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Energetically Unfavorable Amide Conformations for N6-Acetyllysine Side Chains in Refined Protein Structures

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Abstract

The reversible acetylation of lysine to form N6-acetyllysine in the regulation of protein function is a hallmark of epigenetics. Acetylation of the positively charged amino group of the lysine side chain generates a neutral *N*-alkylacetamide moiety that serves as a molecular "switch" for the modulation of protein function and protein-protein interactions. We now report the analysis of 381 N6-acetyllysine side chain amide conformations as found in 79 protein crystal structures and 11 protein NMR structures deposited in the Protein Data Bank (PDB) of the Research Collaboratory for Structural Bioinformatics. We find that only 74.3% of N6-acetyllysine residues in protein crystal structures and 46.5% in protein NMR structures contain amide groups with energetically preferred *trans* or generously *trans* conformations. Surprisingly, 17.6% of N6-acetyllysine residues in protein crystal structures and 5.3% in protein NMR structures contain amide groups with energetically unfavorable *cis* or generously *cis* conformations. Even more surprisingly, 8.1% of N6-acetyllysine residues in protein crystal structures and 48.2% in NMR structures contain amide groups with energetically prohibitive twisted conformations that approach the transition state structure for *cis-trans* isomerization. In contrast, 109 unique *N*-alkylacetamide groups contained in 84 highly-accurate small molecule crystal structures retrieved from the Cambridge Structural Database exclusively adopt energetically preferred *trans* conformations. Therefore, we conclude that *cis* and twisted N6-acetyllysine amides in protein structures deposited in the PDB are erroneously modeled due to their energetically unfavorable or prohibitive conformations.

Keywords

configurational isomer; conformational isomer; crystal structure; NMR structure

INTRODUCTION

Epigenetic regulation of eukaryotic gene expression is achieved in large part by covalent modification of histone proteins by acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, and deimination.[1–5] Unique patterns and combinations of chemical modification comprise a "code" for altering the primary structure of histone proteins and consequently modulating their biological functions. Of these chemical modifications, acetylation is particularly intriguing from both chemical and biological perspectives. The discovery of histone acetylation nearly 40 years ago[6] was the

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first step toward understanding how the reversible acetylation of histone proteins and non-histone proteins can influence the etiology of human disease.[7–10]

Protein acetylation targets the N6-amino group of the lysine side chain to form N6-acetyllysine in a reaction catalyzed by a histone acetyl transferase (also known as a lysine acetyl transferase); to reverse this covalent modification, N6-acetyllysine is hydrolyzed in a reaction catalyzed by a histone deacetylase (also known as a lysine deacetylase).[4] The molecular recognition of N6-acetyllysine in regulatory protein complexes is achieved by a ~110-residue module known as a bromodomain, the first structure of which revealed a novel left-handed four-helix bundle with a hydrophobic pocket that accommodates N6-acetyllysine.[11] Bromodomain-containing proteins function in transcriptional complexes and epigenetic memory, and some serve as targets for drug development.[12–14] An accurate understanding of these biological functions requires that N6-acetyllysine is correctly modeled in experimentally-determined protein structures.

The side chain of N6-acetyllysine contains an amide group that is chemically similar to the amide group of a peptide bond. Due to the partial double bond character of the C-N bond in a sterically unrestrained, planar amide bond,[15,16] two distinct configurational isomers are described as *trans* or *cis* (Figure 1). These configurational isomers are hereafter referred to as conformational isomers; however, they do not freely interconvert, since the activation barrier for isomerization is 20 kcal/mol.[17] Furthermore, the *trans* conformation is more stable than the *cis* conformation and exclusively predominates in simple amides, e.g., as observed in the first crystal structures of *N*-alkylamide peptides such as β-glycylglycine[18] and simple *N*-alkylamides such as *N*-acetylglycine[19] or *N*-methylacetamide.[20] For *N*-methylacetamide, NMR measurements indicate that the *trans* amide linkage is 2.4–2.5 kcal/mol more stable than the *cis* amide linkage,[17,21] consistent with the value of 2.6 kcal/mol determined by *ab initio* molecular orbital calculations.[22] This energy difference suggests that the occurrence of *trans* and *cis* isomers should be 98.8% and 1.2%, respectively (based on the thermodynamic relationship $\Delta G = -RT lnK_{eq}$). This energy difference is even more pronounced in the context of an actual protein backbone, with the *trans* peptide linkage being approximately 5 kcal/mol more stable than the *cis* peptide linkage,[23] thereby accounting for the occurrence of 99.97% *trans* and 0.03% *cis* non-proline peptide linkages in refined protein structures.[24,25]

