Electrostatic Interactions at the C-Terminal Domain of Nucleoplasmin Modulate Its Chromatin Decondensation Activity†

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ABSTRACT: The chromatin decondensation activity, thermal stability, and secondary structure of recombinant nucleoplasmin, of two deletion mutants, and of the protein isolated from *Xenopus* oocytes have been characterized. As previously reported, the chromatin decondensation activity of recombinant, unphosphorylated nucleoplasmin is almost negligible. Our data show that deletion of 50 residues at the C-terminal domain of the protein, containing the positively charged nuclear localization sequence, activates its chromatin decondensation ability and decreases its stability. Interestingly, both the decondensation activity and thermal stability of this deletion mutant resemble those of the phosphorylated protein isolated from *Xenopus* oocytes. Deletion of 80 residues at the C-terminal domain, containing the above-mentioned positively charged region and a poly(Glu) tract, inactivates the protein and increases its thermal stability. These findings, along with the effect of salt on the thermal stability of these proteins, suggest that electrostatic interactions between the positive nuclear localization sequence and the poly(Glu) tract, at the C-terminal domain, modulate protein activity and stability.

Nucleoplasmin (NP)[1] is a key element in chromatin assembly and sperm chromatin remodeling. It belongs to a family of acidic proteins, and was the first identified molecular chaperone due to its ability to mediate nucleosome assembly (*1*, *2*). *Xenopus* NP has 200 amino acids and adopts a pentameric structure in solution (*3*). Each monomer folds into two domains: a protease-sensitive C-terminal domain, also called the "tail" domain, which contains the nuclear localization sequence (NLS) and the poly(Glu) tract within approximately 80 residues, and a protease-resistant N-terminal domain, known as the "core" domain (*3*). The core domain is able to oligomerize into pentamers, is stable against the thermal challenge, and folds into a β-structure, whereas the tail domain is flexible and folds mainly into loops and turns (*4*, *5*). The biological function of nucleoplasmin is based on its ability to interact with and bind basic proteins, specifically histones H2A and H2B and sperm basic proteins (*6*). The poly(Glu) tract is believed to be critical in NP's interaction with these proteins. *Xenopus* sperm chromatin is mainly packed due to histones H3 and H4 and specific basic polypeptides. The higher affinity of NP for the basic proteins allows the exchange of the H2A−H2B dimer, normally associated with NP, by these proteins in an energetically favored reaction that accompanies chromatin decondensation (*7*).

During activation of oocytes to eggs, NP undergoes hyperphosphorylation (*8*, *9*), which enhances the ability of the protein to perform its biological functions, like nucleosome assembly and the decondensation of sperm nuclei (*9*). We have recently found by chemical analysis and mass spectrometry that oNP and eNP have an average number of phosphate groups per protein monomer of 3 and 7−10, respectively (*5*). In the work presented here, we explore how the interaction between different regions of the C-terminal domain of NP modulates its chromatin decondensation activity and conformation. Our data suggest that electrostatic interactions between the poly(Glu) tract and basic residues located in the NLS-containing region control the accessibility of the negatively charged amino acids that bind basic proteins. Although these studies have been performed on rNP and deletion mutants that are not phosphorylated (*5*) (Figure 1), a similar mechanism could, at least in part, account for the phosphorylation-induced activation observed for oNP.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant NP. The coding region of the gene of a nonfusion form of full-length nucleoplasmin was amplified by PCR and cloned into the pET11b expression vector (*5*, *10*). The deletion mutants NPC50 and NPC80, lacking 50 and 80 residues at the C-terminus, respectively, were generated by PCR-based mutagenesis. Competent *Escherichia coli* BL21(DE3) cells

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[1] Abbreviations: DSC, differential scanning calorimetry; CD, circular dichroism; rNP, recombinant nucleoplasmin; NPC50, nucleoplasmin deletion mutant lacking 50 amino acids at the C-terminus; NPC80, nucleoplasmin which lacks 80 residues at the C-terminal domain; oNP, nucleoplasmin isolated from *Xenopus* oocytes; eNP, nucleoplasmin purified from *Xenopus* eggs.

