Havir, E. A., & Hanson, K. R. (1968) *Biochemistry 7*, 1904.
Havir, E. A., & Hanson, K. R. (1975) *Biochemistry 14*, 1620.
Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106.
Hermes, J. D., Tipton, P. A., Fisher, M. A. O'Leary, M. H., Morrison, J. F., & Cleland, W. W. (1984) *Biochemistry 23*, 6263.
Hodgins, D. S. (1971) *J. Biol. Chem. 246*, 2977.
March, J. (1977) *Advanced Organic Chemistry*, pp 895–961, McGraw-Hill, New York.
Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 122, University Park Press, Baltimore, MD.
O'Leary, M. H. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) p 292, Plenum Press, New York.
Porter, D. J. T., & Bright, H. J. (1980) *J. Biol. Chem. 255*, 4772.
Rendina, A. R., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry 23*, 6257.
Schloss, J. V., & Cleland, W. W. (1982) *Biochemistry 21*, 4420.
Schloss, J. V., Porter, D. J. T., Bright, H. J., & Cleland, W. W. (1980) *Biochemistry 19*, 2358.
Snow, M. L., Lavinger, C., & Ressler, C. (1968) *J. Org. Chem. 33*, 1774.
Thode, H. G., Graham, R. L., & Ziegler, J. A. (1945) *Can. J. Res., Sect. B 23*, 40.
Urey, H. C., & Aten, A. H. W., Jr. (1936) *Phys. Rev. 50*, 575.

Circular Dichroic Evidence for an Ordered Sequence of Ligand/Binding Site Interactions in the Catalytic Reaction of the cAMP-Dependent Protein Kinase†

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ABSTRACT: A limiting requirement for substrate specificity of the cAMP-dependent protein kinase is the presence of one or two basic residues located to the N-terminal side of the target substrate serine. Furthermore, circular dichroic (CD) studies have shown that binding of protein substrate involves a series of at least two independent conformational changes in the enzyme, each of which is initiated by a recognition signal on the substrate protein. The present study attempts to elucidate further the complete sequence of enzyme/ligand interactions by using the synthetic substrate peptide Kemptide and analogues differing from it at crucial points in the sequence: the Ala-peptide, where alanine is substituted for the target serine, and D-Ser-Kemptide, where the target serine is in the D rather than the L configuration. Examination of the effects of binding of these substrates on the intrinsic UV CD of the enzyme and the induced CD in the presence of Blue Dextran has revealed a third step in the substrate/enzyme binding interaction. Although sections of the conformational change at the active site are dependent on the basic subsite and the serine hydroxyl group on the peptide, respectively, the complete conformational change requires that the substrate be bound in random coil conformation. Where this does not occur, the kinetics show that the peptide will not act either as substrate or as inhibitor of the enzyme. Further, the interaction between the serine hydroxyl group and an enzyme tyrosine residue, previously observed, appears to be dependent on the correct orientation as well as the mere presence of the target –OH group. Taken together, the data allow one to construct an ordered sequence of conformational changes taking place upon substrate binding leading to the final form of the active site.

The cAMP-dependent protein kinase is the primary, if not sole, effector of hormonally induced actions that are mediated by increases in cellular levels of cAMP. A broad range of proteins has been shown to be phosphorylated both in the cell in response to a cAMP signal and in vitro by the cAMP-dependent protein kinase. Despite this extreme diversity of substrates, a certain degree of specificity must pertain to the kinase in its role as mediator of hormonal control. It is, therefore, to be expected that there exist certain unique features of a protein substrate's structure that are recognized by the kinase and dictate substrate specificity.

Initial investigations of the sequence at the site in phosphorylated proteins led to the use in particular of the heptapeptide Kemptide, whose sequence (Leu-Arg-Arg-Ala-Ser-Leu-Gly) is that of the phosphorylation site of porcine hepatic pyruvate kinase, and of peptide analogues of this sequence. Such studies showed that a limiting requirement in dictating protein kinase substrate specificity is the presence of one or two basic residues optimally located two and three residues away from the target seryl residue on its N-terminal side (Daile et al., 1975; Zetterquist et al., 1976; Kemp et al., 1977; Feramisco et al., 1979; Meggio et al., 1981).

More recent information on the interactions occurring between the protein kinase and its peptide substrates has come from several approaches. Granot, Mildvan, and co-workers

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[see Bramson et al. (1984) for review] have used nuclear magnetic resonance (NMR)[1] and ESR to map the topography of substrate binding and define the reaction coordinates. Their data suggest that the mechanism of catalysis may be dissociative with the formation of a metaphosphate intermediate within the catalytic site prior to phosphorylserine formation. They have also begun to define some of the restrictions that are imposed upon the peptide structure. Kinetic studies (Whitehouse et al., 1983; Whitehouse & Walsh, 1983; Cook et al., 1982; Bramson et al., 1984) have also begun to shed light on binding site interactions occurring during the catalytic process. Most notably, peptide binding is enhanced when nucleotide is present, and thus, the predominant reaction appears to be ordered, with nucleotide binding first. When one considers the interactions of peptides and proteins at the substrate site, however, the picture becomes more complex. Binding of the inhibitor protein of the cAMP-dependent protein kinase is, like Kemptide, enhanced by nucleotide, but binding of the Ala-peptide (a competitive inhibitor analogue of Kemptide in which the phosphorylatable serine is replaced by alanine) is not. Furthermore, a nucleotide-independent binding of Kemptide, analogous to that seen with Ala-peptide, can be observed, albeit with a K_d (0.2–1.0 mM) 2 orders of magnitude weaker than that apparent in the catalytic reaction (K_m = 5 μM). It is clear from such data that despite the similarity between Ala-peptide and Kemptide, there are unique features of the serine in Kemptide that are involved in the interactions at the catalytic site and are not shared by the alanine in the Ala-peptide. Our recent studies (Reed & Kinzel, 1984a,b) using circular dichroism have further explored the nature of the interactions of ligands at the catalytic site. It has been shown that binding of the protein substrate at the active site is followed by conformational changes affecting the enzyme at levels ranging from short-range interactions at the ATP-binding site to changes in the enzyme secondary structure composition. These changes appear to take place in at least two independent steps, each of which is initiated by recognition signals on the substrate protein. Some of these changes are attributable to interactions with the basic subsite on the substrate, whereas others are dependent on the presence of a serine hydroxyl group in the appropriate position.

