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Design of a leucine zipper coiled coil stabilized 1.4 kcal mol^{-1} by phosphorylation of a serine in the **e** position

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Abstract

Using a dimeric bZIP protein, we have designed a leucine zipper that becomes more stable after a serine in the **e** position is phosphorylated by protein kinase A ($\Delta\Delta G^P = -1.4$ kcal mol^{-1} $dimer^{-1}$ or -0.7 kcal mol^{-1} $residue^{-1}$). Mutagenesis studies indicate that three arginines form a network of inter-helical ($i, i' + 5$; $i, i' + 2$) and intra-helical ($i, i + 4$) attractive interactions with the phosphorylated serine. When the arginines are replaced with lysines, the stabilizing effect of serine phosphorylation is reduced ($\Delta\Delta G^P = -0.5$ kcal mol^{-1} $dimer^{-1}$). The hydrophobic interface of the leucine zipper needs a glycine in the **d** position to obtain an increase in stability after phosphorylation. The phosphorylated protein binds DNA with a 15-fold higher affinity. Using a transient transfection assay, we document a PKA dependent four-fold activation of a reporter gene. Phosphorylation of a threonine in the same **e** position decreases the stability by $\Delta\Delta G^P = +1.2$ kcal mol^{-1} $dimer^{-1}$. We present circular dichroism (CD) thermal denaturations of 15 bZIP proteins before and after phosphorylation. These data provide insights into the structural determinants that result in stabilization of a coiled coil by phosphorylation.

Keywords: α-helix; coiled coil; leucine zipper; phosphorylation; PKA; protein stability; serine

An important goal in protein design is to build proteins that change their properties in response to regulatory signals, such as ligand binding or covalent modifications. Leucine zipper coiled coils have been shown to change their properties in response to ligands. Abler's group has shown a benzene dependent shift from dimer to trimer for a GCN4 leucine zipper derivative (Gonzalez et al., 1996). Kim's group has shown that the influenza hemagglutinin coiled coil trimer is extended at low pHs, presumably because repulsion between glutamates is relieved by protonation (Carr & Kim, 1993). We have used the leucine zipper motif to explore energetic changes associated with post-translational phosphorylation.

The leucine zipper is a parallel dimer of amphipathic helices, which have a seven-amino acid repeating structure (O'Shea et al., 1991; Baxevanis & Vinson, 1993) denoted $(abcdefg)_n$. The first (**a**) and fourth (**d**) residues form the hydrophobic interface and the fifth (**e**) and seventh (**g**) residues contain a high frequency of charged amino acids (McLachlan & Stewart, 1975; Vinson et al., 1993). These charged amino acids lie across the hydrophobic core with their methylenes interacting with the hydrophobes of the core (the **a** and **d** positions) (O'Shea et al., 1991). Mutagenesis studies have shown that the **e** and **g** positions are able to regulate dimerization specificity (Nicklin & Casari, 1991; O'Shea et al., 1992; Vinson et al., 1993; Krylov et al., 1994; Zhou et al., 1994): oppositely charged amino acids on opposing helices are attractive and stabilize the leucine zipper dimer while similarly charged amino acids are repulsive and destabilize the dimer. Work in this laboratory, using a double mutant thermodynamic cycle, has shown that arginine in the **g** position interacts with glutamic acid in the following **e'** position ($\mathbf{g} \leftrightarrow \mathbf{e}'$) with a coupling energy of interaction of $\Delta\Delta G_{int} = -1$ kcal mol^{-1} $residue^{-1}$ (Krylov et al., 1994).

We postulated that phosphorylation of serine could mimic the glutamic acid in the $\mathbf{g} \leftrightarrow \mathbf{e}'$ pair and stabilize the leucine zipper dimer via inter-helical interactions with arginine. A phosphorylation-dependent post-translational change in dimerization stability could be used as a switch to regulate a variety of biological processes. We report here the design of a leucine zipper that becomes -0.7 kcal mol^{-1} $residue^{-1}$ more stable after post-translational phosphorylation of a serine in the **e** position by PKA. This change in dimerization stability of a coiled coil increases DNA binding 15-fold, and in transient transfection assays, we observe a four-fold activation of a reporter gene in a PKA-dependent fashion.

Results

Design of a leucine zipper stabilized by phosphorylation of serine in the e position

The amino acid sequence of the leucine zipper derivative of the bZIP protein, vitellogenin binding protein (VBP) (Iyer et al., 1991)

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that is most stabilized by phosphorylation (termed 610) is presented on a schematic of a bZIP protein (Fig. 1). Protein kinase A (PKA) (Kennelly & Krebs, 1991) was used to phosphorylate proteins because its recognition site is compatible with a coiled coil structure when the serine is placed in the **e** position. Additionally, the enzyme can be experimentally regulated (Kennelly & Krebs, 1991), is well studied, and is available commercially. To maintain the amphipathic helices required for a coiled coil structure, the PKA recognition site (RRXS/T) can be introduced with the hydrophobic amino acid (X) in either the **a** or **d** position, which places the serine or threonine in the **b** or **e** position. In this manuscript, we describe the energetic consequences of phosphorylation at the **e** position. The two arginines of the PKA recognition site are on the outside of the dimer in the **b** and **c** positions, and the hydrophobic amino acid (X) is in the **d** position. The name and amino acid sequence of the proteins discussed in this manuscript are shown in Table 1. In a separate manuscript, we describe the destabilizing effects of phosphorylating a threonine or serine in the **b** position, which reflects the α-helical–forming propensities of these phosphorylated amino acids (Szilák et al., 1997).

The PKA recognition sequence (**R**RXS) creates a potential intra-helical interaction ($i, i + 3$) between the phosphorylated serine and the arginine three amino acids N-terminally (shown in bold). Starting with the RRXS protein sequence from the PKA protein recognition site, we used CPK molecular models to aid in the design of a leucine zipper protein sequence that would be stabilized by phosphorylation of serine (Fig. 1). We added additional arginines that were designed to allow favorable inter-helical and intra-helical interactions with the phosphorylated serine. Arginines in the two flanking **g** positions, shown in bold (**R** –RR–S–**R** –), allowed potential attractive inter-helical interactions with the phosphorylated serine position of the other helix ($i, i' + 5$ and $i, i' + 2$). $i, i' + 5$ interactions have previously been observed between arginine and glutamic acid (Vinson et al., 1993; Krylov et al., 1994), while attractive $i, i' + 2$ interactions have not been reported. An arginine, shown in bold (R –RRXS–R –**R**), was introduced to produce a potential $i, i + 4$ intra-helical interaction (Marqusee & Baldwin, 1987).

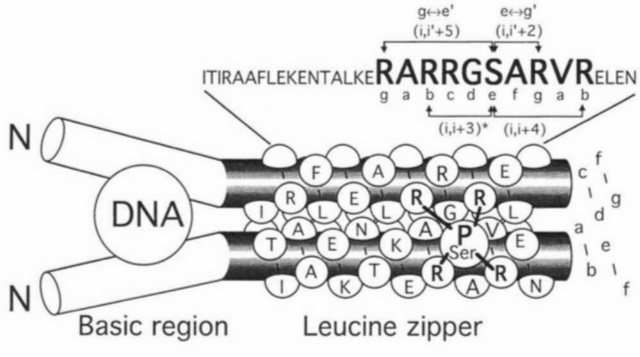

Fig. 1. (**A**) Side view of 610 (R ARRGSAR VR), the bZIP stabilized by phosphorylation of serine in the **e** position ($\Delta\Delta G^{P} = -1.4$ kcal mol^{-1} dimer^{-1}). The top shows a portion of the leucine zipper protein sequence with the critical phosphorylation site in bold. The coiled coil nomenclature is immediately under the phosphorylation sequence. A schematic of a bZIP protein bound to DNA is depicted with the placement of amino acids in the coiled coil denoted. The phosphorylated serine is depicted by a larger circle. Potential intra-helical ($i, i + 3$; $i, i + 4$) and interhelical ($i, i' + 2$; $i, i' + 5$) charged interactions between arginines and the phosphorylated serine are indicated by arrows at the top of the figure and lines in the schematic. The diagram shows the amino acids in the seven positions of a coiled coil (**a**,**b**,**c**,**d**,**e**,**f**,**g**).

Table 1. *List of proteins used in the study*[a]

Protein name	Amino acid sequence
	g abcdefg ab
594	R ARRASAR VR
608	R GRRLSAR VR
610	R ARRGSAR VR
612	R GRRASAR VR
645	R GRRGSAR VR
647	K AKKGSAK VK
654	A ARRGSAA VR
695	R VRRLSAR VR
697	R VRRGSAR VR
718	R ARRGSAR VA
736	A ARRGSAR VR
738	R ARRGSAA VR
796	R ARRGTAR VR
878	R ARRGAAR VR

[a]All proteins were engineered in the background of VBP (see Materials and methods). Only the sequence of the PKA site is shown in each case.