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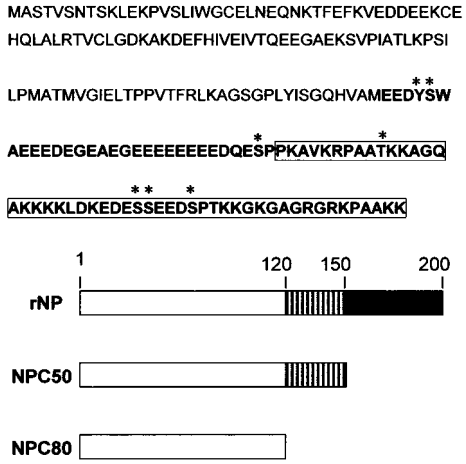

FIGURE 1: Amino acid sequence of nucleoplasmin and schematic representation of the deletion mutants characterized in this study. The residues deleted to generate NPC50 are shown within a box, and those removed in NPC80 are in bold. The asterisks denote the predicted phosphorylatable amino acids at the C-terminal domain of the protein. The C-terminal domain contains two different regions with opposite net charge; the one represented with a black box is positively charged and contains the NLS, and that shown as a striped box bears the poly(Glu) tract.

were transformed with the resultant recombinant plasmids, which contained no errors in the nucleotide sequence. Cell growth, expression conditions, and protein purification were performed as previously described (*5*).

Purification of NP from Xenopus laevis Oocytes. Ovaries were dissected from mature females and treated as reported previously (*11*). Protein purification was carried out as previously described (*5*). The purity of the sample was analyzed by two-dimensional PAGE.

Circular Dichroism. CD spectra were measured at 25 °C on a Jasco 720 spectropolarimeter, using rectangular quartz cuvettes with a path length of 0.01 cm. The protein concentration, as measured by amino acid analysis, was 0.4−0.5 mg/mL in 10 mM Tris-HCl and 50 mM NaCl (pH 7.4). Mean residue ellipticity values were calculated, after buffer subtraction, using the expression $\Theta = \epsilon/10cnl$, where ϵ is the ellipticity (millidegrees), c is the protein concentration (moles per liter), l is the path length of the cuvette, and n is the number of amino acid residues in the protein (200 for rNP and oNP, 150 for NPC50, and 120 for NPC80). CD spectra in the 190−240 nm spectral range were analyzed using LINCOMB with a reference data set of 30 proteins (*12*, *13*), and the neural network-based K2D method (*14*). The molecular masses of the monomers of rNP, NPC50, and NPC80 were 22 043, 16 543, and 13 200 Da, respectively, as calculated from the amino acid sequence of the protein.

Activity Assays. Demembranated sperm nuclei (12 000 nuclei/μL) from *Xenopus laevis* were prepared as reported previously (*15*), and were incubated in 25 mM Tris-HCl, 100 mM KCl, and 2 mM $MgCl_2$ (pH 7.4) in the absence and presence of the desired NP variant (32 μM). Aliquots were removed at specific incubation times and diluted with 4,6-diamidino-2-phenylindole (DAPI) to label DNA, and the unfixed samples were inmediately photographed.

Electrophoretic analyses were also carried out to characterize the basic proteins removed from DNA by nucleoplasmin. Briefly, the corresponding NP (30 μM) and demembranated sperm nuclei were incubated as described above, and centrifuged (13000*g* for 10 min). The resulting supernatants, containing NP and basic proteins removed from DNA, and pellets, containing basic proteins that remained associated with DNA, were analyzed by 5% acetic acid, 15% PAGE in the presence of 2.5 M urea, as reported previously (*4*). The identification of the protamines was carried out by developing each protein band, previously excised and freeze-dried, in Triton X-100−acetic acid−urea gels, as described previously (*16*). The analysis of the chromatin structure using micrococcal nuclease was performed essentially as described before (*17*), with minor modifications. Briefly, sperm nuclei at a concentration of 22 167 nuclei/μL were incubated with the desired NP species (32 μM) for 60 min in 25 mM Tris-HCl buffer, 100 mM NaCl, and 2 mM $MgCl_2$ (pH 7.4). Samples were diluted with 8 volumes of buffer A [15 mM Tris-HCl, 60 mM KCl, 15 mM NaCl, 10 mM β-mercaptoethanol, 0.5 mM spermidine, and 0.15 mM spermine (pH 7.4)] and centrifuged at 13000g for 45 min at 4 °C. Nuclei were then resuspended in 0.2 mL of micrococcal nuclease digestion buffer [15 mM Tris-HCl, 60 mM KCl, 15 mM NaCl, 0.34 M sucrose, 15 mM β-mercaptoethanol, 0.5 mM spermidine, 0.15 mM spermine, and 2 mM $CaCl_2$ (pH 8.5)], to which micrococcal nuclease (Sigma) at 0.08 munit/μL was added, and incubated for 5 min at room temperature. The purified DNA was resolved on a 1.4% agarose gel containing Tris-borate-EDTA buffer.

