Molecular Basis of Histone H3K4me3 Recognition by ING4*[S]

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The inhibitors of growth (ING) family of tumor suppressors consists of five homologous proteins involved in chromatin remodeling. They form part of different acetylation and deacetylation complexes and are thought to direct them to specific regions of the chromatin, through the recognition of H3K4me3 (trimethylated K4 in the histone 3 tail) by their conserved plant homeodomain (PHD). We have determined the crystal structure of ING4-PHD bound to H3K4me3, which reveals a tight complex stabilized by numerous interactions. NMR shows that there is a reduction in the backbone mobility on the regions of the PHD that participate in the peptide binding, and binding affinities differ depending on histone tail lengths Thermodynamic analysis reveals that the discrimination in favor of methylated lysine is entropy-driven, contrary to what has been described for chromodomains. The molecular basis of H3K4me3 recognition by ING4 differs from that of ING2, which is consistent with their different affinities for methylated histone tails. These differences suggest a distinct role in transcriptional regulation for these two ING family members because of the antagonistic effect of the complexes that they recruit onto chromatin. Our results illustrate the versatility of PHD fingers as readers of the histone code.

Regulation of chromatin dynamics dictates the outcome of fundamental nuclear processes such as DNA transcription replication and repair (1–3). It is central to cell homeostasis, because alterations in chromatin structure contribute to the development of cancer and other human diseases (4). The ING[6] family of tumor suppressors consists of five homologous proteins implicated in chromatin remodeling, growth arrest, and, in cooperation with p53, senescence and apoptosis (5–7). They are frequently deregulated in different types of cancer (8) and contain a conserved C-terminal PHD finger (9) that is present in many nuclear proteins involved in gene expression regulation and chromatin remodeling (10). They form stable histone acetylation or deacetylation complexes (11) and are thought to direct them to specific regions of the chromatin through binding of their PHD fingers to histone 3 N-terminal tails trimethylated at lysine 4 (12, 13). These binding properties link ING proteins with actively transcribed genes, because H3K4 trimethylation is a hallmark of active genes (14). The recognition of H3K4me3 by ING2 is critical for the occupancy of the mSin3A-HDAC1 complex at the promoter of the cyclin D1 gene, which results in histone deacetylation and transcriptional repression of the active gene in response to DNA damage (15). This result suggests a general active transcriptional repression role for ING2; nonetheless, the biological outcome of the recognition of methylated histone tails by the other ING proteins is still unclear. Different PHD fingers link H3K4me3 recognition with gene activation, such as the PHD of the bromodomain PHD finger transcription factor, which helps to recruit the nucleosome remodeling factor complex to target promoters modulating transcription initiation (16, 17). Hence, the function of the PHD-H3K4me3 binding event and its effect on transcription are determined by the particular protein reader of this histone code mark.

We have solved the crystal structure of the PHD of ING4 bound to H3K4me3 tail, which remarkably shows a different mode of binding with respect to the previously reported for ING2, with a longer region of the histone tail participating in the interaction with ING4. The structure of the complex provides new insights into the determinants of the different binding affinities measured for the two domains (13) and allows for the interpretation of NMR data showing the stabilizing effect of C-terminal extensions of the histone peptide and a reduced backbone mobility in the PHD on peptide binding. Isothermal titration calorimetry (ITC) measurements show that the dis-

TABLE 1

X-ray data collection and refinement statistics

Data collection[a]	
Environment	ADSC detector, ESRF, beamline ID29
Wavelength	1.072 Å
Cell dimensions (Å,°)	$a = 68.51$, $b = 68.51$, $c = 28.51$, $\alpha = \beta = \gamma = 90$
Resolution (Å)	68.51-1.76 (1.82-1.76)
Space group	P43
Unique reflections	12817
Average multiplicity	3.8 (2.6)
Completeness (%)	96.9 (86.8)
R_{merge}[b]	0.075 (0.38)
$<I/\sigma I>$	9.9 (1.7)
Refinement	
Number of reflections (completeness, %)	12178 (96.92)
Resolution range (Å)	68.51–1.76
R factor/R_{free} (%)	15.62/22.67
Number of protein atoms (average B, Å^2)[c]	929 (30.61)
Number of water molecules (average B, Å^2)[c]	129 (38.31)
Root mean square bond length (Å)	0.012
Root mean square bond angle (°)	1.816
Ramachandran plot outliers (number, %)[d]	3, 2.5

[a] The values in the highest resolution shell are given in parentheses.
[b] $R_{\mathrm{merge}} = \Sigma_{\mathrm{h}\Sigma\mathrm{i}}|I_{\mathrm{h,i}} - <I_{\mathrm{h}}>|/\Sigma_{\mathrm{h}\Sigma\mathrm{i}}|I_{\mathrm{h,i}}|.$
[c] Calculated using MOLEMAN (43).
[d] Calculated with PROCHECK (44).

crimination in favor of the methylated lysine is due to more favorable solvation entropy contributions.

EXPERIMENTAL PROCEDURES

ING4 Proteins and Histone 3 Peptides Sample Preparation—The clone of ING4 PHD finger 188–246 was made from the 188–249 construct using a QuikChange mutagenesis kit (Stratagene). Protein expression and purification was done as previously described (13). Synthetic lyophilized peptides were purchased from NeoMPS and correspond to histone 3 residues 1–10 (ARTKQTARKS) or residues 1–15 with an extra Tyr residue at the C terminus (ARTKQTARKSTGGKAY) (13) with the four possible methylation states at lysine 4. The concentrations of the peptide stock solutions were measured by amino acid analysis (10 residue peptides) or by ultraviolet absorbance (using the absorbance of the aromatic side chain of the tyrosine residue in the 15 + 1-residue-long peptides).

