An active twenty-amino-acid-residue peptide derived from the inhibitor protein of the cyclic AMP-dependent protein kinase

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Digestion with *Staphylococcus aureus* V8 proteinase of the inhibitor protein of the cyclic AMP-dependent protein kinase results in the sequential formation of three active inhibitory peptides. The smallest active peptide has the sequence Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp. This 20-amino-acid-residue peptide has 20–40% of the activity of the native molecule and a K_i of 0.2 nM. Inhibition, as a minimum, appears to be based upon the inhibitor protein containing the recognition sequences that dictate protein-substrate-specificity. This inhibitory peptide also has sequence homology with the phosphorylation site for a protein kinase other than the cyclic AMP-dependent enzyme.

INTRODUCTION

The cyclic AMP-dependent protein kinase appears to be the sole mediator of cyclic AMP, which functions as the second messenger for many extracellular stimuli in eukaryotes. The enzyme has a tetrameric structure, R_2C_2 , containing two catalytic subunits (C) and the dimeric regulatory subunit, R_2 . Activation occurs via dissociation, in accord with eqn. (1), to yield active catalytic subunits:

A potential additional regulator of cyclic AMP-dependent protein kinase activity is the inhibitor protein of this enzyme (Walsh *et al.*, 1971). This protein functions by interacting with the free catalytic subunit (Ashby & Walsh, 1972, 1973) in an $MgATP^{2-}$ -dependent manner (Whitehouse & Walsh, 1983a), most probably at the catalytic site. The inhibitor protein is competitive with protein substrate, with an affinity (approx. 0.5 nM) that is several orders of magnitude more avid than that of any protein substrate (Demaille *et al.*, 1977; McPherson *et al.*, 1979; Whitehouse & Walsh, 1983a). Demaille *et al.* (1979) have described the isolation of an inhibitory penta-decapeptide, derived from the inhibitor protein, with approximately 10% retained activity. We now describe here the use of a similar procedure for fragmentation for the sequential degradation of the inhibitor protein via two intermediary forms to yield a 20-amino acid-residue peptide that has extensively retained inhibitory activity, but appears to be different in composition and size from that described by Demaille *et al.* (1979). The source of this inhibitory peptide is the physiological form of inhibitor protein, designated I (Whitehouse & Walsh, 1982), whereas Demaille *et al.* (1979) used as a starting product a form designated I' (McPherson *et al.*, 1979; Whitehouse & Walsh, 1982). The relationship between the I and I' forms has been suggested to be that of molecular conformers, since clearly their physicochemical attributes are different (McPherson *et al.*, 1979). The difference between the results presented here and those described by Demaille *et al.* (1979) may relate to differences in the starting material that served as the source for the inhibitory peptides, or possibly the latter report was more preliminary in nature.

EXPERIMENTAL

Source, purity and form of the inhibitor protein

The homogeneous physiologically native form of the inhibitor protein (I) was purified from rabbit skeletal muscle (peak 2 isomer) as described previously (Whitehouse & Walsh, 1982, 1983b), with the following exceptions.

(a) Preparation of catalytic subunit–Sephacryl affinity resin. Marked enhancement of the capacity and stability of the (catalytic subunit)–Sephacryl affinity resin, used in the purification of inhibitor protein, was obtained by its preparation as follows. All buffers and vessels used in the preparation of the affinity resin, up to the stage of adding 2-mercaptoethanol, were flushed with N_2 . The entire procedure was carried out at 4 °C. A 6 ml mini-column of Sephadex G-50 was prepared in a 12 ml disposable plastic syringe barrel (containing a bottom support of nylon screen plus nylon membrane) by pipetting a predetermined volume of Sephadex, equilibrated in 50 mM-potassium phosphate buffer, pH 7.5, containing 500 mM-NaCl and 5 mM-EDTA (buffer A), into the column and removing excess buffer by centrifugation of the mini-column at 500 g for 10 min and then 3000 g for 3 min. To this column was applied 2.5 ml of homogeneous bovine heart catalytic subunit (8 mg/ml) in 2.5 mM-Tris/HCl buffer, pH 7.4, containing 0.1 mM-EDTA, 7.5 mM-2-mercaptoethanol, 50 mM-NaCl and 50% (v/v) glycerol, and it was then centrifuged as before. The eluted catalytic subunit was adjusted to 20% (v/v) glycerol and immediately added to 0.75 g of CNBr-activated Sepharose (Pharmacia), activated by washing with 0.1 mM-HCl, and equilibrated in buffer A containing 20% glycerol. The resin plus protein was gently mixed for 2 h at 4 °C, then 0.1 vol. of 5 M-ethanolamine and 0.001 vol. of 15 M-2-mercaptoethanol were added, and the mixing was continued for 2 h at 4 °C. The resin was then washed with three 10 ml volumes of 30 mM-potassium phosphate buffer, pH 6.7, containing 0.1 mM-EDTA and 15 mM-2-mercaptoethanol (buffer B) plus 1 M-NaCl, and equilibrated in buffer B containing 1 mM-ATP and 2 mM-MgCl₂. Columns prepared by this procedure and stored in buffer B containing 0.1% bovine