Differential Scanning Calorimetry (DSC). Differential scanning calorimetry was performed in a VP-DSC microcalorimeter (Microcal, Northampton, MA). Prior to DSC experiments, samples were dialyzed against 10 mM phosphate and 1 mM EGTA (pH 7.0) with or without 150 mM NaCl. Samples and reference solutions were properly degassed and loaded into the calorimeter, and the experiments were carried out under an extra pressure of 1 atm to avoid degassing during heatings. The calorimetric data were analyzed using Origin software provided with the calorimeter. The protein concentration was 0.3−0.6 mg/mL, and the scan rate was 1 °C/min. The molecular masses given above were used in the calculations of the calorimetric enthalpy per protein monomer.

RESULTS

Biological Activity of Natural and Recombinant Nucleoplasmins. The ability to decondense chromatin of natural and recombinant variants of nucleoplasmin is shown in Figure 2. As previously reported, under our experimental conditions, i.e., in the absence of spermine and spermidine, rNP does not significantly decondense sperm nuclei (Figure 2B), in contrast to what is observed with oNP (Figure 2C) (*5*). Removal of 50 residues at the C-terminus activates the recombinant protein so that the unphosphorylated deletion mutant NPC50 becomes competent for chromatin decondensation (Figure 2D). Further removal of 30 residues at this domain, which includes the poly(Glu) tract, yields an inactive protein (NPC80, Figure 2E) which behaves like the full-length recombinant protein (Figure 2B), and shows no effect on chromatin decondensation (see Figure 2A for a control in the absence of protein).

Two types of experiments were carried out to further characterize the functionality of these protein variants. First, the ability of these proteins to remove basic proteins from