If the final active form of the enzyme is reached through a series of interactions between it and its ligands, it seems reasonable to identify as many as possible since each one could act as a potential control point in modulating enzyme activity, reaction rate, and specificity. For this reason, we have used the technique of circular dichroism to compare the interactions of protein kinase with Kemptide, the Ala-peptide, and the D-serine analogue of Kemptide (D-Ser-Kemptide). The results reveal that there is a third step in the substrate/enzyme binding interaction not previously identified and suggest a possible mechanism for dictating enzyme specificity.

Materials and Methods

Peptides. Two types of Kemptide were used in these studies, the native molecule (Leu-Arg-Arg-Ala-Ser-Leu-Gly) and the C-terminal amide derivative (Leu-Arg-Arg-Ala-Ser-Leu-Gly-NH_2). The circular dichroic spectra obtained with these were indistinguishable, and throughout the text those peptides containing only L-amino acids are referred to simply as Kemptide. Native Kemptide was purchased from Peninsula Laboratories, Santa Clara, CA; the purity of this commercial sample has been previously evaluated (Whitehouse et al., 1983). The C-terminal amide derivative was synthesized as previously described (Kemp, 1979). Its purity and composition have been reported, and analysis by HPLC indicated it to be at least 95% pure. Ala-peptide (Leu-Arg-Arg-Ala-Ala-Leu-Gly) was obtained from Peninsula Laboratories, Santa Clara, CA. Two types of D-Ser-Kemptide were used, the native molecule (Leu-Arg-Arg-Ala-D-Ser-Leu-Gly) and the C-terminal amide derivative. As in the case of Kemptide, the two forms gave identical CD spectra and are not further distinguished in the text. Native D-Ser-Kemptide was purchased from Peninsula Laboratories, Santa Clara, CA. The C-terminal amide derivative was synthesized as described for the C-terminal amide derivative of Kemptide with the exception of the use of *t*-BOC-D-serine (Protein Research Foundation, Osaka, Japan). The peptide was purified by SP-Sephadex chromatography. Compositonal analysis was determined both by acid hydrolysis and with aminopeptidase M digestion (Table I); the latter provides evidence that racemization did not occur during the synthesis of the peptide. The D-Ser-Kemptide was demonstrated to be pure by TLC (butanol (15)–acetic acid (3)–pyridine (10)–water (12); R_f 0.53) and more than 92.5% pure by HPLC. The simple substitution of D-serine for L-serine in the peptide produces a conformational change in the peptide sufficient to result in a differential elution of the two on HPLC using a μBondapak column and an acetonitrile gradient elution (data not shown).

Table I: Peptide Compositional Analysis

	C-terminal amide derivative			
	acid hydrolysis[a]		aminopeptidase digestion[b]	
	D-Ser-Kemptide	Kemptide[d]	D-Ser-Kemptide	Kemptide
Leu	1.99	2.00	1.06	1.98
Arg	1.99	1.96	2.04	1.99
Ala	1.01	1.04	0.85	1.08
Ser	1.11	1.01	0	0.85
Gly	1.00	1.01	0	0.59[c]

[a] Lyophilized peptide was hydrolyzed in 5.7 M HCl for 24 h at 105 °C. [b] Hydrolysis conditions: 1 mM peptide and 125 g/mL amino peptidase M for 24 h at 40 °C. [c] This recovery is low because of incomplete digestion of glycinamide by aminopeptidae M. [d] As reported by Kemp (1979).

Other Materials. Catalytic subunit of the cAMP-dependent protein kinase (type II) was prepared either from Sprague-Dawley rat skeletal muscle or bovine cardiac muscle as previously described by Kübler et al. (1979) and Whitehouse & Walsh (1983), respectively. Enzyme used for induced dichroism spectra was purchased from Sigma. Aminopeptidase M was obtained from Protein Research Foundation, Osaka, Japan.

Circular Dichroism Measurements. Circular dichroism spectra were obtained on a Jasco J-500 automatic recording spectropolarimeter coupled with a J-DPY data processor. Automatic slit width control was maintained with a 1.0-nm dispersion. Digitally recorded curves were fed through the data processor for smoothing, signal averaging, and base-line subtraction.

For studies on induced dichroism in the region 300–700 nm, catalytic subunit was dialyzed against 10 mM Tris-HCl, pH 7.5, and bound to freshly prepared Blue Dextran/Sepharose as described earlier (Reed & Kinzel, 1984a). Around 200–300 μg of enzyme protein was bound to 4.0 g of wet-packed beads for a final enzyme concentration of 50–60 μg/mL. Distortion due to light scattering in this system was negligible, as discussed previously (Reed & Kinzel, 1984a). Samples were

[1] Abbreviations: NMR, nuclear magnetic resonance; ESR, electron spin resonance; HPLC, high-performance liquid chromatography; *t*-BOC, *tert*-butoxycarbonyl; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; CD, circular dichroism.