CPK model building suggested that for the phosphorylated serine in the **e** position to interact inter-helically with the arginines in the **g** position, the hydrophobic interface, created by amino acids in the **a** and **d** position, needed to be devoid of bulky hydrophobic side chains. Thus, we placed a glycine in the **d** position and an alanine in the **a** position, shown in bold (R **A**RR**G**S–R –R), to avoid any steric clash between the bulky phosphate group and the hydrophobic interface. The **f** position and second **a** position contained an alanine and valine, respectively (R ARRGS**A**R **V**R). This 10 amino acid protein sequence (termed 610) is essential to produce an increase in stability upon phosphorylation of a leucine zipper coiled coil dimer and occupy the following coiled coil positions (g abcdefg ab). Throughout this manuscript, the protein sequence of individual mutants is presented with a break between the **g** and **a** positions of the coiled coil heptad to aid in identification.

Enzymatic phosphorylation of PKA site in a bZIP protein

We produced in *E. coli* milligram quantities of a derivative of the bZIP protein, VBP, that contained the PKA recognition site designed to be stabilized by phosphorylation. To confirm that PKA phosphorylates the serine or threonine in the PKA recognition site, we phosphorylated the parental protein (VBP) and three derivatives that contain a PKA site with the PKA enzyme and [γ-^{32}P]ATP (Fig. 2). The parental protein, VBP, contains three serines and five threonines (see Materials and methods), but no obvious PKA recognition sites. Phosphorylation of the parental protein showed no incorporation of radioactive phosphate as assayed by SDS gel electrophoresis and subsequent autoradiography (Fig. 2; Autoradiogram). The three proteins containing a PKA site with either a serine (610: R ARRG**S**AR VR; 594: R ARRA**S**AR VR) or a threonine (796: R ARRG**T**AR VR) became radioactive (Fig. 2), suggesting that the phosphate was on the serine or threonine within the PKA recognition site. As an additional control, we mutated the serine of the PKA site to alanine (Fig. 2; Ala) to ensure that the PKA site did not affect phosphorylation outside the recog-

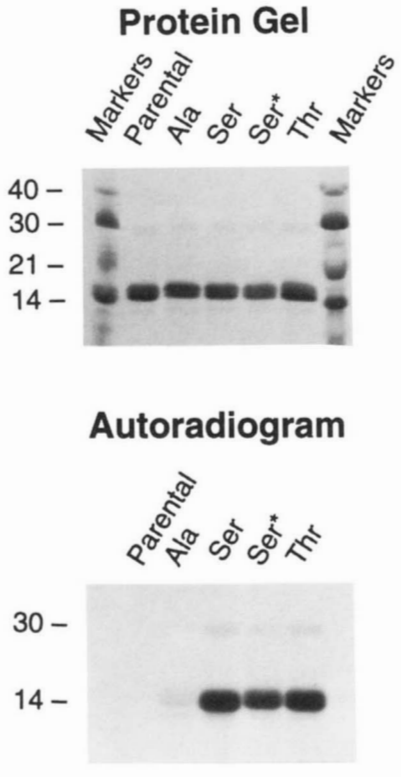

Fig. 2. Protein gel (top panel) and autoradiogram (bottom panel) of proteins phosphorylated with protein kinase A (PKA) and [γ-^{32}P]ATP to determine the specificity of phosphorylation. The top panel represents a 15% SDS acrylamide protein gel containing protein size markers and five samples, the parental protein VBP, a control where the **e** position serine in the PKA recognition site has been mutated to alanine (Ala = R ARRGAAR VR), and three proteins containing the PKA phosphorylation site (Ser: 610; Ser*: 594; Thr: 796). The bottom panel presents an autoradiogram of the dried protein gel seen in the top panel. Neither the parental nor the alanine protein became radioactive after treatment with PKA and [γ-^{32}P]ATP, while the three proteins containing either a serine or threonine in the PKA site became radioactive.

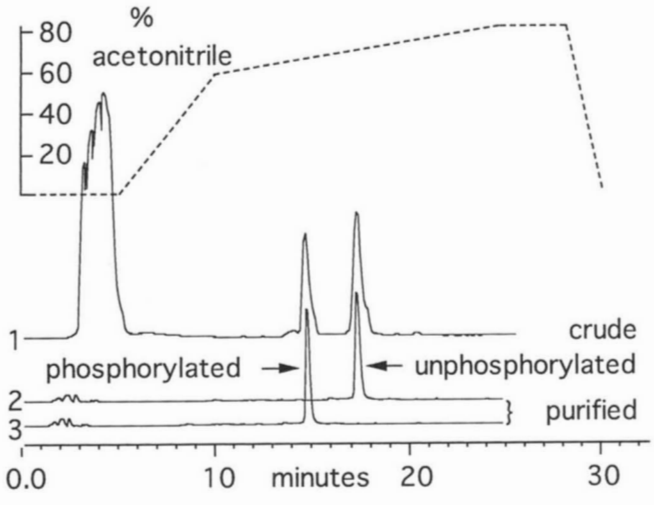

Fig. 3. Purification of phosphorylated proteins by HPLC chromatography. The top dashed trace shows the acetonitrile gradient in 0.1% heptafluorobutyric acid used for the separations. The samples eluted in a shallow portion of the gradient (60% to 80% acetonitrile). Three traces are shown: (1) the crude sample after the phosphorylation reaction of 610; approximately half of the sample is phosphorylated. Chromatogram of the purified (2) unphosphorylated and (3) phosphorylated protein.

nition site. The serine to alanine mutation (878: R ARRGAAR VR) did not become radioactive when phosphorylated with PKA and [γ-^{32}P]ATP. These mapping data indicate that PKA-dependent phosphorylation occurs only at the serine or threonine in the PKA recognition site. Proteins with a lower T_m (<50°C) were more easily phosphorylated than those with a high T_m (>50°C), suggesting that the phosphorylation reaction occurred when the protein was nonhelical, a result expected from analysis of PKA inhibitors (Glass et al., 1995).

Purification of phosphorylated proteins

Phosphorylated proteins were purified by HPLC in 0.1% heptafluorobutyric acid using an acetonitrile gradient (Ohguro & Palczewski, 1995) (Fig. 3). After the PKA phosphorylation reaction, the C_{18} column elution profile produced an additional earlier-eluting peak, which we hypothesized was the phosphorylated protein. To confirm this, a protein sample, phosphorylated in the presence of [γ-^{32}P]ATP, was chromatographed on the HPLC and all the radioactivity eluted with the new peak seen after phosphorylation (data not shown), indicating that only the new peak contained a phosphate group.

Phosphorylation increases the stability of 610

610 (R ARRGSAR VR), the protein most stabilized by phosphorylation, was characterized by analytical equilibrium ultracentrifugation and circular dichroism spectroscopy before and after phosphorylation. Equilibrium analytical ultra-centrifugation indicates that 610 is dimeric before and after phosphorylation (Fig. 4). Certain amino acid changes have been shown to induce a change in multimeric state in coiled coils (Harbury et al., 1993; Krylov et al., 1994), but phosphorylation of a serine in the **e** position does not have this property.

The effect of phosphorylation on thermal stability was studied by circular dichroism (CD) spectroscopy. CD spectra were recorded between 195 and 250 nm at 10 temperatures that covered the range of the thermal denaturation (Fig. 5). An important conclusion from these data is that, at all temperatures, the measured spectra are well represented by the population-weighted superposition of the characteristic spectra for α-helix and random coil conformations. The well-defined isodichroic point at 203 nm is consistent with a system with only two structural conformations present. The content of α-helix is maximum at low temperatures and decreases to zero above the thermal transition. The CD spectra for the unphosphorylated and phosphorylated 610 (R ARRGSAR VR) protein are very similar at 20 °C, and display the characteristic minima at 208 and 222 nm expected for a coiled coil dimer (Fig. 6).

The change in thermal stability of 610 after phosphorylation of the serine in the **e** position was determined by thermal denaturation monitored by circular dichroism at 222 nm. Samples were thermally denatured in two buffers: 10 mM MOPS, 150 mM KCl, 0.25 mM EDTA or 10 mM phosphate, 150 mM KCl, 0.25 mM EDTA. Phosphorylation increased the thermal stability by approximately 5 °C when assayed in either buffer (10 mM MOPS Fig. 7A; 10 mM phosphate Fig. 7B). This represents a change in free energy in MOPS of $\Delta\Delta G^{P} = -1.4$ kcal mol^{-1} dimer^{-1} or -0.7 kcal mol^{-1} residue^{-1} (the fitting parameters for the CD thermal denaturations are shown in Table 2B).

