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The role of the phosphate group for the structure of phosphopeptide products of adenosine 3′,5′-cyclic monophosphate-dependent protein kinase

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By c.d. studies it is shown that liver-pyruvate-kinase-related peptide substrates of cyclic AMP-dependent protein kinase have a high tendency towards non-random structures in non-aqueous media. When phosphorylated, the conformation tendencies decrease. This structural change is explained in terms of the formation of strong intrapeptide phosphate–guanidinium salt links. It is proposed that similar events occur at the catalytic site of protein kinase and that such an interaction could facilitate the removal of the phosphorylated products.

Protein phosphorylation plays a central role in neural and hormonal control of cellular activity (Engström, 1978; Krebs & Beavo, 1979; Cohen, 1982). Multiple basic amino acids, particularly arginine, at the phosphorylation site are prerequisites for an appreciable rate of the phosphorylation by cyclic AMP-dependent protein kinase (Zetterqvist *et al.*, 1976; Kemp *et al.*, 1977; Krebs & Beavo, 1979; Zetterqvist & Ragnarsson, 1982).

Significantly, arginine forms stable complexes with orthophosphate esters due to the combined influence of electrostatic interaction and hydrogen-bonding (Springs & Haake, 1977; Williams, 1979; Tatham *et al.*, 1983). Indeed, two guanidine nuclei can simultaneously interact with a single phosphate entity (Cotton *et al.*, 1974, 1979). Therefore it is plausible that such interactions could occur on the phosphorylated peptides, the newly phosphorylated serine residue forming an intrapeptide interaction with one or more adjacent arginine residues, thus inducing a conformation change in the product. Such interactions would be much weaker with lysine and histidine (Williams, 1979). In this context it is relevant to note that, on phosphorylation of serine-14 in glycogen phosphorylase *a*, the *N*-terminal portion of this protein is involved in a conformation change that brings the phosphorylated serine in close proximity to a number of arginine residues, including arginine-16 (Madsen *et al.*, 1978; Fletterick *et al.*, 1979).

In the present study we have used c.d. in an attempt to investigate the solution conformations of a range of cyclic AMP-dependent protein kinase peptide substrates together with their phosphorylated derivatives.

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Experimental

Preparations of peptides and phosphopeptides

Peptides were synthesized by the solid-phase method as described by Merrifield (1969) with the use of a Beckman model 990 peptide synthesizer, and most of them were purified by ion-exchange chromatography on CM-cellulose (Zetterqvist *et al.*, 1976; Engström *et al.*, 1984). The peptide preparations were analysed for amino acid content and in most cases by h.p.l.c. (Fransson *et al.*, 1982).

The [^{32}P]phosphopeptides were prepared from parent peptides by enzymic phosphorylation with [γ-^{32}P]ATP and cyclic AMP-dependent protein kinase, and were separated from non-phosphorylated parent peptides by CM-cellulose chromatography, as described by Titanji *et al.* (1980).

Phosphorylation experiments

For the determination of kinetic constants, the phosphorylation of synthetic peptides was performed in 0.04ml as described previously (Kemp *et al.*, 1976, 1977; Zetterqvist & Ragnarsson, 1982). [γ-^{32}P]ATP (New England Nuclear, Boston, MA, U.S.A.) was diluted with unlabelled ATP to give a specific radioactivity of 10–50c.p.m./pmol. The catalytic subunit of cyclic AMP-dependent protein kinase of rat liver was isolated as described by

Zetterqvist *et al.* (1976). Each peptide was used over a 10-fold range of concentrations around its K_m value. The enzyme dilutions were made in 10mM-Mes buffer, pH6.9, containing bovine serum albumin (0.5mg/ml), and were selected to yield less than 10% phosphorylation. The separation of the phosphorylated peptide from [γ-^{32}P]ATP was achieved by ion-exchange chromatography (Kemp *et al.*, 1976). ^{32}P was measured as Čerenkov radiation.

C.d. measurements

C.d. spectra were measured with Jasco J40CS instruments with 0.02cm, 0.05cm and 0.2cm cells. Indication of the noise level of each spectrum (190–200nm) is given in the Figures by an error bar. The solubility of the pentapeptide RRASV (in the one-letter notation) was enhanced in the two alcohols by the addition of 1mM-$MgCl_2$.

Estimation of secondary structure content

Estimation of α-helix was based on the method of Greenfield & Fasman (1969). Reverse-turn spectra were classified in accordance with Woody (1974).

Results and discussion

Effects of phosphorylation on peptide structure

RRASV and RRPSV. The c.d. spectrum of RRASV in aqueous solution is typical of coil structure (Greenfield & Fasman, 1969) (Fig. 1*a*). However, when dissolved in either 95% (v/v) trifluoroethanol or 95% (v/v) ethanol, evidence for the formation of defined secondary structure elements was obtained. In view of the size of the peptide, this structure is most probably a reverse turn. The c.d. spectra of reverse turns have been separated into four broad classes (Woody, 1974), and RRASV gives a class B spectrum in the presence of alcohols. Similar results were obtained with RRPSV (Fig. 1*b*), although spectra typical of class A were observed in alcohol-rich media.

