Kinetics of inhibition by 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole on calf thymus casein kinase II

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The adenosine analogue 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) is a specific inhibitor for RNA polymerase II transcription *in vivo* and *in vitro* [Tamm & Sehgal (1978) Adv. Virus Res. 22, 187–258; Zandomeni & Weinmann (1984) J. Biol. Chem. 259, 14804–14811]. The effect on RNA polymerase II-specific transcription seems to be mediated by its inhibition of nuclear casein kinase II [Zandomeni, Carrera-Zandomeni, Shugar & Weinmann (1986) J. Biol. Chem. 261, 3414–3419]. Inhibition studies indicated that DRB acted as a mixed-type inhibitor with respect to casein and as a competitive inhibitor with respect to the nucleotide phosphate donor substrates. The DRB inhibition constant is 7 μ M for the calf thymus casein kinase II, with regard to both ATP and GTP.

INTRODUCTION

By using 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), an analogue of adenosine, it has been possible to establish the involvement of casein kinase II during RNA polymerase II transcription (Zandomeni & Weinmann, 1984). This compound inhibits specific cellular RNA polymerase II transcription without directly affecting other cellular functions (Tamm & Sehgal, 1978). DRB inhibits both specific RNA polymerase II transcription and the casein kinase II contained in the same extracts at identical concentrations (Zandomeni & Weinmann, 1984). When transcription reaction mixtures *in vitro* inhibited with DRB were supplemented with excess pure casein kinase II, the inhibition was partially overcome (Zandomeni *et al.*, 1986). Unphosphorylated phosvitin was able to inhibit transcription after micro-injection of *Chironomus* cells, whereas phosphorylated phosvitin was not. Presumably, all casein kinase II activity was shunted to this exogenous substrate and was thus unable to participate in the transcription process (Egyhazi & Pigon, 1986). Thus, although the phosphorylation targets for casein kinase within the transcriptional machinery have not yet been identified, the role of this enzyme in the DRB inhibition of specific transcription *in vitro* appears to be well established (Egyhazi & Pigon, 1986; Zandomeni *et al.*, 1986).

Since the target for the DRB inhibition appears to be casein kinase II and this enzyme can be readily purified, it becomes important to establish the type of inhibition and its kinetic parameters. Casein kinase II can use either ATP or GTP as a phosphate donor. We have developed a rapid purification procedure for the calf thymus enzyme (Zandomeni *et al.*, 1986). With this purified enzyme the kinetic parameters for ATP, GTP and DRB have now been determined. DRB is a competitive inhibitor for both ATP and GTP for the phosphorylation reaction catalysed by casein kinase II.

MATERIALS AND METHODS

Nucleotides and radiochemicals

ATP and GTP were from Pharmacia. [γ - ^{32} P]ATP and [γ - ^{32} P]GTP were obtained from Amersham Corporation.

Protein substrate

Partially hydrolysed and dephosphorylated casein was purchased from Sigma Chemical Co.

Casein kinase assay

Routine kinase determinations were performed in 20 mM-Tris/HCl buffer, pH 7.9, containing 160 mM-KCl or -NaCl, 8 mM-MgCl₂, 0.1 mM-dithiothreitol and 20 μ M-[γ - ^{32} P]ATP or -[γ - ^{32} P]GTP (5000 c.p.m./pmol) with 2 mg of casein/ml as substrate acceptor in a total volume of 15 μ l. After reaction at 30 °C for 5 min samples of the mixture were spotted on Whatman 3MM paper and washed with 20% (w/v) trichloroacetic acid. Values were corrected for phosphorylation of proteins contained in the kinase fraction when it exceeded 1% of the activity in the absence of casein.

Protein determination

Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as standard.

Enzyme purification

Frozen calf thymus tissue was freeze-dried and pulverized in a Waring blender for 10 s at low speed. The protein was then solubilized by addition of buffer A [50 mM-Tris/HCl buffer, pH 7.9, containing 10% (v/v) glycerol, 0.2 mM-dithiothreitol and 0.5 mM-EDTA] containing 50 mM-KCl, and homogenized, with the temperature being kept below 4 °C.

The homogenate was centrifuged at 10000 *g* in a Beckman JA20 centrifuge with a G10 rotor for 20 min at 0 °C. The pellet was re-extracted in 500 ml of buffer A containing 50 mM-KCl and spun under the same conditions as above. The KCl concentration of the pooled supernatants was adjusted to 550 mM and the mixture was directly mixed with 200 ml of Q-Sepharose (Fast Flow; Pharmacia), previously equilibrated in buffer A containing 550 mM-NaCl. After 30 min adsorption, the resin was transferred to a sintered-glass funnel, and the flow-through was collected and used as a source of enzyme.

Abbreviation used: DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole.