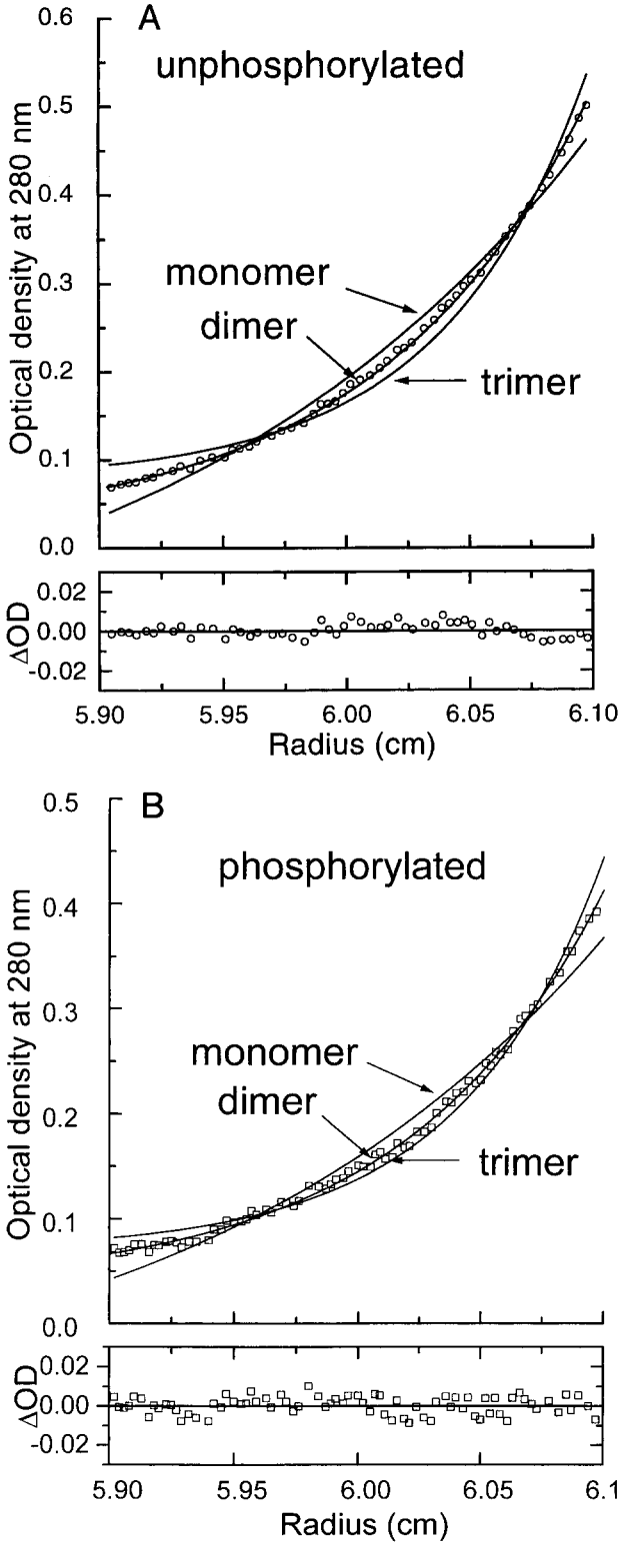

Fig. 4. Molecular weight determination by sedimentation equilibrium of 610: unphosphorylated (circle) and phosphorylated (square) proteins at 10 °C. The samples were in 10 mM potassium phosphate, pH 7.4, 150 mM KCl, 1 mM EDTA. Theoretical curves for monomer, dimer, or trimer molecular weights are plotted as solid lines. The actual data are plotted as circles or squares. Both unphosphorylated and phosphorylated proteins overlap the dimer curve. The bottom panel shows no systematic error for the residuals for either protein from fitting the experimental data to a dimer model.

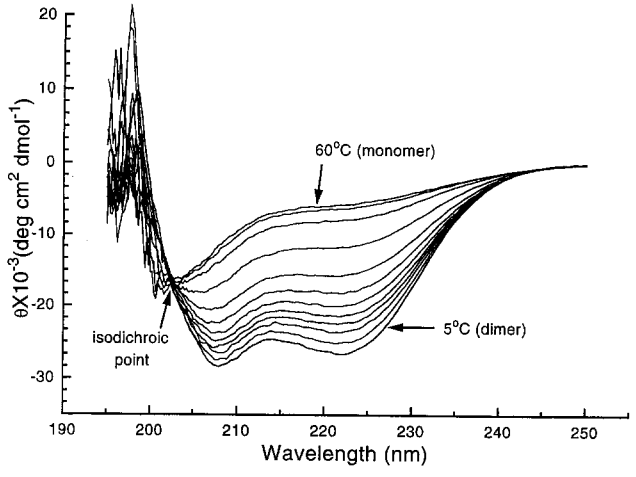

Fig. 5. Far-UV CD spectra of 610 at 4 μM as a function of temperature. The temperatures examined are from the highly ordered (dimer) at low temperature to the random coil (monomer) at high temperatures, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 °C (T_m = 36.0 °C). The minima at 208 and 222 nm, characteristic of α-helix, are seen to disappear as the sample is heated. There is an isodichroic point at 202.7 nm. The sample was in 10 mM potassium phosphate, pH 7.4, 150 mM KCl, 1 mM EDTA.

Threonine phosphorylation is destabilizing

We used a mutagenesis analysis to explore which amino acids in the 610 protein sequence (R ARRGSAR VR) were critical for the increase in stability seen after phosphorylation. Initially, we examined the effect of replacing the serine with threonine (796: R ARRGTAR VR) (Fig. 7C, Table 2A). Before phosphorylation, the serine and threonine proteins display similar stabilities. After phosphorylation, they are dramatically different. Phosphorylation of threonine destabilized the protein ($\Delta\Delta G^P$ = +1.2 kcal mol^{-1} dimer^{-1}) while phosphorylation of serine stabilized the protein ($\Delta\Delta G^P$ = −1.4 kcal mol^{-1} dimer^{-1}) leading to a difference between serine and threonine of $\Delta\Delta\Delta G^P$ = 2.6 kcal mol^{-1}

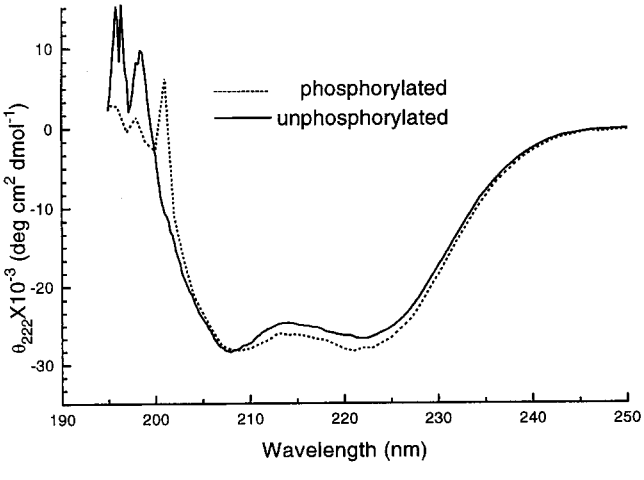

Fig. 6. CD spectra of 610 in 10 mM potassium phosphate, pH 7.4, 150 mM KCl, 1 mM EDTA at 6 °C. The unphosphorylated protein is indicated by a solid line and the phosphorylated protein by a dashed line. Both curves show minima at 208 and 222 nm, suggestive of an α-helical structure.

