Molecular Basis of Histone H3K4me3 Recognition by ING4*§

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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 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 crucial 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

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The atomic coordinates and structure factors (code 2VMF) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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6 The abbreviations used are: ING, inhibitor of growth; PHD, plant homeodomain; H3, histone 3; ITC, isothermal titration calorimetry; ASA, accessible surface area; CSP, chemical shift perturbations; CHD1, chromo-ATPase/ helicase-DNA-binding protein 1; HDAC1, histone deacetylase complex 1; NOE, nuclear Overhauser effect; HSQC, heteronuclear single quantum coherence.
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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 the 188–249 construct using a QuikChange mutagenesis kit. The protein was dialyzed against 10 mM Tris, pH 6.5, 150 mM NaCl and T2 experiments were acquired with eight scans and a representative 1.4 mM. The crystals grew in the hanging drop vapor diffusion method at 5 °C. The pure protein was deposited with the Protein Data Bank (access code 2VNF). Data collection details and statistics of the refinement can be found in Table 1. The coordinates have been deposited with the Protein Data Bank (access 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) 2H2O, 0.01% Na3N 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 H310K4m3 or H315K4m3 were obtained under identical conditions with two samples containing 50 μM PHD and a 4-fold excess of each peptide, which were simultaneously dialed against the same buffer. Backbone 15N T1, T2, and 1H-15N heteronuclear NOE measurements (23) were performed on a Bruker AVANCE 700 spectrometer on a 0.83 mM uniformly 15N 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 to measure the T2 values. T1 and T2 experiments were acquired with eight scans and a rep-
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etition delay of 3 s, whereas each $[^1\text{H}]$-$^{15}\text{N}$ 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 H3K4me3 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 $[^1\text{H}]$-$^{15}\text{N}$ NOE experiment 148 and 128 scans in the case of H310K4me3 and H315K4me3, respectively. To check the reproducibility of the calculation failed when using the errors in $T_1$ and $T_2$ estimated quadric diffusion (A. G. Palmer III, Columbia University). The approaches (27, 28) using the programs r2r1_diffusion and the rotational diffusion anisotropy, was determined by two 1.02 Å, and a contribution from the $^{15}\text{N}$ chemical shift anisotpling with the directly attached proton, with a bond length of 4.2 (30) was used to fit the relaxation data to the model free (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 $[^1\text{H}]$-$^{15}\text{N}$ pair: (i) $S^2$, (ii) $S^2$ and $\tau_\epsilon$, (iii) $S^2$ and $R_{ex}$, (iv) $S^2$, $\tau_\epsilon$, and $R_{ex}$, and (v) $S^2$, $S^2$, and $\tau_\epsilon$, where $S^2$ is the generalized order parameter, $\tau_\epsilon$ is the effective internal correlation time, $R_{ex}$ is the exchange contribution to the transverse relaxation, and $S^2$ 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 H310K4me0 with ING4-PHD, for which the dissociation constant is out of the range measurable directly by ITC, displacement experiments using H310K4me1 as competing ligand were carried out. Briefly, a 65 µM ING4-PHD solution with H310K4me0 at a 1:4.6 molar ratio was placed in the calorimetric cell and titrated with H310K4me1 (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 H310K4me1 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-H310K4me3 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, ∆ASA = 26.93 Å²), and the third one is significantly exposed (carbon atom number 892, ∆ASA = 16.93 Å²), and the third one is significantly exposed (carbon atom number 892, ∆ASA = 26.93 Å²). 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 H315K9me2 and me3 peptides, where the methylated lysine side chains adopt a structure very similar to that in ING4-PHD-H310K4me3 complex (34). For this reason, in the model of H310K4me2 bound to ING4 the methyl group that is exposed the most (C atom number 892) was removed from the structure of H310K4me3, whereas C atoms numbers 892 and 893 were removed to build the model for the H310K4me1 complex.