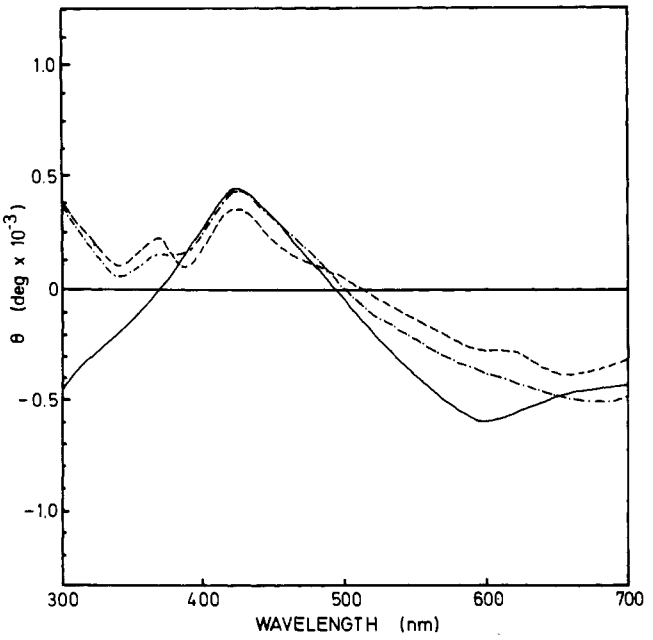

FIGURE 1: Induced CD spectrum of Blue Dextran/Sepharose-bound catalytic subunit in the presence of excess substrate analogues. Curve a (—), enzyme plus Kemptide (518 μM); curve b (---), enzyme plus Ala-peptide (518 μM); curve c (-·-), enzyme plus D-Ser-Kemptide (518 μM).

measured in a dichroically selected quartz cuvette with a 0.5-mm light path at a sensitivity of 0.2 mdeg/cm, a scan speed of 50 nm/min, and a time constant of 2.0 s. A signal average of 16 scans was corrected for base line by subtraction of an equivalent signal-averaged spectrum of Blue Dextran/Sepharose alone.

Spectra in the UV region were measured by using dialyzed enzyme at a concentration of 50–100 μg/mL in 150 mM phosphate buffer, pH 6.8. No ATP or ATP analogue was present. Measurements for secondary structure determination were taken over the region 190–240 nm by using a 0.1-cm quartz cuvette at a sensitivity of 2.0 mdeg/cm, a scan speed of 5.0 nm/min, and a time constant of 1.0 s. Curves are presented as the signal average of four consecutive measurements with a similarly signal-averaged base line subtracted. Secondary structure estimates were obtained from these curves as previously described (Reed & Kinzel, 1984b) by using the Globular Protein Secondary Structure applications package of CONTIN, a program developed for solving linear integral equations (Provencher & Glockner, 1981).

Measurements of the CD of aromatic amino acids were taken from 250 to 300 nm by using a 1.0-cm quartz cuvette and scanning at a sensitivity of 0.2 mdeg/cm and a speed of 10 nm/min with a 2.0-s time constant. Curves are given as the signal average of 32 consecutive measurements with appropriate base line subtracted. All spectra were measured at room temperature.

RESULTS

Induced Dichroism of the Blue Dextran/Sepharose-Bound Enzyme. The circular dichroism spectra of the Blue Dextran bound protein kinase catalytic subunit in the presence of Kemptide, Ala-peptide, and D-Ser-Kemptide are given in Figure 1. The concentrations of each used were those that produced a maximal effect. The Blue Dextran binds to the ATP-binding site of the protein kinase (Witt & Roskoski, 1975; Reed et al., 1983) so that the binding of peptides with this protocol mirrors, at least in part, the ordered sequence of the catalytic reaction. The curve with Kemptide bound to the enzyme is typical of the spectrum induced on binding of any substrate (Reed & Kinzel, 1984a), with a broad, negative ellipticity from 580 to 610 nm and complex positive bands at higher wavelengths dominated by a peak at 420–424 nm.

As previously shown, this type of curve appears to be induced only when both the basic subsite (one or more arginine residues to the N-terminal side of the target serine) and the serine hydroxyl group itself are present on the substrate. With Ala-peptide, which lacks the serine hydroxyl group, although a portion of the CD spectrum, notably the positive ellipticity maximum at 420–424 nm, is similar to that induced by Kemptide, other portions of the spectrum are not, especially at 375 nm where the sign of the maximum appears to be reversed (Figure 1, curve b). The spectrum obtained with Ala-peptide is very similar to that previously reported for a second alanine-substituted analogue peptide, Arg-Gly-Tyr-Ala-Leu-Gly (Reed & Kinzel, 1984a). With each, the positive ellipticity maximum at 420–424 nm, as also observed with Kemptide, is probably actuated by binding of the basic subsite. In contrast to Kemptide, however, both alanine-substituted analogues cause reversal of the sign of ellipticity at 375 nm. This reversal occurs both in protein kinase isolated from rat skeletal muscle as reported previously (Reed & Kinzel, 1984a) and in the commercial beef heart preparation used here; the initial sign of the 375 nm ellipticity, however, is different in the two enzymes so that while the change on substrate binding in the rat muscle protein kinase is from positive to negative, in the beef heart protein kinase it moves from negative to positive. As reported previously, this sign reversal upon peptide binding appears to be attributable to an interaction between the serine hydroxyl group on the substrate and a sensitive site on the enzyme resulting in a reorientation of an externally located tyrosine residue tentatively located at the ATP–binding site of the enzyme. Consistent with this, binding of Kemptide to the enzyme is in fact accompanied by a change in the CD signal of the aromatic amino acids (Reed & Kinzel, 1984a,b). The difference in the initial sign, i.e., that exhibited by the enzyme alone, of the 375-nm ellipticity between the rat muscle and beef heart protein kinases is probably due to a slight difference in the conformation of the unliganded enzyme in the angle of the affected tyrosine with respect to the main chain. Substrate binding in each case results in a sufficient change in this angle to reverse the sign of the associated ellipticity.