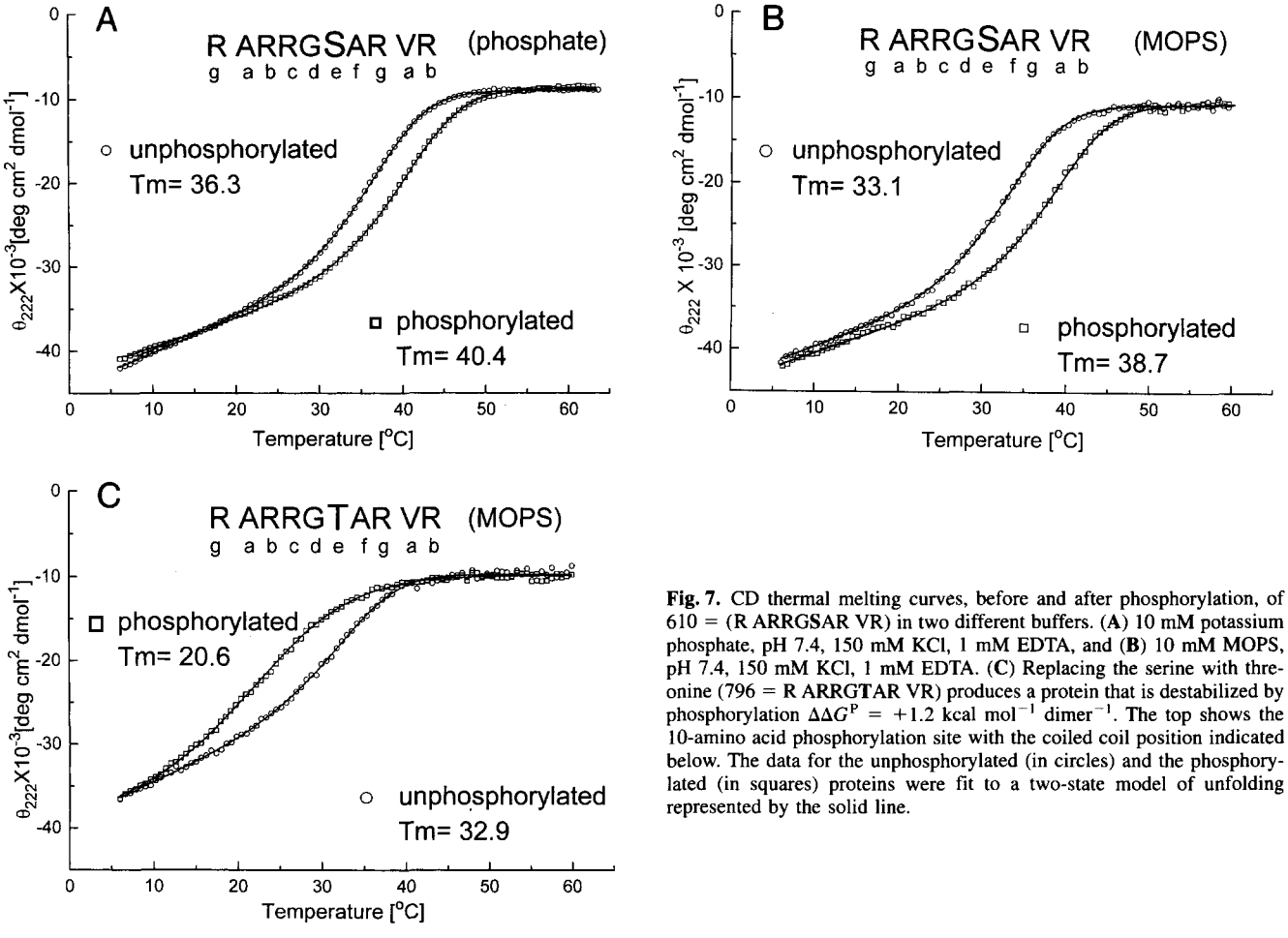

Fig. 7. CD thermal melting curves, before and after phosphorylation, of 610 = (R ARRGSAR VR) in two different buffers. (**A**) 10 mM potassium phosphate, pH 7.4, 150 mM KCl, 1 mM EDTA, and (**B**) 10 mM MOPS, pH 7.4, 150 mM KCl, 1 mM EDTA. (**C**) Replacing the serine with threonine (796 = R ARRGTAR VR) produces a protein that is destabilized by phosphorylation $\Delta\Delta G^P$ = +1.2 kcal mol^{-1} dimer^{-1}. The top shows the 10-amino acid phosphorylation site with the coiled coil position indicated below. The data for the unphosphorylated (in circles) and the phosphorylated (in squares) proteins were fit to a two-state model of unfolding represented by the solid line.

dimer^{-1}. We presume that the additional methylene in threonine sterically constrains the side-chain conformation, thus precluding interaction between the phosphate of threonine and the arginines.

A network of charged interactions with arginines

The arginines near the phosphorylated serine were mutated to alanine to determine their contribution to the increase in stability after phosphorylation of 610 (R ARRGSAR VR). Figure 8 and Table 2A present thermal denaturations for six proteins: 610 (R ARRGSAR VR) and five mutants where the arginines have been changed to either alanine or lysine. 610 (**R** ARRGSA**R** **VR**) has four arginines near the **e** position serine that is phosphorylated; two are in the **b** position and can form potential **intra**-helical interactions ($i,i+3$; $i,i+4$), and two are in the **g** position and can form potential **inter**-helical interactions ($i,i'+2$; $i,i'+5$). We changed the $i,i'+5$ arginine to alanine in 736 (**A** ARRG SAR VR), the $i,i'+2$ arginine to alanine in 738 (R ARRGS **AA** VR), or both arginines to alanine in 654 (**A** ARRGS **AA** VR). We also changed the $i,i+4$ arginine to alanine in 718 (R ARRGSAR **VA**) and all arginines to lysine in 647 (**K** A**KK**GSA**K** **VK**). One arginine, three amino acids N-terminal of the serine in the $i,i+3$ position (R **A**RRGSAR VR), could not be mutated because it is part of the PKA recognition sequence.

Changing the **g** position $i,i'+5$ arginine to alanine in 736 (**A** ARRGSAR VR) produced a protein that was slightly more stable after phosphorylation ($\Delta\Delta G^P = -0.3$ kcal mol^{-1} dimer^{-1}), indicating that the $i,i'+5$ interaction contributed 1.1 kcal mol^{-1} dimer^{-1} to stabilization. The arginine in the $i,i'+2$ position had an even more profound effect: this mutant protein (738: R ARRG SAA VR) was not stabilized by phosphorylation ($\Delta\Delta G^P = 0.0$ kcal mol^{-1} dimer^{-1}). Mutating both inter-helical arginines to alanine in 654 (**A** ARRGSAA VR) again produced a protein that was not stabilized by phosphorylation ($\Delta\Delta G^P = 0.0$ kcal mol^{-1} dimer^{-1}). The absence of additivity in the double mutant (654: **A** ARRGSAA VR) indicates that there is some coordinated interaction between the two inter-helical arginines and the phosphorylated serine. This type of interaction has been reported previously in the natural protein barnase (Horovitz et al., 1990).

Disrupting the potential $i,i+4$ intra-helical interaction by mutating the arginine to alanine in 718 (R ARRGSAR VA) indicated that this arginine is the most critical for stabilization ($\Delta\Delta G^P = +0.3$ kcal mol^{-1} dimer^{-1}). These data demonstrate that all three arginines are critical to achieve phosphorylation induced stabilization. The contributions made by individual or pairs of arginines to the increase in stability after phosphorylation are summarized graphically in Figure 8.

Changing all arginines to lysine decreased the stabilizing effect of phosphorylation ($\Delta\Delta G^P = -0.5$ kcal mol^{-1} dimer^{-1}). These data indicate that the phosphate interact more strongly with the arginines than lysines.

Table 2A. *Effect of arginines on change in stability upon phosphorylation*[a]

Interactions	Protein	Coiled coil (g abcdefg ab)	Unphosphorylated T_m	Unphosphorylated $\Delta H(T_m)$	Unphosphorylated $\Delta G(37)$	Phosphorylated T_m	Phosphorylated $\Delta H(T_m)$	Phosphorylated $\Delta G(37)$	$\Delta\Delta G^P$	$\Delta\Delta\Delta G^P$
	796	(R ARRGTAR VR)	33.0 ± 0.11	−72	−6.8	22.3 ± 0.04	−42	−5.6*	+1.2	+2.6
	610	(R ARRGSAR VR)	33.1 ± 0.06	−72	−6.7	38.8 ± 0.06	−75	−8.1	−1.4	—
$i,i'+5$	736	(A ------R -R)	35.2 ± 0.04	−74	−7.3	36.4 ± 0.06	−77	−7.6	−0.3	+1.1
$i,i'+2$	738	(R ------A -R)	23.1 ± 0.04	−49	−5.4*	22.1 ± 0.04	−44	−5.4*	0.0	+1.4
$i,i'+2$, $i,i'+5$	654	(A ------A -R)	22.2 ± 0.07	−45	−5.5*	22.8 ± 0.07	−43	−5.5*	0.0	+1.4
$i,i+4$	718	(R ------R -A)	32.6 ± 0.08	−71	−6.7	31.9 ± 0.70	−81	−6.4	+0.3	+1.7
	647	(K -KK---K -K)	24.2 ± 0.09	−59	−5.2	27.3 ± 0.10	−61	−5.7	−0.5	+0.9

[a]The table presents energetic calculations derived from thermal denaturation monitored by CD spectroscopy at 222 nm for a variety of mutant VBP proteins before and after phosphorylation. Proteins were melted in 10 mM MOPS, pH 7.4, 150 mM KCl, and 1 mM EDTA. The following parameters are presented: type of interaction between the phosphorylated serine and arginine; protein name; amino acids in PKA site; melting temperature, T_m (°C); dimerization van't Hoff enthalpy at $T = T_m$, $\Delta H(T_m)$ (kcal mol^{-1} dimer^{-1}); dimerization free energy extrapolated to $T = 37$ °C, $\Delta G(37)$ (kcal mol^{-1} dimer^{-1}); change in stability upon phosphorylation ($\Delta\Delta G^P$); contribution of amino acids to the change in stability upon phosphorylation ($\Delta\Delta\Delta G^P$). Asterisk indicates the I fitting parameter was fixed to 120 °C.