Crystallization and Structure Determination—Crystals of ING4(188–246) bound to H3$_{10}$K4me3 peptide were grown by the hanging drop vapor diffusion method at 5 °C. The pure protein was dialyzed against 10 mM Tris, pH 6.5, 150 mM NaCl and 2 mM dithiothreitol and concentrated to 1.4 mM. The H3$_{10}$K4me3 peptide was used at a concentration of 35 mM in water. The complex was formed by mixing the PHD and the peptide in a 1:2 molar ratio. Crystallization drops were set up by mixing 1 μl of protein·peptide complex with 1 μl of reservoir solution of 35% polyethylene glycol 6000 and 0.4 μl of 100 mM CoCl$_2$. Needles grew overnight but were not suitable for diffraction experiments. Good quality crystals appeared after using these needles for seeding in fresh drops that were incubated for 3 h at 4 °C. The crystals grew in ~3 days and reached final dimensions of 0.15 × 0.01 × 0.01 mm^3. Prior to data collection, protein complex crystals were immersed in the precipitant solution containing 5% (v/v) ethylene glycol, followed by rapid cooling in liquid nitrogen. A complete x-ray diffraction data set was collected at the beamline ID 29 (European Synchrotron Radiation Facility, Grenoble, France). The data were indexed, integrated, and scaled with HKL2000 (18). The crystals

belonged to the P43 space group with cell dimensions $a = b = 68.51$ Å, $c = 28.51$ Å, and α, β, $\gamma = 90°$. Matthews coefficient and self-rotation function indicated the presence of two molecules in the asymmetric unit, with a solvent content of 36.78%. Molecular replacement was performed with Phaser (19) using the Protein Data Bank entry 2G6Q as model after removal of the bound peptide. Refinement was carried out with REFMAC5 (20) including rigid body refinement as the first step. Several rounds of iterative rebuilding with O (21) and refinement were performed. Two molecules of 1,4-dithiothreitol and solvent water molecules were placed into the electron density using Arp/Warp (22). The final model contains two complexes in the asymmetric unit: the first was constituted by ING4(195–244)/H3(1–6), and the second was constituted by ING4(191–244)/H3(1–10). Data collection details and statistics of the refinement can be found in Table 1. The coordinates have been deposited with the Protein Data Bank (accession code 2VNF).

NMR Spectroscopy—NMR spectra were recorded at 25 °C in 20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 1 mM dithiothreitol, 5% (v/v) ^{2}H$_2$O$_2$, 0.01% Na$_3$N in a Bruker AVANCE 600 as described (13). Titrations were performed by stepwise addition of concentrated (5–6 mM) peptide stock solutions into 600-μl samples of 50 μM PHD. The dissociation constants were determined as described (13). The spectra to measure the differences in the chemical shift perturbation (CSP) upon ING4 binding to H3$_{10}$K4m3 or H3$_{15}$K4m3 were obtained under identical conditions with two samples containing 50 μM PHD and a 4-fold excess of each peptide, which were simultaneously dialyzed against the same buffer. Backbone ^{15}N T$_1$, T$_2$, and {^{1}H}-^{15}N heteronuclear NOE measurements (23) were performed on a Bruker AVANCE 700 spectrometer on a 0.83 mM uniformly ^{15}N labeled PHD sample with or without 1.66 mM peptide. For free ING4-PHD ten time points (20, 60, 140, 240, 360, 460, 660, 860, 1100, and 1300 ms) were collected for T$_1$ measurements, and a different set of nine time points (16, 32, 63, 110, 158, 190, 222, 270, and 396 ms) was collected to measure the T$_2$ values. T$_1$ and T$_2$ experiments were acquired with eight scans and a rep-

etition delay of 3 s, whereas each {^{1}H}-^{15}N NOE spectrum (both saturated and nonsaturated one) was acquired with 136 scans and with an overall recycling delay of 10 s to ensure the maximal development of NOEs before acquisition and to allow solvent relaxation, thus avoiding transfer of saturation to the most exposed amide protons of the protein from scan to scan (24). Relaxation measurements for ING4-PHD bound to H3K4m3 peptides were carried out under the same conditions as those of the free form, but with one more sample time for T$_1$ experiment (1600 ms) and different number of scans of the {^{1}H}-^{15}N NOE experiment 148 and 128 scans in the case of H3$_{10}$K4me3 and H3$_{15}$K4me3, respectively. To check the reproducibility of the relaxation measurements, the T$_1$ experiments for free ING4-PHD and the {^{1}H}-^{15}N NOE experiment for ING4-PHD bound to H3$_{15}$K4me3 were measured twice yielding the same results. The relaxation times were calculated via least squares fitting of peak intensities to a two parameter exponential function, using the rate analysis routine contained in the NMRView program (25). The heteronuclear NOEs were calculated from the ratio of cross-peak intensities in spectra collected with and without amide proton saturation during the recycle delay. Uncertainties in peak heights were determined from the standard deviation of the distribution of intensities in a region of the HSQC spectra where no signal and only noise was observed. The principal components of the inertia tensor were calculated with the Pdbinertia program (A. G. Palmer III, Columbia University) using the first model of the ensemble of 20 structures determined by NMR (Protein Data Bank entry 2JMQ), which is the best one according to restraint violations data and the x-ray structure for the ING4-PHD·H3K4me3 complex. The estimation of the overall correlation time was obtained from the ratio of the mean values of T$_1$ and T$_2$, which were calculated from a subset of residues with little internal motion and no significant exchange broadening. This subset excluded those residues with NOEs smaller than 0.65 and also those residues with T$_2$ smaller than the average minus one standard deviation, unless their corresponding T$_1$ values were larger than the average plus one standard deviation (26). The diffusion tensor, which describes the rotational diffusion anisotropy, was determined by two approaches (27, 28) using the programs r2r1_diffusion and quadric_diffusion (A. G. Palmer III, Columbia University). The calculations failed when using the errors in T$_1$ and T$_2$ estimated by Monte Carlo simulations, which were unrealistically low. Therefore, the errors were scaled up by the minimum factor that allowed an interpretation of the data in terms of a rotational diffusion tensor. This procedure resulted in average errors of 9.5 and 10% for free and bound ING4-PHD, respectively. The ^{15}N relaxation was analyzed assuming dipolar coupling with the directly attached proton, with a bond length of 1.02 Å, and a contribution from the ^{15}N chemical shift anisotropy with a value of -160 ppm. The program FAST-Modelfree (29), which interfaces with the program MODELFREE version 4.2 (30) was used to fit the relaxation data to the model free formalism of Lipari and Szabo (31). Five different models of internal motion were evaluated for each amide ^{1}H-^{15}N pair: (i) S^2, (ii) S^2 and τ_e, (iii) S^2 and R_{ex}, (iv) S^2, τ_e, and R_{ex}, and (v) S_f^2, S^2, and τ_e, where S^2 is the generalized order parameter, τ_e is the effective internal correlation time, R_{ex} is the exchange contri-

bution to the transverse relaxation, and S_f^2 is related to the amplitude of the fast internal motions.

