Noncovalent Active Site Interactions Enhance the Affinity and Control the Binding Order of Reversible Inhibitors of the cAMP-dependent Protein Kinase*

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The peptides, Leu-Arg-Arg-Ala-Leu-Gly-NH₂, Leu-Arg-Arg-Gln-Ala-Leu-Gly-NH₂, and Leu-Arg-Asn-Ala-Leu-Gly-NH₂, serve as active site-directed inhibitors of the cAMP-dependent protein kinase from bovine cardiac muscle. The Asn-containing peptide is a 10-fold more potent inhibitor than its Ala- and Gln-containing counterparts. All three peptides are linear competitive inhibitors versus a peptide-based substrate and uncompetitive inhibitors versus MgATP. The enhanced inhibitory potency of the Asn-peptide, in conjunction with the observed loss of ATPase activity of the enzyme in the presence of the inhibitor, suggests that asparagine may serve as a through-space isostere of serine. The uncompetitive inhibition pattern displayed by amide-capped peptides versus MgATP indicates that these species bind in an ordered fashion to the cAMP-dependent protein kinase, with MgATP binding first.

Protein kinases have been implicated as participants in a variety of biological phenomena, including the molecular events that constitute memory (1) and carcinogenesis (2). Since substrate recognition is a key element in these complex biochemical pathways, it is not surprising that the noncovalent active site interactions responsible for substrate specificity have come under close scrutiny (3–6). Much information can be gained concerning the nature of these active site interactions through the use of reversible inhibitors. We have developed a protocol to assess the manner in which these inhibitors interact with the active site of the cAMP-dependent protein kinase (“A-kinase”). We have found that the inhibitory efficacy displayed by a peptide-based mimic of a naturally occurring A-kinase inhibitor is dependent upon a crucial active site interaction. Furthermore, a second critical active site interaction appears to control the pathway (ordered versus random) by which peptide-based inhibitors bind to the cAMP-dependent protein kinase.

Materials and Methods

All chemicals were obtained from Aldrich, except for [γ-32P]ATP (Du Pont-New England Nuclear), cAMP (Fluka), protected amino acid derivatives (U. S. Biochemical Corp.), and Universol scintillation mixture (ICN Radiochemical). Phosphocellulose P 81 paper discs were purchased from Whatman, and "Baker-flex" polyethyleneimine-amide gel electrophoresis, which displayed a single band at a molecular mass of 41,000 daltons. Ellman's reagent titrated the cysteine residues to 2.05-2.10 sulfhydryls/molecule of enzyme, which is in excellent agreement with previously reported studies (8) and the known primary structure of the catalytic subunit (9). Kinase assays were conducted according to a previously reported protocol (7).

ATPase Assay—ATPase activity was measured using a procedure analogous to that reported by Kaiser and co-workers (10). Assays were performed in triplicate under previously described conditions (7), except that 50 μM [γ-32P]ATP (80–200 cpm/pmol) and 500 nM A-kinase were employed. The ATPase reaction was initiated by the addition of 10 μl of catalytic subunit from the concentrated stock solution employed for the kinase assay. After 5 min, a 5-μl aliquot was withdrawn and spotted on polyethyleneimine sheets, which had been previously spotted with 5 μl of 125 μM EDTA (pH 7.0) and dried. All spots were then treated with 5 μl of 2 mM nonradioactive ATP for visualization purposes. The polyethyleneimine sheets were developed in a chromatographic tank with 1 M KH₂PO₄ (pH 3.4) for 1 h at room temperature. The ATP spots were visualized under UV light, cut from the sheets with a 5-mm margin, placed in a scintillation vial containing Universol mixture, and counted for radioactivity. The portion of the thin layer chromatography plate containing phosphate (from above the ATP region to the solvent front) was also counted for radioactivity. The counts/min associated with the ATP spot served as a control by enabling us to determine the total radioactivity present. ATPase activity itself was obtained from the phosphate data.