The overwhelming energetic preference for the planar *trans* conformation in peptide linkages and *N*-alkylacetamide linkages leads to the expectation that the *trans* conformation must similarly dominate for the *N*-alkylacetamide side chain of N6-acetyllysine. In other words, using the traditional torsion angle nomenclature for amino acid side chains, we expect to find $\chi_6 = 180°$ for ~99% of the population based on the *trans* isomer being 2.6 kcal/mol more stable than the *cis* isomer (Figure 1). However, nonplanar distortions of *N*-alkylacetamide torsion angles in small molecules are occasionally observed, particularly as they result from steric effects;[26–28] recent studies of protein structures refined at ultrahigh resolution also show that peptide bond torsion angle distortions sometimes exceed 20°.[29] Accordingly, in view of the fact that slight amide distortions are feasible, we suggest the following classification of N6-acetyllysine χ_6 amide bond conformations: *cis*, $0° \le |\chi_6| \le 15°$; generously *cis*, $15° < |\chi_6| \le 30°$; twisted$_{cis}$, $30° < |\chi_6| < 45°$; severely twisted, $45° \le |\chi_6| \le 135°$; twisted*trans*, $135° < |\chi_6| < 150°$; generously *trans*, $150° \le |\chi_6| < 165°$; *trans*, $165° \le |\chi_6| \le 180°$. In the current structural bioinformatics study, these criteria are used to analyze and classify the amide conformations of N6-acetyllysine residues (three-letter abbreviation = ALY) contained in the Protein Data Bank (PDB) of the Research Collaboratory for Structural Bioinformatics (www.rcsb.org). We have additionally used these criteria to analyze and classify *N*-alkylacetamide conformations in highly accurate small molecule crystal structures deposited in the Cambridge Structural Database (CSD).[30]

MATERIALS AND METHODS

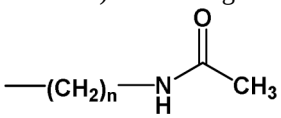
The CSD (version 5.33, November 2011, plus one distributed update) was installed on a Lenovo Y510 personal computer. All searches were performed with the program *ConQuest* (version 1.14)[31] and data analysis was performed with the program *Mercury CSD 3.0*.[31–33] Searches for structures containing *N*-alkylacetamide linkages of the general form $-(CH_2)_n$-$NHC(O)CH_3$ (n = 1–6) were performed using the following criteria: (a) the structure was refined with *R*-factor ≤ 0.05, (b) the structure entry contained a full set of three-dimensional atomic coordinates, (c) the structure exhibited no disorder, and (d) the structure was not flagged with any errors. Hydrogen atoms were specified with "implicit" notation in the CSD fragment search.

The Protein Data Bank (PDB) at the Research Collaboratory for Structural Bioinformatics was accessed online at www.rcsb.org. A total of 90 structures determined by X-ray crystallography and NMR spectroscopy were retrieved that contained one or more N6-acetyllysine (ALY) residues. If a crystal structure contained more than one protein chain in the asymmetric unit, these chains were counted as separate and individual structures for the purposes of calculating amide bond statistics. Similarly, for NMR structures, 20 or 25 independently determined minimum energy structures were deposited, so these structures were also evaluated as separate and individual structures. A program was written to evaluate the N6-acetyllysine side chain torsion angle χ_6 for each of these residues. Each residue was inspected visually to confirm the torsion angle measurement and to study the environment of the side chain.

