Differential responses of cyclic GMP-dependent and cyclic AMP-dependent protein kinases to synthetic peptide inhibitors

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The peptide Arg-Lys-Arg-Ala-Arg-Lys-Glu was synthesized and tested as an inhibitor of cyclic GMP-dependent protein kinase. This synthetic peptide is a non-phosphorylatable analogue of a substrate peptide corresponding to a phosphorylation site (serine-32) in histone H2B. The peptide was a competitive inhibitor of cyclic GMP-dependent protein kinase with respect to synthetic peptide substrates, with a K_i value of $86\ \mu\text{M}$. However, it did not inhibit phosphorylation of intact histones by cyclic GMP-dependent protein kinase under any conditions tested. Arg-Lys-Arg-Ala-Arg-Lys-Glu competitively inhibited the phosphorylation of either peptides or histones by the catalytic subunit of cyclic AMP-dependent protein kinase, with similar K_i values ($550\ \mu\text{M}$) for both of these substrates. The peptide Leu-Arg-Arg-Ala-Ala-Leu-Gly, which was previously reported to be a selective inhibitor of both peptide and histone phosphorylation by cyclic AMP-dependent protein kinase, was a poor inhibitor of cyclic GMP-dependent protein kinase acting on peptide substrates ($K_i = 800\ \mu\text{M}$), but did not inhibit phosphorylation of histones by cyclic GMP-dependent protein kinase. The selectivity of these synthetic peptide inhibitors toward either cyclic GMP-dependent or cyclic AMP-dependent protein kinases is probably based on differences in the determinants of substrate specificity recognized by these two enzymes. It is concluded that histones interact differently with cyclic GMP-dependent protein kinase from the way they do with the catalytic subunit of cyclic AMP-dependent protein kinase.

Synthetic oligopeptides have been used as model substrates to investigate the substrate specificity of cyclic nucleotide-dependent protein kinases (Krebs & Beavo, 1979; Glass & Krebs, 1980). The peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly, which corresponds closely to the site in liver pyruvate kinase that is phosphorylated by cyclic AMP-dependent protein kinase, has been used extensively in studies of this enzyme (Kemp *et al.*, 1977; Feramisco *et al.*, 1980). The determinants of substrate specificity of cyclic GMP-dependent protein kinase have been studied and compared with those of cyclic AMP-dependent protein kinase by using a peptide corresponding to the amino acid sequence around serine-32 in histone H2B (Glass & Krebs, 1979, 1982), the major site of phosphorylation in this protein catalysed by cyclic GMP-dependent protein kinase (Hashimoto *et al.*, 1976). The peptide Arg-Lys-Arg-Ser-Arg-Lys-Glu corresponds to this sequence, and is selectively phosphorylated with favourable kinetic constants by cyclic GMP-

dependent protein kinase (Glass & Krebs, 1979, 1982).

Because no selective inhibitors of cyclic GMP-dependent protein kinase are known, we synthesized an analogue of the peptide substrate Arg-Lys-Arg-Ser-Arg-Lys-Glu in which the serine was replaced by an alanine residue, in an attempt to develop a competitive inhibitor (Glass & Miller, 1980). The resulting synthetic peptide contained the determinants of specificity present in the parent substrate peptide (Glass & Krebs, 1982), but lacked its phosphate-accepting hydroxy group. The present paper concerns the ability of Arg-Lys-Arg-Ala-Arg-Lys-Glu to inhibit the phosphorylation of substrate peptides or histones by the cyclic GMP-dependent or cyclic AMP-dependent protein kinases. Similar approaches to the development of competitive peptide inhibitors of cyclic AMP-dependent protein kinase have been described previously (Kemp *et al.*, 1976; Feramisco & Krebs, 1978). Because one of these, Leu-Arg-Arg-Ala-Ala-Leu-Gly, an analogue of the peptide corresponding to the sequence of the phosphorylation site

Abbreviation used: Boc-, t-butoxycarbonyl-.

in pyruvate kinase, has been reported to be a selective inhibitor of cyclic AMP-dependent protein kinase (Feramisco & Krebs, 1978), the influence of this peptide inhibitor on the phosphorylation of both peptide and histone substrates by cyclic GMP-dependent protein kinase was further examined in the present studies.

