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The H2A-H2B Dimeric Kinetic Intermediate is Stabilized by Widespread Hydrophobic Burial with Few Fully Native Interactions

Paul J. Guyett and Lisa M. Gloss*

School of Molecular Biosciences, Washington State University, Pullman, WA 99164-7520

Abstract

The H2A-H2B histone heterodimer folds via monomeric and dimeric kinetic intermediates. Within ~5 ms, the H2A and H2B polypeptides associate in a nearly diffusion-limited reaction to form a dimeric ensemble, denoted I_2 and I_2^* , the latter being a sub-population characterized by a higher content of non-native structure. The I_2 ensemble folds to the native heterodimer, N_2 , through an observable, first-order kinetic phase. To determine the regions of structure in the I_2 ensemble, 26 Ala mutants of buried hydrophobic residues were characterized, spanning the three helices of the canonical histone folds of H2A and H2B and the H2B C-terminal helix. All but one targeted residue contributed significantly to the stability of I_2 , the transition state and N_2 ; however, only residues in the hydrophobic core of the dimer interface perturbed the I_2^* population.

Destabilization of I_2^* correlated with slower folding rates, implying that non-native structure is not a kinetic trap, but rather accelerates folding. The pattern of Φ values indicated that residues forming intramolecular interactions in the peripheral helices contributed similar stability to I_2 and N_2 , but residues involved in intermolecular interactions in the hydrophobic core are only partially folded in I_2 . These findings suggest a dimerize-then-rearrange model. Residues throughout the histone fold contribute to the stability of I_2 , but after the rapid dimerization reaction, the hydrophobic core of the dimer interface has few fully native interactions. In the transition state leading to N_2 , more native-like interactions are developed and non-native interactions are rearranged.

Keywords

Protein folding; kinetic intermediates; histones; phi-value analysis

The eukaryotic histone heterodimer, H2A-H2B, is an important component of the protein octamer of the nucleosome core particle (NCP), the fundamental repeating unit of DNA packaging in chromatin. The NCP is a dynamic nucleoprotein complex comprised of ~150 bp of DNA wrapped around an octamer of an (H3-H4)₂ tetramer and two H2A-H2B dimers. The NCP is important in the regulation of DNA dependent processes such as transcription, replication and repair.^{1; 2} The structure and stability of the histones are critical in the regulation of these processes that are modulated by post-translational modifications,³

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*Author to whom correspondence should be addressed at School of Molecular Biosciences, Washington State University, Pullman, WA 99164-7520. Phone (509) 335-5859; lmgloss@wsu.edu.

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incorporation of histone variants,⁴ ATP-dependent remodeling complexes,⁵ and interactions with histone chaperones.⁶

The structure of the “histone fold” is conserved across archaea and eukaryotes. The two monomers dimerize in a head-to-tail orientation with extensive inter-molecular interactions along an extended, domain-swapped architecture. The histone fold consists of an ~30 residue central α -helix (α_2) flanked by two β -loops and ~10 residue helices (α_1 and α_3). Eukaryotic histones also have extended N-terminal tails that are sites of post-translational modification important for NCP regulation.³ C-terminal to the canonical histone fold motif, H2B has an ~20 residue α -helix (α_C) which docks on the opposite side of H2A from the major H2A-H2B dimer interface. C-terminal to its histone fold, H2A has a short C-terminal helix followed by an extended tail. Despite a similar core histone fold, H2A and H2B exhibit very little sequence identity.

The H2A-H2B dimer folds through a multi-state kinetic mechanism (Scheme 1), with transient population of monomeric and dimeric intermediate species (2M and I_2 , respectively). Within the ~5 ms dead time of the stopped flow (SF) instrument, the H2A and H2B polypeptides associate to form a dimeric I_2 species, which then folds in an observable, first-order kinetic phase to the native heterodimer, N_2 .⁷ Given this rapid, burst-phase dimerization, with an estimated rate of $\geq 10^8 \text{ M}^{-1}\text{s}^{-1}$, it was not possible to ascertain the degree of folding of the monomers prior to dimerization. However, the individual H2A and H2B monomers in isolation are partially folded at equilibrium and kinetically competent to dimerize to the I_2 species and then further fold to N_2 .^{8;9} Therefore, partially folded monomers, 2M, are included in the kinetic mechanism shown in Scheme 1.

There are three indications of non-native structure (NNS) in the 2M and I_2 species. 1) The m values from equilibrium studies for the isolated, partially folded monomers are greater than expected for simple dissociation of fully folded monomers in the extended conformation present in the native heterodimer.⁸ The monomers appear to be overly collapsed, maximizing burial of hydrophobic surface area by the formation of NNS. 2) Mutation of several surface residues to Ala and Gly also indicated the presence of stabilizing interactions in I_2 that are absent in N_2 .⁹ Specifically, the mutations destabilized I_2 more than N_2 and/or the transition state between I_2 and N_2 , resulting in Φ values > 1 . 3) Below ~0.5 M urea, the folding rate for I_2 to N_2 deviates from a log-linear dependence on the denaturant concentration, indicative of a shift in the transition state or ground state ensembles.⁹ Thus, the I_2 ensemble appears to be heterogeneous, as indicated by I_2^* in Scheme 1, where the I_2^* sub-population is characterized by additional non-native interactions whose rearrangement becomes rate-limiting at low denaturant concentrations. Importantly, the NNS in the I_2 ensemble appears to promote rapid folding. Mutants in this study and a previous study⁹ that destabilize the NNS fold slower than WT. This mutational effect is in contrast to NNS in other model systems, such as Im7¹⁰ and cytochrome c,¹¹ where destabilization of NNS accelerates folding.

Analysis of the SF-CD burst phase signal and the equilibrium and kinetic m values indicate that the I_2 ensemble has ~50% of the α -helical content and ~60% of the buried surface area of the native heterodimer.⁷ The structure of I_2 could be either: 1) partial structure throughout the dimer, or 2) a structured sub-domain that additional structure condenses around in subsequent folding to N_2 . Because H2A-H2B is a heterodimer, it is also uncertain whether the structure of I_2 is pseudo-symmetric. In other words, are the same regions of H2A and H2B folded, suggesting that topology dictates the order of folding, or is the folding landscape influenced by sequence differences such that the two polypeptides contribute differently to the structure and stability of I_2 . To distinguish between these alternatives and further elucidate the contribution of different segments of structure to the folding landscape

of H2A-H2B, hydrophobic residues were mutated to Ala. At least two residues in each helix of the canonical histone fold of H2A and H2B were mutated as well as three residues in α C of H2B. Mutated residues were chosen based on their exclusion from solvent (>75%) in N₂. Comparison of kinetic and equilibrium effects of a mutation report on the contribution of the mutated residue to the stability of I₂ and the subsequent transition state.

Results

WT equilibrium stability and kinetics in GdmCl

Guanidinium chloride (GdmCl) was used as the denaturant in equilibrium and kinetics experiments, rather than urea as in previous studies.^{7; 9} The Ala mutations of this study were quite destabilizing, and thus many mutants did not exhibit a well-defined native baseline in urea-induced equilibrium unfolding experiments. Determination of the native baseline (Y_N in Eq. 6 in Methods) is important for accurate fitting of equilibrium data. The dimers could be stabilized by addition of cosolutes such as TMAO or increasing protein concentration. However, the increased viscosity from molar concentrations of cosolutes complicated stopped-flow (SF) experiments, and the necessary ~10-fold increase in protein concentration was impractical. Instead, the denaturant GdmCl was used because the native baselines were better defined (Fig. 1).