Isothermal Titration Calorimetry—ITC experiments were performed using a high precision MCS titration calorimetric system (Microcal Inc., Northampton, MA). The ING4-PHD domain was extensively dialyzed against the titration buffer. All of the solutions were filtered, properly degassed to avoid bubble formation, and equilibrated to 25 °C prior to each experiment. The protein solution (at 40–65 μM) in the calorimetric cell was titrated with the appropriate ligand (at 600–800 μM) dissolved in the dialysis buffer following a profile of injection volumes from 2.8 to 20 μl to better define the titration curve. The heat evolved after each peptide injection was obtained from the integral of the calorimetric signal. The heat produced by the binding reaction between the PHD and the peptides was obtained as the difference between the heat of reaction and the corresponding heat of dilution, as obtained from independent titrations of the peptides into the buffer. The resulting binding isotherms were analyzed by nonlinear least square fittings of the experimental data to a model corresponding to a single set of identical sites, as described in the supplemental data. For the interactions of H3$_{10}$K4me0 with ING4-PHD, for which the dissociation constant is out of the range measurable directly by ITC, displacement experiments using H3$_{10}$K4me1 as competing ligand were carried out. Briefly, a 65 μM ING4-PHD solution with H3$_{10}$K4me0 at a 1:4.6 molar ratio was placed in the calorimetric cell and titrated with H3$_{10}$K4me1 (at 2 mM) following a profile of injection volumes from 4 to 20 μl. The resulting binding isotherms, corrected for the dilution heats, were analyzed by nonlinear least square fittings of the experimental data to the exact displacement model as described (32) using the binding affinity and binding enthalpy for H3$_{10}$K4me1 obtained from the titrations with this peptide using the same injection profile. The data analysis was done with Microcal Origin (OriginLab Corporation, Northampton, MA) together with software developed in our laboratory.

Solvation Energy Calculations—The differences in solvation entropy were calculated according to Freire's structural parameterization of the energetics (33) calculated using the crystal structure of the ING4-PHD·H3$_{10}$K4me3 complex and two modeled structures for the di- and mono-methylated species. Of the three methyl groups in the crystal structure, one is fully buried at the binding interface (carbon atom number 891, ΔASA = 0 Å^2), the second is somewhat exposed (carbon atom number 893, ΔASA = 16.93 Å^2), and the third one is significantly exposed (carbon atom number 892, ΔASA = 26.93 Å^2). One could reasonably assume that in the dimethylated complex both methyl groups would tend to be as buried as possible. This is what is observed in the complex of the heterochromatin protein 1 chromodomain with H3$_{15}$K9me2 and me3 peptides, where the methylated lysine side chains adopt a structure very similar to that in ING4-PHD·H3$_{10}$K4me3 complex (34). For this reason, in the model of H3$_{10}$K4me2 bound to ING4 the methyl group that is exposed the most (C atom number 892) was removed from the structure of H3$_{10}$K4me3, whereas C atoms numbers 892 and 893 were removed to build the model for the H3$_{10}$K4me1 complex.
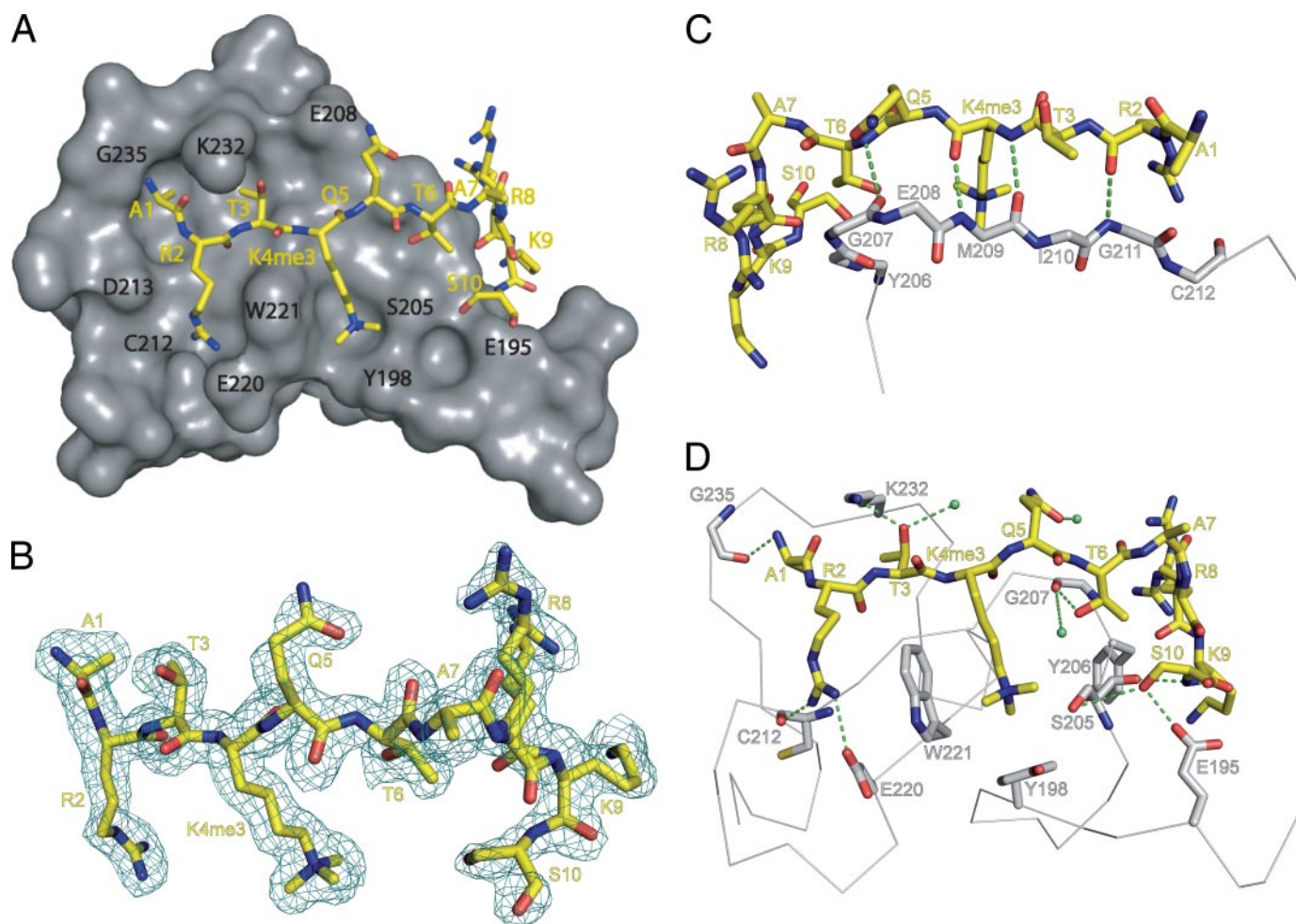
FIGURE 1. **Three-dimensional crystal structure of the complex of ING4-PHD bound to H3$_{10}$K4me3.** *A*, overall structure with ING4-PHD shown in *gray* and H3$_{10}$K4me3 shown in *yellow stick* representation. The location of the ING4 residues lining the binding grooves for histone 3 residues Ala[1], Arg[2], Thr[3], Lys[4], and Ser[10] is indicated with *black labels*. *B*, view of a 2($|F_o| - |F_c|$) omit map at 1.76 Å contoured at 1 σ as a *blue mesh*. The omit map was calculated with the program OMIT in the CCP4 package (45). *C*, details of the β-sheet structure formed by H3 residues Arg[2]–Thr[6] and ING4 residues Gly[207]–Gly[211] with backbone hydrogen bonds in *green*. *D*, details of side chain interactions with hydrogen bonds and water molecules in *green*.
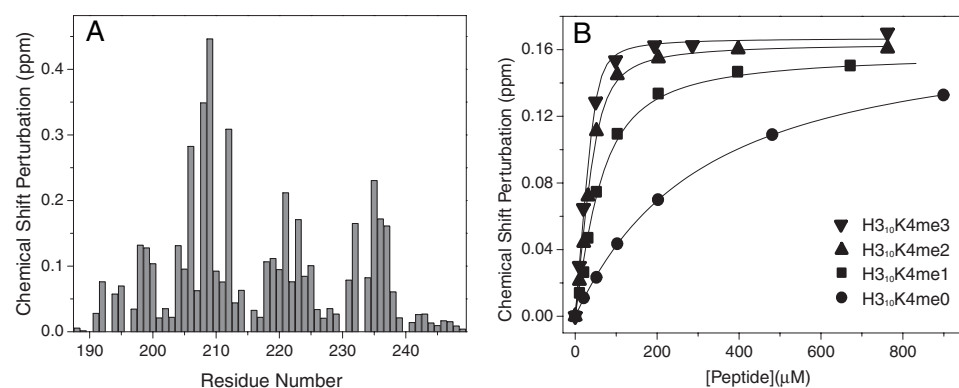

