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**Global unfolding of a substrate protein by the Hsp100 chaperone ClpA**

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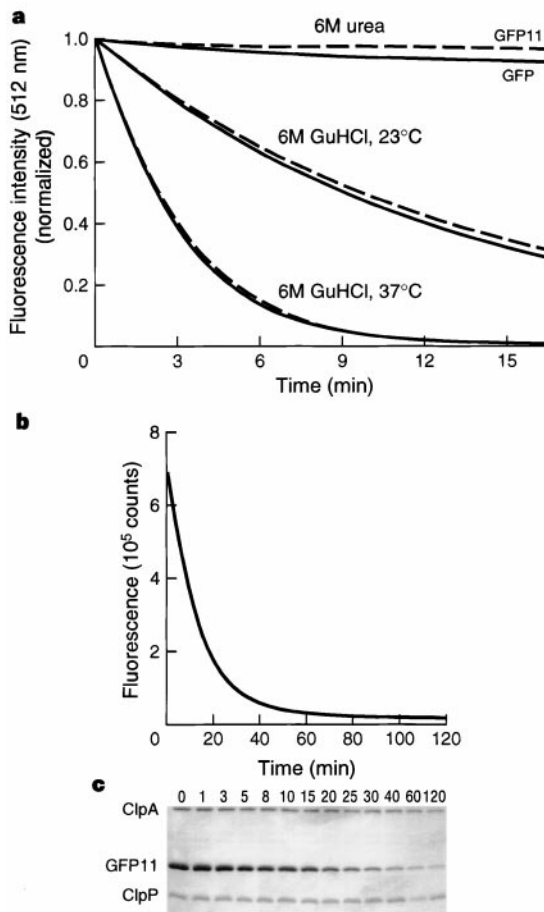
The bacterial protein ClpA, a member of the Hsp100 chaperone family, forms hexameric rings that bind to the free ends of the double-ring serine protease ClpP (refs 1, 2). ClpA directs the ATP-dependent degradation of substrate proteins bearing specific sequences<sup>3–5</sup>, such as the 19S ATPase ‘cap’ of eukaryotic proteasomes functions in the degradation of ubiquitinated proteins<sup>6–8</sup>. In isolation, ClpA and its relative ClpX can mediate the disassembly of oligomeric proteins<sup>9,10</sup>; another similar eukaryotic protein, Hsp104, can dissociate low-order aggregates<sup>11</sup>. ClpA has been proposed to destabilize protein structure, allowing passage of proteolysis substrates through a central channel into the ClpP proteolytic cylinder<sup>12–14</sup>. Here we test the action of ClpA on a stable monomeric protein, the green fluorescent protein GFP, onto which has been added an 11-amino-acid carboxy-terminal recognition peptide, which is responsible for recruiting truncated proteins to ClpAP for degradation<sup>5,15</sup>. Fluorescence studies both with and without a ‘trap’ version of the chaperonin GroEL, which binds non-native forms of GFP<sup>16</sup>, and hydrogen-exchange experiments directly demonstrate that ClpA can unfold stable, native proteins in the presence of ATP.

We first tested whether GFP, a very stable monomeric protein (it is resistant to denaturation by 6M urea, for example; Fig. 1a), could be degraded by the ClpAP machinery once recruited to it as a result of the attachment of an 11-residue *ssrA* peptide to the GFP terminus. Appendage of the peptide did not interfere with the fluorescent properties or stability of the GFP portion: the tagged protein (GFP11) gave the same fluorescence quantum yield and emission spectrum as non-tagged GFP (not shown) and was as stable to denaturant (Fig. 1a). When incubated with catalytic amounts of ClpA and ClpP in the presence of ATP, however, GFP11 was completely degraded, as evidenced both by loss of fluorescence (Fig. 1b) and by the disappearance of the protein on SDS–polyacrylamide gel electrophoresis (Fig. 1c). The kinetics of the two measurements were similar, preventing the resolution of an

unfolding action from degradation. In contrast with the tagged protein, non-tagged GFP gave no change in fluorescence or loss of protein when incubated under similar conditions with ClpA, ClpP and ATP (results not shown). We conclude that even the stable  $\beta$ -barrel of GFP can be destabilized and degraded by the ClpAP machinery when recruited to it as a result of the addition of the *ssrA* sequence.

