Evidence for an Essential Arginine Recognition Site on Adenosine 3':5'-Cyclic Monophosphate-Dependent Protein Kinase of Rabbit Skeletal Muscle

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On the basis of the chemical and structural features of the amino acid sequences in the vicinities of phosphorylatable hydroxyamino acid residues in several of the well-known protein substrates for skeletal-muscle cyclic AMP-dependent protein kinase, it is hypothesized that the phosphorylatable residue at position i and arginine residue at position $i-3$ of these protein substrates are located on a peptide turn on the hydrophilic protein surface. It is further hypothesized that there is an arginine-recognition site near the active centre on the protein kinase. This site is essential for the function of cyclic AMP-dependent protein kinase, for, not only does it recognize specifically the exposed arginine residue of the protein substrate, but, more importantly, via the interaction with arginine- $(i-3)$, it may help to steer the topologically adjacent serine- i into proper orientation on the nearby active centre for phosphorylation. Model-building and kinetic data that provide support for the proposed hypotheses are presented.

Cohen and co-workers have elegantly identified the primary amino acid sequences near the phosphorylated sites in several of the best protein substrates for skeletal-muscle cyclic AMP-dependent protein kinase (Yeaman *et al.*, 1977; Cohen *et al.*, 1977). On inspection of these sequences, some remarkable features of the protein substrates surrounding the phosphorylated site are revealed.

(1) These authors note that two basic amino acids are present on the N -terminal side of the phosphorylated hydroxy amino acid (either serine or threonine). More specifically, if the phosphorylated residue is designated as the i th residue in these sequences, arginine is invariably found at residue $i-3$ in all substrates.

(2) We further note that residues with marked hydrophobic character, such as valine, leucine, isoleucine, phenylalanine and tyrosine, are conspicuously absent from the tetrapeptide sequence from arginine- $(i-3)$ to the phosphorylated i th residue for the great majority, although not all, of the protein substrates.

(3) Moreover, sequences that are well known to form turns on the surface of protein molecules (Lewis *et al.*, 1971; Kuntz, 1972) are persistently found in the tetrapeptide residues $(i-3)-i$ for the great majority of the protein substrates. These are the uninterrupted sequences of three or more of any combination of glycine, proline, aspartate, glutamate, arginine, lysine, serine, threonine, histidine, asparagine, glutamine and tyrosine. Alanine is included if it alone is adjacent to one of the above (Kuntz, 1972).

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From the structural features given above, we suggest the tentative hypothesis that the hydroxy amino acid residue, i , is characteristically located at a peptide turn on the hydrophilic surface of the protein substrate, thus enabling its hydroxy group to be as fully exposed as possible to the cyclic AMP-dependent protein kinase.

We further hypothesize that the occurrence of arginine- $(i-3)$ in the turn not only makes arginine available for possible binding of anionic groups but also contributes to stabilization of the turn. Implicit in this hypothesis is that, in close proximity to the active centre of protein kinase, there is a recognition site of the enzyme that binds to the spatially exposed arginine- $(i-3)$ in the protein substrates.

Unfortunately, secondary or tertiary structures in the best protein substrates for cyclic AMP-dependent protein kinase as listed by Cohen and co-workers (Cohen *et al.*, 1977) are not yet available, hence the proposed topological locations of the functional groups of arginine- $(i-3)$ and serine- i (or threonine- i) exposed simultaneously at a peptide turn remain to be verified. A seemingly favourable sequence does not always ensure that the peptide turn or bend will occur. For instance, sequence Arg₍₂₁₎-Gly-Tyr-Ser₍₂₄₎-Leu-Gly of the native lysozyme, by inspection, seems to exhibit all the necessary features of a protein substrate. However, serine-24 is not a residue characteristically located at a peptide turn in the known secondary structure of native lysozyme (Kuntz, 1972), and it is partially buried in the three-dimensional structure of the protein; hence this segment does not meet the sufficient condition to serve