Phosphocellulose chromatography

The Q-Sepharose effluent was diluted with buffer A to a final concentration of 380 mM-NaCl, and batch-adsorbed on phosphocellulose. After 30 min, the resin was collected by filtration and washed with buffer A containing 380 mM-NaCl. The bound proteins were eluted with a linear gradient of 380–900 mM-NaCl in buffer A. Protein kinase activity sensitive to DRB was eluted at 560 mM-NaCl.

Mono Q f.p.l.c.

Active fractions were pooled and diluted with buffer A to a concentration of 280 mM-NaCl and loaded on to a 1 ml Mono-Q anion-exchange column pre-equilibrated with 280 mM-NaCl in buffer A. After a 20 ml wash, the kinase activity was eluted with a linear gradient of 280–500 mM-NaCl in buffer A. The run was performed at 4 °C with a 0.2 ml/min flow rate.

Data analysis

Data from duplicate reactions for initial-velocity studies were plotted in double-reciprocal form. The kinetic constants were determined from the slope and intercept replots of the initial-velocity data. For the calculation of inhibition constants, the slopes and intercepts of the primary double-reciprocal plots at each concentration of the relevant substrate were plotted against the inhibitor concentrations, and the curves were calculated by the least-squares fit method. All the kinase studies were carried out with homogeneous casein kinase II purified as described. The kinase was more than 98 % pure and was used at a concentration of 2 nM (assuming an M_r of 170 000) in all reactions. Partially hydrolysed and dephosphorylated casein was used as a protein phosphate acceptor, and ATP or GTP was used as the phosphate donor as indicated.

RESULTS

Kinetics of casein kinase II: initial-velocity patterns

Casein kinase II exhibited hyperbolic saturation curves with ATP or GTP and casein at the substrate

Fig. 1. Plot of $1/v$ versus $1/[MgGTP^{2-}]$ at different fixed concentrations of casein

Casein concentrations were 0.22 mg/ml ($\square$), 0.5 mg/ml ($\blacklozenge$), 0.75 mg/ml ($\triangle$) and 2.0 mg/ml ($\diamond$). Inset: replot of the $1/v$ axis intercept versus $1/[casein]$.

Scheme 1.

Abbreviation: PK, protein kinase.

Fig. 2. Plot of $1/v$ versus $1/[casein]$ at different fixed concentrations of $MgGTP^{2-}$

$MgGTP^{2-}$ concentrations were 10 μM ($\square$), 12.5 μM ($\blacklozenge$), 20.5 μM ($\triangle$) and 30.5 μM ($\diamond$). Inset: replot of the $1/v$ axis intercept versus $1/[MgGTP^{2-}]$. In both cases K_m was estimated from the inverse of the abscissa intercept of the replot.

concentrations used in this study. When $[MgGTP^{2-}]$ was varied at different fixed casein concentrations, a family of lines intersecting below the abscissa in double-reciprocal plots was obtained for the calf thymus DRB-sensitive casein kinase (Fig. 1). These results indicate that the enzyme follows a sequential mechanism in which a ternary complex must form before any products are released (Scheme 1). Fig. 1 inset shows a replot of data taken from plots of $1/v$ versus $1/[MgGTP^{2-}]$ at different fixed concentrations of casein. This replot of the $1/v$ coordinate of the intersection points versus $1/[casein]$ was used to calculate a K_m for casein at saturating concentrations of the donor phosphate $[MgGTP^{2-}]$ of 0.2 mg/ml. The family of plots for various concentrations of casein is similar to that for varied $[MgGTP^{2-}]$ (Fig. 2). Replot (inset) of the data in Fig. 2 gives a K_m of 72 μM for $[MgGTP^{2-}]$ at saturating concentrations of casein. A V_{max} of 1 $\mu\text{mol/min per mg}$ of enzyme could be calculated from either replot.

The family of reciprocal plots intersects below the abscissa, indicating that the binding of any one of the

Table 1. Kinetic parameters of casein kinase II

The kinetic parameters presented correspond to two experiments where all the intersections and values perfectly correspond in all the Figures. In all other experiments the DRB dissociation constant was in the 6–8 μM interval. The same enzyme preparation was used for these experiments.

MgATP ²⁻		MgGTP ²⁻	
V_{max}	1.8 $\mu\text{mol/min per mg}$	V_{max}	1 $\mu\text{mol/min per mg}$
α	5	α	4
K_{ATP}	4.2 μM	K_{GTP}	18 μM
K_{m}	21 μM	K_{m}	72 μM
Casein		Casein	
K_{c}	0.28 mg/ml	K_{c}	0.3 mg/ml
K_{m}	1.4 mg/ml	K_{m}	1.2 mg/ml
DRB		DRB	
K_{DRB}	6.8 μM	K_{DRB}	7.2 μM
β	1.5	β	1.4

substrates to the enzyme inhibits the binding of the other. α is the factor by which the binding of one substrate changes the dissociation constant of the other substrate. Intersect replots were used to calculate an α value of 4. Thus the dissociation constant for GTP (K_{GTP}) is 18 μM for the calf thymus casein kinase II.