To test whether we could detect unfolding of GFP11 as a step distinct from the degradation carried out by ClpAP, we incubated GFP11 with stoichiometric amounts of ClpA on its own. In the presence of ATP, but not of ATP- $\gamma$ S, there was a small decrease (20%) in fluorescence intensity (Fig. 2a), indicating that some fraction of the input GFP11 molecules could be unfolded. The observed effect was small, however, compared with the effect of adding a stoichiometric amount of ClpA and ClpP, which caused a complete loss of fluorescence as a result of the total proteolytic degradation of the added GFP11 (Fig. 2a, and results not shown).

We reasoned that, if GFP11 molecules unfolded by ClpA were released from it and then refolded, establishing an equilibrium between unfolding and refolding, then preventing refolding should reveal the unfolding action of ClpA, producing a larger drop in fluorescence. To prevent refolding, we added a GroEL ‘trap’ mutant (D87K, in which an aspartate residue at position 87 is replaced by a



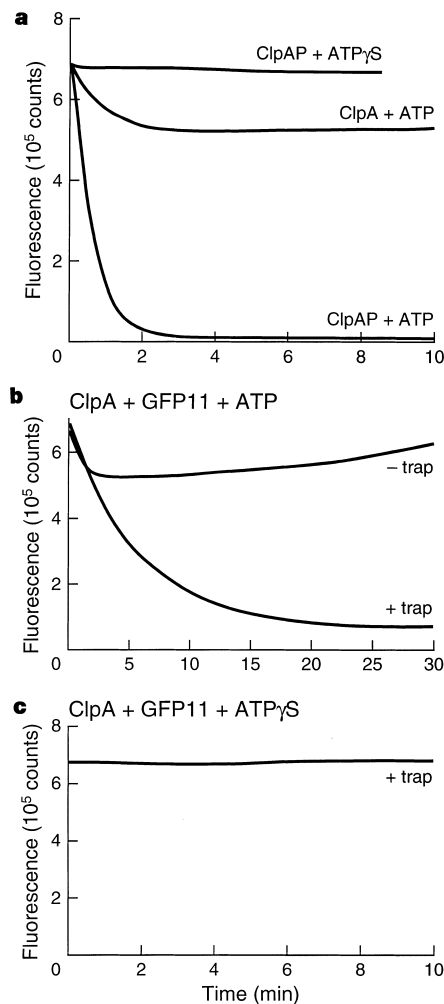
**Figure 1** GFP11, a derivative bearing an 11-amino-acid C-terminal *ssrA* tag recognized by ClpA, exhibits the same stability to denaturants as untagged GFP, and is degraded by ClpA/ClpP in the presence of ATP. **a**, Time course of fluorescence intensity changes of GFP11 and GFP after dilution to 600 nM in 6 M urea at 23 °C (top), 6 M guanidine-HCl at 23 °C (middle), or 6 M guanidine-HCl at 37 °C (bottom). **b**, Time-course of the fluorescence intensity change of 10  $\mu$ M GFP11 incubated with 100 nM ClpA, 50 nM ClpP and 10 mM ATP. Excitation and emission wavelengths were 400 and 510 nm, respectively. **c**, SDS–PAGE analysis of aliquots of the reaction used in **b**, taken at the times indicated.

lysine) to the reaction: this mutant captures but is unable to release non-native forms of GFP. As shown in Fig. 2b, when a 5-fold molar excess of trap is present with ATP in a stoichiometric mixture of GFP11 and ClpA, there is a large and irreversible loss of fluorescence, unlike the small change in the absence of trap, indicating that efficient unfolding can be mediated by ClpA. Unfolding is not effected by the GroEL trap alone, which when incubated with GFP11 gave no fluorescence change (results not shown), nor does it occur in the presence of the non-hydrolysable ATP analogue ATP- $\gamma$ S (Fig. 2c), which is consistent with the inability of this nucleotide to support turnover of GFP11 by ClpAP.