RESULTS

N-Alkylacetamide Conformations in Small Molecule Crystal Structures

A total of 109 unique *N*-alkylacetamide fragments were retrieved from 84 well-determined small molecule crystal structures deposited in the CSD (individual entries are recorded in Table S1). These fragments have the general form shown below, with n = 1, 2, 3, 4, or 6:



Strikingly, 100% of the *N*-alkylacetamide linkages adopt the energetically preferred, planar *trans* conformation, with an average amide torsion angle of 180 ± 4°. No *cis* or significantly twisted amide conformations are observed, which is consistent with the energetic cost of such alternative conformations. It follows that a comparable preference for the *trans* isomer of N6-acetyllysine should predominate in protein structures determined by X-ray crystallography or NMR spectroscopy. A histogram of CSD amide torsion angles is presented in Figure 2a.

N6-Acetyllysine Amide Conformations in Protein Crystal Structures

A total of 166 N6-acetyllysine residues are contained in 79 protein structures determined by X-ray crystallography available as of 5 October 2012 in the PDB (individual entries are recorded in Table S2). Sixteen of these residues are truncated to alanine as a consequence of side chain disorder, and 14 are completely disordered with no coordinates available for main chain or side chain atoms. Accordingly, 30 N6-acetyllysine residues are not subject to amide bond classification. Of the 136 remaining N6-acetyllysine residues found with intact side chains, 101 (74.3%) adopt *trans* or generously *trans* conformations with average χ_6 = 180 ± 5°. While up to 28° distortions from planarity are observed in some of these structures, these amide distortions are thought to be within a reasonable margin of error; additionally,

distortions of this magnitude are not inconsistent with those occasionally observed for peptide bonds in protein structures refined at ultrahigh resolution.[29] Regardless, it is quite surprising that only 74.3% of these amide linkages are found with *trans* conformation, rather than the ~99% expected based on energetic considerations as well as the precedent established by highly-accurate small molecule crystal structures.

Curiously, 24 N6-acetyllysine residues (17.6%) adopt unfavorable *cis* or generously *cis* conformations with average $\chi_6 = 2 \pm 3°$. The high occurrence of *cis* or generously *cis* conformations is far greater than the 1.2% expected for the *cis* conformation based on the energetic difference of 2.6 kcal/mol between *trans* and *cis N*-methylacetamide.[17,21,22] Moreover, there is no precedent for *cis N*-alkylacetamide linkages in highly accurate small molecule crystal structures, as described above (Table S1).

Finally, 11 N6-acetyllysine residues (8.1%) adopt energetically prohibitive twisted or severely twisted conformations. Visual inspection of protein crystal structures containing unfavorable *cis* or twisted N6-acetyllysine residues does not yield compelling structural explanations for unfavorable amide conformations. Indeed, some of these amide groups exhibit pyramidal geometry inconsistent with the sp^2 hybridization of the amide carbonyl carbon atom. There is no correlation between the occurrence of energetically unfavorable *cis* or twisted conformations and the resolution of the structure determinations (Figure S1) or the program used for structure refinement (Figure S2). Amide conformation statistics for protein crystal structures are recorded in Table I and summarized in the histogram presented in Figure 2b.

N6-Acetyllysine Amide Conformations in Protein NMR Structures

Protein structures determined by NMR spectroscopy are deposited in the PDB as ensembles of 20 or 25 sets of protein coordinates resulting from separate and independent refinements against structural restraints determined by NMR. Although only 11 protein structures in the PDB containing N6-acetyllysine residues were determined by NMR spectroscopy, these structures contain 245 independently refined N6-acetyllysine residues (Table S3). Since each of these structures emanates from an independent calculation, they are considered independent measurements, in the same manner that multiple copies of a protein in the asymmetric unit of a crystal structure represent independent measurements. Of the 245 N6-acetyllysine residues contained in protein structures determined by NMR spectroscopy (Table I), we find that only 114 (46.5%) adopt *trans* or generously *trans* conformations with average $\chi_6 = 181 \pm 11°$; 13 (5.3%) adopt *cis* or generously *cis* conformations with average $\chi_6 = -4 \pm 21°$; and 118 (48.2%) adopt twisted or severely twisted conformations. These results are summarized in the histogram presented in Figure 2c. The NMR structures refined with the program Aria contain N6-acetyllysine residues with generally better geometries than structures refined with X-Plor or CNS (Figure S3).

