Novel Mechanism of Surface Catalysis of Protein Adduct Formation

NMR STUDIES OF THE ACETYLATION OF UBIQUITIN*

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Reactivity of surface lysyl residues of proteins with a broad range of chemical agents has been proposed to be dependent on the catalytic microenvironment of the residue. We have investigated the acetylation of wild type ubiquitin and of the UbH68N mutant to evaluate the potential contribution of His-68 to the reactivity of Lys-6, which is about 4 Å distant. These studies were performed using [1-13C]acetyl salicylate or [1,1-13C2]acetic anhydride, and the acetylated products were detected by two-dimensional heteronuclear multiple quantum coherence spectroscopy. The results demonstrate that His-68 makes a positive contribution to the rate of acetylation of Lys-6 by labeled aspirin. Additionally, a pair of transient resonances is observed after treatment of wild type ubiquitin with the labeled acetic anhydride but not upon treatment of the H68N mutant. These resonances are assigned to the acetylated His-68 residue. The loss of intensity of the acetylhistidine resonances is accompanied by an increase in intensity of the acetyl-Lys-6 peak, supporting the existence of a transacetylation process between the acetylhistidine 68 and lysine 6 residues located on the protein surface. Hence, this may be the first direct demonstration of a catalytic intermediate forming on the protein surface.

The formation of covalent adducts by proteins and other cellular macromolecules plays an important role in the mediation of toxicity of both natural (e.g. non-enzymatic glycosylation) and synthetic (e.g. halothane trifluoroacetylation) substances. Hence, developing a mechanistic basis of such adduct formation is central to both an understanding of the structural basis for such toxicity and the development of alternate agents that may retain useful properties while exhibiting reduced tendencies to form undesirable adducts. For perhaps a majority of the examples of stable, covalent adducts that have been reported to form with proteins, linkages to lysine side chains are observed, despite the fact that at physiological pH values the lysyl ε-amino group is fully charged and hence a poor nucleophile. Several lines of evidence suggest that a catalytic microenvironment exists that overcomes the poor reactivity of the lysyl-NH₂ group. Thus, Nigen et al. (1) found that, whereas at neutral pH bromoacetate will modify some of the lysyl residues in myoglobin, no lysyl adducts were formed after incubation of the peptide Gly-Gly-Lys-Gly-Gly with labeled bromoacetate at pH 7.6. In this case, adducts were observed with the N-terminal glycolyl amino group. Analogous observations regarding the formation of thionoacyl adducts of albumin with metabolically generated thionoacetyl fluoride intermediates led to a similar conclusion and to a proposed catalytic effect of histidine or tyrosine side chains, which was studied by the addition of imidazole and phenol to the system (2).

The most extensive investigations of non-enzymatic protein modification have involved protein glycation and the formation of “advanced glycation end products” or “AGE” (3, 4), as shown in Scheme 1. It is clear from such studies that not all lysines behave equivalently, and several of these studies indicate that factors other than accessibility, in particular the existence of a catalytic microenvironment, play an important role in determining the site of glycation and the nature of the subsequent chemistry (5–11). In a number of studies involving glycation and the formation of other adducts, histidine has been proposed to play an important role in determining the reactivity of nearby lysine residues. Shilton and Walton (8) and Shilton et al. (9) have proposed that the imidazole group of His-348 facilitates the Amadori chemistry with Lys-231 in alcohol dehydrogenase via acid-base catalysis. In particular, a base supplied either by the buffer or by a nearby residue can facilitate the Amadori rearrangement by removing the proton from carbon 2 of the Schiff base-linked aldimine, and the subsequent tautomerizations are also subject to acid-base catalysis. Miyata et al. (11) similarly proposed that His-31 catalyzed Amadori rearrangement of the glycated adduct at Ile-1 in human β₂-microglobulin. In bovine ribonuclease A, the highest degree of Amadori chemistry was reported to occur at Lys-41 (6); the Lys-41 amino group is located in close proximity to the His-12 side chain (12). The proposed catalytic role of histidine in this chemistry has led to the hypothesis that the histidine-containing dipeptide carnosine (β-alanyl-histidine) may play a protective role in cells by rapidly forming adducts with excess sugars (13, 14).

As noted above, Hayden et al. (2) found that imidazole and phenol catalyzed adduct formation with metabolically generated thionoacetyl fluorides. Khalifah and Sutherland (15) found that imidazole stimulated alkylation of alcohol dehydrogenase at low concentrations but was inhibitory at high concentrations. Thus, the effects of added molecules can be complex, involving both catalytic activation and competition for modification. Another complicating factor is the unique active site chemistry of each enzyme, which can trap or specifically bind particular agents. In the present study, we have used two-dimensional NMR methods to study the acetylation of a very well characterized protein, ubiquitin. Ubiquitin is a particularly attractive target for such studies because of the wealth of available structural data, the lack of an active site with its specific catalytic chemistry, and the presence of a single histi-
dine residue located in the vicinity of Lys-6. NMR studies of the modification of ubiquitin and its H68N mutant offer a more direct approach to the analysis of the role of surface functionality in protein adduct formation.