The equilibrium GdmCl-induced unfolding of WT H2A-H2B was monitored by far-UV circular dichroism (CD) and intrinsic tyrosine fluorescence (FL). An equilibrium data set of eight titrations was analyzed, including CD and FL unfolding titrations at 6, 10 and 14 μ M monomer and CD and FL refolding titrations at 6 and 14 μ M monomer. GdmCl, through salt effects, affects H2A-H2B Tyr fluorescence at concentrations below 0.5 M GdmCl (Fig. 1 inset), resulting in a curved native baseline.¹² Hence, FL data below 0.5 M GdmCl were not used in the determination of the native baseline during data fitting. The data set of CD and FL titrations as a function of monomer concentration was globally fit to a dimeric two-state model. The $\Delta G^\circ(\text{H}_2\text{O})$ and the m_{Eq} values are reported in Table 1. These values are ~10% lower than those reported previously from a less extensive data set that did not include FL data.¹²

Relative to urea, the GdmCl-induced unfolding transitions were much steeper, as has been reported for other protein folding systems.¹³ However, there is not a compensatory shift in the midpoint of the transition (*i.e.* a lower C_M value in GdmCl). Therefore, the extrapolated $\Delta G^\circ(\text{H}_2\text{O})$ values are higher in GdmCl (Table 1) than in urea.¹⁴ If this effect simply reflected the ionic nature of GdmCl and the relatively high positive charge of histones, one would predict that the effect could be recapitulated by addition of salts to urea-induced equilibrium unfolding experiments. However, the m values for urea unfolding transitions are independent of ionic strength and the salt employed.¹⁴ There appears to be a difference between the relative ΔG values of the native and unfolded states in urea and GdmCl. Since the native states, in the absence of denaturant, must be the same, this difference apparently arises in the unfolded state, which is presumably more unfolded in the generally more potent denaturant GdmCl.

The SF-CD and SF-FL folding and unfolding kinetics of H2A-H2B were determined as a function of GdmCl to verify that the folding mechanism is independent of denaturant. The following features were the same as reported previously^{7; 9} for kinetic studies performed with urea: 1) The observed folding and unfolding kinetic responses were well-described by a single, first-order exponential at all denaturant concentrations. 2) No burst-phase amplitude was observed in the unfolding reaction. 3) There was a significant burst-phase signal detected in 5 ms dead time of SF-CD folding kinetics, consistent with the formation of the I₂ kinetic intermediate. 4) The rates of folding at various denaturant concentrations were

identical whether refolding was initiated from acid or denaturant unfolded species. 5) At urea and GdmCl concentrations below ~0.5 M, the folding rates exhibited “roll-over” and deviation from a log-linear relationship between the observed rate and the denaturant concentration (Fig. 2A). In summary, Scheme 1 adequately describes the folding mechanism of H2A-H2B in both urea and GdmCl.

The fitted parameters for the GdmCl-dependent kinetic reactions are given in Table 2. The I₂ to N₂ folding rate extrapolated to the absence of denaturant, $k_f(H_2O)$, is slightly faster in GdmCl than urea,⁷ while the extrapolated unfolding rate, $k_u(H_2O)$, is much slower (Fig. 2A). These kinetic differences reflect the higher GdmCl $m^\ddagger$ values (Eq. 8, Methods), as indicated by steeper slopes of the fitted lines in Figure 2A, and the larger free energy associated with the interconversion of I₂ and N₂ in GdmCl. This value, $\Delta G^\circ(H_2O)_{I_2-N_2}$, can be calculated from the extrapolated first-order rate constants:

$$\Delta G^\circ(H_2O)_{I_2-N_2} = -RT \ln \left(\frac{k_u(H_2O)}{k_f(H_2O)} \right) \quad (1)$$

The validity of this approach has been verified by complete analysis of the SF-CD burst-phase and final signals obtained from urea folding/unfolding kinetics.⁷ The urea $\Delta G^\circ(H_2O)_{I_2-N_2}$ value was ~25% of the equilibrium $\Delta G^\circ(H_2O)$ for folding of 2U to N₂.⁷ In GdmCl, the $\Delta G^\circ(H_2O)_{I_2-N_2}$ was 5.2 kcal mol⁻¹, or 35% of the equilibrium $\Delta G^\circ(H_2O)$. Similar to the burst-phase signal observed in urea folding kinetics, ~60% of the ellipticity at 222 nm is formed in 5 ms upon refolding at 0 M to 0.5 M GdmCl. Thus, a comparable fraction of the stabilizing interactions are formed in I₂ in both denaturants.

The greater folding $m^\ddagger$ value in GdmCl produces a more pronounced roll-over than observed in urea (Fig. 2A), prompting a re-examination of the relationship between I₂ and I₂^{*} (Scheme 1). Folding-unfolding double jump experiments showed that I₂ (at 1 M urea)⁷ and I₂^{*} (at 0 M)⁹ can fold directly to N₂, and off-pathway mechanisms have been ruled out (see Supplementary Materials for reference⁹). There are five potential on-pathway mechanisms. If I₂ and I₂^{*} are distinct kinetic species, separated by a significant barrier, there could be: 1) two parallel pathways, 2) a sequential mechanism with I₂ preceding I₂^{*}; 3) a sequential mechanism with I₂^{*} preceding I₂. If I₂ and I₂^{*} are not separated by a distinct barrier, but reflect denaturant-dependent shifts in ensemble populations, then there could be: 4) ground state effects, or 5) transition state effects. To aid in distinguishing between mechanisms, chevron plots of the folding and unfolding rates from semi-global fits of SF-CD and SF-FL data for WT in GdmCl (Fig. 2A) and urea as well as a mutant with an extended roll-over, E64A,⁹ were simulated using the program “Chevron Simulator” (available at www.osmanbilsel.net/software/).

A parallel mechanism is excluded for two reasons: 1) for WT and all mutants characterized to date, only one unfolding phase is observed, not the potential two phases (unfolding via I₂ and I₂^{*}); 2) at low denaturant concentrations, two folding rates differing by at least four-fold should be observed. This latter point is confirmed by simulations that suggest that at low denaturant concentrations, the two folding microscopic rate constants should differ by nearly an order of magnitude. A sequential mechanism with I₂ preceding I₂^{*} was discounted based on: 1) the convergence of the log-linear dependence of the rates for I₂ folding and N₂ unfolding in the transition region (suggesting microscopic reversibility and a common transition state for these two reactions); and 2) the predictions from simulations that the microscopic rate constants should yield two distinguishable folding phases below 0.5 M denaturant.