Table 1 summarizes the kinetic parameters for GTP and the results of similar kinetic studies with ATP.

Mechanism of casein kinase II inhibition by DRB

The effect of DRB on the kinase activity was examined at different concentrations of phosphate donor (ATP or GTP). The inhibitory effect of DRB appeared to be dependent upon the nucleotide concentration of either possible donor substrate (ATP or GTP), suggesting that the DRB inhibition affects the enzyme–nucleotide interaction. To characterize further the mechanism of DRB inhibition on the pure calf thymus casein kinase, the enzymic activity was measured at various GTP and casein concentrations in the presence of different DRB concentrations, assuming that DRB competes with GTP in a rapid-equilibrium random system (Sehgal, 1975) (Scheme 1).

β is the value by which the dissociation constant of casein (K_{c}) is modified by the presence of DRB. A plot of $1/v$ versus $1/[\text{MgGTP}^{2-}]$ at different concentrations of DRB is obtained for each concentration of casein. Fig. 3 shows one of these plots for a casein concentration of 2 mg/ml. For every plot at each concentration of casein, the family of lines (each line corresponds to a different concentration of DRB) intersected on the ordinate. Thus DRB is a competitive inhibitor with respect to GTP in the kinase reaction. The DRB dissociation constant, K_{DRB} , was calculated by plotting the slope of the $1/v$ versus $1/[\text{MgGTP}^{2-}]$ plots for each casein concentration versus DRB concentration. Fig. 4 shows such a plot; each line corresponds to a fixed concentration of casein. This family of lines intersected at a point where the DRB

Fig. 3. Competitive inhibition by DRB with respect to $[\text{MgGTP}^{2-}]$: plot of $1/v$ versus $1/[\text{MgGTP}^{2-}]$ at different fixed concentrations of DRB and a constant concentration of casein (2.0 mg/ml)

DRB concentrations were 0 μM ($\square$), 5 μM ($\blacklozenge$), 10 μM ($\blacksquare$) and 30 μM ($\diamond$).

Fig. 4. Replot of the $1/v$ slope versus $[\text{DRB}]$

Each line represents data from a family of reciprocal plots obtained at a different fixed concentration of casein. Casein concentrations were 0.22 mg/ml ($\square$), 0.5 mg/ml ($\blacklozenge$), 0.75 mg/ml ($\blacksquare$) and 2.0 mg/ml ($\diamond$).

co-ordinate represents $-K_{\text{DRB}}$. From this plot a K_{DRB} of 7.2 μM for the calf thymus casein kinase II was calculated. Similar plots and a similar K_{DRB} were obtained with ATP as substrate, indicating that DRB is a competitive inhibitor of ATP as well as of GTP. A plot of $1/v$ versus $1/[\text{casein}]$ at different concentrations of DRB was obtained for each constant concentration of $[\text{MgGTP}^{2-}]$. The plot corresponding to a $[\text{MgGTP}^{2-}]$ concentration of 20 μM is shown in Fig. 5. To calculate β , the factor reflecting how the binding of DRB affects the binding of casein, the $1/v$ axis intercepts (from Fig. 5) and the other reciprocal plots at different concentrations of GTP versus DRB concentration were replotted (Fig. 6). The vertical co-ordinate of the intersection point of the family of lines (each line represents a different concentration of GTP) corresponds to $1/V_{\text{max}}$. The V_{max} calculated from Fig. 6 was 1 $\mu\text{mol/min per mg}$, in agreement with the value calculated in the kinetic analysis (Figs. 1 and 2). A secondary replot (Fig. 6 inset) shows the intercepts of the projection of each line with the horizontal axis (from

Fig. 5. Mixed-type inhibition by DRB with respect to casein: plot of $1/v$ versus $1/[\text{casein}]$ at different fixed concentrations of DRB and a constant concentration of MgGTP^{2-} ($30.5 \mu\text{M}$)

DRB concentrations were 0 μM ($\square$), 5 μM ($\blacklozenge$), 10 μM ($\blacksquare$) and 30 μM ($\diamond$).

Fig. 6. Replot of the $1/v$ axis intercept versus [DRB]

Each line represents data from a family of reciprocal plots obtained at a different fixed concentration of MgGTP^{2-} . MgGTP^{2-} concentrations were 10 μM ($\square$), 12.5 μM ($\blacklozenge$), 20.5 μM ($\blacksquare$) and 30.5 μM ($\diamond$). Inset: secondary replot of the abscissa intercept of the $1/v$ axis intercept versus [DRB].