as a substrate. Indeed, it has been reported that native lysozyme cannot be phosphorylated by protein kinase (Bylund & Krebs, 1975). However, the synthetic fragment Arg-(1)-Gly-Tyr-Ser-(4)-Leu-Gly can serve as a substrate for protein kinase, albeit a poor one (Kemp *et al.*, 1976), and there is a much greater chance that this fragment assumes a β -bend conformation. It is well known that in aqueous solution small oligopeptides do not readily form α -helices, and there is a finite probability of occurrence of β -bends in small oligopeptides (Anfinsen & Scheraga, 1975). It is therefore likely that some of the synthetic fragments may have their arginine-1 and serine-4 functional groups simultaneously exposed in close proximity on β -bends, rendering them more susceptible to recognition and phosphorylation by protein kinase than in the native lysozyme.

The assumption that the guanidinium ion of arginine-(*i*-3) in protein substrates may be specifically involved in stabilizing interactions with the recognition site of cyclic AMP-dependent protein kinase can be tested experimentally. In the present paper, experiments were designed to assess the effect of polyarginine on the enzymic activities of cyclic AMP-dependent protein kinase and its catalytic subunit isolated from rabbit skeletal muscle. We found that polyarginine, but not polylysine, has a profound inhibitory effect on the protein kinase activity, suggesting that the enzyme may indeed have a recognition site for the protein substrate and that the recognition site can be blocked competitively by polyarginine.

Experimental

Materials

DEAE-cellulose (DE-52) and CM-cellulose (CM-52) were purchased from Whatman, Clifton, NJ, U.S.A. Sephadex G-100 was purchased from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. Two batches of poly-L-arginine hydrochloride were used: one (mol.wt. 10000-20000; control no. 12546) was obtained from United States Biochemical Corp., Cleveland, OH, U.S.A., and the other (type V B; mol.wt. 15000-50000) from Sigma, St. Louis, MO, U.S.A. Poly-L-lysine hydrobromide (type V; mol.wt. 25000; lot no. 26C-50051) and histone type IIA were purchased from Sigma. Poly-L-glutamic acid (mol.wt. 4100; lot no. 602) and poly-L-aspartic acid (mol.wt. 4260; lot no. AS43) were purchased from Miles-Yeda, Rehovot, Israel. All the poly-(L-amino acid) solutions were adjusted to pH 7.0 before use.

$^{32}\text{P}_i$ was purchased from New England Nuclear Corp., Boston, MA, U.S.A., and used to prepare [γ - ^{32}P]ATP by the method of Glynn & Chappell (1964).

Purification of cyclic AMP-dependent protein kinase and its catalytic subunit

The cyclic AMP-dependent protein kinase (type I) was purified from rabbit skeletal muscle as described previously (Huang & Huang, 1975). The catalytic subunit of the cyclic AMP-dependent protein kinase (type I) was prepared by the method of Bechtel *et al.* (1977) with the following minor modification. The fresh rabbit skeletal muscle was homogenized with 2.5 vol. of 10mM-potassium phosphate buffer, pH 7.2, containing 4mM-EDTA and 6mM-mercaptoethanol. After the homogenate was centrifuged at 7000g for 30 min, the supernatant was fractionated by $(\text{NH}_4)_2\text{SO}_4$ (50%-saturated). The resultant precipitate was dissolved in 10mM-Pipes (1,4-piperazinediethanesulphonic acid) buffer, pH 7.0, containing 2mM-EDTA and 15mM-mercaptoethanol, and dialysed against 2×10 vol. of the same buffer. The dialysed enzyme was centrifuged at 78000g for 2 h, then the supernatant was applied to a DEAE-cellulose ion-exchange column (8cm $\times$ 30cm). The cyclic AMP-dependent protein kinase (peak I) was then eluted with 100mM-NaCl in the same buffer. The subsequent steps were identical with those described by Bechtel *et al.* (1977).