The chevrons for WT in GdmCl and E64A were recapitulated very well with simulations to a sequential model with I_2^* preceding I_2 , using the observed rates and related m values as follows: k_{OM} , the microscopic rate constant for I_2^* to I_2 ; $k_f(H_2O)$, the microscopic rate constant for I_2 to N_2 ; and $k_u(H_2O)$, the microscopic rate constant for unfolding of N_2 to I_2 . For the WT urea chevron, the required microscopic rate for I_2^* to I_2 was slightly lower than the observed k_{OM} . The only under-determined values in the simulation were the rate and associated m value for unfolding of I_2 to I_2^* . However, there are inconsistencies with this model as well. 1) For WT refolding above ~0.8 M GdmCl, the microscopic rate for I_2^* to I_2 should be at least five-fold faster than for I_2 to N_2 , predicting the detection of a second kinetic folding phase—which was not observed. Even if the signal change between I_2^* to I_2 is minimal, one might expect to observe a lag phase. The caveat is that this prediction of two phases depends on the chosen values for the poorly defined rate and m value for I_2 to I_2^* unfolding; some combinations of these parameters that describe the chevron data reasonably well estimate that I_2^* would be too unstable to be significantly populated at these GdmCl concentrations. 2) For the urea-dependent WT kinetics, the $\Delta G^\circ(H_2O)$ and m value for the burst-phase ensemble have been carefully determined from SF-CD amplitudes, so the $\Delta G^\circ(H_2O)$ and m value expected for conversion of the burst-phase species to N_2 have been determined independently of the observed folding and unfolding rates (see Eq. 1).⁷ Simulations predict values for one or both of these parameters that are at least 2-fold greater than observed. 3) Simulations of the WT GdmCl chevron also over-estimate the m value for a two-step interconversion of I_2^* to N_2 (via I_2). The simulated m value is at least 70% of the equilibrium m value, which is inconsistent with analysis of the SF-CD burst-phase data (data not shown).

In summary, the consistent observation of only one kinetic phase for folding and unfolding for WT and all mutants to date (see below), and the inaccuracy of the $\Delta G(H_2O)$ and m values from simulations of the chevrons argue that there is no kinetic barrier between I_2 and I_2^* . Instead, the two states represent different components of an ensemble population, as concluded previously.⁹ In the previous study, it was unclear whether the ensemble reflected shifting of the ground state populations in a broad well (as shown in Scheme 1) or movement across a rough transition state. The latter scenario is usually accompanied by symmetric roll-over on the folding and unfolding arms of the chevron (for example,¹⁵). A deviation from a log-linear dependence on denaturant concentration of the unfolding rate has not been observed for WT or any mutants in this study or previously.⁹ This suggests that the roll-over at low denaturant results from ground state effects as highlighted in Scheme 1.

Mutant heterodimer stabilities

To determine the contributions of buried hydrophobic side chains to the folding of H2A-H2B, 27 mutations to Ala were analyzed throughout the helices of H2A and H2B. Thirteen of the targeted residues were Leu or Met, so mutations to Ala had little effect on helix propensity. However, Phe, Ile and Val residues were also mutated to Ala, so the effects of side chain truncation may be partially offset by the increased helix propensity of Ala.

The equilibrium stability of each mutant was determined from a data set that included at least four FL and three CD titrations at multiple monomer concentrations between 4 and 20 μ M. As observed for WT H2A-H2B, the CD and FL transitions were coincident, consistent with a two-state unfolding process, and the data set as a function of protein concentration was described well by global fits to a two-state dimeric equilibrium model. The fitted parameters are given in Table 1, and the F_{app} plots of the equilibrium data are shown in Supplemental Figure 1.

The $\Delta\Delta G_{Eq}$ values, the change in the free energy of unfolding caused by the mutation, for the majority of mutants were 1.6 to 4.5 kcal mol⁻¹ (Table 3), corresponding to a

destabilization of ~10 to 30% relative to WT. In general, the most destabilized H2A variants were mutations clustered in the center of H2A- α 2, predominantly Leu residues. The effects of the H2B mutations, which included more Val and Phe residues, did not exhibit any regions with uniformly high sensitivity to Ala substitution. The H2A-L65A mutant was slightly stabilizing; this residue is near the C-terminus of α 2 and is the most solvent accessible residue mutated in this study.

The most destabilizing mutation, H2B-I91A in α 3, was 40% less stable than WT. This residue is at the end of an extended hydrophobic cluster and interacts with several residues of the H2A- α 2 helix. Given the pseudo-symmetry of the histone fold, the structurally equivalent H2A residue is Leu83, and the L83A mutant also exhibited a relatively high $\Delta\Delta G_{\text{Eq}}$, 4.7 kcal mol⁻¹ (data not shown).

The m_{Eq} value in a two-state equilibrium unfolding process is generally proportional to the change in solvent accessible surface area, ΔASA , between the native and unfolded states.¹³ Many mutants exhibited decreased m_{Eq} values; nonetheless, the m_{Eq} values for 25 of the 27 mutants were within 15% of the WT value. The two exceptions were the strongly destabilized H2A-L58A and H2B-I91A mutants, with $\Delta\Delta G_{\text{Eq}}$ values > 5 kcal mol⁻¹. To further assess possible mutational effects on structure, the far-UV CD spectra were examined. At the monomer concentrations used in equilibrium unfolding studies, there is a general trend between decreased mean residue ellipticity (MRE) at 222 nm and decreased stability, presumably because the native state is not fully populated. Under conditions where the native state is fully populated (0.3 to 1 M TMAO), the MRE at 222 nm for the majority of the mutants were within 15% of WT; only H2A-L58A in α 2 exhibited a 30% decrease in MRE. Thus, the mutations alter stability, but there is no evidence for significant alteration of the heterodimer structure.

Mutant unfolding kinetics

The unfolding kinetics of 26 mutants were determined by SF-CD and SF-FL as described for WT. For all mutants, the kinetic responses fit well to a single, first-order exponential with excellent agreement between CD and FL data. Thus, the data were globally fit across at least 6 denaturant concentrations to determine $k_{\text{u}}(\text{H}_2\text{O})$ and $m_{\text{u}}^{\ddagger}$ values (Table 2). As expected for destabilizing mutations, none of the mutants unfold more slowly than WT (Supplemental Fig. 2); however, because of minor effects on $m_{\text{u}}^{\ddagger}$ values, five mutants exhibit $k_{\text{u}}(\text{H}_2\text{O})$ values that are 1.2 to 2.2-fold slower than WT. Fourteen mutants exhibited moderate increases of 1.6 to 7.5-fold in $k_{\text{u}}(\text{H}_2\text{O})$, and four mutants had $k_{\text{u}}(\text{H}_2\text{O})$ values increased by 10 to 50-fold. Nearly 90% of the mutants had $m_{\text{u}}^{\ddagger}$ values that were within 1.3-fold of WT; the remainder were within 1.6-fold of the WT value. The variations in $m_{\text{u}}^{\ddagger}$ values suggest some movement of the unfolding transition state, but these movements are generally minor with Tanford β values (Table 3) from 0.3 to 0.5, compared to 0.3 for WT H2A-H2B.

Mutant folding kinetics

The effect of the 26 mutations on the kinetics of folding from I₂ to N₂ were examined by SF-CD and SF-FL. Folding was initiated from GdmCl-unfolded dimers. As observed for WT H2A-H2B, the kinetic responses were well-described by a single, first-order exponential, and the rates determined from CD and FL methods were in excellent agreement. The SF data were fit globally across at least 5 denaturant concentrations to determine $k_{\text{f}}(\text{H}_2\text{O})$ and $m_{\text{f}}^{\ddagger}$ values (Table 2, Supplemental Fig. 2). The kinetic data for all mutants were consistent with the folding mechanism shown in Scheme 1. In general, the $k_{\text{f}}(\text{H}_2\text{O})$ values were similar to or less than the WT value, as expected for destabilizing mutations. The exceptions were the slightly stabilizing H2A-L65A mutant and H2A-L85A,

which exhibited $k_f(\text{H}_2\text{O})$ values 1.5 and 2.2-fold greater than WT, respectively. These are the only two mutants with $m_f^\ddagger$ values significantly greater than WT. Most other mutants exhibited $m_f^\ddagger$ values $\leq$ WT, with a few mutants exhibiting $m_f^\ddagger$ values that were 2.7-fold less than WT.

