Conformational preferences in the Ser^{133}-phosphorylated and non-phosphorylated forms of the kinase inducible transactivation domain of CREB

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Abstract Phosphorylation of Ser^{133} within the kinase inducible transactivation domain (KID) of the transcription factor CREB potentiates interaction with the KIX domain of coactivator CBP. Heteronuclear NMR spectroscopic analyses reveal that the KID domain is largely unstructured except for residues that comprise the αA helix in the pKID-KIX complex, which populate helical conformations to a significant extent (>50%). The helical content in the αB region is very small in the non-phosphorylated form (~10%) although a small increase is detected upon Ser^{133} phosphorylation. The intrinsic bias towards helical conformations probably facilitates folding of the KID domain upon binding to KIX while the principal role of the phosphate group appears to be largely in mediating the intermolecular interactions in the pKID-KIX complex.

Key words: Protein phosphorylation; Conformational change; Transactivation domain structure; Nuclear magnetic resonance spectroscopy; Protein-protein interaction; Transcription activation

1. Introduction

Eukaryotic transcriptional activators generally possess in addition to a DNA binding domain, one or more transactivation domains that serve as sites for interaction with other nuclear factors [1]. In the most common mechanism of transcription activation, the basal transcription apparatus is recruited by activators either through direct interactions with components of the apparatus or through indirect interactions mediated by coactivators [1,2]. A subset of these activator-coactivator associations is subject to further regulation by reversible covalent modifications such as protein phosphorylation [3]. Little is known, however, about the structural mechanisms by which phosphorylation modulates the affinity of such macromolecular interactions.

The transcription factor CREB plays a role in mediating the transcriptional response evoked by intracellular second messengers such as cAMP and Ca^{2+} in response to extracellular stimuli [4]. The transactivation domain of CREB is bipartite, consisting of a glutamine-rich domain termed Q2 and a domain regulated by cellular kinases termed the kinase inducible domain, KID. The activity of the Q2 domain is constitutive [5] and it relies on interactions with a TATA binding protein associated factor, $hTAF_{II}130$ [6]. By contrast, the activity of KID is dependent on phosphorylation of Ser^{133} by a variety of cellular kinases including protein kinase A (PK-A) and Ca^{2+}/calmodulin-dependent protein kinases I and IV [7,8]. Ser^{133}-phosphorylation in turn, facilitates productive interactions with the KIX domain of the coactivator CBP. When fused to a heterologous DNA binding domain such as that of GAL4, the kinase inducible domain can cooperate *in trans* with the Q2 domain or even with the acidic activation domains of other transcription factors such as GAL4 and GCN4 [5]. These results suggest that the kinase inducible domain contains all of the structural determinants required for protein kinase-A responsive activity.

A direct role for the phosphate group of phosphoserine-133 (pSer^{133}) in the binding of pKID to KIX was indicated by the NMR structure of the pKID-KIX complex ([9]; PDB accession code: 1kdx). Specifically, the phosphate group engages in intermolecular hydrogen bonding interactions with the hydroxyl group of Tyr^{658} and electrostatic interactions with the ε-amino group of Lys^{662} of KIX ([9]; M. Montminy, personal communication). In addition, a number of intramolecular interactions involving the phosphate group were observed in the NMR structure. These interactions could potentially occur within the phosphorylated form of the free polypeptide, which prompted us to examine whether Ser^{133} phosphorylation triggers any conformational transitions within KID. As we noted previously [9], the NMR spectra of both the Ser^{133}-phosphorylated and non-phosphorylated forms of KID indicate that they are largely unstructured polypeptides, although small differences in the vicinity of Ser^{133} were noticeable between the two forms in the ^{1}H-^{15}N HSQC spectrum [9]. We have analyzed these differences in greater detail and in this report present the results of our analyses.