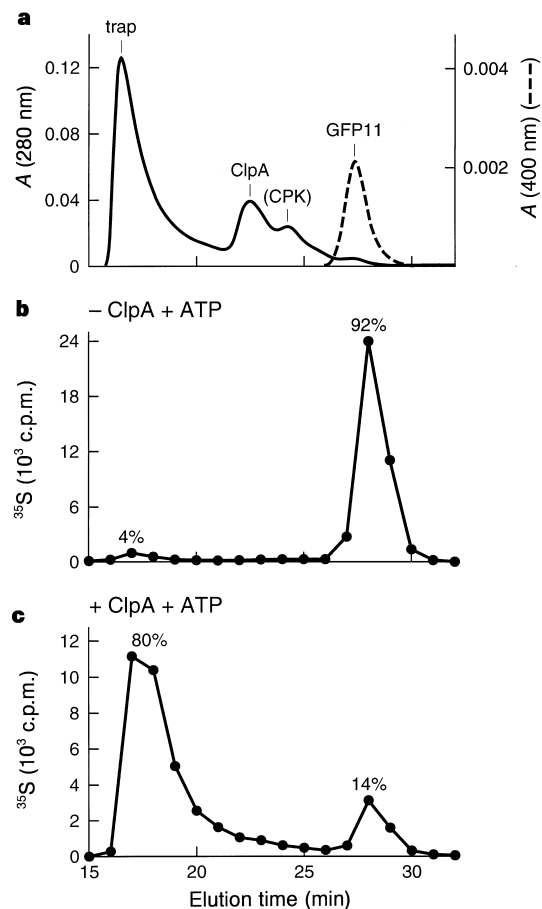
To confirm that ClpA was releasing non-native forms that were captured by the trap, we did the same experiment with  $^{35}$ S-methionine-labelled GFP11, and subjected the mixture to gel filtration. In the absence of ClpA, but in the presence of trap and ATP, virtually all (92%) of the input molecules were found at the same migration position as GFP11 (Fig. 3a, b), which also indicates that the GroEL trap does not bind native GFP11. After addition of ClpA, however, most (80%) of the input molecules migrated at the position of trap (Fig. 3c), in agreement with the loss of fluorescence shown in Fig. 2b. The remaining molecules were found at the same

position as GFP11. They had either failed to be recognized by ClpA during the reaction or had dissociated from the trap during gel filtration. In control incubations, replacement of ATP with ATP- $\gamma$ S or the omission of trap resulted in all the input labelled molecules migrating at the GFP11 position. This is consistent with ATP- $\gamma$ S being unable to promote unfolding, with the refolding of GFP11 in the absence of trap, and with the unstable association of GFP11 with ClpA in gel-filtration chromatography. We found that GFP11 did not stably associate with ClpA even when chromatography was done in the presence of ATP (results not shown). We conclude that ClpA mediates ATP-dependent unfolding of GFP11, producing non-native forms that are devoid of fluorescence and that are readily recognized by the chaperonin GroEL. These non-native forms are probably collapsed globular states that expose hydrophobic surfaces which are recognizable by the GroEL trap apical domains.

To assess the competition between trap and ClpP for the ClpA-unfolded substrate protein, we supplied a stoichiometric amount of ClpP to ClpA with trap in 5-fold molar excess. The  $^{35}$ S label did not elute with the trap in gel filtration but as a broad peak several minutes after GFP11 (result not shown), indicating that GFP11 had been degraded. In the presence of ClpP, therefore, GFP11 that has been unfolded by ClpA is probably released from ClpA into the adjoining ClpP proteolytic cavity, without access to trap, and is committed to degradation.