Results and Discussion

The A-kinase catalyzes the phosphorylation of serine and threonine residues in a vast array of proteins and peptides, including Leu-Arg-Arg-Ala-Ser-Leu-Gly-COO⁻ (“Kemptide”) (6). Recently, several truncated versions of a naturally occurring heat-stable inhibitor (“PKI”) of the cAMP-dependent protein kinase have been synthesized and evaluated (11–14). The region of PKI (-Arg-Arg-Asn-Ala-) that binds to the enzyme active site resembles the primary sequence surrounding the phosphorylatable residue in A-kinase substrates (in which Asn-Ala has replaced Ala-Ser). Based on the known enzyme-bound conformation of Kemptide (15, 16), as well as related studies (17), we reasoned that the asparagine residue in PKI may be interacting with a critical residue in the A-kinase active site (see below). To explore this hypothesis, we prepared the following COOH-terminal amide-capped peptides: Leu-Arg-Arg-Ala-Ala-Leu-Gly-NH₂ (“Ala-peptide”), Leu-Arg-Asn-Ala-Leu-Gly-NH₂ (“Asn-peptide”), and Leu-Arg-Asp-Ala-Leu-Gly-NH₂ (“Gln-peptide”). The Ala-peptide lacks the amide side chain present in its Asn-containing counterpart. The side chain of the glutamine residue in the Gln-peptide does contain an amide moiety, albeit with an additional methylene group relative to that of asparagine. The K₅ values for the three peptides are provided in Table 1 and were obtained from a replot (slope versus [I]) of a double-reciprocal Lineweaver-Burk plot. The Asn-peptide is a 10-fold more potent inhibitor than its Gln- and Ala-
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TABLE I

| Inhibitor   | Inhibition pattern versus Kemptide | $K_i$ | Inhibition pattern versus MgATP | $K_i$ |
|-------------|-----------------------------------|------|---------------------------------|------|
| Asn-peptide | Linear competitive                 | 20.4 ± 0.8 | Linear uncompetitive             | 28.9 ± 1.6 |
| Gln peptide | Linear competitive                 | 183 ± 3.9  | Linear uncompetitive             | 322 ± 14.5 |
| Ala-peptide | Linear competitive                 | 200 ± 10.0 | Linear uncompetitive             | 267 ± 16.0 |

Fig. 1. Lineweaver-Burk plot for the Asn-peptide versus Kemptide. The linear competitive inhibition pattern is also observed for the Ala- and Gln-containing inhibitors (plots for the latter two peptides not shown).

The enhanced inhibitory potency of the Asn-peptide suggests that the asparagine residue plays a key active site role in PKI. This is consistent with a previous study which demonstrated that a truncated PKI peptide analog which lacks the asparagine residue exhibits a reduced affinity for the A-kinase compared with its asparagine-containing counterparts (11). How does the asparagine residue serve to enhance the affinity of peptides for the A-kinase active site? We have recently obtained data which implies that the methyl side chain of the alanine moiety in Leu-Arg-Arg-Ala-Thr-Leu-Arg-Arg-Asn-Ala-Leu-Gly-NH$_2$ can also orient itself toward the site of phosphorylation and in addition reside in the position normally reserved for the hydroxyl moiety of serine. In short, it is possible that the relatively high affinity of the Asn-peptide for the A-kinase may be a consequence of hydrogen bonding between the asparagine side chain and a residue that is either adjacent to or comprises the active site base responsible for hydroxyl group deprotonation (Asp-184 has been implicated in this role; see Ref. 18). To address this question, we monitored the ATPase activity of the enzyme in the presence of the Ala-, Asn-, and Gln-peptides.

In addition to proteins and peptides, the A-kinase catalyzes the phosphorylation of water (i.e. ATPase activity), albeit at a rate 1300 times less than that observed for the kinase reaction (10, 19–21). It occurred to us that ATPase activity, when measured in the presence of kinase inhibitors, could be used to decipher the fashion by which these inhibitors interact with the protein kinase active site. The velocity of ATPase activity as a function of inhibitor concentration is illustrated in Fig. 2. The dashed curve is the calculated inhibition pattern for a hypothetical inhibitor that, when associated with the enzyme, completely blocks ATPase activity. For example, this is the expected result for an inhibitor which interferes with the active site base responsible for hydroxyl group deprotonation, since both the kinase and ATPase reactions appear to be dependent upon the same active site residue (18, 19). The dashed velocity curve for this hypothetical inhibitor is derived from Equation 1,

$$a = \frac{K_m + [S]}{K_m + [S](1 + [I]/K_i)}$$

which expresses the fractional ATPase activity ($a$) as a function of inhibitor concentration. In Fig. 2, inhibitor concentration is plotted in terms of $K_i$, which provides a direct comparison of all inhibitors, irrespective of their actual $K_i$ values. For example, at a concentration of 8 $K_i$, the percentage of enzyme present that contains bound inhibitor is approximately 88% (22). Consequently, if this inhibitor completely eliminates ATPase activity when associated with the enzyme, then 12% residual ATPase activity would be observed.