2. Materials and methods

2.1. Protein production and NMR sample preparation

The kinase inducible domain of rat CREB (residues 101–160 plus an initiator methionine) was expressed in *Escherichia coli* BL21(DE3) and purified to homogeneity as described previously [9]. Uniformly ^{15}N- and ^{15}N,^{13}C-enriched forms of the protein were produced. Phosphorylation was accomplished in vitro by incubating with the catalytic subunit of PK-A as previously described [9]. Samples of non-phos-

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Abbreviations: CREB, cyclic AMP response element binding protein; CBP, CREB binding protein; KID, kinase inducible domain; pKID, Ser^{133}-phosphorylated KID; PK-A, protein kinase-A; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence; 3D, three-dimensional; NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; TOCSY, total correlation spectroscopy; CD, circular dichroism

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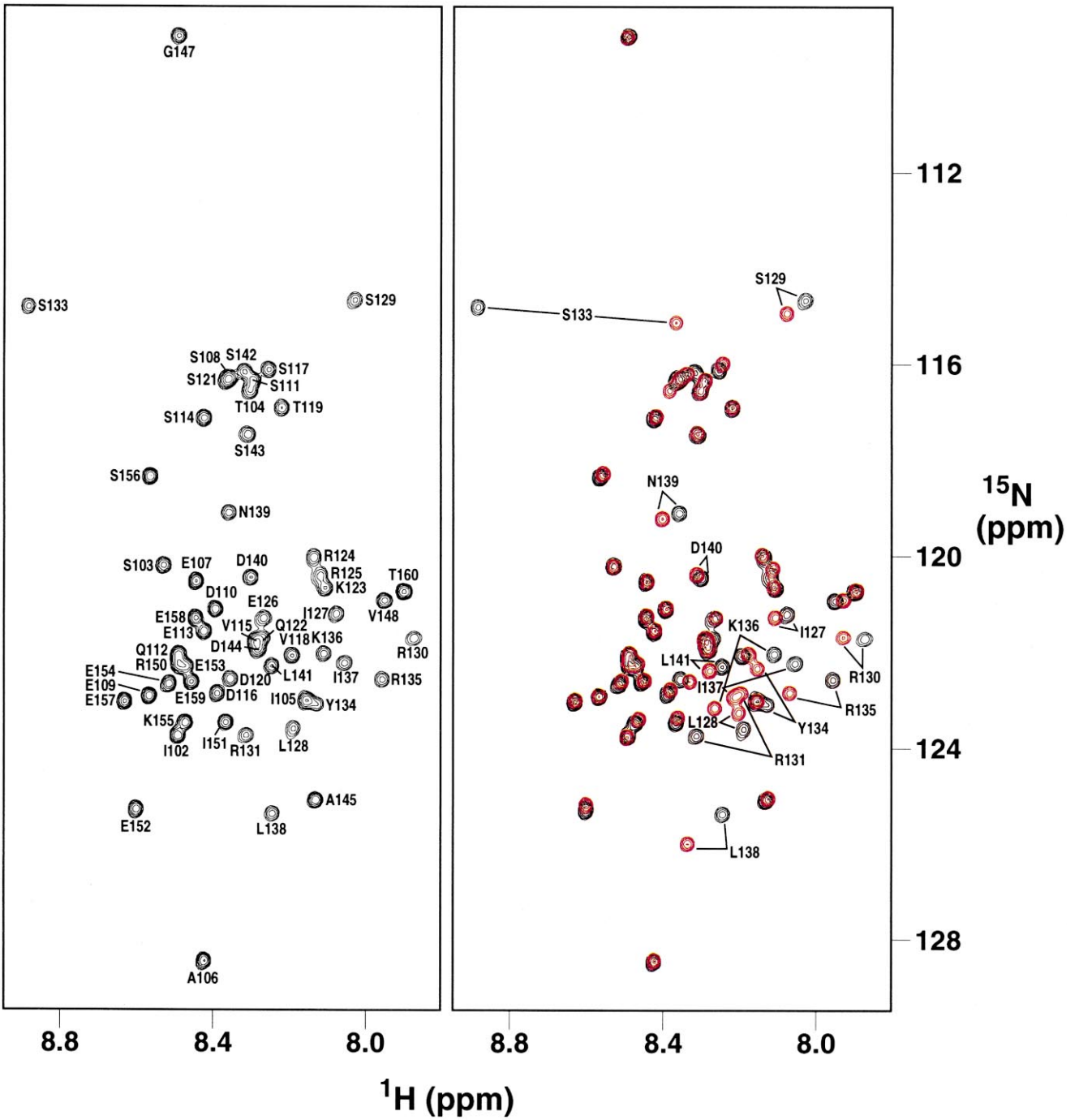