To ascertain the mutational effects on the relative population of I_2^* , refolding to 0 M GdmCl was initiated from dimers unfolded in 10 mM HCl. The kinetic response was also determined for refolding to 0.5 M GdmCl. As reported for WT,⁹ the observed rates at 0.5 M were virtually identical to the rates determined for refolding from GdmCl-unfolded dimers (Supplemental Fig. 2). SF-FL and SF-CD kinetic responses were fit semi-globally, and the observed rates, $k_{0\text{ M}}$ are listed in Table 2.

The extent of deviation from log-linearity, or the amount of roll-over, was assessed by the ratio of $k_f(\text{H}_2\text{O})/k_{0\text{ M}}$ (Table 2). Several H2A and H2B mutants have significantly lower ratios than WT, and some mutants exhibit no roll-over. Diminished roll-over suggests that the mutated residue contributed to the stability of I_2^* . The residues whose mutation reduced the ratio of $k_f(\text{H}_2\text{O})/k_{0\text{ M}}$ to <2 are highlighted in Figure 3, six residues in H2A and ten residues in H2B. The residues are not confined to consecutive segments of structure, but the side chains do interact in the native state through an extended hydrophobic network. Residues with WT-like or greater ratios tend to be more peripheral. The mutational results suggest that the non-native structure of I_2^* is stabilized by hydrophobic tertiary and quaternary interactions dispersed throughout the secondary structural elements of H2A and H2B. Based on the mutations made, it appears that H2B contributes more extensively than H2A to the non-native structure in I_2^* . The H2A residues are predominantly in $\alpha 2$, spanning most of the helix. The H2B residues are distributed across $\alpha 1$, $\alpha 3$ and αC , but only the N-terminus of $\alpha 2$. Thus, the residues involved in the non-native structure of I_2^* are not distributed symmetrically across the two polypeptides. It is hypothesized that this non-native structure may be set up in the partially folded monomers.

Calculation of the stability of I_2

In a previous study of WT H2A-H2B, analysis of the SF-CD burst-phase signal was used to determine the $\Delta G^\circ(\text{H}_2\text{O})$ and m values associated with the folding to the I_2 state.⁷ Similar values were obtained from calculations using fitted parameters from equilibrium and folding/unfolding kinetic data:

$$\Delta G^\circ(\text{H}_2\text{O})_{2\text{U}-\text{I}_2} = \Delta G^\circ(\text{H}_2\text{O})_{\text{Eq}} - \Delta G^\circ(\text{H}_2\text{O})_{\text{I}_2-\text{N}_2} \quad (2)$$

where the $\Delta G^\circ(\text{H}_2\text{O})_{\text{I}_2-\text{N}_2}$ was calculated using Eq. 1. The m value associated with folding to I_2 , $m_{2\text{U}-\text{I}_2}$, can also be calculated using equilibrium and kinetic m values:

$$m_{2\text{U}-\text{I}_2} = m_{\text{Eq}} - (m_f^\ddagger - m_u^\ddagger) \quad (3)$$

The $\Delta G^\circ(\text{H}_2\text{O})$ and m values for the folding of 2U to I_2 derived from equilibrium and kinetic parameters, rather than burst-phase analyses, were used because of greater precision and technical ease. Most mutations destabilized I_2 by more than 1 kcal mol⁻¹ (>20%); the major exception is H2A-L65A, which does not destabilize the native dimer.

$\Delta\Delta G$ and Φ values for the Ala mutations

In Figure 4, the mutants' $\Delta\Delta G$ values are compared to illustrate their effects on overall stability, the burst-phase folding of 2U to I_2 and the I_2 to N_2 transition state. In accord with

standard convention, the free energy of the unfolded states of the WT and mutants were normalized to zero. Thus, the $\Delta\Delta G$ values are typically interpreted as removing a favorable interaction that stabilizes the native state, I_2 or the transition state. However, positive $\Delta\Delta G$ values can also arise from mutations introducing an interaction that stabilizes the unfolded state relative to the native state and/or intermediate species. In either scenario, the relative $\Delta\Delta G$ values for different states along a folding pathway describes the contribution of the mutated residue to the stability of that state. $\Delta\Delta G_{I_2}$ values were calculated using k_f ; similar trends were observed if k_{0M} was used. The $\Delta\Delta G_{I_2}$ and $\Delta\Delta G_{\text{unf}}^\ddagger$ values reported in Table 3 were calculated using the equations:

$$\Delta\Delta G_{I_2} = \Delta G^\circ(\text{H}_2\text{O})_{2\text{U}-I_2}^{\text{WT}} - \Delta G^\circ(\text{H}_2\text{O})_{2\text{U}-I_2}^{\text{mutant}} \quad (4a)$$

$$\Delta\Delta G_{\text{unf}}^\ddagger = -RT \ln[(k_u(\text{H}_2\text{O})_{\text{WT}})/(k_u(\text{H}_2\text{O})_{\text{mutant}})] \quad (4b)$$

where $\Delta G^\circ(\text{H}_2\text{O})_{2\text{U}-I_2}$ values were calculated using Eq. 2.

In general, the $\Delta\Delta G$ values are progressive, *i.e.* the effects on I_2 are less than or equal to the effects on the I_2 - N_2 transition state, and the latter are less than or equal to the effects on the equilibrium stability. Phi-value analysis is a method to quantify the relative contribution of a mutated residue to an intermediate species relative to the equilibrium stability: $\Phi_i = \Delta\Delta G_i / \Delta\Delta G_{\text{Eq}}$, where $\Delta\Delta G_i$ is either the effect on I_2 or the subsequent folding transition state. Phivalues typically vary from 0 to 1. A value near 0 indicates residues that are largely unfolded and do not contribute to the stability of the intermediate species; a value approaching 1 indicates residues that contribute as much to the stability of the intermediate as to the stability of N_2 . The Φ_{I_2} H2B mutants are listed in Table 3 and displayed on the three dimensional structure of the heterodimer in Figure 5. The mutants can be divided into two groups: those with moderate Φ_{I_2} values, between 0.4 and 0.7, and those with high Φ_{I_2} values, >0.7 .

Most mutants have Φ_{TS} values ≥ 0.7 (Table 3), indicating the hydrophobic core of H2A-H2B is well formed in the I_2 - N_2 transition state. The exceptions are H2A-L34A and H2B mutants L42A, F67A and I91A, with Φ_{TS} values between 0.5 and 0.65.

Discussion

H2A-H2B is apparently more stable to GdmCl denaturation compared to urea, but the folding mechanisms are the same. The larger $\Delta G^\circ(\text{H}_2\text{O})$ and m_{Eq} values for GdmCl titrations facilitated characterization of the destabilizing Ala mutations. The side chain contribution to stability was determined for I_2 , I_2^* , the TS and N_2 . Of the 26 residues studied, 25 contributed significantly to the stability of I_2 , the TS and N_2 , though only a subset of mutants affected the I_2^* component of the burst-phase ensemble. Furthermore, residues developed progressively more stabilizing interactions. The average of the Φ_{I_2} values was 0.68, while the average Φ_{TS} value was 0.83. This is in contrast to a previous study⁹ where the mutation of several surface residues to Ala destabilized I_2 more than the TS and N_2 .