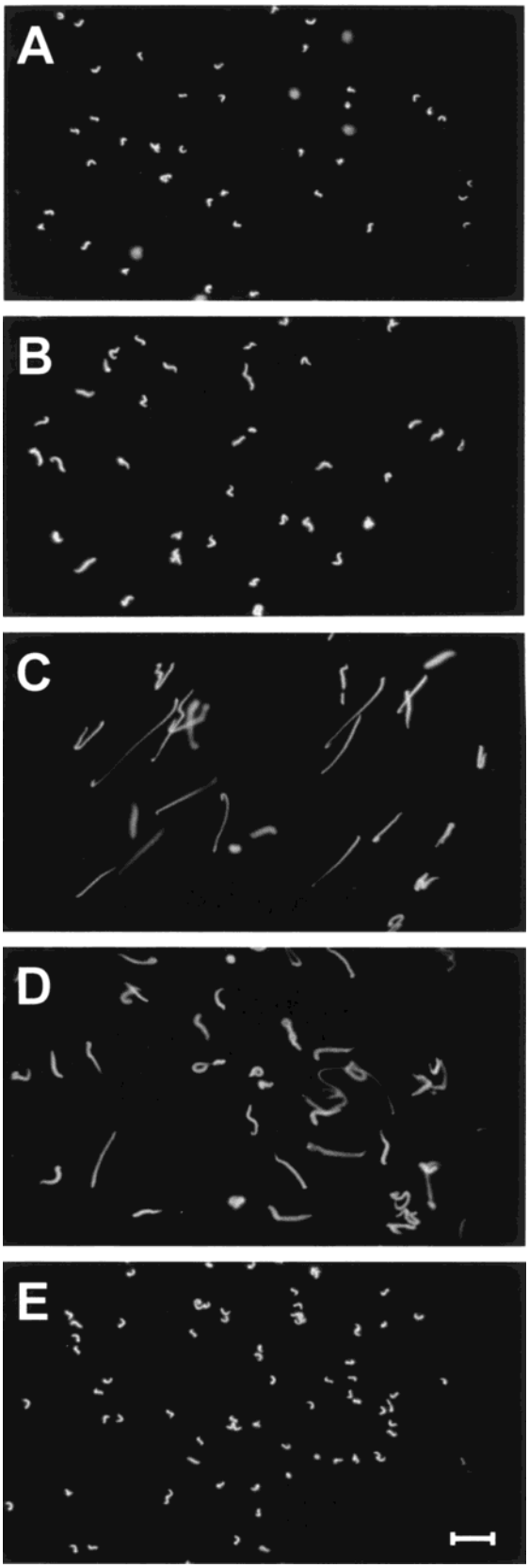

FIGURE 2: Decondensation of sperm nuclei by nucleoplasmin. Demembranated *Xenopus* sperm nuclei were incubated for 15 min in 25 mM Tris-HCl, 100 mM KCl, and 2 mM $MgCl_2$ (pH 7.4) in the absence (A) and presence of 32 μM rNP (B), oNP (C), NPC50 (D), and NPC80 (E). The scale bar is 50 μm.

DNA was analyzed by electrophoresis (Figure 3). After incubation of the desired nucleoplasmin species with demembranated sperm nuclei, the samples were centrifuged to separate basic proteins dissociated from DNA by NP (supernatants, lanes 1−4) from those that remain bound (pellets, lanes 5−9). As expected from the decondensation experiments, neither rNP (lanes 2 and 6) nor NPC80 (lanes 4 and 8) is able to significantly dissociate basic proteins from DNA, although the full-length protein might be able to remove a small amount of sperm basic proteins. In contrast, oNP (lanes 1 and 5) and NPC50 (lanes 3 and 7) clearly dissociate proteins from DNA. In a second set of experiments, the accessibility of chromatin to micrococcal nuclease was tested (Figure 4). The chromatin from fresh sperm has been shown to be extensively digested by the nuclease, since it does not form nucleosomes (*18*). The sizes of the DNA molecules generated by nuclease after incubation with rNP (lane 3) and NPC80 (lane 5) are similar to those observed in the absence of protein (lane 2). However, smaller DNA fragments are detected after incubation with oNP (lane 4) and NPC50 (lane 6), indicating that chromatin decondensation (Figure 2) is caused by removal of basic proteins from DNA (Figure 3), which in turn increases the susceptibility of the DNA molecule to nuclease attack (Figure 4). Taken together, these results indicate that deletion of 50 residues at the C-terminus of rNP confers on the protein a decondensation activity comparable to that of phosphorylated oNP.

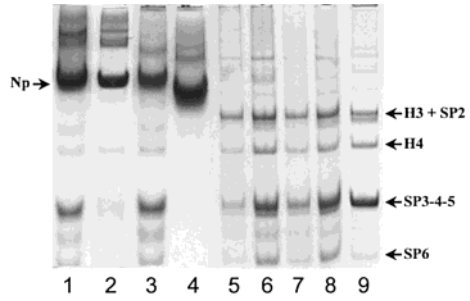

FIGURE 3: Nucleoplasmin-induced removal of basic proteins from DNA. Demembranated sperm nuclei were incubated for 60 min with 30 μM oNP (lanes 1 and 5), rNP (lanes 2 and 6), NPC50 (lanes 3 and 7), and NPC80 (lanes 4 and 8) and centrifuged as described in Experimental Procedures. Supernatants (lanes 1−4) showing the basic proteins that have been dissociated from DNA by the corresponding NP species and pellets (lanes 5−8) containing basic proteins bound to DNA. Pellet obtained in the absence of protein (lane 9). The corresponding supernatant did not exhibit any protein band (not shown). The arrow points to the NP band. Other details as in Figure 2.