Assay of protein kinase activity

Protein kinase activity was assayed radioisotopically as described elsewhere (Huang & Huang, 1975). The assay solution (total volume 90 μ l) contained: glycylglycine buffer, 5 μ mol, pH 7.0; MgCl_2 , 0.5 μ mol; histone type IIA, 0.05mg; [γ - ^{32}P]ATP, 3nmol (specific radioactivity 50-100 c.p.m./pmol); cyclic AMP, 0.1nmol when added; poly(amino acid) at various concentrations as stated in the relevant Figure legend. The enzymic reaction was initiated by adding a portion (10 μ l) of purified protein kinase. After incubation at 30°C for 10 min, 75 μ l of the reaction mixture was spotted on a piece of filter paper. The paper was then washed in 10% (w/v) trichloroacetic acid and counted for radioactivity (^{32}P) as previously described. The reaction of protein kinase with poly-(L-amino acids) was first carried out at room temperature (about 23°C) for 10 min.

Other methods

Purity of the enzyme preparation was checked by polyacrylamide-gel electrophoresis as described previously (Huang & Huang, 1975). Protein concentration was determined by the method of Lowry *et al.* (1951), with albumin as standard.

Results

The cyclic AMP-dependent protein kinase and its catalytic-subunit preparations were highly purified as

judged by polyacrylamide-gel electrophoresis (Huang & Huang, 1975). The position of the enzyme activity on the polyacrylamide gel was identical with the protein position detected by staining with Coomassie Blue in a duplicate gel (Huang & Huang, 1975). The polypeptide chains of the holoenzyme determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were found to have mol.wts. 49000 and 41000, corresponding to the inhibitory and the catalytic subunit respectively (Hoppe & Wagner, 1977). The catalytic-subunit preparation showed only one band after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, of mol.wt. 41000.

The incorporation of radioactivity from [γ - ^{32}P]-ATP into the protein substrate (histone IIA) was linear with time and with the enzyme concentration up to the production of at least 110 pmol of ^{32}P -labelled histone.

Reaction of polyarginine and polylysine with protein kinase

Additions of polyarginine to a cyclic AMP-dependent protein kinase resulted in both inactivation of the enzyme and a conversion of the enzyme from a cyclic AMP-dependent form into an independent form. In contrast, polylysine causes primarily the conversion (Table 1). Similarly to the effect of protamine (Miyamoto *et al.*, 1973), the observation that the cyclic AMP-dependence of the holoenzyme is abolished on addition of polyarginine or polylysine presumably reflects the dissociation of cyclic AMP-dependent protein kinase by the poly(basic amino acid). To simplify our studies of the effect of the poly(amino acid) on enzymic activity and to avoid possible complications caused by the inhibitory subunit, subsequent studies were carried out with the purified catalytic subunit of the cyclic AMP-dependent protein kinase.

The activity of the catalytic subunit was observed to decrease with increasing concentration of polyarginine added, and the inhibition was unchanged when a different batch of polyarginine was used. In contrast with the dramatic inhibitory effect of polyarginine, the catalytic-subunit activity was not inhibited by polylysine (Fig. 1). These results suggest that polyarginine may react specifically with the catalytic subunit.

Polyarginine inhibition

If polyarginine has multiple binding sites that interact with MgATP^{2-} , a substrate for protein kinase, one would expect that a lesser amount of the substrate would be available to the enzyme for catalysis when polyarginine is added, and hence less

Table 1. *Effects of polyarginine and polylysine on the enzymic activity of protein kinase assayed in the presence and absence of cyclic AMP*

Purified cyclic AMP-dependent protein kinase (40 $\mu\text{g}/\text{ml}$ in 0.1 M-glycylglycine, pH 7.0) was preincubated with various amounts of polyarginine (lot 12546) or polylysine, given in the first column, at room temperature for 10 min. A portion (10 μl) of the preincubated enzyme was then added to the assay mixture for measuring the enzymic activity in the presence and absence of cyclic AMP under the assay condition described in the Experimental section. The enzymic activity, given in columns 2-5, is expressed as pmol of ^{32}P incorporated into histone/10 min. The final polyarginine or polylysine concentration in the assay mixture is one-ninth of that in the preincubation mixture.