DISCUSSION

The *trans* conformation of a sterically unrestrained amide bond is overwhelmingly more stable than *cis* or twisted conformations based on established principles of chemical bonding. Accordingly, *trans N*-alkylacetamide conformations are observed exclusively in highly accurate small molecule crystal structures (Figure 2a). In view of the increased stability of the *trans* amide linkage, however, it is surprising that so many examples of *cis* and twisted amide linkages are found for N6-acetyllysine residues in refined protein crystal structures deposited in the PDB (Figures 2b, 2c); examples from each category of amide conformation are shown in Figure S4. Even though a majority of protein crystal structures (74.3%) contains *trans* or generously *trans* N6-acetyllysine amide groups, this ensemble of structures contains a substantial number (17.6%) of unfavorable *cis* or generously *cis* N6-

acetyllysine amide groups. We respectfully suggest that most, if not all, of these amide groups are incorrectly modeled.

As a possible explanation for the occurrence of *cis* N6-acetyllysine amide groups in protein crystal structures, consider that the oxygen atom and the methyl group of the *N*-alkylacetamide moiety are virtually indistinguishable in a "Y"-shaped cloud of electron density at the typical resolution of a protein crystal structure determination. For example, using the structure of an inactivated mutant of histone deacetylase 8 complexed with a tetrapeptide assay substrate determined in our laboratory (PDB accession code 3EWF),[34] the electron density map for a *trans* N6-acetyllysine residue as correctly-fit in the protein coordinates is shown in Figure 3a. However, it is easy to see how this map could be fit nearly satisfactorily, but incorrectly, with a *cis* conformation in Figure 3b. Such an incorrect fit could conceivably result from unintentional manual or computational adjustment of the N6-acetyllysine conformation during refinement in the absence of a suitable planar *trans* restraint on the amide conformation.

It is important to note that the distinction between *trans* and *cis* amide linkages for N6-acetyllysine is not a simple distinction between side chain rotamers, e.g., as routinely encountered for the χ_2 angles of asparagine and histidine or the χ_3 angle of glutamine. These rotamers, too, are indistinguishable in electron density maps, and the rotamer that optimizes hydrogen bond interactions is typically selected. However, these rotamers are approximately isoenergetic, and the barrier for interconversion between rotamers is relatively low, comparable to that for C-C bond rotation in ethane (~3 kcal/mol).[35] In contrast, the *trans* amide conformation of N6-acetyllysine is 2.6 kcal/mol more stable than the *cis* conformation, and the barrier to interconversion is 20 kcal/mol.[17,21,22] Therefore, based on energetic considerations, the planar *trans* amide conformation should dominate exclusively for N6-acetyllysine residues in refined protein structures, just as it dominates exclusively in highly accurate small molecule crystal structures.

The observation of 8.1% twisted N6-acetyllysine amide conformations in protein crystal structures is perhaps more troubling. As previously outlined, the plane of the amide carbonyl approaches a perpendicular relationship with the plane of the amide nitrogen atom for a severely twisted amide conformation. The perpendicular twist of an amide (i.e., $\chi_6 = \pm 90°$) corresponds to the transition state for interconversion between *trans* and *cis* conformations; such a severe conformational distortion is energetically prohibitive. Therefore, we respectfully suggest that N6-acetyllysine amide groups with twisted conformations are incorrectly modeled.

Amide conformations for N6-acetyllysine residues in protein structures determined by NMR spectroscopy are characterized by strikingly different statistics, in that a majority of the amide groups are found with energetically unfavorable or prohibitive conformations. Only 46.5% of these N6-acetyllysine residues contain amide groups with *trans* or generously *trans* conformations. While only a small number (5.3%) of amide groups adopt *cis* or generously *cis* conformations, 48.2% adopt twisted or severely twisted conformations. Here, too, we respectfully suggest that N6-acetyllysine amide groups with *cis* and twisted conformations are incorrectly modeled.