FIGURE 2. **NMR analysis of the binding of ING4-PHD to H3$_{10}$K4meX peptides.** *A*, binding histogram showing the CSP observed for each residue in the ^{1}H-^{15}N HSQC spectra of ING4 PHD in the presence of 1:4 excess of H3$_{10}$K4me3. *B*, the CSP of the Trp[237] amide resonance of ING4(188–249) in ^{1}H-^{15}N-HSQC spectra is represented as a function of peptide concentration. The *continuous lines* are fit to the binding model (13). The height of the symbol indicates the experimental error.

RESULTS AND DISCUSSION

Recognition of H3K4me3 by ING4—The crystal structure of ING4-PHD (residues 188–246) bound to histone 3 (residues 1–10) trimethylated at lysine 4 (H3$_{10}$K4me3) was solved at 1.76

Å resolution (Fig. 1*A*). The ten residues of the peptide were observed in the electron density, including two alternate conformations for H3 Arg[8] that could be modeled without ambiguity into their corresponding densities (Fig. 1*B*). The peptide conformation does not seem to be influenced by the neighboring crystallographically related molecules, and the interactions with ING4 observed in the crystal structure are consistent with solution NMR data (Fig. 2*A*). The structure of the complex shows that the N-terminal half of the histone tail binds to the surface of the PHD finger as a third strand of the anti-parallel β-sheet that forms the core of the PHD (35). The peptide N terminus forms a hydrogen bond with the carbonyl of ING4 residue Gly[235], and H3 residues Arg[2]–Thr[6], which have β-sheet backbone dihedral angles, form backbone hydrogen bonds with

A

B

ING4/H3$_{10}$K4me3   ING2/H3$_{12}$K4me3
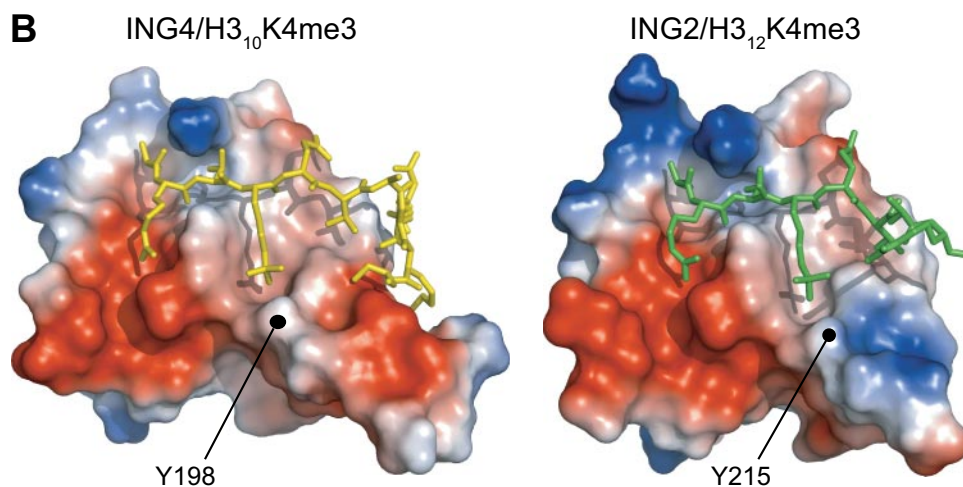