EXPERIMENTAL PROCEDURES

Materials—Ubiquitin was obtained from Sigma. Methylene chloride, pyridine, deuterium oxide, deuterium chloride, and deuterium hydroxide were purchased from Aldrich. Potassium phosphate dibasic and monobasic salts were purchased from Mallinkrodt. For dialysis, 2-kDa molecular mass cutoff slide-A-Lyzer\textsuperscript{\textregistered} cassettes were purchased from Pierce. Salicylic acid was purchased from Fluka (Ronkonkoma, NY). Ethanol was purchased from Amersham Pharmacia Biotech. \([1,1\textsuperscript{3}C_2]\)Acetic anhydride and \([1-13C]\)acetyl chloride were purchased from Isotec, Inc. (Miamisburg, OH).

Synthesis of \([1,1\textsuperscript{3}C]\)Acetyl Salicylic Acid—\([1,1\textsuperscript{3}C]\)Acetyl salicylic acid was synthesized following an approach similar to that described by Geric et al. \((16)\) by the reaction of \([1-13C]\)acetyl chloride with salicylic acid. Salicylic acid (1.5 g) was dissolved in 90 ml of methylene chloride containing 1.8 ml of pyridine. A solution of 20\% \([1,1\textsuperscript{3}C]\)acetyl chloride (2.8 ml) in methylene chloride (11.2 ml) was added (14 ml total volume). The reaction container was capped and allowed to react for 15 h at 22 °C. The reaction container was un-capped, the methylene chloride was evaporated by heating (~45 °C), and the sample was then lyophilized. The purity of the \([1,1\textsuperscript{3}C]\)acetyl salicylic acid was determined by \(1^H\) NMR to be >99\%. The isotopically labeled aspirin was stored in a desiccator at 0 °C.

SCHEME 1. Formation of advanced glycation end products (AGE).

FIG. 1. Time dependence of the peak volumes corresponding to acetyl-Lys-6 and acetyl-Lys-48 derived from two-dimensional \(1^H\)-\(13C\) HMQC spectra of ubiquitin acetylated with \([1-13C]\)acetyl salicylic acid, obtained at the times indicated. Reactions were performed at 37 °C with 0.2 M potassium phosphate buffer in 100\% \(D_2O\) (pH 7.4), 40 mM \([1,1\textsuperscript{3}C]\)acetyl salicylic acid, and 8.3 mM wt ubiquitin or 4.0 mM H68N mutant. Each spectrum consisted of 2,048 complex data points yielding an acquisition of 4,504.5 Hz in the t2 dimension, yielding an acquisition time of 0.445 s with \(13C\) WALTZ16 decoupling during acquisition, an interpulse delay of 0.4 s, a scalar evolution delay of 61 ms, and a 0.4-s presaturation pulse. Typically, the t1 dimension had a spectral width of 628.6 Hz and 64 increments and was collected in phase-sensitive mode. The number of increments and/or transients were varied to obtain the required resolution or signal-to-noise ratio. Two-dimensional spectra were zero-filled to 2,048 points in t1, and apodization consisted of a shifted sine-bell function. Peak volumes were calculated using the Varian VNMR software (version 5.3b) and referenced to aspirin at 174.2 ppm (\(13C\)) and 2.34 ppm (\(1H\)) when visible in the two-dimensional spectrum; all shift values are in ppm. When acetic anhydride was used as the acetylating agent, the position of the acetyl-Lys-33 resonance was set at \(\delta^H\) 4,073 ppm, 174.50 ppm. Lys-33 is one of the more highly acetylated lysine residues in ubiquitin, and its chemical shift is essentially pH-independent between pH 4.95 and 9.8 (17).

The kinetics of aspirin acetylation of wt ubiquitin and H68N mutants were studied using a solution containing 8.3 mM ubiquitin or 0.4 mM H68N mutant and 40 mM \([1,1\textsuperscript{3}C]\)acetyl salicylic acid in 0.2 M phosphate buffer (pH 7.4) in 100\% \(D_2O\) at a temperature of 37 °C. Although a high concentration of ubiquitin was used in this study to facilitate peak quantification, we found that we obtained very similar results at much lower ubiquitin concentrations; i.e., the relative reactivities of the amino groups is approximately independent of concentration \((17)\). In addition to the HMQC parameters described above, an inverse BIRD pulse was placed in front of the HMQC sequence to suppress the increasing acetate signal that co-resonates with the signals of interest. The inverse BIRD sequence is essentially a spin echo with a scalar evolution delay of 78.1 ms, a presaturation pulse of 0.4 s, and a 1.063-s delay before the start of the HMQC sequence. Each spectrum was composed of 32 transients, resulting in a temporal resolution of 3 h. Peak volumes were calculated using the Varian VNMR software (version 5.3b). A threshold...
was chosen that was visually above the noise level, and the volume borders were automatically selected by the Varian software. Both peak volumes and peak heights were calculated based on the peak volume integration calculation and peak picking procedure of the software, respectively. Both measurements gave similar results, but only the peak volumes were used for graphing time courses of the reactions.