The *cis* and twisted amide conformations for N6-acetyllysine residues observed in protein structures are inconsistent with established principles of chemical bonding. Incorrect amide conformations could result from ambiguities in the electron density maps of protein crystal structures, and/or insufficiently strong *trans* planar restraints in the refinement of crystal structures or NMR structures. Unfavorable N6-acetyllysine conformations are found in recently-determined protein structures, so this remains a persistent problem in structural and

chemical biology. While a small population (1.2%) of the *cis* conformation would be predicted based on energetic considerations, there does not appear to be any compelling reason for this less stable conformation in any protein structure determined to date based on our visual inspection. Of course, we cannot rule out the possibility that the rare *cis* conformation might not be important for some as-yet unidentified epigenetic function in a future protein structure determination.

The conundrum of energetically unfavorable *N*-alkylacetamide conformations in refined protein structures extends beyond the N6-acetyllysine residue. For example, the first structure of a bromodomain was reported in complex with *cis*-acetylhistamine bound in the N6-acetyllysine binding pocket.[11] In examples beyond the realm of epigenetic function, consider UDP-*N*-acetylglucosamine (UD1). A total of 49 protein crystal structures deposited in the PDB contain 94 independent UD1 ligands, and only 81 of these ligands (86.2%) contain acetamide linkages with *trans* or generously *trans* conformations; 13 of these ligands (13.8%) contain acetamide linkages with *cis* or twisted conformations (Table S4, Figure S5). One of these coordinate sets (2IU9, chain B) contains a plausible *cis* UD1 conformation in which the acetamide carbonyl group accepts a hydrogen bond from a histidine imidazole, but chain B contains a *trans* UD1 conformation lacking this interaction. None of the other *cis* or twisted acetamide groups engage in interactions that might justify an unstable conformation. Indeed, acetyl groups are ubiquitous in protein structures, and as simple covalent modifications (three-letter abbreviation ACE) to N-termini of protein structures there are 1230 independent examples found in 674 crystal structures deposited in the PDB. Only 83.3% of these acetyl groups adopt a *trans* or generously *trans* conformation; 6.3% adopt a *cis* or generously *cis* conformation, and 10.4% adopt a twisted conformation of some sort (Figure S6).

In summary, properly modeled *N*-alkylacetamide conformations in peptide and non-peptide groups are critical for understanding the molecular basis of biological function, and properly modeled N6-acetyllysine residues in protein structures are critical for understanding the molecular function of this common epigenetic modification. It is hoped that this report will lead to improved refinement protocols to ensure that future structures of acetylated proteins and peptides will contain predominantly *trans* amide groups. Such improvements will in turn yield an improved understanding of the molecular function of *N*-alkylacetamide groups in biology, especially the molecular recognition of N6-acetyllysine and related structure-function relationships underlying the emerging epigenetic code.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

PDB	Protein Data Bank
CSD	Cambridge Structural Database
ALY	N6-acetyllysine

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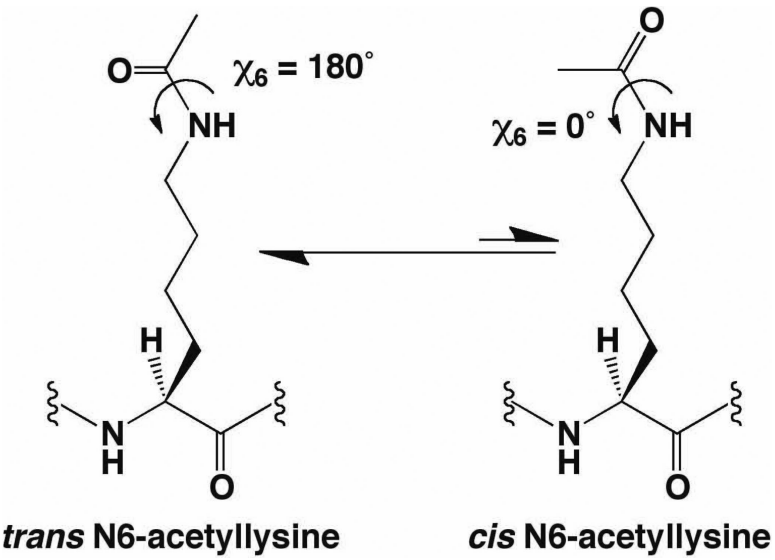

Figure 1.