Y198   Y215
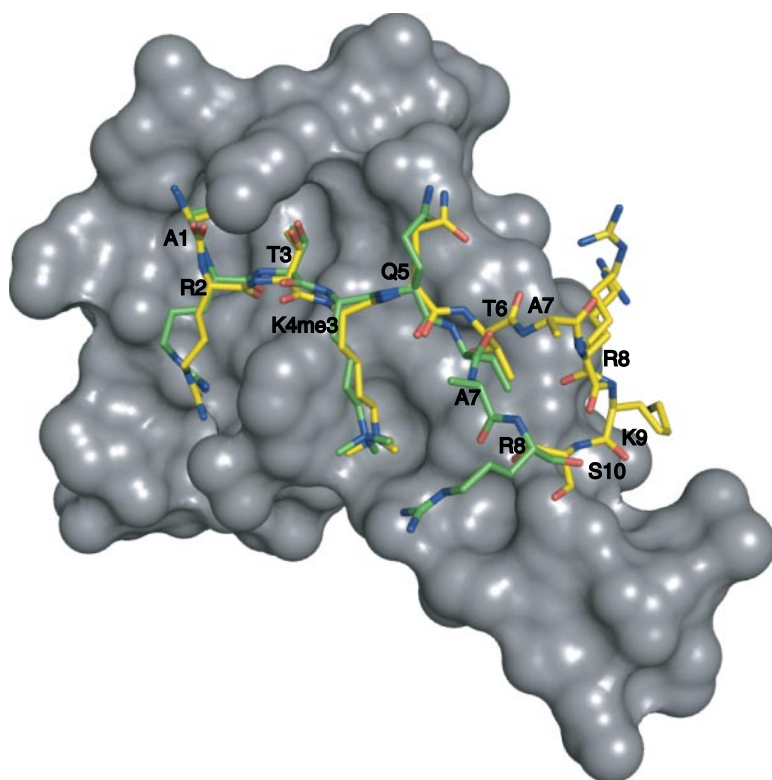
FIGURE 3. **Comparison of the structures of H3K4me3 peptides bound to ING4 and ING2 PHD.** *A*, the crystal structure of ING4-PHD is shown as a *gray surface* with the bound H3$_{10}$K4me3 in *yellow* and the superimposed H3$_{12}$K4me3 from the corresponding ING2-PHD complex structure in *green*. Only H3 residues Ala[1] to Arg[8] are seen in the crystal of the ING2 complex (Protein Data Bank entry 2G6Q). *Black labels* indicate the Cα atoms of the peptide residues, whose positions are very similar in both peptides up to H3 Thr[6]. *B*, surface representations of the PHD fingers of ING2 and ING4 bound to the histone peptides where the electrostatic potential is indicated by a gradient of *red* (negative charge) and *blue* (positive charge) colors. The positions of the homologous tyrosine residues 198 and 215 are indicated in ING4 and ING2, respectively.

H3K4me3 make cation-π interactions with the side chain of Trp[221], which sits between the two basic peptide side chains. H3K4me3 forms another cation-π contact with Tyr[198], which is not essential for recognition because the mutant Y198A still binds the peptide with moderate affinity (13). H3 residue Lys[9] makes a cation-π interaction with Tyr[206], and so does H3 Arg[8] (at least in one of the two alternative conformations). However, these two interactions are probably weaker because whereas the distances between the charges and the aromatic rings are within the range of typical van der Waals' interactions, their relative orientations are not optimal (the charges are off the C$_6$ ring axis C).

The PHD structure resembles an oblate ellipsoid with three grooves or channels in one side that are filled by the backbone and the side chains of H3 residues Ala[1]–Thr[6] (see supplemental Fig. S1). The kink at H3 Ala[7] directs the peptide chain along the rim of the ellipsoid, and H3 Ser[10] occupies a small depression lined by Glu[195]. The shape complementarities between the PHD and the bound peptide structures result in the burial of a large accessible surface area upon complex formation both in the PHD and in the peptide molecules (ASA, 1318 Å^2; see below), with H3 Ala[7] at the kink being the only residue not contributing to the buried ASA.

In the complex with ING4, the methylammonium of H3K4me3 is positioned in a cage formed by two aromatic residues (Tyr[198] and Trp[221]), the hydrophobic side chain of Met[209], and Ser[205]. This binding mode of the methylated lysine is common to ING2 and similar to the bromodomain PHD finger tran-

Cys[212]–Gly[207] (Fig. 1*C*). A kink in the main chain at H3 residues Ala[7]-Arg[8] recovers the extended conformation for H3 residues Lys[9]-Ser[10], with H3 Ser[10] forming a backbone hydrogen bond with Ser[205]. Because of the polar nature of histone 3 tail residues, the interactions are predominantly polar. The side chain of H3 Arg[2] forms a salt bridge with Glu[220], while the side chains of Thr[3], Thr[6], and Ser[10] form hydrogen bonds with Lys[232], Gly[207], and Glu[195], respectively (Fig. 1*D*). The guanidinium group of H3 Arg[2] and the trimethylammonium group of

scription factor, with a cage of four aromatic residues, (17). Overall, the recognition of the N-terminal half of H3$_{10}$K4me3 by ING4 is very similar to that observed in the complex with ING2 (12) but differs markedly in the C-terminal half (Fig. 3 and supplemental Fig. S2). The peptide bound to ING2 is not kinked at H3 Ala[7], and the last two residues observed in the crystal (H3 Ala[7]-Arg[8]) wander off the PHD. The different mode of binding of ING4 is consistent with the different effect of a homologous residue substitution (Y198A in ING4 and Y215A in ING2),

which causes a 3-fold reduction in the affinity of ING4 for $H3_{15}K4me3$ while it strongly destabilizes (more than 3000-fold less affinity) the complex with ING2 (13). The reason for the different effect of the homologous mutation could be the distinct distribution of charges on the surface of the PHD fingers of ING2 and ING4. While in ING4 the N-terminal end of the molecule is predominantly negatively charged on the side closer to K4me3, it is positively charged in ING2 (Fig. 3*B*). The shielding of the aromatic side chain of Tyr[215] in ING2 may reduce the charge repulsion between this region and K4me3, and its elimination destabilizes the complex beyond the already destabilizing effect of removing the favorable cation-π interaction. In the complex with ING4, the removal of the aromatic side chain of Tyr[198] eliminates that same cation-π interaction, but it does not result in unfavorable electrostatic interactions.

ING4 Binding to Histone Tails of Different Lengths—The recognition of the H3 Ala[1] by Gly[235] blocks N-terminal extensions of the peptide chain bound to ING4, which is probably important for the specificity of the binding of $H3_{10}K4me3$ because the

TABLE 2
Dissociation constants (K_D) measured in solution by NMR for the 10- and 15-residue-long histone tails methylated at lysine 4 bound to ING4(188–249)

Ligand[a]	K_D[b]
	μM
$H3_{15}K4me0$	370 ± 20
$H3_{10}K4me0$	274 ± 6
$H3_{15}K4me1$	1.6 ± 0.8
$H3_{10}K4me1$	34 ± 4
$H3_{15}K4me2$	2 ± 1
$H3_{10}K4me2$	9.2 ± 1.4
$H3_{15}K4me3$	3.9 ± 0.7
$H3_{10}K4me3$	3.0 ± 0.6
$H3_{10}R2me2K4me3$	19.2 ± 1.7
$H3_{10}R2me2$[c]	1400 ± 40