None of the 25 destabilizing Ala mutations in this study exhibited anomalously high Φ_{I_2} values, including those residues that were adjacent in sequence to polar residues implicated in NNS.⁹ The analysis of Φ values can indicate the extent to which a residue contributes to the stability of I_2 or the TS. However, it is not possible to assess if the stabilizing

interactions formed by that residue are the same as those found in the native state, particularly if the Φ value is between 0 and 1. Thus, for this set of mutants, it is not possible to state whether the hydrophobic residues contributing to I_2 structure are making native or non-native contacts, only whether these residues are contributing less to the stability of I_2 than N_2 . Similarly, there is the caveat that it can not be determined if the contribution of the residue to the stability of I_2 results from intra- or inter-molecular interactions, only whether the residue forms predominantly intra- or inter-molecular interactions in the native state.

Structural features of I_2

Of the 25 destabilizing mutations, 16 exhibited moderate Φ_{I_2} values, between 0.4 and 0.7, while only nine exhibited values approaching 1, indicative of fully native-like interactions (Figure 5). Seven of these nine mutations were Val-to-Ala substitutions, accounting for all but two of the mutated Val residues. Interpretation of the high Φ values for Val mutations may be complicated because of the contrasting effects of destabilization by decreasing hydrophobic side chain volume and stabilization by increasing helix propensity, which is further complicated by the different impact of these two effects on the stability of I_2 and N_2 . A comparison of Ala and Gly mutations indicated that increased helix propensity typically had a greater impact on equilibrium stability than the stability of I_2 .⁹ In contrast, it is possible that non-native burial of hydrophobic surface area may stabilize I_2 , particularly for those residues that are partially solvent accessible in the native state. Given this caveat regarding the enrichment of Val residues in mutations with high Φ_{I_2} values, the tertiary and quaternary interactions of the residues reveal some common characteristics.

Residues that affect I_2^* (Figure 3) and residues with high Φ_{I_2} values (Figure 5) are mutually exclusive, except for H2B-Val108, which has a modest effect on I_2^* . Residues with high Φ_{I_2} values are on the periphery of the heterodimer, and do not contribute significantly to the extended hydrophobic core dominated by residues either from or interacting with the long α_2 helices. Only four of the nine high Φ_{I_2} value residues (H2A: Val30, Val49, Val87; and H2B-Val95) are completely buried in the native state, and none make extensive inter-monomer interactions.

In three-dimensional space, most residues with high Φ_{I_2} values can be divided into two clusters (Fig. 5). The first cluster contains Leu85 and Val87 on the H2A α_3 helix. Previous studies indicated that near-by residues—Asn89, the C-cap residue of α_3 , and Glu91 of the short α_C —are folded in I_2 and contribute to non-native structure.⁹ The second cluster contains four H2B residues and two H2A residues. H2B Val95 (α_3) and Val108 (α_C) make tertiary contacts, and are proximal to Val115 (α_C) and Leu77 on the C-terminal portion of H2B α_2 that forms predominantly intramolecular contacts with α_3 . The H2A residues, Val30 and Val49, are on segments of α_1 and α_2 , respectively, that make intramolecular interactions, and Val49 is proximal to H2B-Val115.

The partially solvent accessible H2B-Val45, on the C-terminus of α_1 , does not appear to contribute to either cluster. The mutation of a nearby surface residue, H2B-K43A, exhibits a Φ_{I_2} value of 1.3, and appears to stabilize I_2 by non-native interactions.⁹

The clustering of residues with high Φ_{I_2} values, in conjunction with a previous mutational study,⁹ suggest a structural model for I_2 . The helical segments that are stabilized to a greater extent by intramolecular interactions in N_2 contribute native-like stability to I_2 . However, the structure is not a contiguous sub-domain, as observed for many intermediates; rather the regions with high Φ_{I_2} values form a bi-partite structure. These helical regions are peripheral to the dimerization interface and hydrophobic core. These include, at the bottom of Figure 5: 1) the interface between H2A- α_1 and the N-terminus of α_2 ; and 2) the H2B interfaces between the C-terminus of α_2 , α_3 , and α_C ; and at the top of Figure 5: 3) the H2A- α_3 and

short α C helices; and 4) H2B- α 1. In contrast to the partially surface-exposed residues in H2B- α 1 and α 3, buried residues in these helices have moderate Φ_{I_2} values (Table 3). This implies that in the I_2 ensemble, the helices may be folded and partially docked, but buried hydrophobic residues are not packed for optimal stability. This model is reminiscent of a molten globule in hierarchical folding, with secondary structure formation and burial of hydrophobic surface area, but an absence of specific, native-like tertiary/quaternary interactions. The partial Φ_{I_2} values for the residues that affect the population of the I_2^* species supports this model.

Interactions that contribute to I_2^*

Central buried residues with moderate Φ_{I_2} values contribute significantly to the stability of I_2^* (Figure 3). Removal of the side chain by mutation to Ala reduces the population of the I_2^* non-native structure sufficiently to eliminate the characteristic roll-over at low denaturant concentration. The network of interactions includes α 2 residues and extends to residues in peripheral helices that make significant intermolecular interactions with α 2 in the native state (Figure 3). The centrality of these residues, their moderate Φ_{I_2} values and their intermolecular interactions in N_2 suggest that these residues form non-specific, non-native interactions during the nearly diffusion limited association of monomers to maximize burial of hydrophobic surface. The low $m^\ddagger$ value observed in the roll-over at low denaturant suggests that the non-native structure of I_2^* is resolved by an isomerization-like rearrangement to I_2 ,⁹ a dimerize-then-rearrange model. Three of the four residues with moderate Φ_{TS} values, H2A-L34A and H2B-L42A and -I91A, are important for I_2^* stability. It is hypothesized that the NNS of the I_2 ensemble may initially form in the overly collapsed monomers, and thus mutations that destabilize I_2^* may also destabilize the isolated monomers. Studies are in progress to address this hypothesis.

There is a significant correlation between the destabilization of I_2^* (loss of rollover) and decreased $k_{fold}(H_2O)$ values for this set of Ala residues as well as previously described mutations of polar surface residues.⁹ This observation strongly suggests that the non-native structure that must be rearranged from I_2^* to I_2 is not a kinetic trap, but contributes to faster folding.

Implications for rapid, efficient dimerization folding reactions

The dimerization of H2A and H2B to form the I_2 ensemble approaches the rate of diffusion. The current mutational study is one of few that examine the contributions of intra- and inter-molecular interactions to the folding of dimeric proteins, particularly with a nearly-diffusion limited association.