Poly(amino acid) concentration ($\mu\text{g}/\text{ml}$)	Activity			
	Polyarginine		Polylysine	
	-Cyclic AMP	+Cyclic AMP	-Cyclic AMP	+Cyclic AMP
0	1.9	53	1.9	53
6	9	47	28	49
30	43	36	34	42
100	44	43	39	47
300	23	20	50	44
400	14	10	50	45

Fig. 1. *Effect of polyarginine (●) and polylysine (○) on protein kinase activity*

Activity was measured by the method described in the text.

radioactive product would result. Kinetically this type of interaction

would lead to the following competitive Lineweaver-Burk type equation:

$$\frac{1}{v} = \frac{1}{V} \left\{ 1 + \frac{K_A}{\left(1 + \frac{[\text{polyarginine}]}{K_P} \right)} \cdot \frac{1}{[\text{MgATP}^{2-}]} \right\}$$

where V is the maximum velocity, and K the dissociation constant. In the presence of various fixed concentrations of polyarginine, double-reciprocal plots of initial-velocity data for MgATP^{2-} , shown in Fig. 2(a), clearly demonstrated that the polyarginine is non-competitive with respect to MgATP^{2-} . This kinetic behaviour is obviously not compatible with the simple assumption that polyarginine interacts specifically with MgATP^{2-} . However, the non-competitive pattern does imply that polyarginine and MgATP^{2-} bind to separate sites on the catalytic

Fig. 2. Lineweaver-Burk plots of initial velocity versus (a) $[\text{MgATP}^{2-}]$ at a fixed histone concentration (0.55 mg/ml) and (b) $[\text{histone}]$ at a fixed concentration of MgATP^{2-} (33 nmol/ml)

Polyarginine was added as follows: (a) ●, 0 $\mu\text{g/ml}$; Δ , 2.5 $\mu\text{g/ml}$; $\square$, 10 $\mu\text{g/ml}$; (b) ●, 0 $\mu\text{g/ml}$; Δ , 2.5 $\mu\text{g/ml}$; $\blacksquare$, 5 $\mu\text{g/ml}$.

subunit and that MgATP^{2-} cannot donate its γ -phosphate group at the active centre when polyarginine is bound to the other site.

If polyarginine, on the other hand, interacts specifically with the recognition site of the enzyme, which presumably lies near the active centre of the enzyme, as postulated in the introduction, polyarginine and the protein substrate with known Arg-X-Y-Ser sequence would then compete for the same recognition site of the enzyme, and consequently competitive-type kinetics would be expected. Double-reciprocal plots of initial-velocity data for histone, shown in Fig. 2(b), at various fixed concentrations of polyarginine indicated that polyarginine is indeed competitive with respect to histone. This kinetic behaviour is in complete accord with our hypothesis that the recognition site lies near the phosphorylation site of the enzyme and is specific for interaction with an arginine residue of the substrate. Various kinetic parameters calculated from secondary plots of Fig. 2 are given in Table 2.

Substrate interaction with polyglutamate or polyaspartate

If our hypothesis that the arginine residue near the potential phosphorylatable site of the protein substrate is spatially exposed at a peptide turn on the protein surface is a reasonable one, then the protein substrate can be expected to interact with polyglutamates or polyaspartates via its exposed arginine residue. Such complex-formation in the reaction sequences

would lead to a competitive Lineweaver-Burk-type equation that is very similar to that described above for the possible polyarginine $\cdot \text{MgATP}^{2-}$

Table 2. Apparent kinetic constants associated with poly(amino acid) inhibition of the reaction catalysed by the catalytic subunit of protein kinase

The data were calculated from the slopes of Lineweaver-Burk plots which show competitive and non-competitive inhibitors as shown in Figs. 2 and 3 versus inhibitor concentration. Errors are about 10%.