[a] The values for $H3_{15}K4$ peptides are from Ref. 13.
[b] The fitting errors are indicated.
[c] Measured from the CSP of C212 signal instead of Trp[237] (see supplemental Fig. S4).
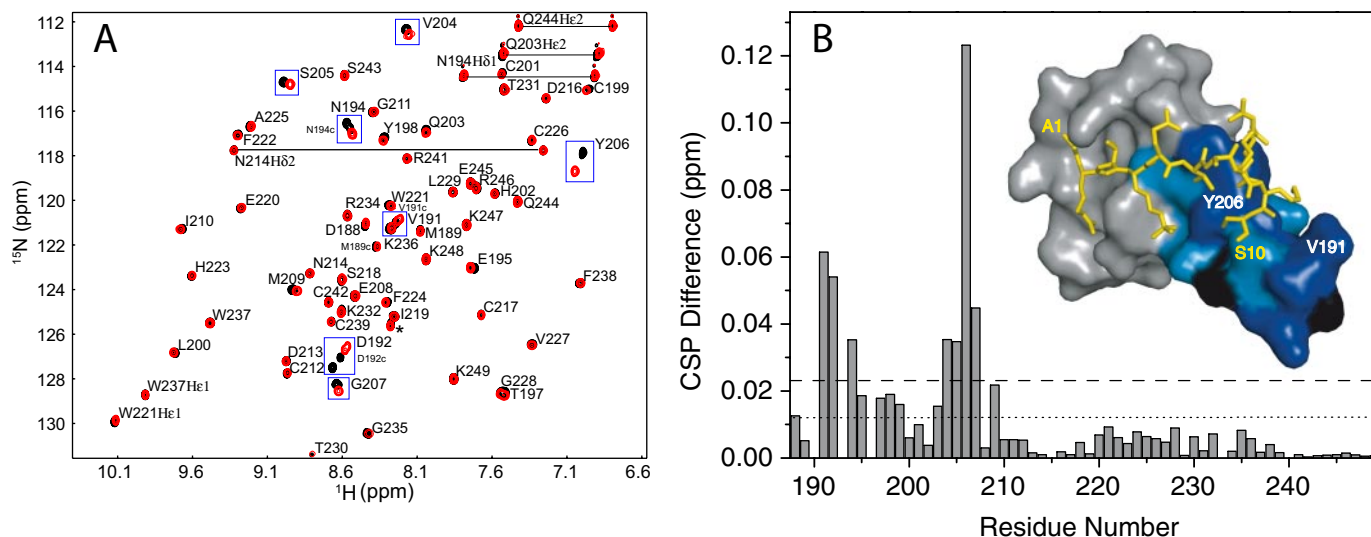
lysine modification is near the N terminus (36). No similar limitation exists at the C terminus, and the extent of the interactions with other regions of the histone tail is unclear.

We have previously characterized the binding of ING4 to 15-residue-long $H3_{15}K4meX$ peptides (13). The affinity for $H3_{10}K4me3$ is, within error, the same as for $H3_{15}K4me3$. Nonetheless, whereas ING4 does not discriminate between $H3_{15}K4me1$, -me2, or -me3, it does so for the shorter peptides, with the affinity increasing with the higher number of methyls (Fig. 2*B* and Table 2), indicating that the additional residues contribute to the interaction with ING4. Crystallization trials with this longer peptide were unsuccessful, and the differences in the binding of $H3_{15}K4me3$ and $H3_{10}K4me3$ were examined in solution by NMR measuring the differences in the CSPs caused on the ING4 signals on binding to the two peptides. These measurements are very sensitive to weak interactions or those involving flexible regions of the ligand and/or receptor. The spectra of ING4 bound to $H3_{10}K4me3$ or $H3_{15}K4me3$ (Fig. 4*A*) show several residues with larger perturbations caused by the longer peptide. Moreover, these perturbations are clustered in a region of ING4 in the vicinity of the C-terminal end of the peptide (Fig. 4*B*), indicating that additional interactions take place, resulting in higher affinities for $H3_{15}K4me1$ and -me2 (Table 2). This finding is consistent with the decreased flexibility of the N-terminal end of ING4-PHD bound to $H3_{15}K4me3$ (see below).

Dynamics and Thermodynamics of the Recognition of H3K4me3 by ING4—The chain termini of ING4 are highly flexible in solution, as indicated by the small values of the order parameters (S^2) measured for the backbone ^{15}N atoms (Fig. 5*A*), which are sensitive to movements of the N-H bond in ps to ns time scales. Residue Gly[235] also shows increased mobility compared with the rest of the chain. Binding to $H3_{10}K4me3$ increases the order parameters at several regions of the ING4

FIGURE 4. **Binding of ING4-PHD to H3K4me3 peptides of different lengths.** *A*, overlay of HSQC spectra of ING4(188–249) bound to $H3_{15}K4me3$ (*black*) or $H3_{10}K4me3$ (*red*). The cross-peaks are labeled with their corresponding residue number and single-letter code. Peaks from amide side chains are connected by *straight lines*. Residues with CSP differences larger than twice the experimental error are *boxed*. Four signals from a minor conformer caused by the *cis-trans* isomerization of the peptidyl-prolyl bonds in the flexible N-terminal region of ING4 are labeled (*M189c*, *V191c*, *D192c*, and *N194c*). A signal from an unknown molecule is marked with an *asterisk*. *B*, binding histogram of the differences in the CSP for each ING4 PHD residue; the experimental error (± 0.012 ppm) is indicated by the *dotted line*, while twice this error is indicated by the *dashed line*. The *inset* is a surface representation of ING4 PHD where residues with 0 > ΔCSP > error or ΔCSP > 2 × error are colored in *light* or *dark blue*, respectively. Proline residues for which no signal can be observed are colored in *black*.
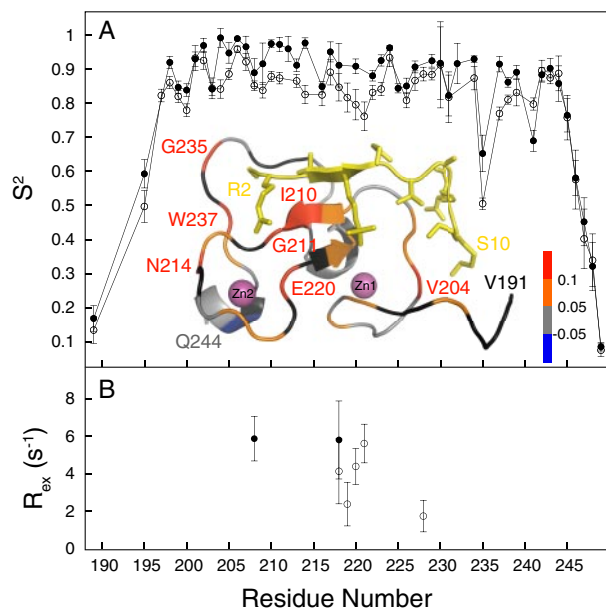
FIGURE 5. **Backbone dynamics of the ING4-PHD backbone.** The backbone ^{15}N nuclei order parameters (*A*, S^2) and the contribution of exchange processes (*B*, R_{ex}) to the relaxation of backbone ^{15}N nuclei are represented *versus* the sequence of ING4, in its free form (*open circles*) and in the complex with H3$_{10}$K4me3 (*filled circles*). The ribbon diagram shows the crystal structure of ING4-PHD bound to H3$_{10}$K4me3 (in *yellow* and with side chains). A color code indicates the change in S^2 for the corresponding ING4 residue on peptide binding. The residues for which the difference could not be measured are colored in *black*.