**Figure 2** ClpA, in the absence of ClpP, mediates ATP-dependent unfolding of GFP11. **a**, Time course of fluorescence intensity changes of 2  $\mu$ M GFP11, incubated with 2  $\mu$ M ClpA and ATP; or with ClpA and ClpP, each 2  $\mu$ M, and ATP or ATP- $\gamma$ S. **b**, Time course of fluorescence intensity changes of 2  $\mu$ M GFP11, in the presence of 2  $\mu$ M ClpA, ATP, and in the absence or presence of 10  $\mu$ M of the GroEL trap mutant D87K. The recovery of fluorescence intensity at later times in the absence of trap probably reflects exhaustion of the ATP-regeneration system. **c**, Lack of fluorescence intensity change of GFP11 in ATP- $\gamma$ S even in the presence of trap, indicating that ATP- $\gamma$ S does not support unfolding of GFP11.



**Figure 3** Transfer of GFP11 to GroEL trap in the presence of ClpA and ATP. **a**, Gel-filtration profile showing positions of migration of GroEL trap, ClpA (dimer) and CPK (from the ATP-regenerating system) (absorbance A at 280 nm) and GFP11 (A at 400 nm). **b**, Elution profile of 2  $\mu$ M  $^{35}$ S-GFP11 after incubation for 40 min in the presence of ATP with a 5-fold molar excess of GroEL trap, showing that most of the GFP11 is uncomplexed. **c**, Elution profile of 2  $\mu$ M  $^{35}$ S-GFP11 incubated with 2  $\mu$ M ClpA and ATP under the same conditions as in **b**, showing transfer of ~80% of the molecules to the trap. In all cases, chromatography was on a Superose-12 gel-filtration column (Pharmacia) and without ATP in the column buffer.

To determine whether the unfolding mediated by ClpA is partial, like the dissociation of a dimer, exposing hydrophobic surfaces at the interface but leaving the core intact, or total, perhaps destabilizing the core of a substrate protein, we used hydrogen–deuterium exchange and mass spectrometry experiments<sup>17</sup>. GFP11 was deuterated (dGFP11) by unfolding it in 6 M guanidine-DCl in D<sub>2</sub>O and then refolded by diluting into D<sub>2</sub>O buffer. Its molecular mass was determined after incubating with ATP in H<sub>2</sub>O buffer (Fig. 4b) to be 106 daltons heavier than fully protonated GFP11 (27,861 daltons; Fig. 4a), reflecting the number of sites that are protected by the core in the folded state. Incubation with ClpA and ATP for 2 h at 23 °C, however, produced a molecular mass (Fig. 4c) that was within 6 daltons of fully protonated GFP11, indicating that nearly complete hydrogen exchange of the stable core of dGFP11 had occurred. By contrast, in a mixture of ClpA with ATP- $\gamma$ S (Fig. 4d), dGFP retained >100 daltons of deuterium. When dGFP11 was incubated with GroEL in the absence of nucleotide, the retention of deuterium was the same (results not shown), reflecting that the chaperonin neither stably binds (Fig. 3b) nor transiently unfolds native GFP11 on this timescale.

We conclude that ClpA in the presence of ATP mediates the unfolding of proteins bound after recognition of specific terminal amino-acid sequences. In association with ClpP protease, these destabilized structures are committed to translocation from the cavity of ClpA into the hydrolytic cavity of the protease, where they are degraded. In the absence of associated ClpP, substrate proteins are released from ClpA into solution, where they may spontaneously refold or become bound, as in the trap experiments described here, by other components such as chaperonins. This action of unfolding and release may also be exerted by other Hsp100 family members: for example, in the dissociation by ClpX of the

MuA transposase tetramer from DNA<sup>10</sup>, and in the dissociation by eukaryotic Hsp104 of low-order aggregates<sup>11</sup>.