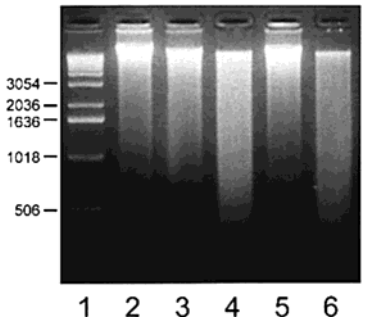

FIGURE 4: Analysis of chromatin structure by nuclease digestion. Demembranated sperm nuclei were incubated with buffer alone (lane 2) and with buffer containing 32 μM rNP (lane 3), oNP (lane 4), NPC80 (lane 5), and NPC50 (lane 6). Lane 1 contains the 1 kb DNA ladder markers. The buffer was the same as that described in the legend of Figure 2.

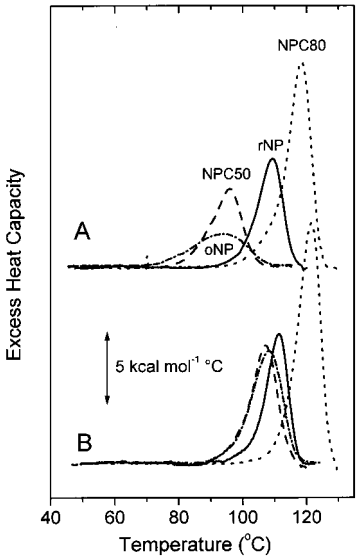

FIGURE 5: Thermal stability of NP. Excess heat capacity function vs temperature of rNP (—), NPC50 (− − −), NPC80 (•••), and oNP (−•−). Experiments were performed in 20 mM phosphate (pH 7.0) in the absence (A) and presence (B) of 150 mM NaCl.

Table 1: Thermodynamic Parameters of NP Thermal Denaturation[a]

	T_m (°C)		ΔH_{cal} (kcal/mol)[b]	
	without NaCl	with NaCl[c]	without NaCl	with NaCl[c]
rNP	109.3	111.3	130	136
NPC50	95.8	107.2	108	130
NPC80	117.7	120.6	250	257
oNP	94.4	108	80	120

[a] Unfolding transition temperatures, T_m, and calorimetric denaturation enthalpies, ΔH_{cal}, of different nucleoplasmins. The maximun standard deviations in the derived parameters for two different protein batches are 1 °C for T_m and 15 kcal/mol for ΔH_{cal}. [b] Estimated for the protein monomer. [c] In the presence of 150 mM NaCl.

Differential Scanning Calorimetry and Circular Dichroism. The thermal stability of these protein variants was measured by high-sensitivity differential scanning calorimetry under different salt conditions. Figure 5A shows the calorimetric profiles of the above samples in 10 mM phosphate and 1 mM EGTA (pH 7.0) in the absence of additional salts. Interestingly, the trace corresponding to rNP displays an endotherm with a T_m value above the boiling point of water, i.e., 109.3 °C. Deletion of 50 residues at the C-terminus of the protein results in a significant destabilization, as seen by the decrease in both the T_m value from 109.3 to 95.8 °C (Figure 5A and Table 1) and the calorimetric enthalpy from 130 to 108 kcal/mol (Table 1). Further removal of 30 residues at the same protein domain of NPC50 to generate the NPC80 deletion mutant reverts the destabilization effect described for the former and yields a remarkably thermostable protein with a T_m of 117.7 °C (Figure 5A) and a calorimetric enthalpy (ΔH_{cal}) of 250 kcal/mol (Table 1). The thermal stability of NP isolated from oocytes of *Xenopus* is also shown in Figure 5A. As judged from the T_m (94.4 °C) and ΔH_{cal} (80 kcal/mol) values, its thermal stability resembles that of NPC50.

Increasing salt concentrations, i.e., in the presence of 150 mM NaCl, significantly stabilized NPC50 and oNP (Figure 5B), as demonstrated by the shift of their T_m values from 95.8 and 94.4 °C to 107.1 and 108.1 °C (ΔT = 11.3 and 13.7 °C, respectively), respectively (Table 1). In contrast, the corresponding values of rNP and NPC80 only increase by 2 and 3 °C, respectively (Figure 5B and Table 1). It is important to note that the secondary structure of all the samples is not sensitive to the same increase in salt concentration (data not shown).