Monomeric proteins that fold through a two-state mechanism often develop kernel(s) of interactions that nucleate the further condensation of structure, *i.e.* the nucleation-condensation mechanism,^{16; 17; 18} for example CI2,^{19; 20} CspB,²¹ and the SH3 domains.^{22; 23; 24} Many monomeric folding intermediates are also characterized by sub-domains of native-like structure with other regions being largely or partially unstructured, for example apomyoglobin,²⁵ ribonuclease H,^{26; 27} and the α -subunit of Trp synthase.²⁸ The H2A-H2B I_2 kinetic intermediate is in contrast to these examples; many residues, spread throughout the structure, contribute partially to the stability of the intermediate. The residues that contribute similarly to the stability of I_2 ensemble and N_2 do not represent an autonomous sub-domain, and the central hydrophobic core is only partially structured and appears to be the hub of non-native structure in the I_2^* sub-population.

Most mutational studies of dimeric proteins have targeted residues in the dimeric interface to engineer lower order oligomers (for example, dimers to monomers^{29; 30} or tetramers to

dimers^{31; 32}) or residues that are implicated in disease states.^{33; 34; 35; 36} It is perhaps most instructive to compare the folding mechanisms of intertwined or domain-swapped dimers. The dimeric P22 Arc repressor, with 53 residues per monomer, has a dimerization interface that exchanges two β -strands, and folds in a two-state manner with an association rate of $\sim 10^7 \text{ M}^{-1}\text{s}^{-1}$.³⁷ Mutational analyses indicated that the dimerization transition state was globally collapsed, but most residues do not form the tight, complementary packing interactions of the native state.³⁸ This is similar to the H2A-H2B I_2 ensemble with a partially formed hydrophobic dimer interface, with more fully native contacts developing in the rate-limiting, first-order conversion to N_2 . It is tempting to speculate that the dimerize-then-rearrange mechanism of H2A-H2B facilitates the near-diffusion limited association. Interestingly, other similarly sized (~ 100 to 130 residues), intertwined, obligatorily domain-swapped DNA-binding dimers, the *E. coli* Factor for Inversion Stimulation³⁹ and Trp repressor,^{40; 41} also exhibit nearly diffusion-limited association reactions that populate an I_2 folding intermediate.

In conclusion, it appears that intramolecular interactions in the peripheral regions contribute native-like stability to I_2 , while the intermolecular interactions remain more fluid until after dimerization. The structure in I_2 is dispersed throughout the dimer, and is not localized to a specific sub-domain. While there is some pseudo-symmetry in the regions of H2A and H2B with high Φ_{I_2} values, the two monomers do not contribute symmetrically to the NNS of I_2^* . These findings suggest that topology dictates the accumulation of native-like interactions in I_2 , but that NNS reflects the differences in sequence and structure⁸ between the H2A and H2B monomers. Further mutational studies on the isolated monomers will elucidate the contribution of monomer folding to the NNS of the I_2 ensemble.

Materials and Methods

Materials

Ultra pure GdmCl was purchased from MP Biomedicals, LLC (Solon, OH). All other buffer components were of molecular biology or reagent grade from J.T. Baker (Phillipsburg, NJ). H2A and H2B mutants were generated from plasmids containing the WT H2A and H2B *Xenopus laevis* genes that have been described elsewhere⁴² using the QuikChange II Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). The presence of the desired substitution and the absence of other mutations were verified by sequencing of the entire gene. Recombinant protein overexpression, purification from inclusion bodies and dimer reconstitution for the mutants were performed as described previously for WT H2A-H2B.¹⁴

Methods

Collection of equilibrium and kinetics data—Buffer conditions were 0.2 M KCl , 20 mM KPi , $\text{pH } 7.2$, $0.1 \text{ mM K}_2\text{EDTA}$, with varying concentrations of GdmCl. Instrumentation, data collection and analysis procedures for equilibrium and kinetics experiments were similar to previous studies.^{7; 9} Equilibrium CD and FL titrations were automated with Hamilton Model 500 titrators. At each GdmCl concentration, samples were incubated for 3 min to allow equilibration. Equilibrium FL unfolding titrations were carried out on an AVIV ATF-105/305 differential/ratio spectrofluorometer monitoring Tyr FL at 308 nm (2 nm bandwidth) with excitation at 280 nm (1 nm bandwidth). Equilibrium CD, SF-CD and SF-FL experiments were performed on an AVIV 202SF spectrophotometer. CD ellipticity was monitored at 222 nm (1 nm bandwidth). Tyr SF-FL was monitored with excitation at 280 nm , and FL emission was measured at 90° to the incident beam using a 295 nm cut-off filter.

SF kinetics experiments were conducted at 10 μ M monomer. The SF flow rates were 2 ml/s, resulting in a mixing dead time of ~5 ms. To improve the signal to noise of SF-CD and SF-FL kinetic responses, 27 and 20 transients were averaged, respectively. At each denaturant concentration, a minimum of two SF-FL and one SF-CD kinetic responses were collected. To measure folding rates at 0 M GdmCl, SF-CD and SF-FL experiments were performed by six-fold dilution of dimer unfolded in 10 mM HCl into folding buffer. As a control, folding from 10 mM HCl or GdmCl unfolded protein were compared at 0.5 M GdmCl.

Data Analysis—Equilibrium CD and FL titrations were globally fit across multiple monomer concentrations to a dimeric two-state model using the data fitting software, Savuka 6.2.32 (software available at www.osmanbilsel.net).⁴³ The standard state free energy of unfolding in the absence of GdmCl, $\Delta G^\circ(\text{H}_2\text{O})$, was determined by linear extrapolation:

$$\Delta G^\circ = \Delta G^\circ(\text{H}_2\text{O}) - m[\text{GdmCl}] \quad (5)$$

where ΔG° is the free energy of unfolding at each GdmCl concentration and m reflects the sensitivity of the transition to GdmCl.⁴⁴ The apparent fraction of unfolded monomers, F_{app} , is related to K_{Eq} , the equilibrium constant, by the equation:

$$F_{app} = \frac{\sqrt{K_{Eq}^2 + 8K_{Eq}P_T} - K_{Eq}}{4P_T} = \frac{Y_i - Y_N}{Y_U - Y_N} \quad (6)$$

where P_T is the total monomer concentration, Y_N and Y_U are the GdmCl dependence of the spectral properties of the folded and unfolded protein, respectively, and Y_i is the signal at each GdmCl concentration.

WT and mutant folding and unfolding individual kinetic traces were fit locally to a single, first-order exponential function:

$$Y(t) = Y_\infty + \Delta Y \times \exp(-k_{obs}t) \quad (7)$$

where Y_∞ is the final equilibrium signal of the reaction, ΔY is the change in signal associated with the kinetic phase, and k_{obs} is the observed rate. The rates determined by both SF-CD and SF-FL were in excellent agreement, thus the data were semi-globally fit across all SF-CD and SF-FL kinetic traces at each GdmCl concentration. To determine the folding and unfolding rate constant in the absence of denaturant, $k(\text{H}_2\text{O})$, the SF-CD and SF-FL kinetics data were globally fit across all GdmCl concentrations using the equation:

$$k_i = k(\text{H}_2\text{O}) \times \exp\left(\frac{-m^\ddagger[\text{GdmCl}]}{RT}\right) \quad (8)$$

where k_i is the local rate of each trace and $m^\ddagger$ is the dependence of rate on the concentration of GdmCl.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

$\alpha 1, \alpha 2, \alpha 3$	the first, second and third helices, respectively, in the canonical histone fold
αC	the C-terminal helix outside the canonical histone fold
ΔASA	the change in accessible surface area
CD	circular dichroism
C_M	concentration of denaturant at the midpoint of the unfolding transition
$\Delta G^\circ(H_2O)$	the free energy of unfolding in the absence of denaturant
FL	fluorescence
GdmCl	guanidinium chloride
I_2	H2A-H2B dimeric kinetic folding intermediate
I_2^*	H2A-H2B dimeric kinetic folding intermediate ensemble species observed by roll-over in chevron at <0.5 M denaturant
$k_{0\ M}$	the observed rate of folding in 0 M denaturant
$k_f(H_2O)$	extrapolated folding rate in the absence of denaturant
$k_u(H_2O)$	extrapolated unfolding rate in the absence of denaturant
m_{Eq} value	parameter describing the sensitivity of the unfolding transition to the [denaturant]
$m^\ddagger$ value	parameter describing the dependence of folding or unfolding rate on [denaturant]
MRE	mean residue ellipticity
N_2	native H2A-H2B dimer
NCP	nucleosome core particle
NNS	non-native structure
SF	stopped-flow
TS	transition state between I_2 and N_2
WT	wild-type H2A-H2B

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Figure 1.