Inhibitor	Variable substrate	Fixed substrate	K_i ($\mu\text{g/ml}$)
Polyarginine	ATP	Histone	2.4
	Histone	ATP	3.7
Polyglutamate	ATP	Histone	30.0
	Histone	ATP	20.5
Polyaspartate	ATP	Histone	33.3
	Histone	ATP	13.9

Fig. 3. Lineweaver-Burk plots of initial velocity versus (a) histone at a fixed concentration of $[\text{MgATP}^{2-}]$ (33 nmol/ml) and (b) $[\text{MgATP}^{2-}]$ at a fixed histone concentration (0.55 mg/ml)

Polyglutamate was added as follows: (a) ●, 0 $\mu\text{g/ml}$; Δ , 12.5 $\mu\text{g/ml}$; $\square$, 25 $\mu\text{g/ml}$; (b) ●, 0 $\mu\text{g/ml}$; $\blacktriangle$, 20 $\mu\text{g/ml}$; $\blacksquare$, 40 $\mu\text{g/ml}$.

complex-formation. The inhibition pattern for polyglutamate against histone is shown in Fig. 3(a). A similar pattern is also observed for polyaspartate. These reciprocal plots indicate that these polypeptides function as linear competitive inhibitors with respect to histone. Secondary slope plots are linear and the values for $K_{i,\text{slope}}$ are given in Table 2. These competitive inhibitions with respect to histone are consistent with our assumption that an arginine residue is located on the surface of the protein substrate; moreover, it is essential for the enzymic activity.

Investigations were also made of the inhibition by polyglutamate and polyaspartate when the concentration of MgATP^{2-} was varied. Results indicate that these polypeptides are non-competitive inhibitors with respect to MgATP^{2-} (Fig. 3b).

Discussion

The cyclic AMP-dependent protein kinase in mammalian tissue appears at present to be a specific enzyme through which cyclic AMP carries out its function as a ubiquitous 'second messenger' in response to hormonal stimuli. In the hormonal regulation of glycogen metabolism in skeletal muscle, for instance, cyclic AMP-dependent protein kinase catalyses the phosphorylation of phosphorylase kinase, glycogen synthase and phosphoprotein phosphatase III inhibitor I by transferring the terminal phosphate group of MgATP^{2-} to serine or threonine residues of these enzymes or proteins. The amino acid sequence at the phosphorylated site of some of these enzymes and proteins involved in glycogen metabolism and other proteins has been documented (Cohen *et al.*, 1977). Of particular interest in these sequence studies is the observation that an arginine residue is always found at the third residue from the *N*-terminal side of the phosphorylated site. This specific location raises the question of the molecular basis for the importance of this arginine residue.

On the basis of the common chemical and structural features of the amino acid sequence at the site of phosphorylation, we propose that the specific arginine residue and the serine (or threonine) residue are located at peptide turns on the surface of the protein substrate. One such turn, a type-I β -bend (Venkatachalam, 1968), is shown diagrammatically in Fig. 4. Serine-*i* and arginine-(*i*-3) residues are in spatial proximity to each other. In fact, the main-chain geometry of the β -bend is fixed in such a way that the backbone amide -NH group of serine-*i* forms a hydrogen bond with the backbone amide C=O group of arginine-(*i*-3); moreover, the aliphatic hydroxy oxygen of serine-*i* may also form a hydrogen bond with the -NH group of the guanidinium ion of arginine-(*i*-3).