Table 2B. *Fitting parameters for proteins in Table 2A*

Protein	Coiled coil (g abcdefg ab)	Unphosphorylated I	Unphosphorylated θ_{Do}	Unphosphorylated θ_M	Phosphorylated I	Phosphorylated θ_{Do}	Phosphorylated θ_M
796	(R ARRGTAR VR)	66.4	−78.1	−195.3	120.0*	−81.2	−20.8
610	(R ARRGSAR VR)	93.6	−86.7	−21.4	98.9	−87.7	−21.2
736	(A ------R -R)	155.9	−73.8	−19.8	101.2	−73.6	−19.9
738	(R ------A -R)	120.0*	−80.7	−19.6	120.0*	−80.9	−22.6
654	(A ------A -R)	120.0*	−79.1	−24.2	120.0*	−83.6	−23.9
718	(R ------R -A)	83.2	−73.4	−18.6	76.7	−70.1	−19.3
647	(K -KK---K -K)	79.0	−80.8	−19.8	70.2	−79.7	−19.5

Table 3 presents thermodynamic parameters derived from thermal denaturations for four proteins in either phosphate or MOPS buffers. Both buffers produce similar results.

The hydrophobic interface: Glycine is required in the **d** *position*

Table 4A presents the thermal stability of seven proteins, before and after phosphorylation, that contain different hydrophobic amino acids in the hydrophobic core (**a** and **d** position). From CPK model building, we predicted that bulky hydrophobic amino acids would prevent the inter-helical interaction between the phosphorylated serine and the arginines on the opposite helix. We, therefore, engineered 610 (R ARRGSAR VR) with an ala in the **a** position and a gly in the **d** position ($\Delta\Delta G^P = -1.1$ kcal mol^{-1} dimer^{-1} in phosphate buffer and -1.4 kcal mol^{-1} dimer^{-1} in MOPS buffer). We determined the importance of three different amino acids in the **a** position, 610 with ala (R ARRGSAR VR), 645 with gly (R GRRGSAR VR), and 697 with val (R VRRGSAR VR), on the change in stability after phosphorylation. 645 and 610 (gly and ala) gave a similar increase in stability ($\Delta\Delta G^P = -1.2$ kcal mol^{-1} dimer^{-1}), while the more bulky hydrophobic amino acid valine in 697 was deleterious ($\Delta\Delta G^P = -0.5$ kcal mol^{-1} dimer^{-1}), indicating that a small hydrophobic amino acid is critical in the **a** position of the hydrophobic interface.

Four additional proteins were examined that contained a variety of combinations of hydrophobic amino acids in the **a** and **d** positions. 594 (R ARRASAR VR) replaces the **d** position gly with ala, and 612 (R GRRASAR VR) replaces the **d** position gly with ala and the **a** position ala with gly. The stability of these proteins was unchanged after phosphorylation, indicating clearly that the gly in the **d** position is critical to achieve an increase in stability on phosphorylation. Two additional proteins, 608 (R GRR LSAR VR) and 695 (R VRRLSAR VR), indicate that larger hydrophobes in the interface produce proteins whose stability is not affected by phosphorylation.

Phosphorylation of 610 (R ARRGSAR VR) increases DNA binding and trans-activation

The phosphorylated 610 (R ARRGSAR VR) protein binds DNA 15-fold better than the unphosphorylated 610 protein when examined using a gel shift assay (Fig. 9). The increase in DNA binding is an indirect result of the increase in dimerization strength after phosphorylation. The ability of the phosphorylated protein to bind DNA demonstrates that phosphorylation does not dramatically alter the leucine zipper conformation of the dimer. The phosphorylated protein bound to DNA migrates slightly more rapidly through the gel, presumably due to the negative charge from the phosphate group.

Phosphorylation of 610 (R ARRGSAR VR) increases the dimerization from $K_d = 7$ μM to $K_d = 0.7$ μM at 37 °C. To determine if this 10-fold change in dimerization affinity can affect biological processes, we introduced 610 coupled to a trans-activation

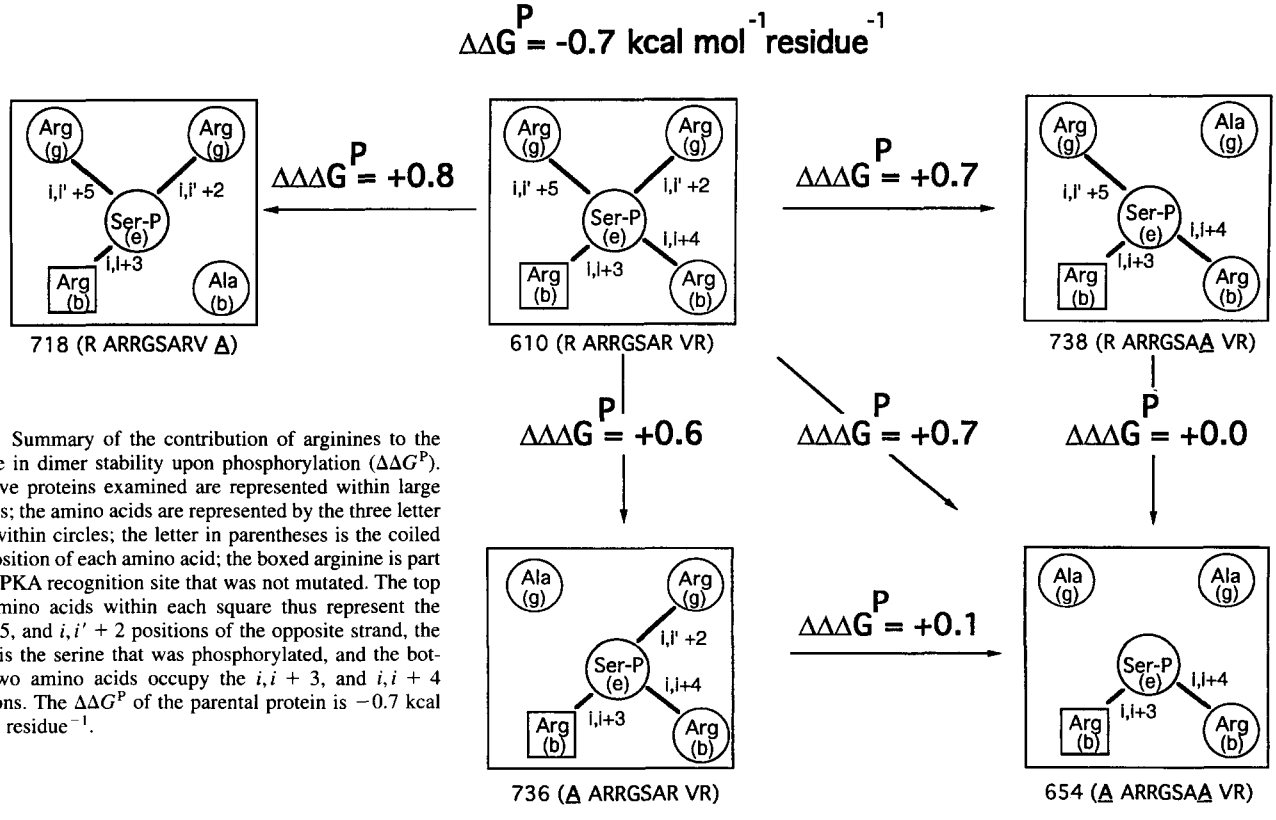

Fig. 8. Summary of the contribution of arginines to the change in dimer stability upon phosphorylation ($\Delta\Delta G^P$). The five proteins examined are represented within large squares; the amino acids are represented by the three letter code within circles; the letter in parentheses is the coiled coil position of each amino acid; the boxed arginine is part of the PKA recognition site that was not mutated. The top two amino acids within each square thus represent the $i, i' + 5$, and $i, i' + 2$ positions of the opposite strand, the Ser-P is the serine that was phosphorylated, and the bottom two amino acids occupy the $i, i + 3$, and $i, i + 4$ positions. The $\Delta\Delta G^P$ of the parental protein is -0.7 kcal mol^{-1} residue^{-1}.

domain, into cells that are deficient in PKA activity (A123.7) due to the expression of a dominant negative to the PKAI regulatory subunit (Ginty et al., 1991). This cell line allowed us to document a change in the ability of 610 to trans-activate a reporter gene in a PKA dependent fashion (Fig. 10). The reporter pVBPCAT that contains a DNA binding site for 610, either alone (lane 2), or with 610 (lane 3), or PKA (lane 4), has no trans-activation potential in this PKA-deficient cell type. The addition of the reporter pVBP-CAT, 610, and PKA together (lane 5) increases the activity of the reporter fourfold, demonstrating the PKA-dependent nature of 610 function. In HepG2 cells that contain endogenous PKA activity, 610 is able to trans-activate the reporter (lane 8), but there is no increase in reporter gene activity with the addition of PKA (lane 10). We interpret this to suggest that endogenous PKA activity phosphorylates 610, thus precluding our ability to demonstrate any exogenous PKA dependency to trans-activation.