The geometry of unfolding and translocation by ClpA remains unclear. Presumably unfolding is driven by multivalent contacts between the surrounding ClpA subunits and the substrate protein. Although the nature of these contacts is unknown, ATP binding or hydrolysis probably drives a conformational change in ClpA that pulls the protein structure apart, in an action analogous to the transient unfolding of Rubisco after GroES and ATP bind to a Rubisco–GroEL binary complex<sup>18</sup>. ClpA releases the unfolded substrate, either into the bulk solution, as indicated by the trap experiment, or into the proteolytic cylinder of ClpP when it is present, whereas the unfolded protein released into the GroEL–GroES *cis* cavity is committed to a time-limited attempt at folding to the native state in this sequestered and hydrophilic environment. But how directional translocation through the ClpA cavity occurs during the unfolding action remains unknown. □

Methods

Proteins.

ClpA, ClpP, and GFP11 (Q80R) were all overproduced from T7 expression plasmids and purified by ion-exchange chromatography or as described (ClpP)<sup>19</sup>. GroEL D87K was expressed and purified as described<sup>20</sup>. Concentrations refer to the fully assembled species.

Fluorescence.

Fluorescence kinetic measurements were made on a PTI Quantmaster QM-1 fluorimeter connected to a PC-based data acquisition program, with excitation at 400 nm and emission at 510 nm. In reactions with catalytic amounts of ClpA and ClpP (Fig. 1), reaction buffer was 50 mM HEPES, pH 7.5, 0.3 M NaCl, 20 mM MgCl<sub>2</sub>, 0.5 mM DTT, 10% glycerol, plus 10 mM ATP. For all other reactions, where substrate and ClpA/ClpP were stoichiometric, the reaction buffer contained 0.05 U  $\mu$ l<sup>-1</sup> creatine phosphokinase, 30 mM phosphocreatine, 5 mM ATP. In all reactions, ClpA, with ClpP where indicated, was incubated with ATP and all other components for 20–30 s to enable ClpA and ClpAP complexes to assemble before addition of GFP11.

Hydrogen–deuterium exchange.

Deuterated GFP11 (dGFP11) was prepared by two cycles of exchange of protein into D<sub>2</sub>O. In the first cycle, GFP11 was diluted 15-fold with D<sub>2</sub>O and dried to its original concentration. In the second cycle, the protein was unfolded by mixing with 5 vol. 6 M guanidine-DCl (pD 7.5) and incubating at 55 °C for 2 min, then at 23 °C for 10 min. Complete unfolding was verified by total loss of fluorescence. The protein was refolded by 50-fold dilution into deuterated refolding buffer (25 mM Tris, pD 7.5, 2.5% glycerol, 4 mM DTT), followed by incubation for 2 h at 23 °C. Aggregates were removed by ultrafiltration (Centriprep 100). The filtrate was concentrated to ~100  $\mu$ M by ultrafiltration (Microcon 10). All quoted pH measurements in D<sub>2</sub>O buffers (pD) are uncorrected pH meter readings.

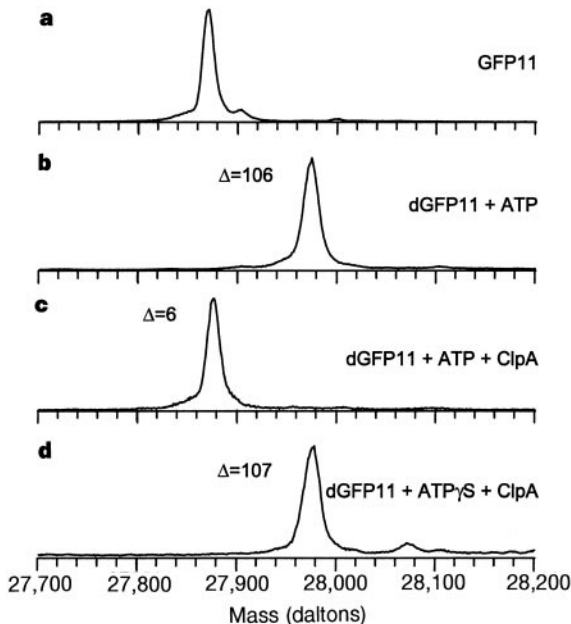
dGFP11 was diluted 50-fold into H<sub>2</sub>O reaction mixtures, giving a final deuterium content of 2%. Reactions were carried out for 2 h at 23 °C. ClpA was then precipitated by 3-fold dilution with 7.5 mM Tris, pH 7.5, and removed by centrifugation. The supernatant was filtered through a Centricon 100 to remove residual ClpA, and the filtrate was concentrated to ~30  $\mu$ M GFP11 with a Microcon 10. Buffer was exchanged into 7.5 mM Tris, pH 7.5, using a Microcon 10 before mass spectrometric analysis. These steps of sample preparation were carried out at 23 °C and required ~6 h.