It is reasonable to assume that triggering of this portion of the conformational change is closely dependent on the orientation of the serine hydroxyl group on binding. For this reason an analogue of Kemptide was tested in which the target serine was the D isomer so that the hydroxyl group, while present, was in an altered position.

The CD spectrum of the Blue Dextran bound protein kinase in the presence of excess D-Ser-Kemptide (Figure 1, line c) is almost identical with that of the Ala-peptide. The 424-nm positive peak is retained, the longer wavelength region changes, and the sign of the ellipticity maximum at 375 nm is reversed from that observed with Kemptide. That portion of the substrate-induced conformational change which is dependent on the basic subsite occurs, but that apparently dependent on the serine hydroxyl group does not take place. The mere presence of a serine the correct distance on the C-terminal side of the basic subsite appears an insufficient trigger; a precise alignment of the hydroxyl group is necessary if interaction resulting in tyrosine CD reversal is to occur.

Near-UV Intrinsic Dichroism. Ealier work in this laboratory has shown that binding of Kemptide to the catalytic subunit

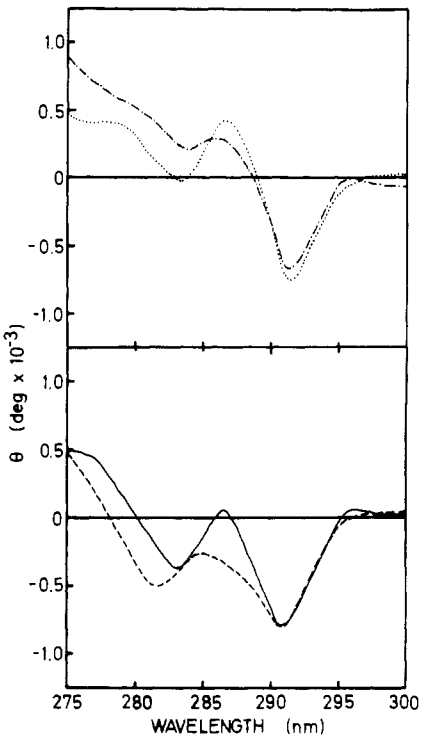

FIGURE 2: Near-UV CD spectrum of catalytic subunit in the presence of substrate analogues. Curve a (- · -), enzyme plus Ala-peptide (518 μM); curve b (···), enzyme plus D-Ser-Kemptide (518 μM); curve c (- - -), enzyme plus Kemptide (518 μM); curve d (—) enzyme alone.

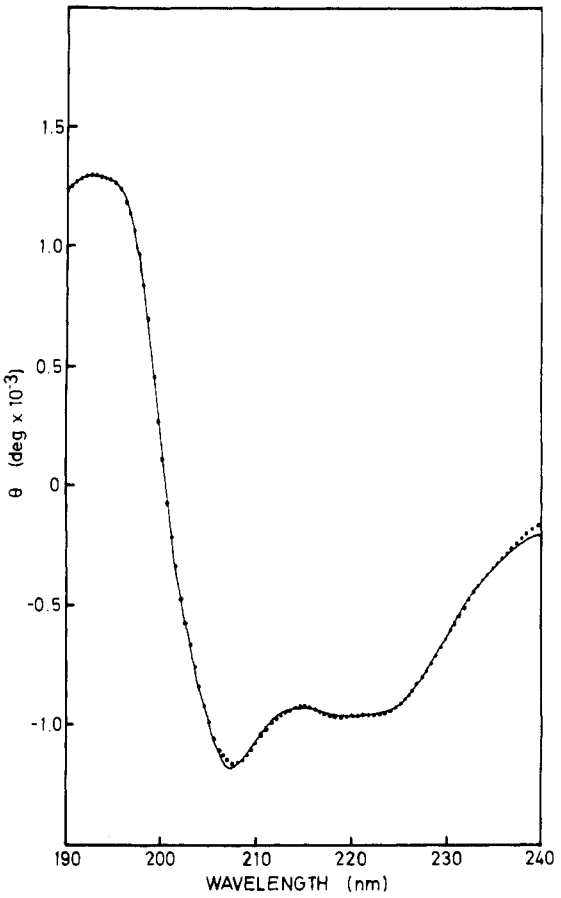

FIGURE 3: Far-UV CD spectrum of catalytic subunit plus Ala-peptide. (—) Measured spectrum; (···) CONTIN-generated fit.

produces an alteration in the UV CD spectrum between 275 and 300 nm that was attributed to a change in the orientation of an externally located tyrosine residue on the enzyme (Reed & Kinzel, 1984b). As mentioned above, this alteration in tyrosine position with respect to the main chain is the probable source of the sign dependency of the 375-nm ellipticity maximum in the induced CD spectrum. To be more certain of this we have examined the behavior of the near-UV CD spectrum on binding of the Ala-peptide and D-Ser-Kemptide to the enzyme to see if it also reflects the effects of these analogues on the 375-nm peak. (The substrate analogue Arg-Gly-Tyr-Ala-Leu-Gly used previously is not suitable for studies in the UV region due to the presence of an aromatic amino acid residue on this peptide.)