Discussion

Critical amino acids for 610 (R ARRGSAR VR) design

We have engineered the leucine zipper of the VBP bZIP protein to become stabilized by phosphorylation of a serine in the **e** position ($\Delta\Delta G^P = -1.4$ kcal mol^{-1} dimer^{-1} or -0.7 kcal mol^{-1} residue^{-1}). Two aspects of the leucine zipper design are critical for

Table 3. *Effect of phosphate or MOPS on thermal denaturation*[a]

Protein	Coiled coil (g abcdefg ab)	Unphosphorylated T_m	Unphosphorylated $\Delta H(T_m)$	Unphosphorylated $\Delta G(37)$	Phosphorylated T_m	Phosphorylated $\Delta H(T_m)$	Phosphorylated $\Delta G(37)$	$\Delta\Delta G^P$
610-P	(R ARRG**S**AR VR)	36.0 ± 0.06	−75	−7.5	41.0 ± 0.05	−71	−8.6	−1.1
610-M	(R ARRG**S**AR VR)	33.1 ± 0.06	−72	−6.7	38.8 ± 0.06	−75	−8.1	−1.4
594-P	(- ---A--- --)	47.4 ± 0.05	−56	−9.5	47.9 ± 0.02	−54	−9.5	0.0
594-M	(- ---A--- --)	45.6 ± 0.07	−75	−9.7	45.2 ± 0.07	−70	−9.5	+0.2
654-P	(A ------A --)	23.5 ± 0.59	−43	−6.3	20.5 ± 0.60	−34	−6.3	0.0
654-M	(A ------A --)	20.8 ± 0.63	−40	−6.0	19.8 ± 0.49	−37	−6.0	0.0
647-P	(K -KK---K -K)	28.0 ± 0.10	−58	−6.0	30.3 ± 0.32	−53	−6.5	−0.5
647-M	(K -KK---K -K)	24.2 ± 0.09	−59	−5.7	27.3 ± 0.10	−61	−6.2	−0.5

[a] See Table 2A for explanation of column headers. Comparison between thermal melts of four proteins, in phosphate or MOPS buffer, before and after phosphorylation. -P denotes that the protein was in phosphate buffer, while -M that it was in MOPS buffer.

Table 4A. *Effect of amino acids in the* ***a*** *and* ***d*** *position on the change in stability upon phosphorylation*[a]

Protein	Coiled coil (g abcdefg ab)	Unphosphorylated T_m	Unphosphorylated $\Delta H(T_m)$	Unphosphorylated $\Delta G(37)$	Phosphorylated T_m	Phosphorylated $\Delta H(T_m)$	Phosphorylated $\Delta G(37)$	$\Delta\Delta G^P$
610	(R ARRG**S**AR VR)	36.0 ± 0.06	−75	−7.5	41.0 ± 0.05	−71	−8.6	−1.1
645	(- G--G--- --)	29.6 ± 0.07	−63	−6.2	35.2 ± 0.25	−45	−7.4	−1.2
697	(- V--G--- --)	46.1 ± 0.06	−68	−9.6	45.7 ± 0.06	−86	−10.1	−0.5
594	(- A--A--- --)	47.4 ± 0.05	−56	−9.5	47.9 ± 0.02	−54	−9.5	0.0
612	(- G--A--- --)	39.5 ± 0.05	−78	−8.3	40.0 ± 0.03	−62	−8.3	0.0
608	(- G--L--- --)	56.3 ± 0.02	−107	−14.0	56.9 ± 0.04	−105	−14.0	0.0
695	(- V--L--- --)	77.2 ± 0.02	−110	−20.3	77.2 ± 0.04	−109	−20.3	0.0

[a] See table 2A for explanation of column headers. Proteins were melted in 10 mM phosphate pH 7.4, 150 mM KCl, and 1 mM EDTA. The top three proteins, 610, 645, and 697, all contain glycine in the **d** position and three different amino acids in the **a** position. The second pair of proteins both contain alanine in the **d** position and different amino acids in the **a** position. The final pair of proteins contain leucine in the **d** position and different amino acids in the **a** position.

Table 4B. *Fitting parameters of proteins in Table 4A*

Protein	Coiled coil (g abcdefg ab)	Unphosphorylated I	Unphosphorylated θ_{Do}	Unphosphorylated θ_M	Phosphorylated I	Phosphorylated θ_{Do}	Phosphorylated θ_M
610	(R ARRG**S**AR VR)	88.5	−82.0	−19.2	87.7	−79.5	−20.5
645	(- G--G--- --)	78.5	−83.0	−18.7	108.0	−85.0	−17.4
697	(- V--G--- --)	111.3	−78.0	−18.0	86.4	−76.0	−17.9
594	(- A--A--- --)	128.1	−72.8	−19.8	147.5	−84.4	−20.2
612	(- G--A--- --)	87.5	−82.5	−21.5	104.8	−79.0	−20.1
608	(- G--L--- --)	109.2	−76.3	−17.6	122.2	−81.1	−19.8
695	(- V--L--- --)	181.9	−70.1	−20.3	196.4	−70.9	−20.3

stabilization upon phosphorylation. First, the phosphorylated serine must form both inter-helical and intra-helical attractive interactions with arginine. Mutagenesis studies indicate that the phosphorylated serine forms a coordinated interaction with three arginines, two in the **g** position on the opposite helix form $i,i' + 5$ and $i,i' + 2$ interactions, and one in the **b** position on the same helix forms an $i,i + 4$ interaction. Secondly, the hydrophobic interface between the two helices of the leucine zipper must contain amino acids with small side chains. The **d** position, immediately adjacent to the phosphorylated serine must contain a glycine, while the **a** position can contain either a glycine or alanine. CPK model building suggests that to optimize inter-helical interactions between the phosphorylated serine and arginines, a void must exist between the two helices. This void is created by placing the smallest amino acid glycine in the **d** position. Similar $\Delta\Delta G^P$ values are obtained when we replace the **d** position glycine with alanine or we replace either or both inter-helical arginines with alanine. This suggests that **d** position alanine totally abolishes any inter-helical interaction between phosphorylated serine and arginine.

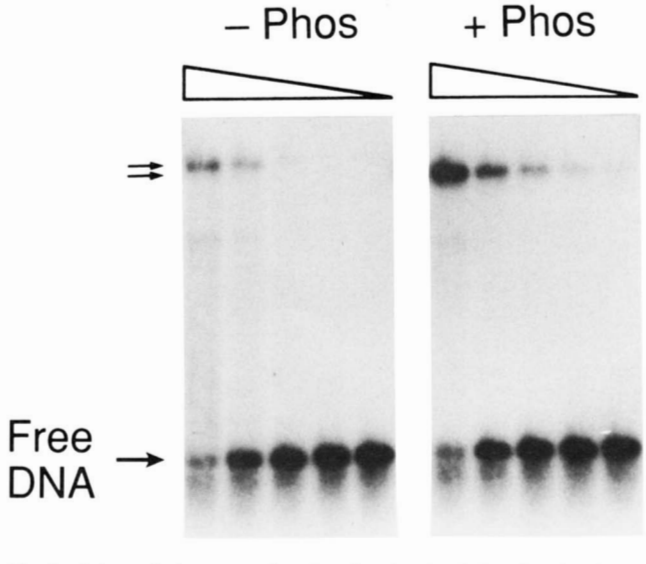

Fig. 9. Gel retardation assay of unphosphorylated and phosphorylated proteins. The left panel shows a gel retardation assay of 610 binding to a specific DNA probe 28 bp long. The right panel shows the same for the phosphorylated protein. In both experiments, the initial concentration of the proteins was 2.5 ng and was diluted fourfold in successive lanes. The phosphorylated protein can bind the DNA 15-fold better than the unphosphorylated one.