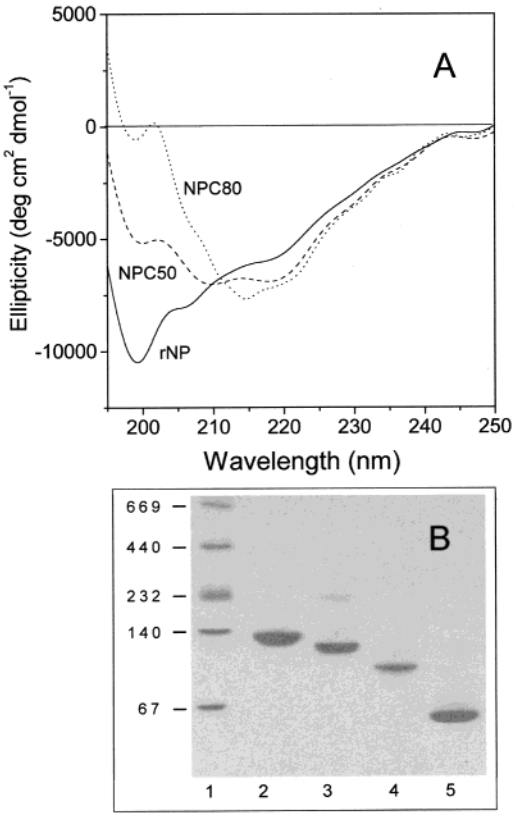

FIGURE 6: Secondary structure and oligomeric organization of NP in solution. (A) Circular dichroism spectra of rNP (—), NPC50 (− − −), and NPC80 (•••). Spectra were recorded in 10 mM Tris-HCl and 50 mM NaCl at pH 7.4 and 25 °C. (B) Native PAGE (8 to 25% gradient) of oNP (lane 2), rNP (lane 3), NPC50 (lane 4), and NPC80 (lane 5). Lane 1 contains molecular mass markers.

CD analyses reveal that the progressive deletion of amino acids at the C-terminal domain of the protein decreases the ellipticity value at 200 nm and increases that at around 215 nm (Figure 6A). This spectroscopic behavior is consistent with a reduction in the amount of nonperiodic structures (loops and turns) and an increase in the amount of β-structure, and indicates that the two deleted regions of the C-terminal domain of NP fold into similar secondary structure elements. The β-structure contents estimated from the CD spectra of rNP, NPC50, and NPC80 are 37, 42−47, and 45−51%, respectively. The last value is in good agreement with the amount of β-structure observed in the crystal structure of the core domain of NP (49%; *19*). The above-mentioned increase in the β-structure content is paralleled by a similar reduction in the percentage of turns. None of these differences are due to changes in the oligomerization state of the NP variants, since all of them remain pentameric in aqueous solution (Figure 6B).

DISCUSSION

In the absence of "helper" proteins, unproductive interactions between negatively charged DNA and positively

charged histones result in an irreversible aggregation. To avoid this undesired effect, nuclear chaperones electrostatically interact with histones and mediate an ordered transfer of basic proteins to DNA, thus allowing the assembly of nucleosomes. Nucleoplasmin, the first identified nuclear chaperone, shares this mechanism of action which is believed to be mediated by several clusters of acidic amino acids at the C-terminal domain. This domain might be divided into two different regions. The first one, including residues 120−150, contains the poly(Glu) tract that seems to mediate the chromatin decondensation activity of the protein, and the second, from residue 150 to the last (residue 200), includes the positively charged NLS that directs the protein into the nucleus.