Figure 2 shows the CD spectrum from 275 to 300 nm of the catalytic subunit in the presence of excess Ala-peptide (curve a) and D-Ser-Kemptide (curve b) contrasted with the spectra of the enzyme plus Kemptide (curve c) and the enzyme alone (curve d). Neither the Ala-peptide nor D-Ser-Kemptide causes the change in CD signal in this region typical of substrate binding. In neither case is there an increase in negative amplitude of the lower wavelength extremum, nor does the wavelength of this complex peak shift from 283.5 to 282 nm, as it does on binding Kemptide. Both spectra display a weaker negative ellipticity at 284 nm than that observed either with Kemptide bound or with the enzyme alone. Thus, the inability of a peptide to produce the normal substrate signal at 375 nm in the induced CD spectrum correlates directly with its inability to bring about the shift from ca. 284 to 282 nm together with its attendant amplitude increase in the aromatic region. In both cases the peptide involved has a missing or misaligned serine hydroxyl group. It appears, therefore, that it is not simply the presence of an -OH group on the C-terminal side of the basic subsite that induces a change in the position of an enzyme tyrosine, but that of one in the correct orientation, and that this change is necessary for the complete conformational change seen on the binding of substrate.

Far-UV Intrinsic Dichroism. As previously reported (Reed & Kinzel, 1984b), the CD spectrum of the enzyme in the region 190–240 nm gives secondary structure composition values of 49 ± 2% α helix, 20 ± 4% β sheet, and 31 ± 3% remainder. When Kemptide is bound this changes radically to 31% α helix, 55% β sheet, and 15% remainder. Binding of the substrate is thus accompanied by a net increase in secondary structure; this increase is in the form of β sheet and occurs at the expense of the disordered portion of the molecule together with some decrease in α helix. Figure 3 gives the CD spectrum from 190 to 240 nm of the catalytic subunit in the presence of excess Ala-peptide and the CONTIN-generated fit to these data. The curve fit corresponds to a secondary structure composition of 33% α helix, 20% β sheet, and 47% remainder. Thus, with Ala-peptide bound, the enzyme loses roughly the same amount of α helix as when Kemptide is bound, but in the absence of the second recognition subsite—a phosphorylatable hydroxyl group on the target serine—the gain in β-pleated sheet structure does not take place. This evidence also supports the idea that separate portions of the complete substrate-induced conformational changes are independently controlled by specific triggering groups on the substrate.

The changes in enzyme secondary structure following binding of D-Ser-Kemptide cannot be consistently resolved by these methods with the computer-generated fit to these curves being poor. The CONTIN program seems unable to find a stable solution to the type of curve generated by enzyme in the presence of D-Ser-Kemptide. Enzyme in the presence of the D-Ser analogue always exhibits a lower α-helix content that that of the enzyme alone, but the amount by which helical structure is reduced appears to vary widely, and no reprodu-

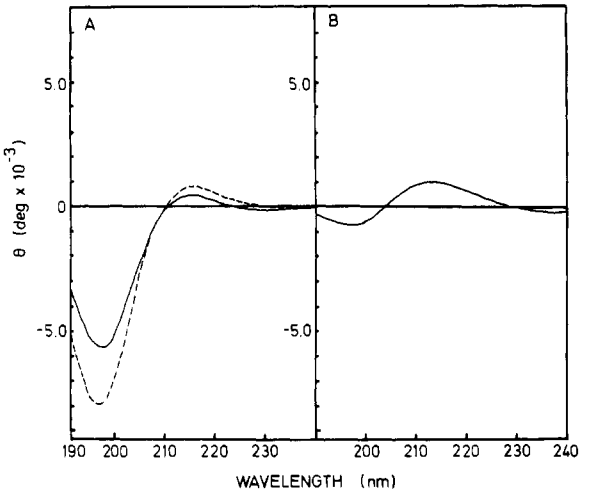

FIGURE 4: Far-UV CD spectra of Kemptide and analogues. (A) (—) 2.6 mM Kemptide; (---) 2.6 mM Ala-peptide. (B) (—) 2.6 mM D-Ser-Kemptide.

cible pattern of the other classes of structure arises even after repeated measurements.

Far-UV CD of Kemptide and Analogues. The far-UV CD spectra of Kemptide, the Ala-peptide, and D-Ser-Kemptide are reproduced in Figure 4. The spectra of Kemptide and its Ala analogue are typical of those exhibited by model polypeptides in a random coil form (Greenfield & Fasman, 1969; Adler et al., 1968), the amplitude of the ellipticity maxima at 198 and ca. 215 nm being somewhat greater for the Ala-peptide than for Kemptide. The CD spectrum of D-Ser-Kemptide presents a completely different picture. The negative and positive extrema are of almost equal magnitude, whereas for the classic random coil structure the negative extremum is much greater than the positive. The conformation of D-Ser-Kemptide in solution is clearly very different from that of Kemptide itself (and also of Ala-peptide), a fact supported by their different elution on HPLC (see Materials and Methods).

It should be borne in mind that the near-UV CD measurements are made on the enzyme alone, while the induced CD measurements are made on enzyme bound to Blue Dextran. The former can only show movements of aromatic amino acids in response to binding of a particular peptide, and not the larger scale conformational changes seen in the induced CD spectra. For this reason it cannot be determined at this point whether the large-scale conformational change is dependent on the presence of a ligand at the ATP binding site. However, the far-UV CD spectra show that some secondary structure changes can evidently proceed when the site is vacant.