chain containing residues directly involved in histone recognition (Glu[195], Gly[211], Glu[220], and Gly[235]). The increased order around Glu[220] on peptide binding is consistent with the conformational exchange detected for this residue in free ING4 but not in the complex (Fig. 5*B*). Conformational exchange in free ING4 is also detected for Trp[221], which interacts with H3 Arg[2] and H3 Lys[4]. These results show that the recognition of H3$_{10}$K4me3 is not made by a static PHD molecule but that binding involves regions of ING4 with different degrees of mobility in its free form that become more rigid upon binding to the peptide. Relaxation data on ING4 bound to H3$_{15}$K4me3 show that the flexibility at the N-terminal region is further reduced on binding to this longer peptide (supplemental Fig. S3), which is consistent with the observed contribution to the binding of the longer histone tails. The NMR spectra also show that the free peptides are highly flexible in solution, and conformational order is induced on binding to ING4 (data not shown). A detailed account of the analysis of the backbone ^{15}N relaxation data can be found in the supplemental data.

The energetics of the ING4 binding to H3$_{10}$K4meX was characterized by isothermal titration calorimetry. Low to moderate binding affinities, with a large increase when the first methyl is introduced, were observed in good agreement with those measured by NMR (Fig. 6). The interaction is driven by a markedly exothermic binding enthalpy partially opposed by unfavorable entropic contributions (Fig. 7). This thermodynamic signature is consistent with the high density of hydrogen bonds and polar interactions at the binding interface and with the burial of apolar and polar ASA upon complex formation (798 and 520 Å^2, respectively, with a ratio $\Delta ASA_{apolar}/\Delta ASA_{polar} = 1.5$). None-

theless, the methylation state of the lysine residue does not have a significant effect on the enthalpic contributions to the binding affinity, which is very similar for the four peptides, so that the increment in the binding affinity upon methylation is entirely due to changes in the entropic contributions (Fig. 7). It is interesting to note that the opposite situation has been proposed for chromodomains (37). Lysine methylation leads to a polarization of the C_ϵ-N_ζ bond increasing the cationic character of the methylammonium group and strengthening the cation-π interactions with the aromatic cage but also results in an increment on the hydrophobic character of an otherwise highly polar side chain. The introduction of the methyl groups leads to an increased burial of apolar ASA on complex formation, which results in more favorable solvation entropies. This interpretation is supported by the agreement between the measured differences in the entropic contributions to binding ($-T\Delta\Delta S = -1.9, -2.8,$ and -3.3 kcal·mol^{-1} for H3$_{10}$K4me1, -me2, and -me3, respectively, referenced to H3$_{10}$K4me0), and the differences in solvation entropy were calculated (33) in terms of changes in accessible surface area ($-T\Delta\Delta S_{solv} = -1.7, -2.9,$ and -2.6 kcal·mol^{-1}).

ING4 Reading of the Histone Code—The structural basis for the binding of ING4 to histone 3 N-terminal tail lies on numerous interactions that occur when the N-terminal histone residues occupy a depression on one side of the PHD. This results, however, in a low binding affinity that increases on methylation at H3 Lys[4]. The relevance of the differences in the affinities for mono-, di- or trimethylated peptides is, however, unclear because peptide elongation at the C terminus stabilizes the complexes blurring those differences. This is in contrast with ING2, which does discriminate between the different methylation states of H3$_{12}$K4meX, with ~10-fold affinity increments for every additional methyl (14). The differences in the binding mode between the two ING proteins discussed above may explain these dissimilar affinities, which could be related with a different function. ING2, as part of the mSin3A·HDAC1 complex, links H3K4me3 recognition with transcriptional repression through histone deacetylation. ING4, involved in a complex containing HBO1 (the histone acetyltransferase binding to ORC 1) (11), may link the recognition of H3K4me3 (and of H3K4me2 and -me1) with transcriptional activation through histone acetylation.