Examinations of Pauling-Corey-Koltun models of the type-I β -bend structure of Arg-(*i*-3)-X-Y-Ser-*i* suggest that a hydrogen bond between the side chains of arginine-(*i*-3) and serine-*i* can be achieved most readily if another arginine residue is located at the X position or the (*i*-4) position. This is due to the fact that the positive charge of guanidinium ion is much more delocalized than the charge on the primary amine group of lysine. Hence electrostatic repulsions between the two adjacent guanidinium ions will shift the side chain of arginine-(*i*-3) closer to the side chain of serine-*i* and thus have the effect of bringing the -NH group of guanidinium ion and the oxygen atom of the hydroxy group into close proximity, allowing for stable hydrogen-bonding. From similar electrostatic reasoning, the sequence Arg-Lys-Y-Ser will have a higher probability of occurrence of a β -bend than Arg-N-Y-Ser, where N is a neutral residue. Consequently, the most interest-

Fig. 4. Schematic diagram illustrating the type-I β -bend structure of Arg-X-Y-Ser

ing observations that two adjacent basic amino acids are present on the *N*-terminal side of the phosphorylated sites of protein kinase substrate are expected, provided that the phosphorylatable residue at position *i* and the arginine residue at *i*-3 are adopting a side-chain (*i*-3) $\rightarrow$ *i* hydrogen-bonded conformation in a β -bend structure as hypothesized in Fig. 4.

By using probability factors listed by Chou & Fasman (1974), it has been calculated by Daile *et al.* (1975) that several phosphorylatable segments, Arg-X-Y-Ser, from protein substrates of cyclic AMP-dependent protein kinase have a high probability of occurrence as β -bends. Since similar higher probabilities have also been obtained for sequences around serine residues that are not phosphorylatable, Daile *et al.* (1975) did not emphasize their calculation. It should be noted, however, that these empirical computations are based on the assumption that the four residues in the β -bend structure behave independently. If the extra stabilization energy for the β -bend structure as provided by the hydrogen bond between the side chains of arginine and serine (Fig. 4) is taken into account, the tetrapeptide around the potential phosphorylatable site of protein kinase substrate, especially if it is Arg-Arg-Y-Ser and Arg-Lys-Y-Ser, may have an even higher probability of occurrence as a β -bend than has been realized previously.

Kemp *et al.* (1977) reported that the synthetic heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly, corresponding to a segment of the phosphorylated sequence

in pig liver pyruvate kinase, serves as a better substrate than the synthetic peptide Leu-Arg-Lys-Ala-Ser-Leu-Gly for the protein kinase. This result is consistent with our proposed model of the secondary structure of protein substrates near the phosphorylatable site. As discussed above, replacement of lysine by arginine in position X of Arg-X-Y-Ser would promote hydrogen-bond formation between the side chains of arginine-(*i*-3) and serine-*i*; hence this substitution would increase the probability of occurrence of β -bends for the synthetic heptapeptide in solution. Since arginine residing in the β -bend structure is, according to our hypothesis, recognized by protein kinase, one would expect the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly to be a better substrate than Leu-Arg-Lys-Ala-Ser-Leu-Gly. Furthermore, one can predict that the synthetic peptide Leu-Arg-Lys-Ala-Ser(*i*)-Leu-Gly can serve as a much better substrate for protein kinase than Leu-Arg-Ala-Ala-Ser(*i*)-Leu-Gly for the following two reasons. (1) Since the side chain of alanine-(*i*-2) in the second peptide is neutral, it cannot repel electrostatically the side chain of arginine-(*i*-3), whereas the side chain of lysine-(*i*-2) in the first peptide can repel electrostatically, albeit not as effectively as if it were arginine-(*i*-2), the side chain of arginine-(*i*-3); the former is thus expected to have a higher probability in forming a β -bend structure in solution. (2) The synthetic heptapeptide Leu-Arg-Ala-Ala-Ser(*i*)-Leu-Gly does not possess the chemical characteristics of peptide sequences that are well known to form turns on the protein surface, as discussed in the introduction. In other words, owing to the presence of leucine-(*i*-4), Ala-Ala and leucine-(*i*+1) in the sequence, the probability of having a β -bend structure for this synthetic heptapeptide is likely to be extremely low. Consequently, the synthetic peptide Leu-Arg-Lys-Ala-Ser(*i*)-Leu-Gly should serve as a substantially better substrate than the heptapeptide Leu-Arg-Ala-Ala-Ser(*i*)-Leu-Gly for protein kinase. Experimental data obtained by Kemp *et al.* (1977) indeed bear this out.