Experimental

Materials

Specifically protected Boc-amino acids and 1% polystyrene resin were purchased from Peninsula Laboratories, San Carlos, CA, U.S.A. Mixed histone (type II-A) was from Sigma, St. Louis, MO, U.S.A., and AG 1-X8 ion-exchange resin (acetate form) was from Bio-Rad, Richmond, CA, U.S.A. [32 P]P_i was purchased from ICN, Irvine, CA, U.S.A. The [γ - 32 P]ATP was prepared by the method of Glynn & Chappell (1964).

Purification and assay of protein kinases

Cyclic GMP-dependent protein kinase and the catalytic subunit of cyclic AMP-dependent protein kinase were purified to homogeneity from bovine lung (Glass & Krebs, 1979) and rabbit skeletal muscle (Bechtel *et al.*, 1977) respectively. Protein kinase assays were performed as described previously (Glass & Krebs, 1979, 1982). Phosphotransferase activity of cyclic GMP-dependent protein kinase was determined in 25 mM-Tris/HCl (pH 7.4), containing 1 μ M-cyclic GMP, 0.4 mM- $[\gamma$ - 32 P]ATP (50–200 c.p.m./pmol), 2 mM-magnesium acetate, 0.3 mg of bovine serum albumin/ml, 3 mM-2-mercaptoethanol, peptide or histone substrate and peptide inhibitor as indicated, and 0.4 μ g of purified enzyme/ml. Catalytic subunit of cyclic AMP-dependent protein kinase was assayed in 50 mM-Mes (4-morpholine-ethanesulphonic acid) (pH 6.9), 0.4 mM- $[\gamma$ - 32 P]ATP (50–200 c.p.m./pmol), 2 mM-magnesium acetate, 0.3 mg of bovine serum albumin/ml, 3 mM-2-mercaptoethanol, peptide or histone substrate and peptide inhibitor as indicated, and 0.3–3.0 μ g of purified enzyme/ml, depending on the substrate used. In cases where a fixed concentration of phosphate-accepting substrate was used, it was used at its previously determined K_m value (Glass & Krebs, 1979), so inhibition by competitive peptide inhibitors could be observed. Reactions were initiated with enzyme and conducted in a final volume of 0.08 ml at 30°C for 2 min. Reactions were terminated by the addition of acetic acid (final concn. 30%, v/v), and the amount of phosphate incorporated into substrate was determined by anion-exchange chromatography as described previously (Kemp *et al.*, 1976). Phosphotransferase assays were linear with respect to time

and to amount of protein kinase. Data from initial enzyme velocity measurements were fitted to the Michaelis–Menten equation by weighted linear least squares and plotted in double-reciprocal form. The K_i values were determined from secondary replots of the slopes versus inhibitor concentration (Segel, 1975).

Purification of histones

Histone H1 and histone H2B were prepared from calf thymus by the method of Johns (1964). Histone H2B was further purified as described by Oliver *et al.* (1972) and was approx. 99% pure as analysed by electrophoresis in acid/urea/polyacrylamide gels (Panyim & Chalkley, 1969) or by amino acid analysis.

Purification of the protein inhibitor of cyclic AMP-dependent protein kinase

The heat-stable protein inhibitor of cyclic AMP-dependent protein kinase was prepared as described by Walsh *et al.* (1971).

Synthesis of substrate and inhibitor peptides

Peptides were synthesized manually by solid-phase techniques (Gutte & Merrifield, 1971) by using the following amino acid derivatives: Boc-Ala, Boc-(N^G -NO₂)-Arg, Boc-(*O*-benzyl)Glu, Boc-Gly, Boc-Leu, Boc-(N^G -*o*-chlorobenzylloxycarbonyl)Lys, Boc-(*O*-benzyl)Ser, Boc-(*O*-benzyl)Tyr and Boc-Val. Deprotection, neutralization and coupling steps were performed with the reagents and protocols previously described (Glass & Krebs, 1979, 1982; Feramisco & Krebs, 1978). Completed peptides were cleaved from the resin with HBr in trifluoroacetic acid and deprotected by catalytic hydrogenation as described previously (Glass & Krebs, 1979, 1982). Synthetic peptides were purified by ion-exchange and gel-filtration chromatography as described by Kemp *et al.* (1976). Purified peptides were analysed by high-voltage electrophoresis on paper at pH 1.9, by t.l.c. on cellulose sheets in several different solvent systems described previously (Kemp *et al.*, 1977), and by quantitative amino acid analysis. All peptides were pure by the criteria of ninhydrin staining of chromatograms and electrophoretograms. Amino acid analyses of duplicate acid hydrolysates of Arg-Lys-Arg-Ala-Arg-Lys-Glu gave values (mol of amino acid/mol of peptide) of Glu (1.03), Ala (0.97), Lys (1.95) and Arg (3.05). Similar analysis of Leu-Arg-Arg-Ala-Ala-Leu-Gly gave Gly (0.93), Ala (2.04), Leu (1.98) and Arg (2.05). The amino acid compositions determined for the other synthetic peptides used as substrates have been published previously (Glass & Krebs, 1979) or were as expected (results not shown).