Fig. 6) versus the GTP concentrations, from which a β value of 1.4 is calculated. Since $\beta \neq 1$, DRB is a mixed-type inhibitor with respect to casein. Similar studies were done with ATP as the phosphate donor substrate (Table 1).

DISCUSSION

Casein kinase II has been found in all eukaryotic cells from yeast to man, and is highly conserved with respect to structure and general enzymic requirements (Walinder, 1973; Dahmus, 1976, 1981; Thornburg *et al.*, 1978; Kumon & Ozawa, 1979; Biovin & Galand, 1979; Rose *et al.*, 1981; Cochet *et al.*, 1981, 1983; Glover *et al.*, 1983). Immunological similarities between the *Drosophila* and

the bovine enzymes have been reported (Dahmus *et al.*, 1984). Furthermore, the kinetic constants of the *Drosophila* protein kinase and of the calf thymus enzyme are very similar (Glover *et al.*, 1983; the present work). This similarity in kinetic parameters is consistent with the low degree of change during evolution of this protein kinase, in turn suggesting a conserved function in eukaryotic cells.

The use of DRB as a dead-end inhibitor independently confirmed the enzymic mechanism for casein kinase II indicated in the initial-velocity studies by variation of the concentrations of both substrates. DRB is a competitive inhibitor of ATP and GTP with a dissociation constant of 7 μM . A β factor equal to 1.4 reflects the change in the association constant of casein by the presence of DRB. Thus the binding of DRB to the enzyme affects the binding of casein, indicating that DRB is a mixed-type inhibitor with regard to casein. All kinetic parameters are consistent with a rapid-equilibrium Random Bi Bi mechanism for the phosphotransferase reaction catalysed by casein kinase II.

The fact that DRB competes with ATP and GTP means that inhibition by this drug will not be observed whenever there is a high concentration of ATP and GTP, as there is in the nucleus (Rapaport *et al.*, 1979). However, since DRB is able to inhibit nuclear protein phosphorylation *in vivo* (Egyhazi & Pigon, 1983; Zandomeni & Weinmann, 1984), the conditions in nuclei must be such that casein kinase II activity is still diminished by the drug even when ATP and GTP concentrations are high. A solution to this contradiction may rest in the high concentration of ADP and GDP in nuclei (Rapaport, 1980). ADP and GDP, as products of the phosphotransferase reaction (Scheme 1), act as competitive inhibitors with regard to the phosphate donor (Glover *et al.*, 1983). Thus high ADP and GDP concentrations in nuclei will directly inhibit the phosphotransferase reaction and have an additive effect with DRB. Furthermore, the presence of additional modulators of casein kinase II activity (Dahmus, 1976; Mäenpää, 1977; Rapaport, 1980; Feige *et al.*, 1980; Farron-Furstenthal, 1980) could also contribute to create an environment in which DRB can inhibit protein phosphorylation by this enzyme.

DRB is a specific inhibitor of RNA polymerase II transcription *in vivo* (Egyhazi, 1974; Tamm & Sehgal, 1978; Zandomeni *et al.*, 1982) and *in vitro* (Zandomeni *et al.*, 1982, 1986). Several lines of evidence indicate that casein kinase II is involved in the DRB inhibition of RNA polymerase II transcription *in vivo* (Egyhazi & Pigon, 1986) and *in vitro* (Zandomeni *et al.*, 1986). The DRB inhibition of transcription appears to result from its inhibition of casein kinase II activity. The fact that DRB competes with the phosphate donor explains the consistent observation in previous studies that the complete inhibition of specific transcription *in vivo* and *in vitro* was never achieved by increasing the concentration of the inhibitor (Tamm & Sehgal, 1978; Zandomeni & Weinmann, 1984; Zandomeni *et al.*, 1986). In a specific transcription reaction *in vitro* after seconds of incubation all four ribonucleoside triphosphates are degraded to the respective nucleoside diphosphates. The reaction *in vitro* reached very rapidly a triphosphate/diphosphate ratio of 1.5:1, which remains constant during the incubation period (Wilkinson *et al.*, 1983; R. O. Zandomeni, unpublished work) and is virtually the same low ratio value

observed in nuclei (Rapaport, 1980). Thus the specific transcription system *in vitro* seems to reproduce the nuclear environment in which DRB inhibition of protein phosphorylation and RNA polymerase II transcription are possible. Furthermore, greater DRB inhibition of specific transcription *in vitro* was always observed at low concentrations of ATP and GTP.

In the cell, variations of ADP and GDP concentration due to ATPases or GTPases could regulate the total casein kinase II activity, and, indirectly, the extent of RNA polymerase II transcription. These may be additional points of regulation of mRNA synthesis in eukaryotic cells. Further studies are needed to determine whether cells can regulate transcription by regulating casein kinase II activity.

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