Fig. 1. a: ^{1}H-^{15}N HSQC spectrum of Ser^{133}-phosphorylated KID recorded under conditions described in the text. Residue-specific assignments are indicated. b: An overlay of the ^{1}H-^{15}N HSQC spectra of Ser^{133}-phosphorylated (black) and non-phosphorylated (red) forms of KID. Residues affected by Ser^{133} phosphorylation are labeled.

phorylated and phosphorylated forms of ^{15}N and ^{15}N,^{13}C-labeled KID in the concentration range 1.2–1.5 mM were prepared in 90% H_2O/10% D_2O buffer (20 mM Tris-d_{11} acetate-d_4, pH 6.6, 50 mM NaCl, 0.2% NaN_3) for NMR studies.

2.2. NMR spectroscopy

All NMR spectra were recorded at 15°C on Bruker AMX500 and AMX600 spectrometers equipped with triple-axis shielded gradient triple-resonance probes. NMR data processing and analysis were performed using Felix95 and Felix97 (Molecular Simulations Inc., San Diego) software packages. Backbone resonances for non-phosphorylated and Ser^{133}-phosphorylated forms of KID were assigned sequence-specifically from 3D HNCACB, CBCA(CO)NH, HNCO, HCACO and ^{15}N-edited TOCSY-HSQC spectra [10,11]. 3D ^{15}N-edited NOESY-HSQC (τ_m = 150 ms) spectra were recorded in order to confirm resonance assignments and also to obtain structural information. The resonance assignment strategy for the Ser^{133}-phosphorylated form of KID in the pKID-KIX complex was described previously [9]. These experiments were performed at pH 5.5.

2.3. Chemical shift analyses

Secondary shifts were calculated using published random coil values for H^{α} and C^{α} [12]. Appropriate corrections to the random coil shifts were made for residues preceding a proline residue [13]. The H^{α} ran-

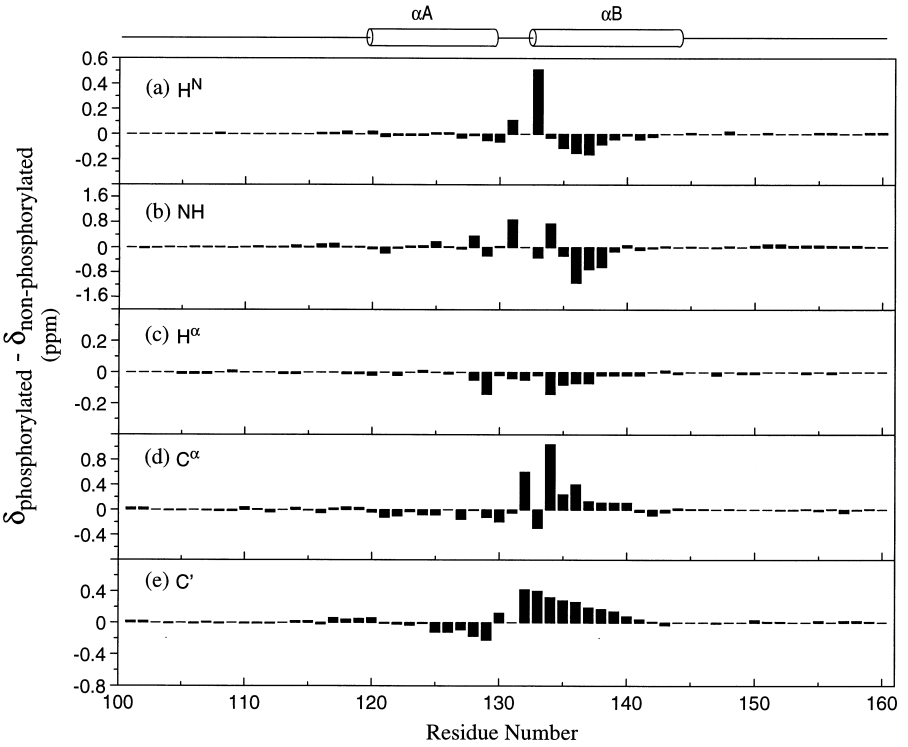

Fig. 2. Chemical shift differences between the Ser[133]-phosphorylated and non-phosphorylated forms of KID plotted by residue for the (a) amide protons (H^N), (b) amide nitrogens (NH), (c) alpha protons (H^α), (d) alpha carbons (C^α) and (e) carbonyl carbons (C′). The cartoon at the top of the figure depicts the location of the αA and αB helices in the pKID-KIX complex.