Kinetics of D-*Ser-Kemptide.* We have previously reported in detail the kinetic constants for Kemptide and Ala-peptide (Whitehouse et al., 1983). In brief, Kemptide has a K_m value of 4.7 ± 0.7 μM for an ordered reaction mechanism with nucleotide binding first and a K_d by direct binding of 0.18 mM in the absence of nucleotide. Ala-peptide exhibits competitive inhibition kinetics vs. Kemptide as substrate (K_i = 0.32 mM) and noncompetitive vs Mg-ATP^{2-} (K_i = 0.77 mM). D-Ser-Kemptide has been tested as a potential inhibitor of the cAMP-dependent protein kinase by two approaches. In the first (Figure 5a), by analogy with conditions under which the inhibitor protein of the cAMP-dependent protein kinase is maximally effective (Whitehouse & Walsh, 1983), D-Ser-Kemptide was incubated with protein kinase and Mg-ATP prior to initiation of the reaction with Kemptide. No inhibition by D-Ser-Kemptide was observed. Further, D-Ser-Kemptide

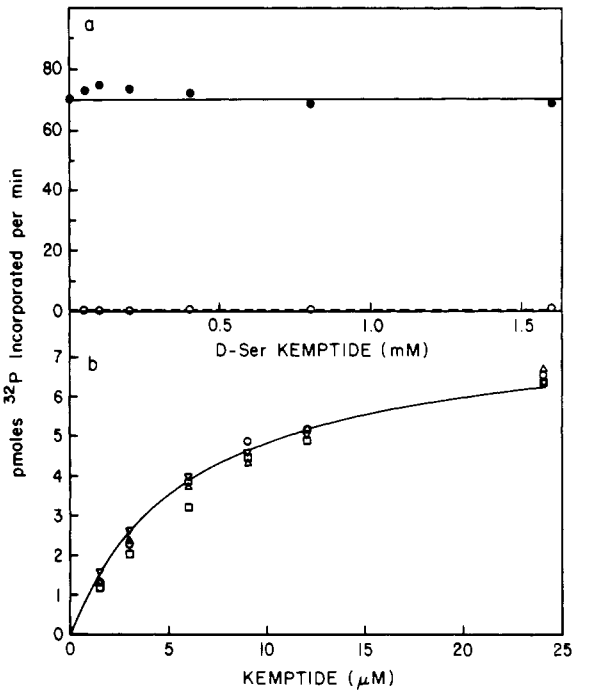

FIGURE 5: Effect of D-Ser-Kemptide on protein kinase activity. In (a) the effect of D-Ser-Kemptide on protein kinase activity was evaluated by the procedure used previously that was best suited to measure inhibition by the protein kinase inhibitor protein (Whitehouse & Walsh, 1983). For the data denoted by solid symbols the indicated concentrations of D-Ser-Kemptide were incubated with protein kinase, 0.25 mM ATP, and 1 mM magnesium acetate for 10 min at 30 °C prior to initiation of the assay by addition of 32.5 μM Kemptide. For the data denoted by open symbols there was no preincubation, and the reactions contained as substrate only the indicated concentrations of D-Ser-Kemptide. In (b) the effect of D-Ser-Kemptide on protein kinase activity was evaluated by the procedure used previously that was best suited to measure inhibition by Ala-peptide (Whitehouse et al., 1983). All reactions contained the indicated concentrations of Kemptide, 30 μM ATP, and either no D-Ser-Kemptide (▽) or 0.4 (△), 0.8 (○), or 1.2 mM D-Ser-Kemptide (□), respectively. The reaction was initiated by addition of protein kinase. The line on the graph is the computer fit to the Michaelis–Menten equation for the data obtained in the absence of D-Ser-Kemptide by using the weighting factors described by Cleland (1967). The apparent K_m value for Kemptide was 6.13 ± 0.5 μM, identical with that determined previously under similar conditions (Whitehouse et al., 1983).

does not serve as a substrate for the protein kinase. In a second approach (Figure 5b), conditions were employed that were similar to those used to evaluate Ala-peptide inhibition, namely, using low concentrations of Kemptide, low concentrations of ATP, and a high ratio of D-Ser-Kemptide to Kemptide. With these conditions, likewise, no significant level of inhibition was detected. At the lowest concentration of Kemptide used (one-fourth the K_m), there was possibly a slight trend for the values in the presence of D-Ser-Kemptide to be lower. Even if this trend were correct, the minimal K_i value for D-Ser-Kemptide would be at least 10 mM, a value some 30-fold higher than the K_i for Ala-peptide and over 1000-fold higher than the K_m for Kemptide. These data demonstrate that the substitution of D-serine for either L-serine of Kemptide or L-alanine of the Ala-peptide very markedly diminishes the interaction of the peptide with the protein kinase.

DISCUSSION

The significance of the unusual far-UV CD spectrum for D-Ser-Kemptide in solution can best be understood in terms of the exact nature of the structure which gives rise to the rotational properties of the "random coil" conformation. In order to fit the known CD curve for random coil polypeptides, Aebersold & Pysh (1970) initially used an equation generating

the rotational strength at each wavelength for a model octapeptide that was allowed to rotate freely at each N–C_α bond and C_α–C′ bond. The CD curve generated had a negative extremum on the high energy side of the absorption maximum, but the positive and negative bands were of approximately equal magnitude. In order to generate a curve in which the negative extremum was much greater than the positive, as seen in the case of random coil structures, it was found necessary to put severe constraints on the rotational freedom of the bond angles. The limitations invoked were consistent with a model in which the chain contains short segments of loosely regular helical structure separated by kinks. The classic random coil spectrum exhibited by Kemptide and the Ala-peptide is thus the product of a peptide showing a fair degree of regular secondary configuration, though by no means so tightly organized or extensive as the α-helix or β-sheet forms.