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified inhibitor protein

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed in accordance with the method of Weber & Osborn (1969), with 12% polyacrylamide gels. Immediately after electrophoresis, gels were sliced into 2 mm slides, which were placed into 3 ml plastic tubes containing 200 μ l of distilled deionized (Milli-Q) water. The tubes were sealed and shaken on an orbital shaker for 16 h at 25 °C. Samples were assayed for inhibitor protein as described in the Experimental section. R_m values are with reference to the migration of Bromophenol Blue. The arrows denote the migration of standards of the I and I' forms (McPherson *et al.*, 1979; Whitehouse & Walsh, 1982). In the inset, 4 μ g of inhibitor protein was applied and the protein band detected by staining with 0.25% Coomassie Blue R-250.

serum albumin and 0.02% NaN_3 at 4 °C have been stable with repeated use for over a year, and bind up to 250 μ g of inhibitor protein.

(b) Affinity chromatography of the inhibitor protein. The inhibitor protein was prepared from 4 kg of rabbit skeletal muscle in accordance with the method of McPherson *et al.* (1979) up to and including chromatography on DEAE-cellulose (Whatman DE-52) at pH 5.0; it was then further purified by a modification of the (catalytic subunit)-Sephacrose affinity-chromatography method described by Demaille *et al.* (1977). Briefly, the peak 2 isomer, eluted from DEAE-cellulose, was neutralized (to pH 6.8), dialysed for 16 h against buffer B with the use of Spectrapor 1 tubing, then adjusted to 1 mM-ATP and 2 mM- MgCl_2 and applied to the affinity resin at a flow rate of 1.25 ml/min. The column was washed with 10 ml of buffer B containing the same concentrations of ATP and MgCl_2 , next washed with a 50 ml 0–150 mM-KCl linear gradient in buffer B containing ATP and MgCl_2 , and the inhibitor protein then eluted with 40 ml of buffer B containing 1 M-KCl, 0.3 M-guanidinium chloride and 5 mM-EDTA. The fractions containing the inhibitor protein were pooled, dialysed against four changes (10 litres each) of 1 mM-Mes/NaOH buffer, pH 6.8, over a 24 h period and then freeze-dried immediately.

(c) Inhibitor protein preparation characteristics. The inhibitor protein preparation procedure takes advantage of our previous observation (Whitehouse & Walsh, 1983a) that its interaction with the catalytic subunit is enhanced in the presence of MgATP^{2-} . With this procedure the yield from 4 kg of tissue was 150–300 μ g of protein with a specific activity in the range 3×10^5 – 5×10^5 units/mg of protein. When the preparation is accomplished rapidly (less than 1 week), the final product contains only a single band of protein (Fig. 1), which corresponds to the physiological I form (R_m relative to Bromophenol Blue 0.57; Whitehouse & Walsh, 1982). The I' form of the inhibitor protein stains poorly with Coomassie Blue, but elution of the sodium dodecyl sulphate/polyacrylamide gel and assay (Fig. 1) showed that the preparation contained at least 98% form I, with little or no form I' detectable (R_m 0.74). The inhibitor protein was also shown to be pure by h.p.l.c. analysis (see below in the Results section).