RRAS(P)V and RRPS(P)V. In water, the structure of both the phosphorylated pentapeptides [S(P) represents phosphoserine] is virtually identical with that of the non-phosphorylated peptide (Fig. 1). In contrast, in alcohol solutions, a difference was displayed such that the tendency to non-random conformation was markedly diminished for both phosphorylated peptides as compared with the parent compounds.

GVLRRASVA. In aqueous solution the nonapeptide GVLRRASVA favours coil formation (Fig. 2). In alcoholic solutions some ordered structure is induced. Although the spectrum is not

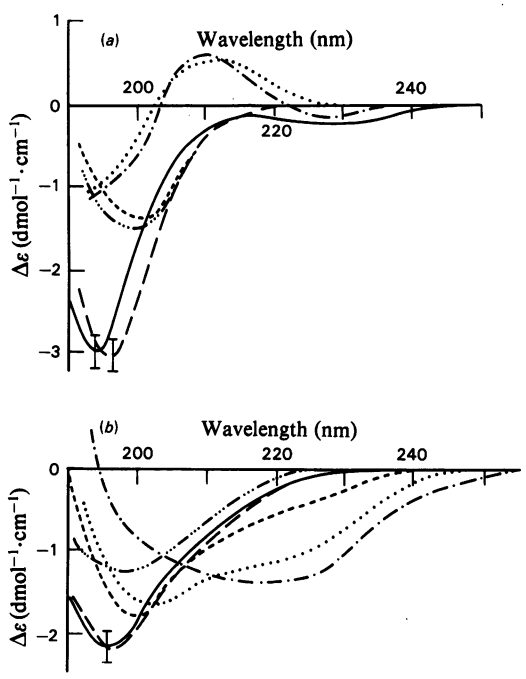

Fig. 1. *Comparison between the c.d. spectra of phosphorylated and non-phosphorylated pentapeptides (0.5mg/ml, 0.2mm-pathlength cell)*

(*a*) ——, RRASV in water, pH7.0; ——, RRAS(P)V in water, pH7.0; ·—·—, RRASV in 95% ethanol; ——, RRAS(P)V in 95% ethanol; ·····, RRASV in 95% trifluoroethanol; -----, RRAS(P)V in 95% trifluoroethanol. (*b*) ——, RRPSV in water, pH7.0; ——, RRPS(P)V in water, pH7.0; ·—·—, RRPSV in 95% ethanol; ——, RRPS(P)V in 95% ethanol; ·····, RRPSV in 95% trifluoroethanol; -----, RRPS(P)V in 95% trifluoroethanol.

typical of a reverse turn, it should be noted that a 100% formation of one single turn would only represent four-ninths of the peptide. Indeed, the spectrum can be readily interpreted in terms of mixtures of coil and reverse turn, possessing a class C spectrum (Fig. 2 inset; Woody, 1974).

GVLRRAS(P)VA. In water, there was virtually no difference in the secondary structure of the phosphorylated and non-phosphorylated peptide as judged by c.d. However, a difference between the two peptides was observed in their response to phosphate counterions in 95% ethanol. Whereas the non-phosphorylated peptide increased its ordered structure appreciably, the phosphorylated derivative failed to do so (Fig. 3).

It is thus clear that phosphorylation of the serine residue disrupts the preferred secondary structure of the non-phosphorylated peptide in favour of an alternative, non-reverse-turn, non-helical conformation. These findings are readily explained by

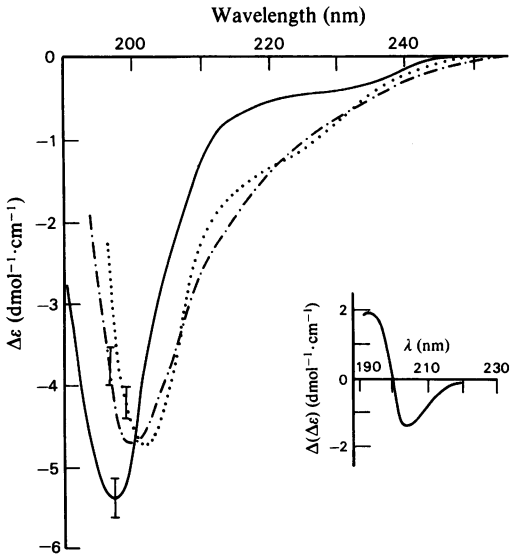

Fig. 2. *C.d. spectra of GVLRRASVA (0.5 mg/ml, 0.2 mm-pathlength cell)*

——, GVLRRASVA in water, pH 2.0 and 7.0; ·—·—·, GVLRRASVA in 95% ethanol; ·····, GVLRRASVA in 95% trifluoroethanol. Inset: c.d. difference spectrum between GVLRRASVA in 95% ethanol and GVLRRASVA in water.