The CD spectrum from 190 to 240 nm of D-Ser-Kemptide is nearly identical with the original curve generated by Aebersold and Pysh, i.e., opposite and nearly equal low amplitude extrema with the negative band shifted upward from the 191-nm absorption maximum. Thus, the D-Ser analogue CD spectrum is consistent with optical activity arising from a heptapeptide with nearly complete rotational freedom around the N–C_α and C_α–C′ bonds and essentially no stable three-dimensional configuration in solution.

The results with Kemptide and its analogues allow one to draw several conclusions about the nature of specific substrate-induced conformational changes at the active site.

(1) Initial electrostatic binding of the basic subsite alone has a noticeable effect on the secondary structure of the enzyme. Binding of the Ala-peptide is accompanied by a reduction in α-helical content roughly equal to that produced on binding of Kemptide. There is some indication of a similar effect in the presence of the D-Ser-Kemptide as well. However, the full effect of substrate binding on enzyme conformation, with changes in β-sheet content as well, is not observed with Ala-peptide.

(2) The analogue work reenforces the thesis presented earlier that the serine hydroxyl group is critical not only as the phosphorylation site but also for enhanced substrate binding (Whitehouse & Walsh, 1983; Reed & Kinzel, 1984b). With both the Ala-peptide and the D-Ser-Kemptide, neither of which has a correctly aligned hydroxyl group, there was a failure to bring about the 375-nm sign change in the induced CD spectrum coupled with failure to produce the change in the aromatic amino acid intrinsic CD spectrum, both of which are seen with peptides that are substrates. Thus, the substrate/enzyme interaction detected from aromatic amino acid intrinsic CD that we have suggested to be a result of repositioning of an enzyme tyrosine seems dependent not merely on the simple presence of the serine –OH group but also on its being in the correct orientation.

(3) There is probably a third signal besides the basic subsite and the serine hydroxyl group that is necessary to induce the full conformational change seen when Kemptide is bound. Any peptide tested thus far which has the basic subsite produces the 420–424-nm positive ellipticity in the induced CD spectrum of the enzyme; however, the D-Ser-Kemptide, which has the basic subsite and brings about the 420–424 nm induced CD peak, does not act as a competitive inhibitor whereas the Ala analogue of Kemptide does. Thus, whatever happens with the Ala analogue to produce enzyme inhibition—presumably occupation of the binding site by the peptide—does not happen with the D-Ser-Kemptide. Granot et al. (1981) from NMR studies of enzyme-bound peptides drew the conclusion that "...if the protein kinase has an absolute requirement for a specific secondary structure, then this structure must be a coil". Clearly from the CD and kinetic studies there must be a second requirement for effective binding which is fulfilled by the Ala-peptide but which the D-Ser-Kemptide lacks. The obvious candidate for this third signal is that the substrate, or effective inhibitor, assume a random coil configuration. This is the one property that the D-Ser-Kemptide lacks but that is shared by both the Ala-peptide and Kemptide.

Taken together, the data suggest the following picture of the order of events taking place on substrate binding:

(A) Initial electrostatic interactions must occur for the substrate to achieve probational recognition by the enzyme. It is at this point only lightly bound by ionic forces alone. The reaction between negative charges on the enzyme [possibly glutamic acid carboxyl groups (Matsuo et al., 1980)] and the positively charged guanidino groups of the basic subsite may be sufficient to cause the shift in secondary structure seen by lowering of the α-helix signal.

(B) If the substrate conformation is random coil, or it can assume such a conformation in association with the enzyme, the firmer binding seen with Ala-peptide or Kemptide takes place. This probably results from a closure over the substrate, which has been suggested as a general phenomenon with kinases.

(C) If then a serine or threonine hydroxyl group is in the right position, interaction resulting in a reorientation of an enzyme tyrosine occurs. If, however, this last step does not occur but the first two steps have been completed, the peptide is in a position to act as inhibitor. It has persuaded the enzyme to hold it in the active site, but the complete conformational alteration of the active site has not taken place. Since Kemptide binding is enhanced by ATP but not AMP-PNP, and Ala-peptide binding is not enhanced by either nucleotide (Whitehouse & Walsh, 1983), it is possible that ATP binding in some manner augments the seryl hydroxyl induced change.

The difficulty experienced in finding a single solution for secondary structure of the enzyme in the presence of the D-Ser analogue is explained if electrostatic interaction between its basic subsite and the enzyme can occur, initiating some conformational change, but the tight binding which would normally follow is prevented by the absence of the random coil fit so that the peptide is easily displaced. This would mean that there was no one dominant change induced by whatever interaction occurred with the D-Ser analogue, and the resulting CD spectrum would be due to a mixture of intermediate forms—a curve which could not be reliably analyzed by a CONTIN-generated fit. This type of behavior is supported by the failure of the D-Ser analogue to act as an inhibitor.

A clearer picture of the role of small modifier proteins in enzyme control is beginning to emerge. At the moment it appears that for the enzyme to function normally a minimum of three independent interactions between its active site and the protein substrate must occur. Since the complete substrate-induced conformational change takes place in small increments, it is not difficult to imagine how binding of modifiers might change the affinity or specificity of the enzyme for one or more of its ligands. Since the question of control of substrate specificity within the cell is central to our understanding of the function of cAMP-dependent protein kinase in metabolic regulation, wider studies on the effects of known modifier proteins on the ligand/enzyme interactions just described are in progress.