Amino acid and sequence analyses

Samples of peptides, obtained by an 8 h digestion of inhibitor protein with proteinase (see the Results section), were purified by h.p.l.c. with the same solvent and gradient as indicated in Fig. 3 (below), and the solvent was removed by freeze-drying. Amino acid analyses were performed for a 24 h acid hydrolysate with a Durrum D-500 analyser. The full peptide amino acid sequence was determined by automated Edman degradation with a Beckman 890M instrument with the 0.1 M-Quadrol programme 031281, with the use of 2 mg of Polybrene carrier (Niall, 1973). Amino acid residues were identified as their phenylthiohydantoin derivatives separated by h.p.l.c. as described by Bhowan *et al.* (1978) and, in addition, by t.l.c. (Kulbe, 1974). For the first eight stages of cleavage the yield of amino acids was in excess of 80% of the amount of peptide applied.

Assay for the inhibitor protein

The procedure for assay was a minor modification of that described previously (Whitehouse & Walsh, 1982). Briefly, except in assays for the kinetic analyses for determinations of K_i value, the catalytic subunit and the pure inhibitor protein or inhibitory peptide fragments (diluted in 10 mM-Mes/NaOH buffer, pH 6.8, containing 0.5 mg of bovine serum albumin/ml) were preincubated in 60 μ l of 33.3 mM-Mes/NaOH buffer, pH 6.8, containing 5 mM-2-mercaptoethanol, 6.6 mM- MgCl_2 and 0.167 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 10 min and the reaction was then initiated by the addition of 20 μ l of a 30 mg/ml solution of histone IIA (Sigma Chemical Co.). The reaction was for 10 min at 30 °C. Measurement of the extent of phosphorylation and the definition of units of inhibitor activity were as previously described (Whitehouse & Walsh, 1983b). For the fractions obtained from h.p.l.c., the solvents were removed by freeze-drying before assay.

Materials and other methods

Protein concentration was determined by amino acid analysis.

Bovine heart cyclic AMP-dependent protein kinase was purified to homogeneity as described previously (Whitehouse & Walsh, 1983a).

Staphylococcus aureus V8 proteinase was from Miles Laboratories.

Fig. 2. Time course of *Staphylococcus aureus*-proteinase digestion of the inhibitor protein

Purified inhibitor protein (1.4 mg/ml) was incubated in 0.1 M-NH₄HCO₃, pH 7.8, plus 0.16 mg of *Staphylococcus aureus* V8 proteinase/ml for the indicated periods of time at 37 °C. The reaction was terminated by a 60-fold dilution of the reaction mixture into 1.0% trifluoroacetic acid, and inhibitory activity was determined by the standard procedure. Activity was measured either directly (▲), or from the added activities (●) of peptide P28, peptide P36, peptide P41 and inhibitor protein, after their separation by h.p.l.c. (Fig. 3). The inset shows the amounts of peptide P28 (○) and peptide P36 (△) during the course of proteolytic digestion as determined after h.p.l.c. separation (Fig. 3) by integration of the peak of 214 nm absorbance as described by Matthews (1984).

RESULTS

Proteinase digestion of inhibitor protein

Staphylococcal-proteinase digestion of inhibitor protein resulted in an initial rapid fall in inhibitory activity to approx. 50% of the starting value by 10 min, followed by a subsequent slower decline (Fig. 2, ▲ symbols). With 10–15 h of digestion, 30–15% of the original activity still remained, but nevertheless there continued to be a slow inactivation. The peptides produced during this digestion were separated by h.p.l.c. and tested for inhibitory activity. [Peptides have been named on the basis of elution time (Fig. 3).] In the standard gradient, the pure inhibitor protein (Fig. 3a) and pure proteinase (Fig. 3c) were eluted at 43–44 min and 45–46 min retention times respectively. After a 10 min proteolytic digestion (Fig. 3d), the bulk of inhibitor protein had been converted primarily into two major peptides (P33 and P36) plus one minor fragment (P41). Of these, peptide P33 was inactive, peptide P36 had retained approx. 40% of the specific activity of the original inhibitor protein, and the minor fragment, P41, had an activity similar to that of the undigested protein. Further digestion (Figs. 3e–3i) resulted in the rapid degradation of peptide P41 and the slow conversion of peptide P36 into a second active fragment (P28), whose activity was approx. 30% of that of the original inhibitor protein. On prolonged digestion, peptide P28 was further degraded and inactivated. During the 16 h of proteolysis, several other peptides were generated, but none had activity. The combined