dom coil value for phosphoserine was taken from [14]. Since the C^α random coil value for phosphoserine is not known, the C^α secondary shift for pSer[133] was calculated using the random coil value for unmodified serine. Helix populations within specified residue ranges were calculated from the ratios of the cumulative C^α secondary shifts in the free and bound states [15,16].

3. Results

The backbone resonances of both Ser[133]-phosphorylated and non-phosphorylated forms of KID were assigned using triple-resonance experiments. The amide proton resonances are poorly dispersed (Fig. 1) in both forms of KID, indicating a largely unstructured polypeptide. Because of the excellent dispersion of the amide nitrogen resonances, however, almost complete backbone resonance assignments for both forms of the protein were possible. Since our primary goal was to derive structural information, we performed these studies at a relatively low temperature (15°C) in order to reduce the rates of amide proton exchange and that of molecular reorientation for improved sensitivity of the NOESY experiments.

The chemical shifts of backbone amide resonances for most of the residues within the KID polypeptide remain unchanged upon Ser[133] phosphorylation. However, significant differences (>0.2 ppm, ^{15}N and ≥ 0.03 ppm, ^{1}H) are readily apparent for residues that are located in the vicinity of Ser[133] in the primary structure (Figs. 1b and 2). These changes propagate beyond a few residues on either side of the phosphorylation site. Moreover, these perturbations are not restricted to amide proton and nitrogen resonances; the alpha proton (H^α), alpha carbon (C^α) and carbonyl carbon (C′) resonances belonging to the same residues are affected as well (Fig. 2) suggesting that the perturbations may not be exclusively due to the magnetic anisotropy and electrostatic effects of the phosphate group. Because the chemical shift changes could also be explained by changes in the conformational ensemble as a result of phosphorylation, we extended our analysis by calculating the secondary chemical shifts for the backbone nuclei.

The secondary chemical shift of a particular nucleus is defined as the observed chemical shift less its random coil value. Theoretical calculations and empirical correlations have established that the secondary chemical shifts of certain backbone nuclei are largely determined by the local (ϕ, ψ) dihedral angles ([17]; reviewed in [12]). This approach has been found to be extremely useful for identifying secondary structural elements within folded, globular proteins [12] and for estimating the secondary structural content within unfolded proteins [15] and peptides [16]. It is particularly suitable for the KID polypeptide because in many ways it behaves like an unfolded protein in the absence of KIX (see below).

We chose to analyze the H^α and C^α secondary chemical shifts because, unlike the other backbone nuclei, they are not influenced by hydrogen bonding effects [12] and are the most sensitive to secondary structure. The secondary shifts for the non-phosphorylated and phosphorylated forms of KID are shown in Fig. 3; shifts for the KIX-bound form are included for comparison. In the pKID-KIX complex, residues in both the αA (residues 120–129) and αB (residues 134–144) helices [9], exhibit large (>0.2 ppm), negative (i.e. upfield) H^α secondary shifts and large (>0.7 ppm) positive (i.e. downfield) C^α shifts. The same residues exhibit greatly diminished secondary shifts in both non-phosphorylated and phosphorylated forms in the absence of KIX. Residues outside this