There is one implicit assumption in the foregoing discussion that should be emphasized, namely that, in addition to the β -bend structure of a segment of four amino acids in the protein substrate from arginine-(*i*-3) to the phosphorylatable residue *i*, the protein kinase has an arginine-recognition site near its active centre. This site is essential for the function of the enzyme, because not only does it act as a site for recognition of the exposed arginine on the surface of the protein substrate, but also, via the interaction with arginine-(*i*-3), it may help to steer the spatially adjacent serine-*i* into a proper orientation on the nearby active centre for phosphorylation. One experimental approach that we have taken to test the hypothesis of a specific arginine-recognition site on protein kinase is kinetic studies of the enzyme under various conditions, as elaborated below.

Comparative studies of catalytic-subunit activity in the presence of polyarginine and polylysine indicate that it is specifically inhibited by polyarginine (Fig. 1). These data are consistent with the assumption that arginine, but not lysine, is recognized by the catalytic subunit; hence polyarginine can inactivate the catalytic subunit by binding to the arginine-recognition site of the enzyme. This interpretation is further supported by the kinetic data (Fig. 2b), which show that polyarginine functions as a linear competitive inhibitor with respect to histone as protein substrate. Furthermore, the possibility that the inhibition reaction is due to rapid interaction between polyarginine and MgATP^{2-} is ruled out by our kinetic data, as illustrated in Fig. 2(a).

Additional experimental data are shown in Fig. 3. These results can be taken as evidence that the guanidinium ion of the arginine residue near the potentially phosphorylatable hydroxy amino acid of the enzyme substrate must be exposed on the protein surface, and hence reaches out to interact with a carboxy group of the added polyglutamates or polyaspartates. In addition, the competitive inhibition by polyglutamates or polyaspartates suggests that the interaction of arginine-($i-3$) of the protein substrate with polyglutamates or polyaspartates will prevent the protein substrate from specific recognition and phosphorylation by protein kinase.

Finally, it should be pointed out that our kinetic data are consistent with our hypothesis about the secondary structure of Arg-X-Y-Ser in the protein kinase substrate; they are also internally consistent with the assumption that there is a recognition site for arginine, but not lysine, near the active centre of the protein kinase. However, these kinetic data do not prove these assumptions. Detailed structural studies are needed for the verification of our hypotheses. We are also aware that the structural model presented in Fig. 4 is not a complete one. For instance, we did not discuss the effect of various Y residues in the sequence Arg-($i-3$)-X-Y-Ser(i) on the stability of the β -bend structure, although it is reasonable to believe that glycine and alanine, which do not have hydrogen-bond acceptors or donors in their side chains and hence cannot compete with the guanidinium group of arginine-($i-3$) for the hydroxy group of serine- i , are preferable at the Y-position for stable β -bend structures. In addition, we are aware that the model may be a gross over-simplification of a true secondary structure of the protein substrate, when the three-dimensional tertiary structure of the

protein substrate is taken into account. Despite all of the shortcomings, the overall hypothesis does appear to be a reasonable one to explain, at least to a first approximation, the substrate specificity of cyclic AMP-dependent protein kinase. Moreover, one potential usefulness of the model lies in its predictions. For example, the third residue from the N -terminal site of the phosphorylated site 2 of glycogen synthase has not yet been identified as arginine or lysine (Proud *et al.*, 1977). Our structural model suggests that it is most likely to be an arginine residue. Only future experiments can tell the validity of our prediction.

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