Comparison of urea and GdmCl induced equilibrium unfolding transitions. WT H2A-H2B (black) and H2A-L51A mutant heterodimer (grey) titrations in urea (circles) or GdmCl (squares). Solid lines represent global fits to a two-state dimeric unfolding model with native baselines shown as dashed lines. Inset: Representative fluorescence titration illustrating curvature of the native baseline below 0.5 M GdmCl. Conditions were 25 °C, 0.2 M KCl, 20 mM KPi (pH 7.2), 0.1 mM K₂EDTA.

Figure 2.

Denaturant dependence of the folding and unfolding rates. Folding, circles; unfolding, squares. Data points represent semi-global fits of multiple SF-CD and SF-FL kinetic traces at a given GdmCl concentration. Lines represent the global fits to Eq. 8 for kinetic data at denaturant concentrations that exhibit a log-linear dependence (*i.e.* excluding the roll-over regions). (a) Comparison of WT H2A-H2B kinetics in GdmCl (black) compared to kinetics in urea (dashed gray lines).^{7,9} (b) Representative mutants H2A-L85A (red) and H2B-M59A (blue) kinetics. WT kinetic data are shown as grey dashed lines. Conditions were 10 μ M monomer, 25 °C, 0.2 M KCl, 20 mM KPi (pH 7.2), 0.1 mM K₂EDTA.

Figure 3.

Ribbon diagram of the H2A-H2B dimer highlighting the effects of Ala mutations on the ratio of $k_f(\text{H}_2\text{O})/k_{0M}$. The H2A and H2B monomers are shown in pale red and blue, respectively. The Ca atoms of residues whose mutation to Ala result in WT-like or greater ratios of $k_f(\text{H}_2\text{O})/k_{0M}$ are shown as magenta spheres. The side chains of residues whose mutation to Ala result in 2-fold or greater reduction in the $k_f(\text{H}_2\text{O})/k_{0M}$ ratio are shown in red and blue for H2A and H2B, respectively. The figure was generated with PyMol (Delano Scientific, LLC, San Carlos, CA) using the coordinates of H2A-H2B in the NCP X-ray crystal structure (1AOI.pdb).⁴⁵

Figure 4. Comparison of the $\Delta\Delta G$ values for the H2A and H2B mutants. $\Delta\Delta G_{I2}$, white bars; $\Delta\Delta G_{TS}$, grey bars ($\Delta\Delta G_{TS} = \Delta\Delta G_{Eq} - \Delta\Delta G_{unf}^\ddagger$); $\Delta\Delta G_{Eq}$, black bars. The error bars depict the propagated error from the fitted data. (a) H2A $\Delta\Delta G$ values. (b) H2B $\Delta\Delta G$ values.

Figure 5.

Structure of the H2A-H2B dimer displaying the Φ_{12} values. The backbone of the H2A and H2B monomers are shown in pale red and blue, respectively. The poorly structured N- and C-terminal tails have been truncated for clarity. The C α atoms of mutated residues are shown as spheres. The H2A and H2B residues with Φ_{12} values ≥ 0.75 are highlighted in red and blue, respectively. The H2A and H2B residues with Φ_{12} values between 0.4 and 0.7 are shown in magenta and cyan, respectively. The figure was generated as described in the legend of Figure 3.

Scheme 1.
Working mechanism for the kinetic folding of H2A-H2B.

Table 1

The $\Delta G^\circ(\text{H}_2\text{O})$ and m values for equilibrium unfolding and the dimeric kinetic intermediate.

	$\Delta G^\circ(\text{H}_2\text{O})_{\text{Eq}}$ (kcal mol ⁻¹)	$\Delta G^\circ(\text{H}_2\text{O})_{2\text{U}-12}$ (kcal mol ⁻¹) ^a	m_{Eq} (kcal mol ⁻¹ M ⁻¹)	$m_{2\text{U}-12}$ (kcal mol ⁻¹ M ⁻¹)
WT	15.0	9.8	5.2	1.9
<i>H2A α1</i>				
V30A	13.4	8.6	5.5	1.9
L34A	10.5	8.0	4.6	2.3
<i>H2A α2</i>				
V49A	13.2	8.4	4.9	1.6
L51A	10.6	7.1	4.9	1.1
L55A	10.2	6.7	4.4	1.1
L58A	9.7	7.1	3.7	0.7
I62A	10.5	8.0	4.9	2.2
L63A	10.8	7.5	5.0	1.8
L65A	15.3	9.7	5.6	1.7
<i>H2A α3</i>				
L85A	13.3	7.6	4.5	0.6
V87A	12.1	7.7	4.7	1.5
<i>H2B α1</i>				
V38A	11.9	8.1	4.9	1.5
V41A	11.8	7.8	4.5	1.2
L42A	10.8	7.8	4.5	1.5
V45A	12.5	7.6	4.9	1.1
<i>H2B α2</i>				
M56A	12.6	8.2	5.0	1.4
M59A	11.5	8.1	5.4	2.1
F62A	11.2	7.9	4.7	1.6
F67A	13.4	9.2	4.8	1.3
L77A	13.4	8.0	5.1	1.2
<i>H2B α3</i>				
I91A	8.8	6.8	3.4	0.9
V95A	12.4	7.7	5.0	1.0
L99A	11.1	8.0	4.8	1.7
<i>H2B αC</i>				
L103A	10.5	6.7	4.5	1.2
V108A	13.2	8.2	4.8	1.4
V115A	12.5	7.3	4.9	1.0

Conditions: 25 °C, 0.2 M KCl, 20 mM KPi, pH 7.2, 0.1 mM K₂EDTA. Errors at one standard deviation were calculated for each fitted parameter, but are not listed for brevity. The $\Delta G^\circ(\text{H}_2\text{O})_{\text{Eq}}$ errors were less than 5% with an average of 2%; m_{Eq} value errors were less than 10% with an average of 4%.

^a $\Delta G^\circ(\text{H}_2\text{O})_{2\text{U}-\text{I}2}$ was calculated using Eq. 2.

Table 2
Folding and unfolding kinetic parameters of WT and mutant H2A-H2B in GdmCl.