From Fig. 2 it is evident that both the Gln- and Ala-peptides serve as only partial inhibitors of the ATPase reaction, demonstrating that phosphoryl transfer can still proceed when these peptides are bound to the enzyme. In contrast, the curve for the Asn-peptide indicates that this inhibitor completely
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eliminates ATPase activity when enzyme-bound. The latter result is consistent with the notion that a critical active site residue is totally compromised in the presence of the Asn-peptide. The critical residue may be the active site base itself or a functional group that is positioned in the immediate vicinity of the active site base. Under these conditions, the water molecule is either unable to interact with the appropriate active site residue or, if this interaction can take place, is unable to attack the γ-phosphate of ATP. Furthermore, the observed loss of ATPase activity in the presence of Leu-Arg-Arg-Asn-Ala-Leu-Gly-NH₂ appears to be a direct consequence of the asparagine side chain, since the corresponding Ala-peptide does not exhibit this behavior (this assumes that these inhibitory peptides bind in an otherwise identical fashion to the enzyme). In addition, the elimination of ATPase activity is not simply due to steric factors, since the Gln-peptide, with its longer side chain, is also unable to shut down ATPase action. Indeed, the dramatic difference in behavior between the Gln- and Asn-peptides reveals the exquisite specificity inherent in the A-kinase active site. This specificity is consistent with the predicted ability of the asparagine side chain NH to secure the active site position (proximal to the active site base) ordinarily reserved for the serine OH moiety in the enzyme-bound peptide. In contrast, molecular models suggest that the amide NH of the glutamine residue can only attain this position if the requisite active site conformation of the peptide backbone is seriously disrupted.

Interestingly, while the amide-capped Ala-, Gln-, and Asn-peptides are competitive inhibitors with respect to Kemptide, they serve as uncompetitive inhibitors versus MgATP (Fig. 3). However, the corresponding Ala-peptide with a free COOH terminus is reported to exhibit a noncompetitive inhibition pattern with respect to MgATP (19, 23). We have confirmed this observation (data not shown). These results are intriguing since they have a direct bearing on the kinetic mechanism of the cAMP-dependent protein kinase. In 1982, Cook and his co-workers (24) reported their studies on the mechanism of the A-kinase-catalyzed phosphorylation of Kemptide. By using a series of dead-end inhibitors, they concluded that the kinetic mechanism is steady-state random in the direction of peptide phosphorylation, followed by an ordered release of the products (Scheme I). Since this time, Kong and Cook (25) have described direct studies on Kemptide itself which appear to validate their earlier conclusions. In contrast, Krebs, Walash, and their colleagues reported in 1983 (23) that the mechanism is a fully ordered steady-state process, with MgATP binding first (Scheme II). A comparison of the expected inhibition patterns for the two mechanisms is provided in Table II. It is clear that only the pattern obtained from the variation of the Kemptide analog (i.e. nonphosphorylatable heptapeptide) versus MgATP allows one to distinguish between these two mechanistic pathways. The amide-capped peptides, with their characteristic uncompetitive inhibition pattern, suggest an ordered process with MgATP binding first. In contrast, the free COOH terminus peptide, Leu-Arg-Arg-Asn-Ala-Leu-Gly-COO⁻, with its noncompetitive pattern implies a random mechanism. These results demonstrate that an unequivocal conclusion concerning the nature of a mechanistic pathway, based solely on dead-end inhibition patterns, is not always possible. This is undoubtedly due to the fact that it is often unclear if a particular dead-end inhibitor is an appropriate substrate analog (for an especially germane discussion see Ref. 23). We simply note that PKI itself is an uncompetitive inhibitor versus MgATP (23, 26). The region of PKI which binds to the A-kinase active site is far removed from the COOH terminus, suggesting that the amide-capped peptides more fully mimic this naturally occurring inhibitor than Leu-Arg-Arg-Asn-Ala-Leu-Gly-COO⁻.

Interestingly, Leu-Arg-Arg-Asn-Ala-Leu-Gly-COO⁻ ($K_\text{D} = 60 \mu M$), which contains a free COOH terminus, behaves analogously to its amide-capped counterparts (i.e. it is a linear competitive inhibitor versus MgATP). This implies that the asparagine amide side chain is able to control the binding.