Similarities and differences between phosphorylated serine and glutamate

Our previous work with the leucine zipper motif has documented a coupling energy of -1.0 kcal mol^{-1} residue^{-1} between a glutamate in the **e** position and an arginine in the previous **g′** position

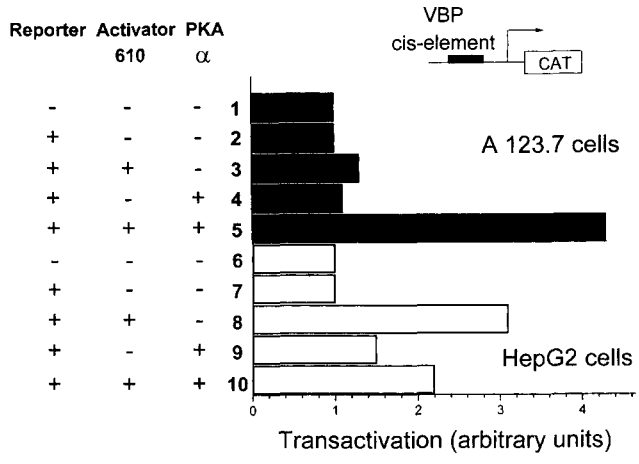

Fig. 10. Trans-activation of 610 is PKA dependent. CAT reporter activity in a PKA deficient cell line A123.7 is shown on the top half of the figure (black bars), and in a PKA containing cell line HepG2 in the bottom half of the figure (open bars). Cells were mock transfected with 20 μg of salmon sperm DNA (lanes 1 and 6) or co-transfected with up to three plasmids, the CAT expression (pVBPCAT), the trans-activator consisting of the 610 bZIP domain with a trans-activation domain (CMV′-CRP2-610), and the PKA expression vector, (RSV-CHO-PKA C-α) as indicated (lanes 2–5 and 7–10).

(denoted $i, i' + 5$ or $\mathbf{g}' \leftrightarrow \mathbf{e}$) The results from the present design indicate that phosphorylated serine in the **e** position can stabilize a coiled coil by interacting with the previous $\mathbf{g}'$ position arginine with a contribution of -0.55 kcal mol^{-1} residue^{-1} (Table 2A, Fig. 8). Attractive interactions between glutamate and arginine are not thought to occur between an **e** position and the following $\mathbf{g}'$ position ($i, i' + 2$) (Vinson et al., 1993), but our mutagenesis studies indicate that such an interaction can exist between phosphorylated serine and arginine with an energy of -0.7 kcal mol^{-1} residue^{-1}, but only if a gly is in the **d** position.

The similarities we observe between glutamate and phosphorylated serine probably reflect their charged properties. Phosphorylated serine and glutamate are both negatively charged but are structurally very different. Glutamate has two methylenes followed by a carboxylic acid. The methylenes are seen in the crystal structure to be interacting favorably with the hydrophobes of the core (O'Shea et al., 1991). Phosphorylated serine contains a methylene followed by a phosphate with four attached oxygens. This short side chain of phosphorylated serine precludes having the bulky leucine in the interface. The detailed structural differences help rationalize why the hydrophobic core needed to be so different to achieve attractive inter-helical interactions.

Phosphorylated threonine is de-stabilizing

Phosphorylation of threonine de-stabilized the coiled coil by $+0.6$ kcal mol^{-1} residue^{-1} compared to the serine stabilization by -0.7 kcal mol^{-1} residue^{-1}. The additional methylene found in threonine decreased the effect of phosphorylation by $+1.3$ kcal mol^{-1} residue^{-1}. Recent work in this laboratory has shown that phosphorylating a threonine or serine in the **b** position of a coiled coil decreases stability by $+2.3$ and $+1.0$ kcal mol^{-1} residue^{-1}, respectively, a result that we have interpreted to mean that phosphorylation changes the α-helix forming propensities of amino acids (Szilák et al., 1997). For threonine and serine in the **e** position, we expect a similar decrease in helical propensity; however, this is offset by interactions with arginines. Interestingly, the 1.3 kcal mol^{-1} residue^{-1} difference in stability between phosphorylated threonine and serine in the **e** position exactly equals the difference seen in α-helical propensities.

If phosphorylation of serine decreases coiled coil stability 1 kcal mol^{-1} residue^{-1} because of a change in helix forming propensities, the observed 0.7 kcal mol^{-1} stabilization after phosphorylation actually represents 1.7 kcal mol^{-1} of stabilizing interaction energies. This suggests that 1 kcal mol^{-1} of stabilizing interactions would be required to achieve no net change in stability upon phosphorylation. Thus, the mutant (654) with interhelical interactions disrupted and $\Delta\Delta G = 0.0$ kcal mol^{-1}, must retain 1 kcal mol^{-1} residue^{-1} of stabilization through the remaining intra-helical interaction with arginine.

Prolonging a molecular switch?

The length of time a protein is phosphorylated in vivo depends on the relative affinity of the PKA and phosphatase for the unphosphorylated and phosphorylated substrate, respectively. Our work with PKA sites in coiled coils indicates that PKA phosphorylates the recognition site when it is not helical. We conclude this because the lower the T_m, the easier it is for PKA to phosphorylate the substrate. We also presume that the phosphatase that dephosphorylates the PKA site acts on an non-helical substrate. Therefore, the stabilization of helical leucine zippers by phosphorylation could have interesting implications for molecular memory. In the cell, the PKA-dependent phosphorylation signal would last longer in the example of 610 presented here because the stabilized phosphorylated coiled coil would be refractory to dephosphorylation. Therefore, the length of time the PKA signal would last might be more extended for the 610 leucine zipper than for others. This suggests that the equilibrium between the phosphorylated and dephosphorylated proteins may depend on the type of change in secondary structure that accompanies phosphorylation. In the case of 610, the stabilization of the coiled coil by phosphorylation may preclude the dephosphorylation reaction, thus leading to a prolonged PKA signal.

Phosphorylation-dependent regulation of gene expression

Typically, the effect of phosphorylation of transcription factors on DNA binding is inhibitory. This is thought to occur via a direct mechanism where the phosphate group interferes with the ability of the basic region to bind DNA (Lin et al., 1992). Phosphorylation of 610, in contrast, enhances DNA binding 15-fold (Fig. 9). The increase in DNA binding in this case is by an indirect mechanism: stabilization of the dimer facilitates DNA binding.

We were interested in determining if the PKA-dependent change in DNA binding could be utilized in a cellular environment to regulate gene expression. We, therefore, examined the ability of 610 to change gene expression in two cell types, one with and the other without endogenous PKA activity. The ability of 610 to activate a reporter gene containing a single *cis* element was different in the two cell types. It did not activate a reporter in the cell with no PKA activity. In contrast, 610 is able to activate the same reporter in a cell line containing PKA activity (Fig. 10). To determine if the difference in the trans-activation properties of 610 is dependent on PKA phosphorylation, the catalytic subunit of PKA was co-transfected with 610 and the reporter into PKA deficient cells. In this situation, 610-dependent reporter activity is increased fourfold. This suggests that PKA is phosphorylating 610, which

increases dimerization strength leading to increased DNA binding and gene expression.

The change in K_d of 610 by phosphorylation is from 7 μM to 0.7 μM. The results of the transfection experiments indicates that this change is biologically relevant. A wonderful design property of the leucine zipper coiled coil is its cooperative folding. Thus, we can design the PKA phosphorylation sequence of 610 in the background of a leucine zipper that has any desired final dimerization strength. It would be interesting to change the absolute dimerization strength of 610 by changing amino acids outside of the PKA site and determine the consequent effect on gene expression. For example, we would like to know whether biological systems are more responsive to a 10-fold change in dimerization strength in the μM or the nM range.

bZIP proteins bind a variety of different abutted palindromic DNA sequences (Vinson et al., 1989; Hurst, 1995). The structural separation between leucine zipper dimerization and bZIP protein DNA binding suggests that the PKA site in 610 could be placed in any bZIP protein without modifying DNA recognition specificity. This would extend the DNA sequences that the 610 PKA site could regulate in a PKA-dependent fashion. The ability of bZIP proteins to bind non-palindromic DNA sequences (Olive et al., 1996) extends even further possible DNA targets for PKA-dependent gene regulation.