The results presented here strongly suggest that in the nonphosphorylated, recombinant protein the oppositely charged residues located in these regions interact electrostatically, and that this interaction shields the negatively charged poly(Glu) sequence so that the protein remains inactive in removing basic proteins from DNA. Deletion of the NLS-containing region would leave in the pentamer exposed and mutually repulsive poly(Glu) tracts, and therefore would destabilize the oligomeric protein structure. This is indeed what is experimentally observed in NPC50 as a decrease in the temperature corresponding to the maximum of the heat capacity change ($\Delta T = 13$ °C). A similar unfavorable contribution of the electrostatic interactions to the stability of a protein oligomer has also been observed in GroES. The presence of two acidic clusters in each of the neighboring monomers of the protein heptamer destabilizes the oligomeric structure of the cochaperonin (*20*, *21*).

As expected from the initial hypothesis, removal of the positive counterion would allow the negatively charged poly(Glu) tract to interact with basic ligands, such as sperm proteins, and thus to decondense chromatin. Both the destabilizing and the activating effects are detected upon removal of the NLS-containing region at the C-terminus. It also follows from the above interpretation that deletion of the poly(Glu) region from NPC50 would have a stabilizing effect on the pentameric core, and would inactivate the chromatin decondensation ability of the protein, as experimentally observed. The fact that the highest thermostability corresponds to the core pentameric protein (NPC80) supports the view of the ordered N-terminal domain as being responsible for the remarkable stability of NP. The effect of salt concentration on the thermal stability of the recombinant proteins reinforces the idea that the destabilization observed in NPC50 is due to repulsive forces between exposed poly(Glu) tracts within the pentameric structure. It also indicates that in the full-length rNP the negatively charged residues are, at least partially, neutralized by basic amino acids located in the NLS-containing region.

NP provides an interesting example of a protein that exerts its biological function at physiological temperatures, as a mesophilic protein, and shows a thermal stability beyond the boiling point of water, a characteristic of hyperthermophilic proteins (*22*−*24*). Although a detailed analysis of the thermodynamic properties of NP is currently underway, we point here to some properties shared by NP and other thermophilic polypeptides: (1) a high content in Pro (5.5%), which might confer rigidity to the structure, and therefore stability (*25*); (2) a high level of charged amino acids, as found in the genomes of thermophiles which could form stabilizing ion pairs (*23*); and (3) an oligomeric structure that might reduce the level of exposure of hydrophobic residues at the monomer−monomer interface (*23*, *26*). The recently published X-ray structure of the protein confirms that extensive hydrophobic contacts at the oligomerization interfaces stabilize the pentamer (*19*).

The comparison of the natural protein isolated from *Xenopus* oocytes, oNP, and the recombinant proteins indicates that both the T_m of the unfolding transition and the chromatin decondensation activity of NPC50 and oNP are similar, as is the effect of salt concentration on their thermal stability. In contrast to rNP, oNP is phosphorylated, containing on average three phosphate groups per protein monomer (*5*). Although the precise location of the phosphorylatable residues within the protein sequence is at present unknown, prediction methods (*27*) suggest that, among others, three serines and one threonine might be phosphorylated at the C-terminal domain, being the Ser residues flanking the nuclear localization sequence (see Figure 1). A simple way to rationalize the above-mentioned experimental findings is to assume that phosphorylation of some of the predicted residues at the C-terminus might increase the negative charge around the NLS, and therefore would weaken the electrostatic interactions that these positively charged residues seem to establish with the poly(Glu) tract. This, in turn, would expose the poly(Glu) region that could interact with basic proteins and induce chromatin decondensation, as described for NPC50. Similarly, weakening of the electrostatic interactions, as a consequence of phosphorylation, would enhance repulsive forces between acidic residues that are reflected in a protein destabilization that can be restored by an increase in the ionic strengh of the medium. Other phosphorylatable residues exist at the N-terminal domain of the protein, of which the effect on protein activity and stability is not considered in this interpretation. A phosphorylation-induced activation mechanism similar to that outlined above for NP, which would include a movement of a loop bearing the phosphorylatable sites relative to the rest of the protein molecule, has been found for glycogen phosphorylase (*28*) and proposed for phenylalanine hydroxylase (*29*, *30*). A significant finding of this work is that protein phosphorylation induces a conformational change in NP that results in a lower stability against the thermal challenge, while it does not modify its secondary structure (*4*, *5*), indicating that this post-translational modification affects the tertiary and/or quaternary structure of NP.

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