The *trans* conformation of N6-acetyllysine is approximately 2.6 kcal/mol more stable than the *cis* isomer, based on measurements and calculations with *N*-methylacetamide.[17,21,22] The conformation of the *N*-alkylacetamide group of N6-acetyllysine is described by side chain torsion angle χ_6. At the transition state for *trans-cis* interconversion, where the plane of the carbonyl group is perpendicular to the plane of the amino group ($\chi_6 = \pm 90°$), the twisted conformation is approximately 20 kcal/mol higher in energy than the *trans* conformation.[17]

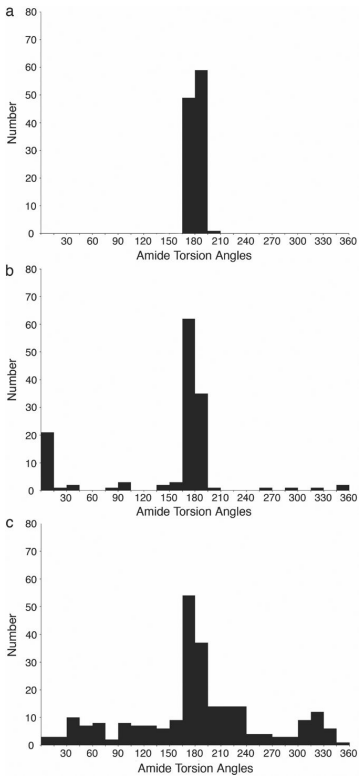

Figure 2.

Histograms illustrating: (a) *N*-alkylacetamide conformations in highly accurate small molecule crystal structures; (b) N6-acetyllysine conformations (side chain amide torsion angle χ_6) in refined protein structures determined by X-ray crystallographic methods; (c) N6-acetyllysine conformations (side chain amide torsion angle χ_6) in refined protein structures determined by NMR methods.

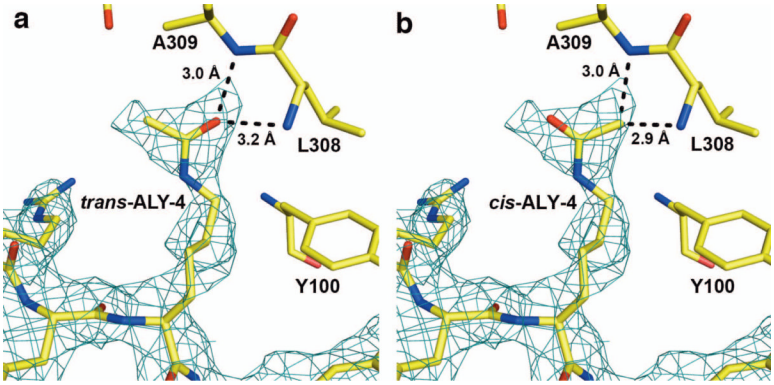

Figure 3.

Electron density map calculated with Fourier coefficients $2|F_o| - |F_c|$ and phases calculated from the final model less the side chain atoms of the first N6-acetyllysine residue in a tetrapeptide assay substrate bound to human histone deacetylase 8 (PDB accession code 3EWF, chain L, residue ALY-4, atomic color-coding C = yellow, N = blue, O = red).[34] The side chain amide group of N6-acetyllysine appears in the PDB coordinate file correctly modeled with a *trans* conformation (a), but it is easy to see how it could be inadvertently modeled with an incorrect *cis* conformation (b). However, only the correct *trans* conformation in (a) allows for a hydrogen bond between the N6-acetyllysine carbonyl oxygen and the backbone NH groups of L308 and A309; the incorrect *cis* conformation in (b) would result in steric clashes with the N6-acetyllysine methyl group.

Table I

Side Chain Amide Conformations for N6-Acetyllysine in Refined Protein Structures

	cis	**generously *cis***	**twisted$_{cis}$**	**severely twisted**	**twisted$_{trans}$**	**generously *trans***	***trans***
X-ray crystallography	23 (16.9%)	1 (0.7%)	3 (2.2%)	6 (4.4%)	2 (1.5%)	4 (2.9%)	97 (71.3%)
NMR spectroscopy	4 (1.6%)	9 (3.7%)	22 (9.0%)	76 (31.0%)	20 (8.2%)	23 (9.4%)	91 (37.1%)