activities of peptides P28, P36 and P41, recovered from h.p.l.c., fully accounted for the overall time course of inactivation. This is shown in Fig. 2 by the comparison of the data presented by ▲ and ● symbols. Also shown in the inset of Fig. 2 are the time courses of formation of peptides P36 and P28 and subsequent degradation as taken from the h.p.l.c. profiles.

Most probably peptide P41 represents a rapid digestion of inhibitor protein to a slightly smaller species, since the inhibitor protein has consistently displayed a 1–2 min higher retention time. (As illustrated by the different panels in Fig. 3, retention times in the h.p.l.c. separations were readily reproducible.) There has been insufficient peptide P41 for further evaluation as yet. The combined data of Figs. 2 and 3 clearly suggest a pattern of digestion of: inhibitor protein → peptide P41 → peptide P36 → peptide P28. The first two cleavages appear to occur rapidly, with the second accounting for the initial rapid decline in activity in the first 10–20 min. After this, there is a slower conversion of peptide P36 into peptide P28, but this results in only a small further loss of activity, since the specific activity of peptide P28 is only slightly lower than that of peptide P36.

Composition and activity of peptides P28 and P36

Amino acid analyses for the two peptides are presented in Table 1. Peptide P28, from these analyses, is a peptide of 20–22 amino acid residues. Peptide P36 contains all of the amino acids of peptide P28, including, of note, the single tyrosine, phenylalanine and histidine residues of the original protein. Peptide P36, a peptide of about 47 amino acid residues, appears to comprise about half of the native molecule (Whitehouse & Walsh, 1982). From sequence determination, by the procedures indicated in the Experimental section, peptide P28 was shown to be a 20-amino acid-residue peptide composed of:

A direct comparison of peptide P28, peptide P36 and native inhibitor protein, as inhibitors of the cyclic AMP-dependent protein kinase, is provided by Fig. 4. When tested in the standard inhibitor-protein assay (which uses near-saturating concentrations of histone IIA as substrate for the protein kinase), similar titration patterns were observed for all three (Fig. 4a). The specific activities of peptides P36 and P28, by these titrations, were 40–60% and 30–40% respectively of that of the original protein. The K_i value for peptide P28 was determined by a Henderson (1972) analysis for high-affinity inhibitors, as has also been utilized for the inhibitor protein (Demaille *et al.*, 1977; Whitehouse & Walsh, 1983a). For the Henderson analyses (Figs. 4b and 4c), reactions were performed with Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as the varied-concentration substrate and ATP at 8.0 μ M; this is a less than saturating concentration of ATP because the inhibitor protein has previously been shown to be an uncompetitive inhibitor versus nucleotide (Whitehouse & Walsh, 1983a). The primary Henderson plot for peptide P28 is depicted in Fig. 4(b). Secondary replots of these data (slope versus substrate concentration) are given in Fig. 4(c) for peptide P28 (from the data provided in Fig. 4b) and for inhibitor protein (from data obtained under identical conditions). The direct proportionality of the slope from the initial plots versus substrate concentration shows that both

Fig. 3. H.p.l.c. separation of the proteolytic products obtained by staphylococcal-proteinase digestion of inhibitor protein