Other methods

Purified kinases were quantified by the Lowry protein assay (Lowry *et al.*, 1951) with bovine serum albumin as standard.

Results

The substrate analogue Arg-Lys-Arg-Ala-Arg-Lys-Glu inhibited the activity of cyclic GMP-dependent protein kinase with respect to the substrate peptide (Fig. 1a). Concentrations of peptide inhibitor that caused 50% inhibition of kinase activity were in the range of 85–100 μM when several different peptides were used as substrate, including those having sequences different from that around the serine-32 site in histone H2B (results not shown). These other substrates included a heptapeptide corresponding to the serine-36 site in histone H2B and an undecapeptide containing both serine-32 and serine-36 in histone H2B (Glass & Krebs, 1979), as well as the heptapeptide corresponding to the site of phosphorylation in pyruvate kinase (Kemp *et al.*, 1977). Unexpectedly, Arg-Lys-Arg-Ala-Arg-Lys-Glu, at concentrations that inhibited the phosphorylation of peptides by 90%, did not inhibit the phosphorylation of histone H2B (Fig. 1a). Under these conditions of initial rates of phosphorylation, the major site phosphorylated in histone H2B by cyclic GMP-dependent protein kinase is serine-32 (Hashimoto *et al.*, 1976; Yamamoto *et al.*, 1977), the exact sequence after which the inhibitor peptide is modelled. In addition, no inhibition of cyclic GMP-dependent protein kinase by the inhibitor peptide was observed when histone H1 or

mixed histones were used as substrates at or below their K_m values (results not shown).

Although the serine-32-containing heptapeptide Arg-Lys-Arg-Ser-Arg-Lys-Glu is a poorer substrate for cyclic AMP-dependent protein kinase than for cyclic GMP-dependent protein kinase (Glass & Krebs, 1979), the corresponding inhibitor peptide analogue was tested as an inhibitor of cyclic AMP-dependent protein kinase with both peptide and histone substrates. The concentrations of Arg-Lys-Arg-Ala-Arg-Lys-Glu that inhibited cyclic AMP-dependent protein kinase by 50% were in the range of 350–460 μM , whether peptide or histone H2B was used as substrate (Fig. 1b). Similar results were observed with other peptide substrates and other histone subfractions (results not shown). Cyclic GMP-dependent protein kinase and the catalytic subunit of cyclic AMP-dependent protein kinase obviously respond differently to this inhibitor peptide when histones are used as substrate.

To test if the above results were unique to Arg-Lys-Arg-Ala-Arg-Lys-Glu, the pyruvate kinase-sequence inhibitor peptide (Feramisco & Krebs, 1978) was investigated as an inhibitor of cyclic GMP-dependent protein kinase. Leu-Arg-Arg-Ala-Ala-Leu-Gly inhibited the phosphorylation of a peptide substrate, but not of histone H2B, by cyclic GMP-dependent protein kinase (Fig. 2a). This same inhibitor peptide prevented the phosphorylation of either peptide substrate or histone H2B by cyclic AMP-dependent protein kinase (Fig. 2b). These latter results are consistent with the data of Feramisco & Krebs (1978). However, in their studies these investigators used only histone H2B,

Fig. 1. Effect of the synthetic Arg-Lys-Arg-Ala-Arg-Lys-Glu on the phosphorylation of histone H2B or synthetic peptide substrates by cyclic GMP-dependent (a) and cyclic AMP-dependent (b) protein kinases

(a) Substrates were (O) histone H2B (2 μM) and (●) Arg-Lys-Arg-Ser-Arg-Lys-Glu (20 μM). (b) Substrates were (O) histone H2B (6 μM) and (▲) Arg-Lys-Glu-Ser-Tyr-Ser-Val (100 μM). Kinase activity was measured by the method described in the Experimental section.