Mass spectrometry.

Spectra were collected using a Micromass LCT electrospray time of flight mass spectrometer. Atmospheric pressure ionization was performed using borosilicate glass capillaries drawn and sputter coated in-house. All spectra show the +13 charge state expressed on a mass scale, but mass and  $\Delta$ -mass determinations were made using a minimum of three charge states. All spectra were collected over a mass range of 200 to 5,000 *m/z*, signal-averaged for 1 min with no smoothing applied. 25–30 point external calibrations were done using 20 mM CsI in H<sub>2</sub>O ionized under matched instrument conditions. GFP11 samples were kept on ice until analysis and diluted 10–50-fold with H<sub>2</sub>O immediately before loading into the capillaries. The mass spectrometer ionization source temperature was kept at 20 °C.

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**Figure 4** Mass spectra of GFP11 subjected to hydrogen–deuterium exchange followed by incubation of the deuterated protein in H<sub>2</sub>O buffer with or without ClpA and nucleotide. **a**, Native GFP11, giving a mass of 27,861 daltons. This compares well with the calculated mass of 27,867 daltons. **b**, dGFP11 incubated in H<sub>2</sub>O buffer with ATP for 2 h at 23 °C, giving a mass of 27,967 daltons. **c**, dGFP11 incubated as in **b**, but with ClpA and ATP, giving a mass of 27,867 daltons, amounting to a nearly complete loss of deuterium label. **d**, dGFP11 incubated as in **c**, but with ATP- $\gamma$ S instead of ATP, giving a mass of 27,968 daltons. In **b–d**, mass differences ( $\Delta$ ) are shown with respect to the mass measured in **a**. A representative experiment is shown, with all samples being measured on the same day. The mass changes shown here are within 6 daltons of the average difference in mass derived from three independent experiments.

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**Structure of *Tetrahymena* GCN5 bound to coenzyme A and a histone H3 peptide**

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**Gene activation is a highly regulated process that requires the coordinated action of proteins to relieve chromatin repression and to promote transcriptional activation. Nuclear histone acetyltransferase (HAT) enzymes provide a mechanistic link between chromatin destabilization and gene activation by acetylating the ε-amino group of specific lysine residues within the amino-terminal tails of core histones to facilitate access to DNA by transcriptional activators<sup>1,2</sup>. Here we report the high-resolution crystal structure of the HAT domain of *Tetrahymena* GCN5 (tGCN5) bound with both its physiologically relevant ligands, coenzyme A (CoA) and a histone H3 peptide, and the structures of**

**nascent tGCN5 and a tGCN5/acetyl-CoA complex. Our structural data reveal histone-binding specificity for a random-coil structure containing a G-K-X-P recognition sequence, and show that CoA is essential for reorienting the enzyme for histone binding. Catalysis appears to involve water-mediated proton extraction from the substrate lysine by a glutamic acid general base and a backbone amide that stabilizes the transition-state reaction intermediate. Comparison with related N-acetyltransferases indicates a conserved structural framework for CoA binding and catalysis, and structural variability in regions associated with substrate-specific binding.**