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 (H310K4me3) was solved at 1.76 Å resolution (Fig. 1A). The ten residues of the peptide were observed in the electron density, including two alternate conformations for H3 Arg8 that could be modeled without ambiguity into their corresponding densities (Fig. 1B). 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. 2A). 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 (35). The peptide N terminus forms a hydrogen bond with the carbonyl of ING4 residue Gly235 and H3 residues Arg2–Thr6, which have β-sheet backbone dihedral angles, form backbone hydrogen bonds with...
Cys\textsuperscript{212}–Gly\textsuperscript{207} (Fig. 1C). A kink in the main chain at H3 residues Ala\textsuperscript{7}–Arg\textsuperscript{8} recovers the extended conformation for H3 residues Lys\textsuperscript{9}–Ser\textsuperscript{10}, with H3 Ser\textsuperscript{10} forming a backbone hydrogen bond with Ser\textsuperscript{205}. Because of the polar nature of histone 3 tail residues, the interactions are predominantly polar. The side chain of H3 Arg\textsuperscript{2} forms a salt bridge with Glu\textsuperscript{220}, while the side chains of Thr\textsuperscript{3}, Thr\textsuperscript{6}, and Ser\textsuperscript{10} form hydrogen bonds with Lys\textsuperscript{232}, Gly\textsuperscript{207}, and Glu\textsuperscript{195}, respectively (Fig. 1D). The guanidinium group of H3 Arg\textsuperscript{2} and the trimethylammonium group of H3K4me3 make cation–π interactions with the side chain of Trp\textsuperscript{221}, which sits between the two basic peptide side chains. H3K4me3 forms another cation–π contact with Tyr\textsuperscript{198}, which is not essential for recognition because the mutant Y198A still binds the peptide with moderate affinity (13). H3 residue Lys\textsuperscript{9} makes a cation–π interaction with Tyr\textsuperscript{206}, and so does H3 Arg\textsuperscript{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\textsubscript{α} 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\textsuperscript{1}–Thr\textsuperscript{6} (see supplemental Fig. S1). The kink at H3 Ala\textsuperscript{7} directs the peptide chain along the rim of the ellipsoid, and H3 Ser\textsuperscript{10} occupies a small depression lined by Glu\textsuperscript{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\textsuperscript{2}; see below), with H3 Ala\textsuperscript{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\textsuperscript{198} and Trp\textsuperscript{221}), the hydrophobic side chain of Met\textsuperscript{209}, and Ser\textsuperscript{205}. This binding mode of the methylated lysine is common to ING2 and similar to the bromodomain PHD finger transcription factor, with a cage of four aromatic residues, (17). Overall, the recognition of the N-terminal half of H3\textsubscript{9}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\textsuperscript{7}, and the last two residues observed in the crystal (H3 Ala\textsuperscript{7}–Arg\textsuperscript{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<sub>10</sub>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. 3B). The shielding of the aromatic side chain of Tyr<sup>215</sup> 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<sup>198</sup> 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<sup>1</sup> by Gly<sup>235</sup> blocks N-terminal extensions of the peptide chain bound to ING4, which is probably important for the specificity of the binding of H3<sub>10</sub>K4me3 because the 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<sub>15</sub>K4meX peptides (13). The affinity for H3<sub>10</sub>K4me3 is, within error, the same as for H3<sub>15</sub>K4me3. Nonetheless, whereas ING4 does not discriminate between H3<sub>15</sub>K4me1, -me2, or -me3, it does so for the shorter peptides, with the affinity increasing with the higher number of methyls (Fig. 2B 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<sub>15</sub>K4me3 and H3<sub>10</sub>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<sub>10</sub>K4me3 or H3<sub>15</sub>K4me3 (Fig. 4A) 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. 4B), indicating that additional interactions take place, resulting in higher affinities for H3<sub>10</sub>K4me1 and -me2 (Table 2). This finding is consistent with the decreased flexibility of the N-terminal end of ING4-PHD bound to H3<sub>10</sub>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<sup>2</sup>) measured for the backbone<sup>15</sup>N atoms (Fig. 5A), which are sensitive to movements of the N-H bond in ps to ns time scales. Residue Gly<sup>235</sup> also shows increased mobility compared with the rest of the chain. Binding to H3<sub>10</sub>K4me3 increases the order parameters at several regions of the ING4