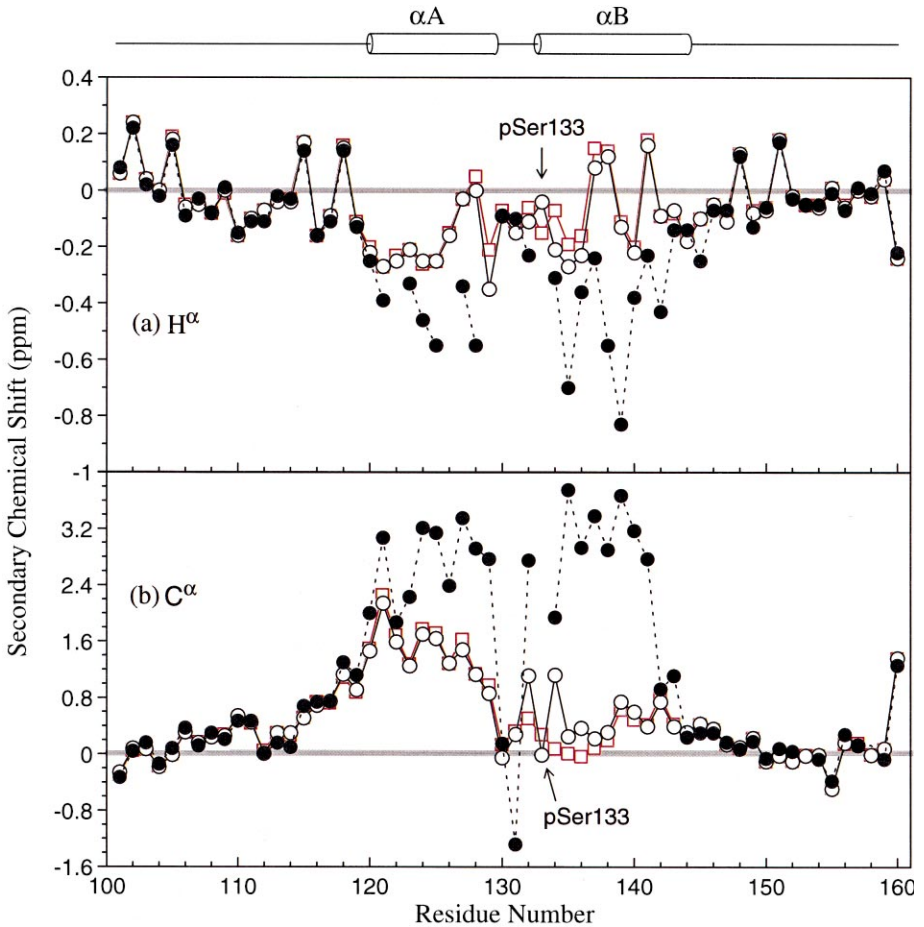

Fig. 3. H^{α} (a) and C^{α} (b) secondary chemical shifts plotted by residue for non-phosphorylated (□), Ser^{133}-phosphorylated (○), and KIX-bound (●) forms of KID. The cartoon at the top of the figure depicts the location of the αA and αB helices in the pKID-KIX complex. The C^{α} secondary shift for $pSer^{133}$ was calculated using the C^{α} random coil value for an unmodified serine residue. H^{α} secondary shifts for residues Gln^{122}, Glu^{126}, Ser^{129} and Ser^{133} and C^{α} secondary shifts for residues Ser^{133}, Glu^{153} and Glu^{158} in the KIX-bound form were not calculated because the corresponding resonances are either not assigned or their positions are unresolved due to overlap.

region however exhibit shifts that are close to zero, characteristic of a random coil, in all three states of KID (Fig. 3). Collectively, these observations reinforce our earlier findings that the domain is largely unstructured when free in solution [9].

Although residues comprising helices αA and αB in the pKID-KIX complex exhibit significant chemical shift differences between the free and bound states (see above), these differences are not uniformly distributed along the sequence (Fig. 3). They are less pronounced for residues within helix αA than in helix αB. The helical population in non-phosphorylated KID estimated for residues 120–129 is 50–60% while that for residues 134–144 is about 10%. The substantial difference in helical content between these two regions is probably due to differences in sequence composition. Indeed, residues 120–129 are predicted to populate helical conformations to a much greater extent than residues 134–144 by the program AGADIR, which predicts the helical behavior of peptides in solution [18].