Fig. 2. Effect of the synthetic peptide *Leu-Arg-Arg-Ala-Ala-Leu-Gly* on the phosphorylation of histone H2B or synthetic peptide substrates by cyclic GMP-dependent (a) and cyclic AMP-dependent (b) protein kinases

(a) Substrates were (○) histone H2B ($2\mu\text{M}$) and (●) Arg-Lys-Arg-Ser-Arg-Lys-Glu ($20\mu\text{M}$). (b) Substrates were (○) histone H2B ($6\mu\text{M}$) and (▲) Arg-Lys-Glu-Ser-Tyr-Ser-Val ($100\mu\text{M}$). Kinase activity was measured by the method described in the Experimental section.

Fig. 3. Competitive inhibition of cyclic GMP-dependent protein kinase by the analogue peptide *Arg-Lys-Arg-Ala-Arg-Lys-Glu*

The inhibitor peptide concentrations were (μM); 0 (●), 70 (○), 140 (▲) and 280 (■). The variable substrate was Arg-Lys-Arg-Ser-Arg-Lys-Glu. A secondary plot of the slopes of the lines as a function of inhibitor peptide concentration is given in the inset: $K_i = 72\mu\text{M}$. Kinase activity was measured by the method described in the Experimental section.

and not peptide, as substrate for cyclic GMP-dependent protein kinase, which made *Leu-Arg-Arg-Ala-Ala-Leu-Gly* appear to be an absolutely selective inhibitor of the cyclic AMP-dependent enzyme. Neither of the peptide inhibitors used in the present

work inhibited cyclic GMP-dependent protein kinase when histone was used as substrate, even though both peptides inhibited this enzyme (albeit with varying potencies) when peptide was used as substrate.

The inhibition of cyclic GMP-dependent protein kinase by Arg-Lys-Arg-Ala-Arg-Lys-Glu was competitive with respect to peptide substrate (Fig. 3). The inhibition of cyclic AMP-dependent protein kinase by Arg-Lys-Arg-Ala-Arg-Lys-Glu and the inhibition of cyclic GMP-dependent protein kinase by the pyruvate kinase sequence inhibitor peptide were also competitive with respect to peptide substrate (results not shown). The apparent K_i values for inhibition of either cyclic GMP-dependent or cyclic AMP-dependent protein kinase by both of the peptide inhibitors are summarized in Table 1. Arg-Lys-Arg-Ala-Arg-Lys-Glu was the most potent inhibitor of cyclic GMP-dependent protein kinase; *Leu-Arg-Arg-Ala-Ala-Leu-Gly* was approx. 10-fold less potent in this respect. Although inhibition of cyclic AMP-dependent protein kinase was less selective, *Leu-Arg-Arg-Ala-Ala-Leu-Gly* was the best inhibitor of this enzyme. The K_i value of this peptide for cyclic AMP-dependent protein kinase compares favourably with the value reported by Feramisco & Krebs (1978). Neither peptide was an absolutely specific inhibitor of either of the protein kinases, but the relative selectivity that they did exhibit presumably reflects known differences in the substrate-specificity determinants of cyclic GMP-dependent and cyclic AMP-dependent protein kinases (Glass & Krebs, 1979, 1980, 1982).

Table 1. Apparent K_i values of inhibitor peptides for cyclic GMP-dependent and cyclic AMP-dependent protein kinases. For cyclic GMP-dependent kinase, variable substrates were synthetic peptides. No inhibition was observed when histones were used as substrates. For cyclic AMP-dependent kinase, variable substrates were synthetic peptides and histones. The values are means $\pm$ s.e.m. The numbers in parentheses indicate the numbers of separate determinations of K_i . The underlined residues in the inhibitor peptides are those that were substituted for phosphate-accepting serine residues in the corresponding substrate peptides.