The structure of the nascent tGCN5 HAT domain was determined by multiple anomalous dispersion phasing, and the structures of the binary tGCN5/acetyl-CoA and ternary tGCN5/CoA/histone H3 peptide complexes were determined by molecular replacement using the refined tGCN5 model (Table 1). The tGCN5 HAT domain contains a mixed α/β structure with a globular fold and an overall structure that is very similar to its yeast (yGCN5)<sup>3</sup> and human (hP/CAF)<sup>4</sup> homologues (Fig. 1a). The central core region has sequence and structural homology to a group of over 50 N-acetyltransferases called GNAT (GCN5-related N-acetyltransferases)<sup>5</sup>, and the amino- and carboxy-terminal domains share sequence and structural divergence with several GNAT members<sup>6–9</sup> (Fig. 1a). Between the N- and C-terminal segments of the protein are two pronounced and roughly orthogonal surface clefts forming an L-shape (Fig. 1b). In the binary tGCN5/acetyl-CoA complex, this cleft is occupied by acetyl-CoA, whereas in the ternary tGCN5/CoA/histone H3 peptide complex, the larger cleft is also occupied by the histone H3 peptide.

The ternary complex contains an 11-residue peptide from yeast histone H3 centred around the reactive Lys 14 and containing the sequence K<sub>9</sub>-S<sub>10</sub>-T<sub>11</sub>-G<sub>12</sub>-G<sub>13</sub>-K<sub>14</sub>-A<sub>15</sub>-P<sub>16</sub>-R<sub>17</sub>-K<sub>18</sub>-Q<sub>19</sub>. The side chains for residues 9, 10 and 11 are not visible in the final electron-density map and are modelled as Ala<sub>9</sub>-Ala<sub>10</sub>-Gly<sub>11</sub> (Fig. 2a). The 11-residue histone H3 peptide is bound above the β<sub>4</sub> strand of the core domain and is flanked on opposite sides by the α<sub>1</sub>-turn-α<sub>2</sub> N-terminal segment and the β<sub>5</sub>-turn-α<sub>4</sub> and α<sub>5</sub>-turn-β<sub>6</sub> C-terminal segments (Fig. 1a). The peptide adopts a random-coil structure and buries a total of 1,562 Å<sup>2</sup> of solvent-exposed surface area upon complex formation.

Most of the protein-peptide interactions are mediated through the backbone of the histone H3 peptide (Fig. 2b), and about 75% of these interactions involve Lys 14 and the five residues C-terminal to it. Protein-peptide interactions are distributed roughly evenly between hydrogen bond and van der Waals contacts, with most of the peptide interactions mediated by residues 159–165 (excluding residue 161) of the β<sub>5</sub>-turn-α<sub>4</sub> region in the C-terminal protein segment. Residues 77–80 within the α<sub>1</sub>-turn N-terminal segment also make peptide interactions, and all of these interactions (except for Pro 78) are with the backbone of the peptide. Tyr 192 of the α<sub>5</sub>-β<sub>6</sub> loop C-terminal segment also contacts the peptide backbone, and CoA directly contacts Pro 16 of the peptide. The reactive Lys 14 residue of the histone H3 peptide is directly anchored to the substrate binding site by van der Waals interactions with Val 123 and Leu 126 in the β<sub>4</sub> strand at the base of the peptide cleft and by the CoA cofactor, and by Nζ hydrogen bonds to the backbone carbonyl group of Ala 124 and the terminal sulphur atom of CoA (Fig. 2b). Significantly, the region of the β<sub>4</sub> strand that interacts with the Lys 14 residue of the histone H3 peptide has a pronounced kink (Fig. 1a) which appears to be essential to facilitate these protein-substrate interactions as well as to point the backbone carbonyls of residues Val 123 and Ala 124 into the histone-binding cleft to facilitate an electrostatic attraction for the lysine substrate. The β<sub>4</sub> kink is found in each of the structures presented here, as well as in the related yGCN5 (ref. 3) and hP/CAF/CoA (ref. 4) structures and in the analogous strands of the functionally divergent GNAT

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