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**TABLE 2**

| Ligand<sup>a</sup> | K<sub>D</sub><sup>b</sup> (μM) |
|----------------|-----------------|
| H3<sub>10</sub>K4me0 | 370 ± 20 |
| H3<sub>15</sub>K4me0 | 274 ± 6 |
| H3<sub>10</sub>K4me1 | 1.6 ± 0.8 |
| H3<sub>15</sub>K4me1 | 34 ± 4 |
| H3<sub>10</sub>K4me2 | 2 ± 1 |
| H3<sub>15</sub>K4me2 | 9.2 ± 1.4 |
| H3<sub>10</sub>K4me3 | 3.9 ± 0.7 |
| H3<sub>15</sub>K4me3 | 3.0 ± 0.6 |
| H3<sub>10</sub>R2me2K4me3 | 19.2 ± 1.7 |
| H3<sub>15</sub>R2me2K4me3 | 1400 ± 40 |

<sup>a</sup> The values for H3<sub>15</sub>K4 peptides are from Ref. 13.

<sup>b</sup> The fitting errors are indicated.

<sup>c</sup> Measured from the CSP of C212 signal instead of Trp<sup>237</sup> (see supplemental Fig. 5A).

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**FIGURE 4.** Binding of ING4-PHD to H3K4me3 peptides of different lengths. A, overlay of HSQC spectra of ING4(188–249) bound to H3<sub>10</sub>K4me3 (black) or H3<sub>15</sub>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 peptide-prolyl bond 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.
ING4 is also detected for Trp221, which interacts with H3 Arg2
mobility in its free form that become more rigid upon binding
binding involves regions of ING4 with different degrees
H310K4me3 is not made by a static PHD molecule but that
not in the complex (Fig. 5
mational exchange detected for this residue in free ING4 but
around Glu220 on peptide binding is consistent with the confor-
mational order is induced on binding to ING4 (data not shown).
the differences in the entropic contributions to binding (−TΔΔS = −1.9, −2.8, and −3.3 kcal mol−1 for H3p,K4me1, -me2, and -me3, respectively, referenced to H3p,K4me0), and the differ-
ences in solvation entropy were calculated (33) in terms of changes in accessible surface area (−TΔΔS_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 numer-
ous interactions that occur when the N-terminal histone resi-
dues occupy a depression on one side of the PHD. This results
however, in a low binding affinity that increases on methylation
H3 Lys4. 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 methyla-
tion states of H312K4meX, 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 com-
plex, links H3K4me3 recognition with transcriptional repression
through histone deacetylation. ING4, involved in a com-
plex 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 Arg2 lies in a groove that is separated by
Trp221 from another groove occupied by H3R2me3 (Fig. 1A), as
was described for H3p,K4me3 bound to the double chromo-
domain 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 Arg2
(H3R2me2a) occurred on the histone 3 tail. In ING4, the side
chain of H3 Arg2 interacts with the side chain of Glu220,
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 his-
tone 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

chain containing residues directly involved in histone recogni-
tion (Glu195, Gly211, Glu220, and Gly235). The increased order
around Glu220 on peptide binding is consistent with the confor-
mational exchange detected for this residue in free ING4 but
not in the complex (Fig. 5B). Conformational exchange in free
ING4 is also detected for Trp221, which interacts with H3 Arg2
and H3 Lys4. These results show that the recognition of
H3p,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 H315,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 confor-
mational order is induced on binding to ING4 (data not shown).
A detailed account of the analysis of the backbone 15N relax-
ation data can be found in the supplemental data.

The energetics of the ING4 binding to H3t0,K4meX was char-
acterized 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 meas-
ured 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 apo-
polar and polar ASA upon complex formation (798 and 520 Å2,
respectively, with a ratio ΔASAapolar/ΔASApolar = 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 inter-
esting to note that the opposite situation has been proposed for
chromodomains (37). Lysine methylation leads to a polariza-
tion of the Cα-Nε bond increasing the cationic character of the
methylammonium group and strengthening the cation−π inter-
actions 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 interpreta-
tion is supported by the agreement between the measured dif-
fferences in the entropic contributions to binding (−TΔΔS = −1.9, −2.8, and −3.3 kcal mol−1 for H3p,K4me1, -me2, and
-me3, respectively, referenced to H3p,K4me0), and the differ-
ences in solvation entropy were calculated (33) in terms of changes in accessible surface area (−TΔΔS_solv = −1.7, −2.9, and −2.6 kcal mol−1).

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FIGURE 5. Backbone dynamics of the ING4-PHD backbone. The backbone
15N nuclei order parameters (A, S2) and the contribution of exchange pro-
cesses (B, R_ex) to the relaxation of backbone 15N nuclei are represented versus
the sequence of ING4, in its free form (open circles) and in the complex with
H3p,K4me3 (filled circles). The ribbon diagram shows the crystal structure of
ING4-PHD bound to H310K4me3 (in yellow and with side chains). A color
indicates the change in S2 for the corresponding ING4 residue on peptide
binding. The residues for which the difference could not be measured are
colored in black.
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 Arg2 inhibits the recognition of H3K4me2 by the Spp1-PHD, a subunit of the Set1p histone methylation complex (40), thus regulating trimethylation at H3 Lys4. Sequence homology of H3 Arg2 inhibits the recognition of H3K4me2 by the Spp1-PHD, a subunit of the Set1p histone methylation complex (40), indicating that dimethylation of H3 Arg2 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 Lys232, with which a favorable electrostatic interaction could be established (Fig. 1A). 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 Tyr206 (Fig. 1D) and probably reducing the affinity of ING4 binding to H3K4me3K9ac. Phosphorylation of H3 Ser10 would disrupt the hydrogen bond of its side chain with Glu195 (Fig. 1D) 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 side chain can adopt two

![Bar diagram showing the thermodynamic parameters of the ING4-PHD/H3K4meX binding.](https://example.com/bar_diagram.png)

**FIGURE 7.** Thermodynamic parameters of the ING4-PHD/H3K4meX binding. The bar diagram shows the change in free energy ($\Delta G$), enthalpy ($\Delta H$), 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%.

![Graphs showing calorimetric titrations of ING4-PHD with H3$_{10}$K4meX peptides.](https://example.com/heat_diagram.png)

**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 correspond 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.
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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