The conditions of proteinase digestion were as presented in Fig. 2 legend. After digestion, the samples were adjusted to 1.0% trifluoroacetic acid and applied to a reverse-phase μ Bondapak C_{18} column. The column was washed with solvent A (0.1% trifluoroacetic acid in water) for 10 min at 1 ml/min and then eluted with a linear gradient of solvent A and solvent B (0.1% trifluoroacetic acid/90% acetonitrile in water), starting with 100% solvent A/0% solvent B, and proceeding to 40% solvent A/60% solvent B, in 60 min at 1 ml/min. Fractions (1 ml) were collected, freeze-dried to remove the solvent, and then assayed for inhibitory activity by the standard procedure. (a) Shows the original inhibitor protein (2 μ g), (b) shows inhibitor protein (2 μ g) plus proteinase with the reaction mixture quenched immediately, (c) shows the proteinase alone, and (d)–(i) show 7.5 μ g of inhibitor protein digested by proteinase for the indicated periods of time. The upper trace in each (—) is the A_{214} absorbance, the lower trace (●—●) is inhibitory activity, and the gradient of acetonitrile is denoted by ----. For some separations (b, for example) the column was not washed initially for 10 min with solvent A before initiation of the gradient. This did not affect the concentration in the gradient at which the peptides were eluted, but the broad peak in the earlier fractions is due to absorbance of the Mes buffer in which the inhibitor protein was stored. Retention time is indicated from the start of the gradient.

peptide P28 and inhibitor protein are competitive inhibitors versus protein substrate, as has been shown for the latter previously (Demaillie *et al.*, 1977; McPherson *et al.*, 1979). From the intercepts of the secondary replots, the K_i values of peptide P28 and inhibitor protein were determined to be 0.2 nM and 0.04 nM respectively. [This value for inhibitor protein is somewhat lower than previously measured (Whitehouse & Walsh, 1983a), which may reflect the more consistent preparations of inhibitor protein that can now be obtained.] These data demonstrate that peptide P28 is a potent inhibitor of the

protein kinase, with an activity approx. 20% that of the native molecule.

DISCUSSION

A previous paper by Demaille *et al.* (1979) reported the isolation of a pentadecapeptide from a proteolysed inhibitor protein that retained approx. 10% inhibitory activity. That peptide appears similar to the smallest inhibitory peptide described in the present paper (P28), but the two do not appear to be identical. From the amino

Table 1. Amino acid composition of peptides P28 and P36

The peptides P28 and P36 were isolated by h.p.l.c. after digestion of inhibitor protein for 8 h. Analyses were as given in the Experimental section. Values are averages for three determinations.

	Amino acid composition (mol of residue/mol)			
	Peptide P28		Peptide P36	
	Found	Integer	Found	Integer
Asx	3.35	3	7.56	7-8
Thr	3.07	3	3.98	4
Ser	1.3	1	6.12	6
Glx	0.39	0-1	2.92	3
Pro	0.14	0	0.40	0-1
Gly	2.5	2-3	5.28	5
Ala	3.37	3	6.12	6
Val	0.14	0	1.36	1
Met	0	0	0	0
Ile	1.97	2	4	4
Leu	0.2	0	3.5	3-4
Tyr	1.06	1	0.86	1
Phe	0.97	1	1.13	1
His	0.98	1	1	1
Lys	0.18	0	1.22	1
Arg	3.2	3	3.88	4
Cys	0	0	0	0

acid analysis of the pentadecapeptide provided by Demaille *et al.* (1979), the closest match would appear to be in the sequence of residues 5-18 of peptide P28, with the exception of the absence of one of the three arginine residues, plus additional threonine and glutamate/glutamine residues present in the amino acid analyses given by Demaille *et al.* (1979), but unaccounted for by this sequence in peptide P28. In our studies, with sequential degradation, no peptide smaller than P28 has been observed that has retained activity. Possibly, the apparent discrepancies between the two investigations are because Demaille *et al.* (1977, 1979) used, as a source of the peptide, inhibitor protein that had already been degraded to the I' form (McPherson *et al.*, 1979). Alternatively, with a lack of material, the analyses by those investigators may have been incomplete. In the present paper we describe the cleavage of the I form of the inhibitor protein; this is the only species detected in initial cell extracts (McPherson *et al.*, 1979). We have obtained the peptide P28 in yields of 20-30%. In preliminary work this peptide has now been obtained by direct synthesis and is potently inhibitory (B. E. Kemp, H.-C. Cheng & D. A. Walsh, unpublished work).

The results given in the present paper permit an initial assessment of what constitutes the domain of interaction between the inhibitor protein and the catalytic subunit of the protein kinase. This is of importance and interest because the affinity of the catalytic subunit for the inhibitor protein is exceptionally high (K_i 0.04-0.5 nM); only that for the regulatory subunit is higher (K_d 0.01-0.20 nM; Beavo *et al.*, 1975; Hoffman, 1980; Rymond & Hoffman, 1982), whereas the best protein substrate binds with an affinity four orders of magnitude weaker (Whitehouse *et al.*, 1983; Mendicino *et al.*, 1978).