Registry No. Kemptide, 65189-71-1; Ala-peptide, 68451-04-7; D-Ser-Kemptide, 96092-86-3; protein kinase, 9026-43-1.

REFERENCES
Adler, A. J., Hoving, R., Potter, J., Wells, M., & Fasman, G. D., (1968) *J. Am. Chem. Soc. 90*, 4736–4741.
Aebersold, D., & Pysh, E. S. (1970) *J. Chem. Phys. 53*, 2156–2163.
Bramson, H. N., Kaiser, E. T., & Mildvan, A. S. (1984) *CRC Crit. Rev. Biochem. 15*, 93–124.
Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1–32.
Cook, D. F., Neville, M. E., Vrana, K. E., Hartl, F. T., & Roskoski, R. R. (1982) *Biochemistry 21*, 5794–5799.
Daile, P., Carnegie, P. R., & Young, J. K. (1975) *Nature (London) 257*, 416–418.
Feramisco, J. R., Glass, D. B., & Krebs, E. G. (1979) *J. Biol. Chem. 255*, 4240–4245.
Granot, J., Mildvan, A. S., Bramson, H. N., Thomas, N., & Kaiser, E. T. (1981) *Biochemistry 20*, 602–610.
Greenfield, N. J., & Fasman, G. D. (1969) *Biochemistry 8*, 4180–4186.
Kemp, B. E. (1979) *J. Biol. Chem. 254*, 2638–2642.
Kemp, B. E., Graves, D. J., Benjamini, E., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 4888–4894.
Kübler, D., Gagelmann, M., Pyerin, W., & Kinzel, V. (1979) *Hoppe-Seyler's Z. Physiol. Chem. 360*, 1421–1423.
Matsuo, M., Huang, C.-H., & Huang, L. (1980) *Biochem. J. 187*, 371–379.
Meggio, F., Chessa, G., Borin, G., Pinna, L. A., & Marchiori, F. (1981) *Biochim. Biophys. Acta 662*, 94–101.
Provencher, S. W., & Glockner, J. (1981) *Biochemistry 20*, 33–37.
Reed, J., & Kinzel, V. (1984a) *Biochemistry 23*, 968–973.
Reed, J., & Kinzel, V. (1984b) *Biochemistry 23*, 1357–1362.
Reed, J., Gagelmann, M., & Kinzel, V. (1983) *Arch. Biochem. Biophys. 222*, 276–284.
Whitehouse, S., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3682–3692.
Whitehouse, S., Feramisco, J. R., Casnellie, J. E., Krebs, E. G., & Walsh, D. A. (1983) *J. Biol. Chem. 258*, 3693–3701.
Witt, J. J., & Roskoski, R. (1975) *Biochemistry 14*, 4503–4507.
Zetterquist, O., Ragnarsson, U., Humble, E., Berglund, L., & Engstrom, L. (1976) *Biochem. Biophys. Res. Commun. 76*, 696–703.

Hydrogen Exchange Kinetics of Core Peptide Protons in *Streptomyces* Subtilisin Inhibitor†

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ABSTRACT: The hydrogen isotope exchange kinetics of the 10 slowest exchanging resonances in the ^{1}H NMR spectrum of *Streptomyces* subtilisin inhibitor (SSI) have been determined at pH 7–11 and 30–60 °C. These resonances are assigned to peptide amide protons in the β-sheet core that comprises the extensive protein–protein interface of the tightly bound SSI dimer. The core protons are atypical in that their exchange rates are orders of magnitude slower than those for all other SSI protons. When they do exchange at temperatures >50 °C, they do so as a set and with a very high temperature coefficient. The pH dependence of the exchange rate constants is also atypical. Exchange rates are approximately first order in hydroxyl ion dependence at pH <8.5 and >9.5 and pH independent between pH 8.5 and 9.5. The pH dependence and temperature dependence of the SSI proton exchange rates are interpreted by the two-process model [Woodward, C. K., & Hilton, B. D. (1980) *Biophys. J. 32*, 561–575]. The results suggest that in the average solution structure of SSI, an unusual mobility of secondary structural elements at the protein surface is, in a sense, compensated by an unusual rigidity and inaccessibility of the β-sheet core at the dimer interface.

Streptomyces subtilisin inhibitor (SSI) inhibits alkaline proteases of bacterial origin (Sato & Murao, 1973). In its native form, SSI is an elongated dimer composed of two identical polypeptides, each containing 113 amino acids and 2 disulfide bonds (Ikenaka et al., 1974). The secondary structure of the monomer consists of a five-stranded β-sheet and two short α-helices, and the dimer interface involves extensive contacts between β-sheet strands of the monomers (Mitsui et al., 1979). The accessible area buried upon dimerization is 786 Å^2 per monomer, 12.7% of the total static solvent-accessible surface area of dissociated (hypothetical) monomer (Satow et al., 1980). In neutral aqueous solution, SSI dimer is tightly bound with a dissociation constant on the order of 10^{-13} M (Akasaka et al., 1982a). SSI has a p*I* of 4.3 (Sato & Murao, 1974) and is unusually stable against thermal and alkaline denaturation (Akasaka et al., 1982b,c; Takahashi & Sturtevant, 1981; Fujii et al., 1981; Akasaka, 1978). SSI has two regions of exposed peptide segments per subunit, residues 1–7 and 64–75. The latter carries the binding loop for proteases (Hirono et al., 1979). Increased mobility of the exposed peptide segments relative to the rest of the protein

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