexpected, bearing in mind that acidic proteins are substrates of both PK60S and CK-II [18]. Moreover, the specificities of the two enzymes are partially overlaping: the ubiquitous and pleiotropic CK-II phosphorylates all five of the pp13 kDa ribosomal acidic proteins, whereas only three of them (YP1β, YP1β′, YP2α) are substrates for PK60S [19]. It has elsewhere been pointed out that, apart from CK-I and CK-II, there are four additional Ser/Thr protein kinases characterized as "acidophilic" [30]. One of these, rhodopsin kinase, has in fact been reported to be inhibited by one of the inhibitors shown in Table 1, *viz.* DRB [31, 34]. To this list yeast PK60S should now be added.

The site(s) of phosphorylation of the pp13 kDa polypeptides by CK-II are supposedly Ser residues in the vicinity of their C-termini [29] surrounded by a cluster of glutamic and aspartic acids [9]. Such an arrangement of amino acids in a polypeptide chain determines the recognition of the substrate by CK-II [35]. Moreover, the target residues for CK-II in mammalian 60S ribosomal acidic proteins are also located at the C-terminal regions of the polypeptides [36]. The sequences embracing the remaining serine residues in yeast ribosomal acidic proteins are differentiated and located within and closer to the N-termini of the polypeptide chains [2, 16]. The latter serines appear to be the targets for PK60S, an inference derived from the fact that synthetic peptides, with sequences corresponding to those in the vicinity of the C-termini of the ribosomal acidic proteins, are good substrates for CK-II, but poor ones for PK60S [29].

Bearing in mind the role of phosphorylation of the ribosomal acidic proteins in the assembly of active ribosomes (see introduction), the use of inhibitors which can, at least partially, discriminate between different kinases *in vivo* and *in vitro* provides a useful tool for studies on the regulation of phosphorylation. It should be noted from Table 1 that DiBr-DRB, although not as potent an inhibitor as TetraBr-2-azaBz, discriminates even more effectively between CK-II and PK60S, with K_i values of 19 μM and over 200 μM, respectively; and, in contrast to TetraBr-2-azaBz, is more effective *vs* CK-II. The use of both inhibitors, consequently, may offer additional advantages.

In the foregoing we have not taken into consideration the possibility that the ribosomal acidic proteins may be subject to sequential phosphorylation by the two kinases. In fact, preliminary experiments have demonstrated

that this may, indeed, be so, *viz.* prior phosphorylation of the isolated partially phosphorylated ribosomes by either kinase was followed by additional phosphorylation by the other kinase. It is consequently also conceivable that prior phosphorylation by one kinase may be, at least in part, a prerequisite for subsequent phosphorylation by the second enzyme. This aspect is presently the object of ongoing studies.

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