The extensive work of Kemp *et al.* (1974, 1977), Zetterquist *et al.* (1976) and Feramisco *et al.* (1980) has shown that an Arg-Arg basic subsite, situated on the N-terminal side and with one residue between this pair and the phospho-acceptor serine residue, is one major recognition point on peptide and protein substrates by the catalytic site of the protein kinase. This basic subsite is a feature of all substrates for this enzyme, although often one of the amino acid residues in the pair is a lysine (Cohen *et al.*, 1983). That this basic subsite only in fact provides a relatively weak interaction point, however, is illustrated by the observation that the substitution of alanine for serine in the peptide Kemptide results in its conversion from an effective substrate (K_m 5 μ M) into being a fairly weak inhibitor (K_i 0.2 mM). This affinity for the catalytic subunit of the alanine-substituted peptide is almost six orders of magnitude weaker than that of the inhibitor protein (Whitehouse *et al.*, 1983). Similarly, the substitution of D-serine for the L-serine in Kemptide results in a peptide that is neither a substrate nor an inhibitor ($K_i > 5$ mM), even though it contains the pair of basic arginine residues (Reed *et al.*, 1985). Kinetic and c.d. studies (Whitehouse *et al.*, 1983; Reed & Kinzel, 1984a, b; Reed *et al.*, 1985) have led to the proposal that the interaction of peptides or protein substrates at the catalytic site of the protein kinase involves a minimum of three steps: firstly, an ionic interaction with the basic subsite; secondly, the assumption by the substrate of a random coil conformation in the active site around which there is closure; thirdly, an interaction with the serine hydroxy group at the catalytic site, which induces a conformational shift in the enzyme before the hydroxy group then serving as the phospho-acceptor site.

The sequence of the inhibitory peptide P28 shows that it, too, possesses the Arg-Arg basic subsite, suggesting that for it, as well, this may be the first recognition point by the catalytic subunit. Of potential interest, the sequence Gly-Arg-Waa-Xaa-Arg-Arg-Yaa-Zaa in peptide P28 is shared with the bovine type II regulatory subunit, where Zaa in the latter is the serine site of autophosphorylation (Takio *et al.*, 1982). In studies by Feramisco *et al.* (1980), peptides having the Arg-Arg residues at increasing distances from the phospho-acceptor serine residue were tested as substrates. The peptide with the pair of arginine residues at positions 5 and 6 on the N-terminal side of the serine residue was not as good a substrate as the peptide with the two arginine residues at positions 2 and 3 (as in Kemptide), but it had a higher affinity for the protein kinase than those peptides with arginine residues in the intervening positions. This suggests that, in the inhibitor protein and regulatory subunit, the optimum situation for binding to the catalytic subunit may, in part, be a consequence of the presence of arginine residues in all three positions. Of interest, the sequence in the inhibitor protein of Arg-Thr-Xaa-Arg-Arg is homologous to the sequence in the epidermal-growth-factor receptor and the *V-erb-B*-gene product that is phosphorylated on the threonine residue by the Ca^{2+} /phospholipid-dependent protein kinase (Hunter *et al.*, 1984).

The nature of the other features of the amino acid sequence that result in the inhibitor protein and the inhibitory peptide P28 having such avid affinities for the catalytic subunit are not obvious by inspection. It is of interest that alanine, a non-phosphorylatable residue, but of similar size to serine, is located where serine would be

Fig. 4. Inhibition of protein kinase activity by inhibitor protein, peptide P28 and peptide P36