The side chain of H3 Arg[2] lies in a groove that is separated by Trp[221] from another groove occupied by H3K4me3 (Fig. 1*A*), as was described for H3$_{15}$K4me3 bound to the double chromodomain of CHD1 (38). The affinity of the chromodomain binding to H3K4me3 was found to experience a 4-fold reduction when the simultaneous asymmetric dimethylation at H3 Arg[2] (H3R2me2a) occurred on the histone 3 tail. In ING4, the side chain of H3 Arg[2] interacts with the side chain of Glu[220], whereas the corresponding position in CHD1 is occupied by a smaller glycine residue. Thus steric clashes may cause an even larger reduction in the affinity of ING4 for K4me3 when the simultaneous H3R2me2a modification occurs on the same histone site. The relevance of this discrimination lies in the mutual exclusion of H3K4me3 and H3R2me2a recently reported in actively transcribed genes (39, 40). The measurement of the binding of ING4 to H3R2me2aK4me3 shows a 6-fold reduction
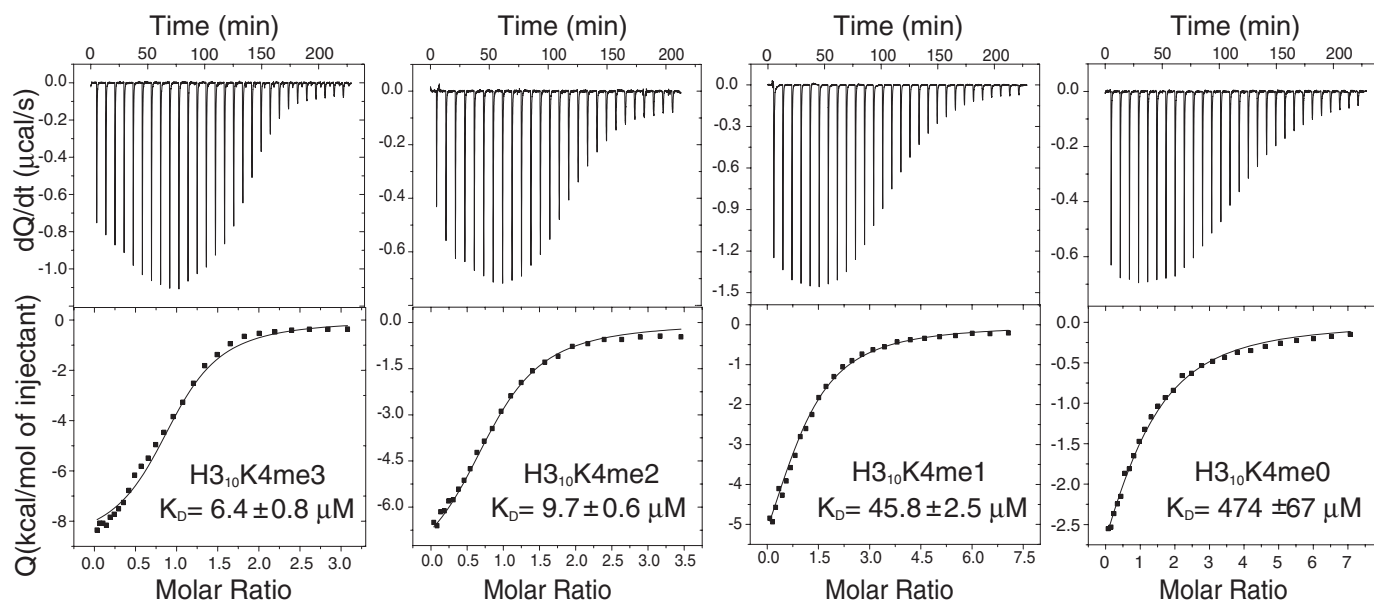
FIGURE 6. **Calorimetric titrations of ING4-PHD with H3$_{10}$K4meX peptides.** Shown are examples of the direct titrations of the H3$_{10}$K4me3, H3$_{10}$K4me2, and H3$_{10}$K4me1 peptides together with the displacement experiment for H3$_{10}$K4me0 using H3$_{10}$K4me1 as competing ligand. In all cases, the *upper panels* represent the heat effect associated with the peptide injections, and the *lower panels* represent the ligand concentration dependence of the heat released upon binding, after normalization and correction for the heats of dilution. The *symbols* correspond to the experimental data, and the continuous *line* corresponds to the best fit to a model of one set of identical binding sites (as described in the supplemental materials) for H3$_{10}$K4me3, -me2, and -me1 and to a complete displacement model for H3$_{10}$K4me0.
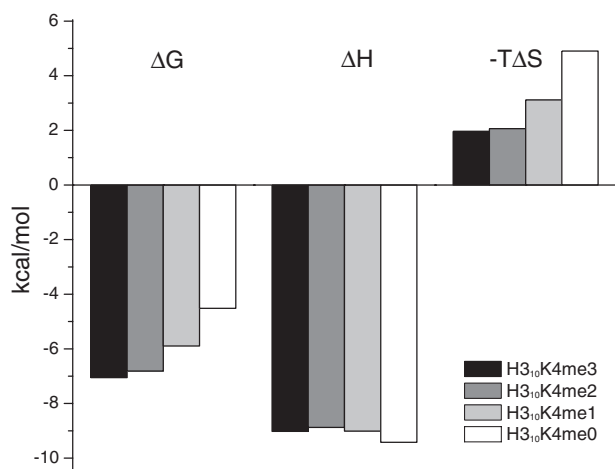

FIGURE 7. **Thermodynamic parameters of the ING4-PHD/H3K4meX binding.** The *bar diagram* shows the change in free energy (ΔG), enthalpy (ΔH), and in the contribution of entropy to the free energy ($-T\Delta S$) of the binding of H3$_{10}$K4meX peptides to ING4(188–249), as determined by ITC. The uncertainty in the experimental values is estimated to be ~5%.

in binding affinity (Table 2 and supplemental Fig. S4), indicating that ING4 can discriminate between chromatin regions enriched in one or both histone modifications. Dimethylation of H3 Arg[2] inhibits the recognition of H3K4me2 by the Spp1-PHD, a subunit of the Set1p histone methylation complex (40), thus regulating trimethylation at H3 Lys[4]. Sequence homology suggests that the binding site for H3 Arg[2] in Spp1 is similar to ING4, indicating that dimethylation of H3 Arg[2] will also inhibit binding of ING4 to H3K4me3, which is consistent with the measured reduced affinity for the corresponding histone fragment. In this way, H3R2me2 may help to maintain the chromatin silent not only by inhibiting the recruitment of methyltransferases but also that of histone acetyltransferase complexes

such as HBO1. Therefore, understanding how different combinations of histone modifications are read by their recognition modules may be critical to appreciating the regulatory mechanisms exerted through those modifications, as illustrated by the antagonizing role of H3K9me3 and H3S10ph in the recruitment of heterochromatin protein 1 to discrete regions of the chromatin, thereby regulating gene expression (41, 42).

The double chromodomain of CHD1 binds to H3K4me3 with similar affinities when combined with either H3K9me3, H3K9ac, or H3S10ph, but with a 20-fold reduced affinity when H3T3ph is present (38). Based on the structure of the ING4·H3K4me3 complex, phosphorylation of H3T3, a modification correlated with transcription activation, would generate a larger and negatively charged residue that could be accommodated by a conformational change at the side chain of Lys[232], with which a favorable electrostatic interaction could be established (Fig. 1*A*). H3K9me3 binds to ING4 with the same affinity as unmodified H3 (13), suggesting the compatibility of methylation at both lysine residues. Acetylation, however, removes the positive charge of H3K9, precluding its interaction with the aromatic side chain of Tyr[206] (Fig. 1*D*) and probably reducing the affinity of ING4 binding to H3K4me3K9ac. Phosphorylation of H3 Ser[10] would disrupt the hydrogen bond of its side chain with Glu[195] (Fig. 1*D*) and introduce an unfavorable electrostatic interaction, suggesting that it would be incompatible with H3K4me3 recognition. A similar effect, though reduced, might occur if H3T11 is the phosphorylated residue because its location on the PHD is close to the negatively charged N-terminal region of the PHD (Figs. 3*B* and 4*B*). On the other hand, monomethylation in Arg[8], which has been associated with repressed transcription, is probably of little relevance for H3K4me3 recognition because its side chain can adopt two

different conformations (Fig. 1), and the structure of the complex does not suggest additional interactions with H3R8me1. Our results suggest that H3K4me3 recognition by ING4 is compatible with H3T3ph, H3R8me1, and H3K9me3, whereas it is not compatible with, or is impaired by, H3R2me2a, H3K9ac, H3S10ph, and H3T11ph. Further experimental studies on PHD fingers and other protein modules will provide more insights into the impact of multiple combinations of histone modifications and their role in the dynamic regulation of chromatin.

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