Materials and methods

Proteins

The sequence of the 96 amino acid host bZIP protein VBP is ASMTGGQQMGRDPLEE-KVFVPD EQKDEKY WTRRKKN NVAAKRS RDARRLK ENQITIR AAFLEKE NTALRTE VAELRKE VGRCKNI VSKYETRYGPL. The first 16 amino acids are from ϕ10 (Studier & Moffatt, 1986) and the remaining 80 amino acids are the C-terminus of VBP (Iyer et al., 1991) separated into heptads (a,b,c,d,e,f,g). The **d** or "leucine" positions of the leucine zipper are in bold type (**I, L, L, L, C**). These 80 amino acids contain the entire bZIP region of the protein and are able to bind to DNA as a dimer in a sequence specific manner (Vinson et al., 1993). The underlined amino acids were changed to contain a PKA phosphorylation site as shown below ASMTGGQQMGRDP-LEE-KVFVPEQKDEKWTRRKKNVAAKRRDARRLK ENQITIR AAFLEKE NTALKER ARRGSAR VRELENI VSKYETR YGPL. A **d** position cysteine was changed to leucine to avoid covalent dimerization via disulfide bond formation (Krylov et al., 1994).

Genetic construction of mutant proteins

Amino acid substitution mutants were introduced into VBP by the four primer PCR mutagenesis method (Ho et al., 1989). DNA sequencing was performed on double-stranded templates using the Sanger dideoxynucleotide method (Sanger et al., 1977).

Protein expression and purification

Proteins were expressed in *Escherichia coli* using the phage T7 expression system (Studier & Moffatt, 1986). Bacterial cultures (500 mL) at an optical density of 0.6 at 600 nm were induced with 1 mM IPTG (isopropyl-β-d-thiogalactopyranoside) for two hours. Cells were recovered by centrifugation, resuspended in 3 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM benzamidine, 1 mM dithiothreitol (DTT), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)), frozen, thawed, and gently brought to 1 M KCl by the addition of 1 mL of 4 M KCl. The isolated supernatant was then heated to 65 °C for 15 min, centrifuged, and the supernatant again isolated. The proteins were dialyzed into 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, and 1 mM DTT and loaded onto a 1 mL heparin agarose (Pharmacia) column. The column was washed with 4 mL of lysis buffer containing 100 mM KCl, followed by a 400 mM KCl wash, and eluted with buffer containing 1 M KCl. The 1 M KCl fraction was dialyzed against 10 mM phosphate pH 7.4, 1 mM EDTA, 50 mM KCl. The typical yield of purified protein was 2 to 3 mg of protein per 500 mL initial culture.

Phosphorylation of proteins

One milligram of protein (100 nmol) in 1.5 mL was phosphorylated with 500 units of PKA catalytic subunit (Sigma) in 10 mM potassium phosphate pH 7.4, 1 mM EDTA, 50 mM KCl, 10 mM DTT, 10 mM $MgSO_4$, 1 mM ATP for 3 h at 37 °C. After addition of 0.1% heptafluorobutyric acid, the reaction mixture was chromatographed on a Rainin HPLC system over a C_{18} column using an acetonitrile gradient in 0.1% heptafluorobutyric acid (Ohguro & Palczewski, 1995). The phosphorylated proteins eluted earlier than the unphosphorylated proteins. Trifluoroacetic acid (0.1%) was not effective in C_{18} column separation of phosphorylated proteins using an acetonitrile gradient. Typical yields of phosphorylated protein were 30% to 50%, depending on the T_m of the protein. The lower the T_m, the greater the extent of phosphorylation. Purified proteins were lyophilized and resuspended in the appropriate buffer.

Equilibrium sedimentation

Equilibrium sedimentation measurements were performed using a Beckman XL-A Optima Analytical Ultracentrifuge equipped with absorbance optics and a Beckman An-60Ti rotor. Samples were loaded at three concentrations, 10 μM, 20 μM, and 40 μM (0.1, 0.2, and 0.4 OD at 280 nm), into a six-hole centerpiece and spun at 25,000 rpm for 24 h at 10 °C. Twenty scans were collected for each concentration. Data from the three concentrations were jointly fit to calculate an apparent molecular weight. Compositional partial specific volumes for the proteins were calculated according to Zamyatnin (Zamyatnin, 1984), with the partial specific volume of phosphorylated serine (threonine) assumed to be the same as serine (threonine).

Circular dichroism studies

Circular dichroism (CD) studies were performed using a Jasco J-720 spectropolarimeter with a 10-mm cylindrical CD cell at 4 μM protein concentration. Temperature scans were performed by scanning continuously from 6 to 80 °C at a scan rate of 1 °C min^{-1}. Nonlinear fitting of the thermal denaturation data was done as described earlier (Krylov et al., 1994) using the five parameters T_m, ΔH, I, θ_M, and θ_{Do}, with the equation:

$$\theta = (\theta_M - \theta_{Do}) * \left(1 - \frac{t}{I}\right)$$

$$* \left(1 + \frac{1 - \sqrt{8 * \exp\left(\frac{1}{R} * \Delta H * \left(\frac{1}{T_m + 273} - \frac{1}{t + 273}\right)\right) + 1}}{4 * \exp\left(\frac{1}{R} * \Delta H * \left(\frac{1}{T_m + 273} - \frac{1}{t + 273}\right)\right)}\right) + \theta_{Do};$$

where θ is the ellipticity of the sample as a function of temperature (t, °C); θ_{Do} is the ellipticity of the dimer at 0 °C, θ_M is the ellipticity

of the monomer. The assumption is that the ellipticity of the dimer is a linear function of temperature ($\theta_D = \theta_{Do} + \alpha t$) and the ellipticity of the monomer is constant. I is the temperature where the ellipticity of the dimer and monomer are equal, ΔH is van't Hoff transition enthalpy, T_m is the melting temperature and R is the gas constant (1.9872 cal $°K^{-1}$ mol^{-1}). T_m and ΔH were converted to $K_d(37)$ and $\Delta G(37)$ using a ΔC_p of -1.2 kcal mol^{-1} $°C^{-1}$ determined from the relationship of T_m and ΔH for all the proteins described in the manuscript. The relationship of T_m and ΔH was the same for the unphosphorylated and phosphorylated proteins. T_m and ΔH are presented in Tables 2A, 3, and 4A, and I, θ_M, and θ_{Do}, are in Tables 2B and 4B. I, reflecting the slope of the baseline of the dimer was fixed to 120 °C for proteins with a low T_m. Fixing I, raised the T_m by 1.5 to 2.0 °C but had no effect on the calculated ΔG (marked with asterisk in Tables 2A and 2B). All thermal melts were reversible. All protein concentrations were determined by absorbance at 280 nm, and calculated with ϵ_{280} = 10,000 mol^{-1} at pH 7.4, using a Hewlett Packard 8425A spectrophotometer.

DNA binding assay

Protein was mixed in 20 μL of the gel shift reaction buffer (25 mM Tris-HCl pH 8.0, 50 mM KCl, 0.5 mM EDTA, 2.5 mM DTT, 1 mg/mL bovine serum albumin, 10% glycerol), heated to 65 °C for 2 min and cooled for 10 min to room temperature. Double-stranded DNA probe (GTCAGTCAGATTACGTAATATCGGTCAG) was added for an additional 10 min and the samples were analyzed on a 5% polyacrylamide gel in 0.5 × TBE at room temperature.

Transfection assay

The PKA deficient cell line A123.7 was grown as described (Ginty et al., 1991). Cells were co-transfected by the calcium phosphate procedure with up to three plasmids: the reporter gene driving CAT expression (pVBPCAT), the trans-activator consisting of the 610 bZIP domain (CMV′-CRP2-610), and the PKA expression vector (RSV-CHO-PKA C-α) (Maurer, 1989). To make a VBP responsive reporter, the oligos 5′-AGCTTAGATCGAGCCCC**ATTACGTAAT** CATAGA-3′ and 5′-GATCTCTATG**ATTACGTAATG**GGGCTCG ATCTA-3′ (the consensus VBP binding site is in bold) were annealed and inserted into the *Hin*dIII-*Bgl*II sites of the reporter gene p35-alb-CAT (Olive et al., 1996) to produce pVBPCAT. To construct the trans-activator plasmid, the polylinker of the plasmid pRc/CMV (Invitrogen) was deleted at *Hin*dIII and *Apa*I, and a 79 mer containing an N-terminal FLAG epitope with a nuclear localization signal (Krylov et al., 1995), followed by recognition sequences for *Not*I, *Xba*I, *Nde*I, and *Hin*dIII was inserted to make the vector CMV′. The trans-activation domain of CRP2 (Williams et al., 1991) with *Not*I and *Xba*I ends and the bZIP domain of 610 with *Nde*I and *Hin*dIII ends were generated by PCR (Ho et al., 1989). The two PCR products were inserted into the *Not*I-*Xba*I and *Nde*I-*Hin*dIII sites of CMV′, respectively, to make the final trans-activator construct CMV′-CRP2-610. Ten micrograms of the reporter (pVBPCAT), 3 μg of PKA, and 1 μg of the trans-activator were transfected into 100 mm plates of PKA^+ or PKA^- cells, either alone or in combination and assayed for CAT activity after 48 h.

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