(a) Comparison of inhibition by inhibitor protein (■), peptide P36 (○) and peptide P28 (□). Protein kinase activity was determined with the standard inhibitor protein assay, with near-saturating concentrations of histone IIA as substrate. From three equivalent titrations peptides P28 and P36 have exhibited specific activities in the ranges 30–40% and 40–60% respectively of that of the inhibitor protein. (b) Henderson analysis of inhibition by peptide P28. Protein kinase activity was determined in the presence and in the absence of peptide P28 at Kemptide concentrations of 15 μM (▲), 8 μM (△), 4 μM (●) and 2 μM (○) and an ATP concentration of 8 μM . Data are plotted in accordance with Henderson (1972), where $[I]_t$ is total peptide concentration, and V_t and V_0 are the reaction velocities in the presence and in the absence of peptide P28 respectively. Peptide P28 concentrations used ranged from 2.4 to 18.2 nM, catalytic-subunit concentration was 1.36 M, and other conditions were as follows. Peptide and catalytic subunit were diluted in 10 mM-Mes buffer, pH 6.8, containing 0.5 mg of bovine serum albumin/ml. The diluted peptide solution (20 μl) was added to tubes containing 40 μl of 50 mM-Mes buffer, pH 6.8, containing 5 mM-MgCl₂, 3.75 mM-2-mercaptoethanol and 16 μM -[γ -³²P]ATP (250–300 d.p.m./pmol) plus the appropriate concentration of Kemptide. Tubes were preincubated for 6 min at 30 °C, and the reaction was initiated by addition of 20 μl of catalytic subunit. The reaction was stopped after 3 min by the addition of 60 μl of 50% (v/v) acetic acid, and the reaction tubes were placed on ice. The extent of phosphorylation was determined with the use of phosphocellulose paper as described previously (Whitehouse & Walsh, 1983a). Total phosphorylated substrate was never greater than 10% of total substrate available. (c) Replot of slopes of Henderson plots for either peptide P28 (○) or inhibitor protein (●). For peptide P28, the slopes from the data presented in (b) were obtained by linear-regression analysis. For inhibitor protein, Henderson-plot analyses were determined exactly as indicated in (b) with concentrations of inhibitor protein in the range 1.1–3.2 nM. K_i values, determined from the intercept of the ordinate axis were: peptide P28, 0.2 nM; inhibitor protein, 0.04 nM.

in relationship to the basic arginine subsite, were the sequence that of a substrate. Clearly, the presence of alanine is not of consequence as an entity enhancing binding, since, as discussed above, synthetic peptides with alanine in this location are poor inhibitors. It is of possible interest that Demaille *et al.* (1979) reported that the modification of the single tyrosine residue in the inhibitor protein by acetylation, or cleavage by chymotrypsin, which will most typically occur at tyrosine or phenylalanine, markedly diminished inhibitory activity. As noted from the sequence, the single tyrosine and phenylalanine residues of the inhibitor protein are both included in the inhibitory peptide P28, but are at some distance from the Arg-Arg basic subsite. Further degradation of peptide P28 resulted in inactivation, which, given the specificity of the proteinase used, may be expected to have removed the five *N*-terminal amino acid residues, including the tyrosine residue. Given the previous data, obtained independently by kinetic analyses (Whitehouse *et al.*, 1983) and c.d. (Reed & Kinzel, 1984a, b), that the hydroxy group of serine is important, not only as the phospho acceptor, but also to enhance binding, a possible role for the tyrosine residue in the inhibitor may be envisioned. Were, in fact, the conformation assumed by the inhibitor protein to result in the tyrosine hydroxy group being in a position to mimic the serine hydroxy

group in terms of binding, but not as a phospho acceptor, this might account for a significant component of the strength of binding of the inhibitor protein to the catalytic subunit. In support of this, Demaille *et al.* (1979) have noted that acetylation of this single tyrosine residue in the native inhibitor protein decreased the inhibitory activity. The distance of the tyrosine residue from the basic subsite may thus be required to permit such an orientation of binding of the inhibitor protein. Knowledge of the sequence of peptide P28 now permits such possibilities to be investigated.

Note added in proof (received 16 August 1985)

Concurrent studies by Scott *et al.* (1985), using a different proteolytic strategy, isolated from the inhibitor protein a 20-residue peptide. This latter peptide is different from peptide P28, having the sequence initiated by Ile-7 and having six additional C-terminal amino acid residues. In contrast with peptide P28, this latter peptide had a reported K_i of 0.8 μM , thus emphasizing the importance of the *N*-terminal six amino acid residues of peptide P28 in its binding to the protein kinase.

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