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1. It is interesting to note that Gly-Arg-Thr-Gly-Arg-Asn-Ser-Ile-NH₂ is an excellent A-kinase substrate (11), demonstrating that phosphoryl transfer to the hydroxyl group in this peptide can proceed in spite of the presence of the asparagine residue. This suggests that for this peptide substrate, the asparagine NH group is simply unable to prevent the serine OH moiety from attaining the requisite position for phosphorylation in the enzyme active site.

2. We have found that peptides of the general structure Leu-Arg-Arg-Asn-Ala-X-Leu-Gly-COO⁻ (where X is a nonphosphorylatable residue) all exhibit a linear noncompetitive inhibition pattern versus variable MgATP (M. Mandelbaum, D. Hernandez, M. Prosek, A. Salerno, and D. S. Lawrence, manuscript in preparation).
order of the peptide in spite of the presence of the negatively charged COOH terminus. Nevertheless, the free carboxylate does have some influence on the binding behavior of Leu-Arg-Asn-Ala-Leu-Gly-COO\(^-\) since this peptide, unlike its amide-capped counterpart, is not able to effect a complete elimination of ATPase activity when bound to the enzyme active site. Instead, only a partial reduction in activity was observed (similar to that obtained for the amide-capped Oin-Anda-peptides). This clearly demonstrates that the chemical nature of the COOH terminus has a profound influence on the fashion by which peptides interact with the A-kinase.

Koshland (27) first proposed that kinases envelop their substrates in order to exclude water from the active site and therefore reduce any inherent ATPase activity that may compete with the kinase reaction. It is now recognized that many kinases undergo conformational changes upon binding substrate ligands (28). For example, hexokinase possesses a deep substrate binding cleft as a consequence of its bilobal three-dimensional structure (29). Upon complexation with glucose, hexokinase undergoes a conformational change in which the two lobes envelop the substrate moieties prior to phosphoryl transfer. Although the three-dimensional structure of the A-kinase has not yet been reported, solution studies have revealed that the enzyme undergoes large scale conformational changes upon interaction with both ATP (32) and Kemptide (33-36). In the latter case, a three-step binding process has been proposed (36). The nonphosphorylatable peptide, Leu-Arg-Arg-Ala-Leu-Gly-COO\(^-\), is able to induce only the first two of these conformational changes (36). Interestingly, we found that this peptide has no effect on ATPase activity, even at a concentration of 4 \(K_i\) (i.e. 4.8 mM), indicating that water can enter the active site and undergo phosphorylation as readily in the A-kinase-Leu-Arg-Arg-Ala-Leu-Gly-COO\(^-\) binary complex as in the free enzyme itself. Such behavior may be indicative of an active site that is in an open conformation when Leu-Arg-Ala-Ala-Leu-Gly-COO\(^-\) is bound. In contrast, its amide-capped counterpart reduces ATPase activity, which suggests that water has been at least partially excluded from the active site. This implies that the active site has assumed a closed conformation in the presence of Leu-Arg-Arg-Ala-Leu-Gly-NI\(_2\) as well as the other amide-capped peptides. However, any final conclusions concerning peptide-induced conformational changes in the A-kinase must await crystallographic verification.

In summary, we have found that the inherent ATPase activity of the A-kinase can be used to assess the manner in which reversible inhibitors interact with the enzyme’s active site. An inhibitory peptide, in which the Ala-Ser dyad of the substrate has been replaced by Asn-Ala, binds to the A-kinase active site in a highly specific fashion, resulting in the elimination of ATPase activity. In conjunction with molecular modeling, these results appear to suggest that asparagine may be serving as a through-space isostere of serine. In this capacity, the Asn-residue potentiates the affinity of the peptide-based inhibitor for the enzyme and, therefore, probably contributes to the observed inhibitory potency of PKI itself. In addition, noncovalent active site interactions also control the binding order of A-kinase reversible inhibitors. The inhibition pattern of the amide-capped peptides versus MgATP indicates that these species bind in an ordered fashion to the cAMP-dependent protein kinase. In contrast, Leu-Arg-Arg-Ala-Leu-Gly-COO\(^-\) binds via a random pathway with respect to MgATP. Finally, the observed reduction in ATPase activity in the presence of several of the nonphosphorylatable peptides described above appears to be consistent with the notion that the active site of the A-kinase assumes a closed conformation prior to phosphoryl transfer.

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