	$k_f(\text{H}_2\text{O})$ (s^{-1})	k_{0M}^a (s^{-1})	$k_f(\text{H}_2\text{O})/$ k_{0M}^b	$k_u(\text{H}_2\text{O})$ $\times 10^{-3}$ (s^{-1})	$m^{\ddagger}_f$ (kcal mol $^{-1}$ M $^{-1}$)	$m^{\ddagger}_u$ (kcal mol $^{-1}$ M $^{-1}$)
WT	12.4	3.2	3.5	2.0	2.0	-1.4
<i>H2A $\alpha 1$</i>						
V30A	16.1	2.8	5.8	4.9	2.0	-1.7
L34A	5.0	2.8	1.8	70.	1.0	-1.4
<i>H2A $\alpha 2$</i>						
V49A	12.0	3.9	3.1	3.6	1.7	-1.6
L51A	1.75	1.0	1.8	4.6	1.6	-2.1
L55A	0.92	1.0	0.9	2.5	1.1	-2.3
L58A	0.44	0.5	0.9	5.8	1.3	-1.7
I62A	1.51	1.3	1.1	23	1.2	-1.5
L63A	3.31	2.9	1.1	12	1.3	-1.9
L65A	18.0	2.0	8.8	1.3	2.4	-1.5
<i>H2A $\alpha 3$</i>						
L85A	27.2	2.0	13.7	1.7	2.6	-1.4
V87A	5.77	2.5	2.3	3.2	1.6	-1.6
<i>H2B $\alpha 1$</i>						
V38A	4.4	2.3	1.9	7.6	1.6	-1.8
V41A	7.2	3.8	1.9	8.8	1.7	-1.6
L42A	4.3	4.0	1.1	28	1.5	-1.5
V45A	13.2	3.5	3.8	3.4	2.0	-1.8
<i>H2B $\alpha 2$</i>						
M56A	8.9	2.1	4.2	5.2	2.2	-1.4
M59A	3.57	4.1	0.9	12.	1.6	-1.7
F62A	2.1	1.1	1.9	8.0	2.1	-1.0
F67A	6.3	1.3	4.7	5.1	2.3	-1.2
L77A	7.9	1.5	5.2	0.87	2.2	-1.8
<i>H2B $\alpha 3$</i>						

	$k_f(\text{H}_2\text{O})$ (s^{-1})	$k_0 \text{ M}^a$ (s^{-1})	$k_f(\text{H}_2\text{O})/$ $k_0 \text{ M}^b$	$k_u(\text{H}_2\text{O})$ $\times 10^{-3}$ (s^{-1})	$m^\ddagger_f$ ($\text{kcal mol}^{-1} \text{ M}^{-1}$)	$m^\ddagger_u$ ($\text{kcal mol}^{-1} \text{ M}^{-1}$)
I91A	3.1	3.3	0.9	105	0.7	-1.8
V95A	5.0	3.5	1.4	1.7	2.1	-1.9
L99A	2.78	3.0	0.9	15	1.5	-1.6
<i>H2B αC</i>						
L103A	3.7	3.0	1.2	5.9	1.1	-2.2
V108A	9.2	4.7	1.9	1.9	2.1	-1.4
V115A	8.2	2.7	3.0	1.2	2.1	-1.8

Conditions: 10 μM monomer, 25 $^\circ\text{C}$, 0.2 M KCl, 20 mM KPi, pH 7.2, 0.1 mM K₂EDTA. Errors at one standard deviation were calculated for each fitted parameter, but are not listed for brevity. All $k(\text{H}_2\text{O})$ errors were less than 16%, with an average of 3%. The $m^\ddagger$ value errors were less than 9%, with an average of 2%.

^a k_0 M values are the rates observed at 0 M GdmCl for folding of dimers unfolded in 10 mM HCl, as described in Materials and Methods.

^b $k_f(\text{H}_2\text{O})/k_0 \text{ M}$ is the extent k_0 M deviates from the extrapolated value, $k_f(\text{H}_2\text{O})$, at 0 M GdmCl. A value of 1 indicates no roll-over was observed.

Table 3

Effect of the mutations on equilibrium and kinetic parameters.

	$\Delta\Delta G_{\text{Eq}}^a$	$\Delta\Delta G_{\text{unf}}^+$	$\Delta\Delta G_{12}$	Φ_{12}^b	Φ_{TS}^c	βd
<i>H2A $\alpha 1$</i>						
V30A	1.6	0.5	1.2	0.77	0.67	0.3
L34A	4.5	2.1	1.9	0.41	0.53	0.3
<i>H2A $\alpha 2$</i>						
V49A	1.8	0.4	1.4	0.80	0.81	0.3
L51A	4.4	0.5	2.8	0.62	0.89	0.4
L55A	4.8	0.1	3.1	0.65	0.97	0.5
L58A	5.3	0.6	2.7	0.51	0.88	0.5
I62A	4.5	1.5	1.8	0.40	0.68	0.3
L63A	4.2	1.1	2.4	0.56	0.75	0.4
L65A	-0.3	-0.3	0.2	$_{-e}$	$_{-e}$	0.3
<i>H2A $\alpha 3$</i>						
L85A	1.7	-0.1	2.3	1.33	1.06	0.3
V87A	2.9	0.3	2.2	0.75	0.90	0.3
<i>H2B $\alpha 1$</i>						
V38A	3.1	0.8	1.7	0.55	0.75	0.4
V41A	3.2	0.9	2.0	0.63	0.73	0.4
L42A	4.2	1.6	2.0	0.48	0.63	0.3
V45A	2.5	0.3	2.2	0.89	0.87	0.4
<i>H2B $\alpha 2$</i>						
M56A	2.4	0.6	1.6	0.68	0.76	0.3
M59A	3.5	1.1	1.7	0.49	0.70	0.3
F62A	3.8	0.8	1.9	0.51	0.78	0.2
F67A	1.6	0.6	0.6	0.40	0.65	0.3
L77A	1.6	-0.5	1.8	1.14	1.31	0.3
<i>H2B $\alpha 3$</i>						
I91A	6.2	2.4	3.0	0.49	0.62	0.5
V95A	2.6	-0.1	2.2	0.83	1.04	0.4

	$\Delta\Delta G_{\text{Eq}}^a$	$\Delta\Delta G_{\text{unf}}^\ddagger$	$\Delta\Delta G_{\text{I2}}$	Φ_{I2}^b	Φ_{TS}^c	β^d
L99A	3.9	1.2	1.8	0.47	0.69	0.3
<i>H2B aC</i>						
L103A	4.5	0.6	3.1	0.70	0.86	0.5
V108A	1.8	0.0	1.7	0.92	1.02	0.3
V115A	2.5	-0.3	2.6	1.02	1.12	0.4

^a $\Delta\Delta G_{\text{Eq}} = \Delta G^\circ(\text{H}_2\text{O})_{\text{WT}} - \Delta G^\circ(\text{H}_2\text{O})_{\text{mutant}}$; a positive value indicates the mutant is less stable than WT.

^b $\Phi_{\text{I2}} = \Delta\Delta G_{\text{I2}} / \Delta\Delta G_{\text{Eq}}$.

^c $\Phi_{\text{TS}} = 1 - (\Delta\Delta G^\ddagger / \Delta\Delta G_{\text{Eq}})$.

^d The Tanford β value, $\beta = m^\ddagger_{\text{u}}/m_{\text{Eq}}$, describes the position of the unfolding TS relative to N2.

^e Φ values were not calculated for H2A-L65A because of its low $\Delta\Delta G_{\text{Eq}}$ value.