Inhibitor peptide	Kinase ...	Apparent K_i value (μ M)	
		Cyclic GMP-dependent	Cyclic AMP-dependent
Arg-Lys-Arg- <u>Ala</u> -Arg-Lys-Glu		86 $\pm$ 5 (13)	550 $\pm$ 89 (12)
Leu-Arg-Arg- <u>Ala</u> - <u>Ala</u> -Leu-Gly		800 $\pm$ 78 (8)	376 $\pm$ 36 (7)

Fig. 4. Effect of the heat-stable inhibitor protein of cyclic AMP-dependent protein kinase on the phosphorylation of histone H2B and synthetic peptide substrates by cyclic GMP-dependent (●, ▲, ■) and cyclic AMP-dependent (○, △, □) protein kinases

Substrates were used at their K_m values for each kinase and were (○) 6 μ M- or (●) 1.5 μ M-histone H2B, (△) 100 μ M- or (▲) 25 μ M-Arg-Lys-Arg-Ser-Arg-Lys-Glu, and (□) 15 μ M- or (■) 185 μ M-Leu-Arg-Arg-Ala-Ser-Leu-Gly. Units of inhibitor protein activity were as defined by Walsh *et al.* (1971). Kinase activity was measured by the method described in the Experimental section.

did the inhibitor peptide used in the present study (see above). The inhibitor protein had no effect on cyclic GMP-dependent protein kinase, even if peptides were used as substrate, under conditions where the activity of the cyclic AMP-dependent protein kinase was essentially totally inhibited (Fig. 4). Thus the apparent selectivity of the inhibitor protein for cyclic AMP-dependent protein kinase is real, and its inability to inhibit cyclic GMP-dependent protein kinase is not due to a unique interaction between this latter enzyme and histone substrates.

Discussion

The inability of Arg-Lys-Arg-Ala-Arg-Lys-Glu to inhibit phosphorylation of histones, as opposed to peptides, by cyclic GMP-dependent protein kinase appears to be due to a unique interaction of histones with the enzyme and not merely to a difference between the use of proteins (histones) and oligopeptides as substrates. This inhibitor peptide inhibits cyclic GMP-dependent protein kinase when non-histone proteins such as phosphorylase kinase, troponin-I or the type-I regulatory subunit of cyclic AMP-dependent protein kinase are used as substrates or when autophosphorylation of cyclic GMP-dependent protein kinase is measured (Glass & Miller, 1981). However, nucleotide-free regulatory subunit of cyclic AMP-dependent protein kinase (type I) has been reported to inhibit competitively phosphorylation of either histone H2B or a synthetic peptide substrate by cyclic GMP-dependent protein kinase (Geahlen & Krebs, 1980). Therefore the type-I regulatory subunit is a better inhibitor of cyclic GMP-dependent protein kinase with respect to histone phosphorylation than is Arg-Lys-Arg-Ala-Arg-Lys-Glu, presumably because it has additional sites of interaction with the catalytic domain of cyclic GMP-dependent protein kinase.

It is not entirely clear why the peptide Arg-Lys-Arg-Ala-Arg-Lys-Glu did not inhibit histone phosphorylation by cyclic GMP-dependent protein kinase, but did inhibit histone phosphorylation by

the cyclic AMP-dependent enzyme. However, it is apparent that histones interact differently with cyclic GMP-dependent protein kinase from the way they do with the free catalytic subunit of cyclic AMP-dependent protein kinase. Walton & Gill (1980, 1981) demonstrated an interaction of histones with cyclic GMP-dependent protein kinase that resulted in a loss of cyclic GMP binding, and hence cyclic GMP-dependency, as well as a decreased total activity of the enzyme. Peptide substrates did not produce these same effects. Histones apparently produce these effects by binding at an anionic region of the enzyme, designated the poly(L-arginine) binding site (Walton & Gill, 1981), which is distinct from the active catalytic site. An interaction of histones at both of these sites on cyclic GMP-dependent protein kinase could result either in a conformational change in the active site of the enzyme or in the formation of a histone-enzyme complex of unusually high affinity, owing to multiple points of attachment. Either situation could prevent an oligopeptide such as Arg-Lys-Arg-Ala-Arg-Lys-Glu from inhibiting the phosphorylation of histones by cyclic GMP-dependent protein kinase. An anionic poly(L-arginine)-binding site occurs on cyclic AMP-dependent protein kinase also, but it is on the regulatory subunit of the enzyme (Walton & Gill, 1981; Taylor *et al.*, 1981; Takio *et al.*, 1980). Since activation of cyclic AMP-dependent protein kinase involves dissociation of the subunits (Krebs, 1972), the free catalytic subunit binds histones only via the substrate-binding site. Under these conditions, peptide inhibitor can readily compete with the histone substrate.

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