The effect of Ser^{133} phosphorylation is manifested in backbone chemical shift changes that extend over several residues in the vicinity of the phosphorylation site (Fig. 2). These changes translate to a small increase in the population of α-region dihedral angles at the beginning of the αB helix (Fig. 3). The helical population as estimated from C^{α} secondary shifts for residues 134–144 is about 15% in phosphorylated KID - a small increase in helical content over the non-phosphorylated form. Consistent with this trend towards increased helicity, the H^{N} and NH resonances within this region shift upfield while the C′ resonances shift downfield upon Ser^{133} phosphorylation (Fig. 2). These effects decline almost monotonically along the chain starting from the phosphorylation site. Small changes in chemical shift are also observed N-terminal to the phosphorylation site, which suggest some change in the local conformational propensities of the polypeptide backbone in the segment connecting the αA and αB regions.

Further evidence for phosphorylation-induced conformational changes comes from analysis of NOESY data. Interproton NOEs are the single most important source of structural information because they rely on spatial separations of hydrogen atoms – a feature that is utilized routinely in NMR structure determination [19]. The interpretation of NOEs in unstructured proteins and peptides is not straightforward because they represent population weighted averages [20]. Nevertheless, when used in conjunction with chemical shift information, they can provide valuable insights. The NOESY spectra of the free KID polypeptide are characterized by strong, sequential $d_{\alpha N}(i,i+1)$ NOEs throughout, indicating

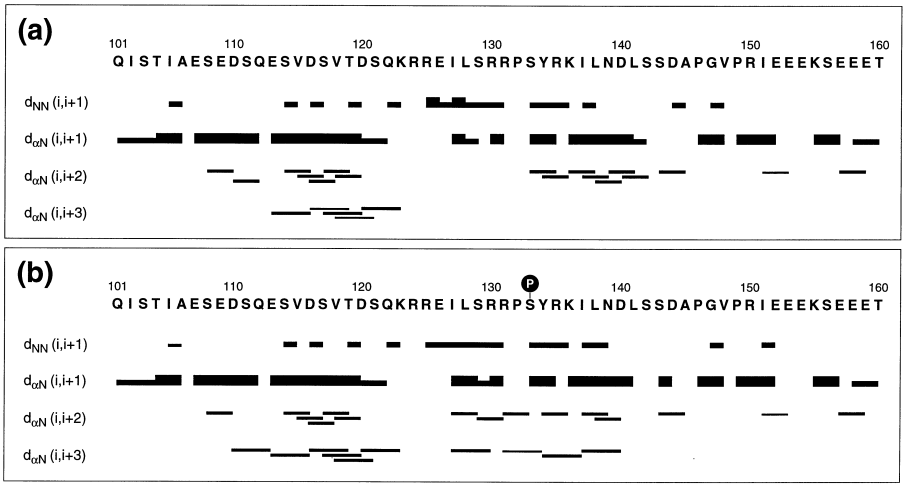

Fig. 4. NOE connectivity diagram summarizing sequential and medium-range correlations observed in 3D ^{15}N-edited NOESY ($\tau_m = 150$ ms) spectra of the (a) non-phosphorylated and (b) Ser^{133}-phosphorylated forms of KID. The thickness of the bars reflects the intensity of the correlations.

that the polypeptide backbone preferentially populates the β region of (ϕ, ψ) space (Fig. 4). However, the added presence of moderately intense $d_{NN}(i,i+1)$ NOEs, especially within regions corresponding to the αA and αB helices, indicates that some regions of the peptide also populate the α region. A number of weak $d_{\alpha N}(i,i+2)$ NOEs are observed throughout the domain, but mostly concentrated in the region corresponding to the αB helix. The presence of both $d_{NN}(i,i+1)$ and $d_{\alpha N}(i,i+2)$ NOEs implies the presence of nascent helical conformations in this region [20]. A few weak or very weak $d_{\alpha N}(i,i+3)$ NOEs can be detected within the αA helix and for the segment immediately N-terminal to it, in accord with the pattern of secondary shifts which implied somewhat greater helical content in this region (Figs. 3 and 4). The region in the middle of this helix is characterized by severe H^{α} resonance overlap, which precluded observation of additional NOEs of this kind.

The NOE patterns in both phosphorylated and non-phosphorylated forms of the domain are very similar except in the vicinity of the phosphorylation site. A few weak or very weak NOEs could be detected in phosphorylated KID that are not present in the non-phosphorylated form. Unambiguous NOEs include, I127H^{α}-S129H^{N}, I127H^{α}-R130H^{N}, R131H^{α}-pS133H^{N}, R131H^{α}-Y134H^{N}, Y134H^{α}-I137H^{N} and I137H^{α}-D140H^{N} (Fig. 4). These NOEs are consistent with the pattern of chemical shift changes that suggest an increase in helical content near the N-terminus of the αB helix and also indicate some restructuring of the loop segment.