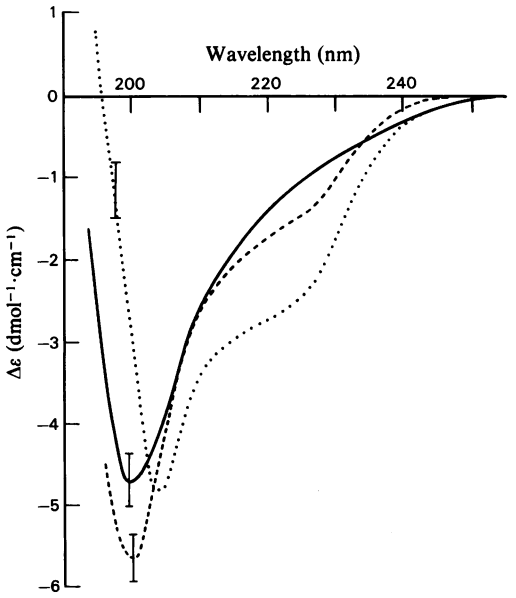

Fig. 3. *Comparison between the spectra of phosphorylated and non-phosphorylated GVLRRASVA (50 μg/ml, 1 mm-pathlength cell)*

——, GVLRRASVA in 95% ethanol; ·····, GVLRRASVA in 95% ethanol in the presence of triethylammonium phosphate (250 mM); -----, GVLRRAS(P)VA in 95% ethanol in the presence and in the absence of triethylammonium phosphate (250 mM).

the formation of an intrapeptide arginine–phosphate salt link.

This property may have relevance to events at the active site of cyclic AMP-dependent protein kinase. Once the substrate is phosphorylated, a new intramolecular binding site for the arginine moieties would be introduced and competition would be possible between the phosphate group and presumed carboxylate anions at the active site. As phosphate–guanidinium complexes are considerably more stable than carboxylate–guanidinium complexes (Springs & Haake, 1977) co-ordination to the phosphate would be favoured. Such an event could facilitate the release of the product from the enzyme.

In apparent contrast with the above findings, Granot *et al.* (1981) were unable to demonstrate a significant difference between the phosphoserine residues of the phosphopeptide LRRAS(P)LG and *O*-phospho-L-serine when the ^{31}P chemical shifts were measured as a function of pH. However, Granot *et al.* (1981) studied the peptide in aqueous solution and, as demonstrated in the present work, no appreciable difference in the secondary structure of phosphorylated and non-phosphorylated peptides was observed in this medium. In contrast, the microenvironment provided by the active site of the protein kinase will not be richly hydrated, and thus the alcoholic solutions adopted in the present work are likely to provide a superior mimic of this environment than is water.

Phosphorylation of peptides by cyclic AMP-dependent protein kinase

Kinetic parameters for the phosphorylation of the range of peptides included in this study by cyclic AMP-dependent protein kinase are presented in Table 1. The $V_{max.}$ for each peptide was virtually the same, despite the apparent K_m changing through a 20-fold range. There was a trend of decreasing apparent K_m with increasing length, which is compatible with the earlier data (Zetterqvist *et al.*, 1976; Kemp *et al.*, 1977).

Effects of $MgCl_2$ on the secondary structure of peptides

The change in K_m as described above is not readily correlated to the secondary structural trends of the peptides. Thus the addition of $MgCl_2$ had quite different effects on the secondary structure of the various peptides. The pentapeptide RRPSV changed to coil structure in alcoholic solutions upon increasing the $MgCl_2$ concentration (Fig. 4). Whereas the β-turn tendencies of the hexa- and hepta-peptide were not significantly changed upon the addition of $MgCl_2$ (results not shown), the octa- and nona-peptide displayed a strong α-helical tendency (Fig. 5).

Table 1. *Kinetic constants (±2S.D.) of synthetic peptides representing the phosphorylatable site of rat liver pyruvate kinase*

Phosphorylation was performed as described in the Experimental section. The parameters were determined by fitting the data to the Michaelis–Menten equation by use of a data program for non-linear regression (James & Roos, 1976), adapted to the Nord-10 computer at the Biomedical Center, Uppsala, by Dr. M. Hakman and Dr. T. Högland (unpublished work). The data for each peptide covered at least 15 rate determinations and five different substrate concentrations.