4. Discussion

We have characterized the conformational features of both the Ser^{133}-phosphorylated and non-phosphorylated forms of the kinase inducible domain of CREB using NMR spectroscopy in aqueous solution. Our results suggest that the domain lacks persistent secondary structure in both phosphorylated and non-phosphorylated forms when free in solution. Consistent with these results, the kinase inducible domain has been found to be highly susceptible to the action of proteases [21,22]. For example, the two predominant products from limited tryptic digests of full-length CREB were mapped to Arg^{125} and Arg^{135}, both of which are located within the kinase inducible domain [21]. Further evidence for the absence of significant secondary structure within this domain comes from circular dichroism studies performed on both full-length CREB and shorter peptides containing the KID domain [22,23]. The DNA binding domain and the leucine zipper dimerization domain together account for the bulk of the signal at 222 nm (characteristic of α-helical structure) in the CD spectrum of the intact protein, whereas, the N-terminal 265 residues of CREB and a peptide containing the KID region show only a weak signal at this wavelength. The propensity of residues within KID to populate helical conformations, as revealed by our studies, probably accounts for this weak CD signal.

The principal finding of our studies is that the KID domain, whether phosphorylated or non-phosphorylated, is intrinsically unstructured prior to binding to the KIX domain of CBP. In the non-phosphorylated KID domain, the αA region has a relatively strong tendency to populate helical conformations, in contrast to the αB region, which exhibits only a very small helical propensity. Ser^{133} phosphorylation leads to an increase in the population of helical states in the αB region but overall this increase is quite small and is localized near the phosphorylation site. Phosphorylation also appears to have a small effect on the conformation of the segment linking the αA and αB regions; the rest of the domain is relatively unperturbed by the modification. Collectively, these results reinforce the notion that phosphorylation per se does not lead to a stable, folded conformation for the KID domain.

In the solution structure of the pKID-KIX complex, the phosphate group can engage in helix 'capping' interactions with its own amide proton [9]. Also, by virtue of its location near the N-terminus of the αB helix, an interaction of the negative charge with the positive pole of the helix dipole is possible [24]. Such interactions may also be operative in free Ser^{133}-phosphorylated KID, and could well lead to a threshold population of a conformation that is 'competent' to interact with KIX. While the detailed mechanism by which the folding transition of pKID is coupled to KIX binding is not

yet understood, the intrinsic bias of the polypeptide backbone towards the conformations that are stabilized in the complex probably facilitates the process. One can draw an analogy with protein folding, where the secondary structure of the native state is frequently observed to be sampled preferentially in unfolded states, i.e. the conformational search is biased by the local amino acid sequence towards the native-like secondary structure [25]. The strong intrinsic propensity of the αA region to populate helical conformations ($>50\%$ in the unbound state) might well be important for nucleation of folding of the remainder of the pKID domain upon binding to KIX. Although the αA helix makes relatively few contacts with the surface of KIX (contributing only $\sim 20\%$ to the total buried surface area [9]), mutation of Leu^{128} to alanine severely impairs binding (M. Montminy, personal communication), consistent with a possible role for this helix in initiation of folding.

Reversible serine/threonine phosphorylation is a recurring mechanism for regulating protein function [26]. In a number of cases, including, glycogen phosphorylase from rat muscle and yeast [27,28], cyclin-dependent protein kinase CDK2 [29], the phosphate group plays an active role in remodeling the local structure while inducing conformational changes at a global level. The role of Ser^{133} phosphorylation within the kinase inducible domain of CREB differs from these mechanisms in two respects: (i) global conformational changes are absent and (ii) local conformational changes do not lead to persistent secondary structure until the KID domain interacts with its cognate binding site [9]. Thus Ser^{133} phosphorylation alone is insufficient to stabilize the folded structure of the free KID domain, implying a principal role for the phosphate group in mediating intermolecular interactions in the complex.

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