Peptide	K_m (μM)	$V_{max.}$ (relative rate)
RRASV	24±3	1.00±0.04
RRPSV	35±6	1.02±0.05
RRASVA	3.2±0.5	0.89±0.04
LRRASVA	2.3±0.3	0.99±0.03
VLRRASVA	2.5±0.3	1.01±0.03
GVLRRASVA	1.3±0.5	0.95±0.06

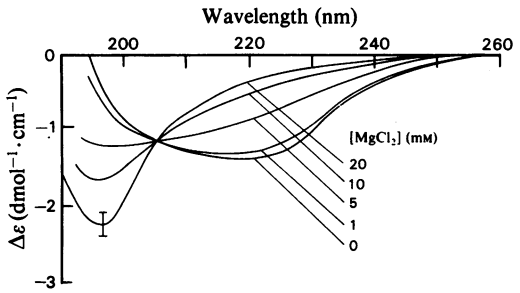

Fig. 4. *C.d. spectra of RRPSV in 95% ethanol in the presence of various concentrations of $MgCl_2$*

The concentration of peptide was 0.5 mg/ml and the cell pathlength was 0.2 mm.

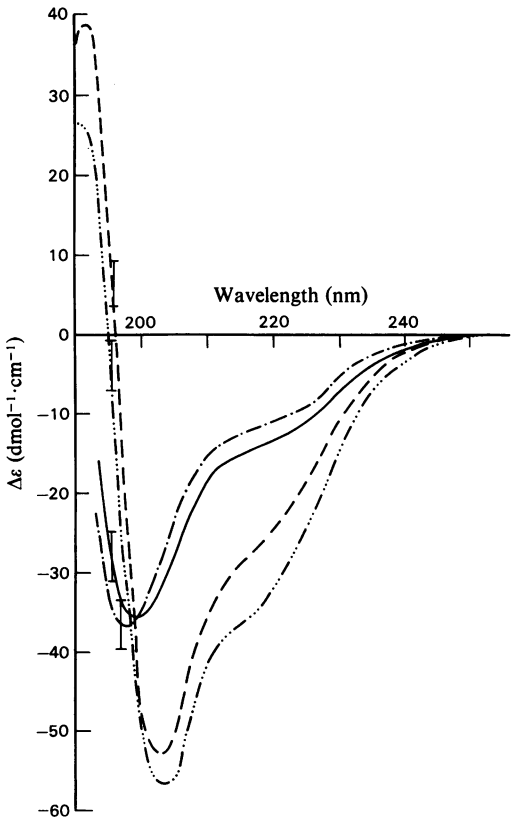

Fig. 5. *Influence of $MgCl_2$ on the c.d. spectra of VLRRASVA and GVLRRASVA dissolved in 95% trifluoroethanol*

The concentration of peptides was 50 μg/ml and the cell pathlength was 2 mm. ——, GVLRRASVA in the absence of $MgCl_2$; ——, GVLRRASVA in the presence of 2 mM-$MgCl_2$; ·—·—, VLRRASVA in the absence of $MgCl_2$; ·····, VLRRASVA in the presence of 2 mM-$MgCl_2$. That the $n\rightarrow\pi^*$ transition at 220 nm is proportionally lower than the $\pi\rightarrow\pi^*$ transition at 205 nm in c.d. spectra of the peptides is similar to that reported for other short helical peptides in trifluoroethanol (Mutter *et al.*, 1976; Schmitt *et al.*, 1982).

The effect on RRPSV can be interpreted as $MgCl_2$ disrupting an intrapeptide salt link, which contributes towards the stability of the reverse turn in solution. A similar observation has been made with a tetrapeptide where a glutamate–arginine salt bridge makes a critical contribution to the stability of the reverse turn (Mayer & Lancelot, 1981). The existence of intramolecular salt links would presumably interfere with the interaction of one or other of the arginine residues with the active site of the protein kinase. Significantly, the K_m values of the pentapeptides RRASV and RRPSV are an order of magnitude larger than for longer peptides (Table 1).

In the presence of $MgCl_2$, an α-helical conformation of VLRRASVA and GVLRRASVA is apparently stabilized (Fig. 5) and makes an appreciable contribution to the total number of conformers present in solution. By use of the method of Greenfield & Fasman (1969), the percentage contribution of this conformer was determined as 34% for VLRRASVA and 43% for GVLRRASVA. Using the same calculations, Mutter *et al.* (1976) determined a value of 20% for deca-alanine in trifluoroethanol.

In conclusion, firstly, no single secondary structural tendency observed in the present study would appear to parallel the kinetic constants. This is compatible with the suggestion by Granot *et al.* (1981) that the structure of the peptide substrate at the active site of protein kinase is a coil. Secondly, the series of c.d. spectra supports the view that the intrapeptide phosphate–guanidinium bond is sufficiently strong, in a milieu of low water content, to

overcome hydrogen bonds or carboxylate–guanidinium bonds that may participate in the binding of the substrate peptide to the protein kinase.

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