Time-dependent studies of acetic anhydride acetylation of wt ubiquitin and the H68N mutant were performed using a solution containing 4 mM ubiquitin or 0.66 mM H68N mutant and 110 mM [1,1\textsuperscript{13}C\textsubscript{2}]acetic anhydride in 0.22 M phosphate buffer in D\textsubscript{2}O (uncorrected meter reading of pH 7.4) at a temperature of 25 °C. A preliminary kinetic study was performed to determine the optimum temporal spacing of data points. The same two-dimensional HMQC NMR parameters as described above were used. Addition of the stock acetic anhydride solutions to the protein samples resulted in initial pH values of 6.85 and 7.2 for the wt and H68N samples, respectively, which decreased to 6.50 and 6.40 after 10 h and 18 h, respectively. Additional kinetic studies were performed with 4 mM ubiquitin and 4 M or 8 M [1,1\textsuperscript{13}C\textsubscript{2}]acetic anhydride in 0.25 M phosphate buffer (pH 5.9) in 100% D\textsubscript{2}O at a temperature of 25 °C to evaluate the effects of higher levels of acetylation. In each of these experiments, the transient resonances assigned to acetyl-His-68 were observed.

Unequivocal assignment of the acetyl-Lys-6 resonance was achieved by reacting 20 mM [1-\textsuperscript{13}C]acetyl salicylate with the 0.4 mM UbK6R mutant. Acetate and unreacted aspirin were removed by dialysis, and a \textsuperscript{1}H-\textsuperscript{13}C HMQC spectrum of the resulting acetylated UbK6R mutant was obtained, confirming our previous assignment (17).

RESULTS

As shown previously (17), incubation of wt ubiquitin with [1-\textsuperscript{13}C]acetyl salicylate results in the time-dependent formation of six adducts that give rise to resolved resonances in two-dimensional HMQC spectra. These adduct resonances have been previously assigned, and it was found that Lys-6 is the most rapidly acetylated residue, followed by Lys-48 and Lys-63. Although the order of acetylation parallels the surface availability of the residues, differences in calculated surface contact areas derived using the Connolly algorithm are in general insufficient to explain these differences. Lys-6 and Lys-48 are located near His-68 and Tyr-59, respectively. This might result in enhanced acetylation by aspirin due to pi-pi bonding or Van der Waals interactions between the aspirin and the aromatic residues. Alternatively, the proximity of Lys-6 to His-68 (the Lys-6 \textsuperscript{\epsilon2}-amino is 4.7 Å from the imidazole C-2 carbon in the crystal (18)) introduces several alternative possible mechanisms for enhancing adduct formation. A series of HMQC spectra corresponding to 3-h accumulation periods was obtained for both the wt ubiquitin and the H68N mutant in the presence of 40 mM [1-\textsuperscript{13}C]acetyl salicylate. Unexpectedly, the substitution of asparagine for histidine resulted in very little shift perturbation for the adduct acetyl resonances. We therefore confirmed our previous assignment (17) of acetyl-Lys-6 by analysis of the acetylated UbK6R mutant, as described under “Materials and Methods.” This result indicates that there is no strong interaction between these side chains at the surface of the protein. The time-dependent HMQC resonance intensities for the Lys-6 and Lys-48 adducts obtained using both the wild type protein and the H68N mutant are shown in Fig. 1. From these data, it is apparent that, in contrast to the chemical shift results noted above, the nearby histidine residue does make a significant contribution to the reactivity of Lys-6. Thus, the rate of modification of Lys-6 in the H68N mutant is found to be fairly similar to the rate for Lys-48 adduct formation. These results indicate a significant promotion of the adduct formation...
at Lys-6 resulting from the nearby His-68 residue (Fig. 2).

The use of acetic anhydride as a protein acetylating agent is expected to result in a greater degree of acetylation of less accessible target groups, as well as more extensive acetylation of histidine residues (19, 20). In a study of the acetylation of prothrombin by acetic anhydride, acetylation of one of the histidyl residues was found to proceed so rapidly that substantial amounts of the diacetylated species were formed, with the doubly acetylated imidazole ring decomposing to yield a di-N-acetylated side chain with an opened imidazole ring and loss of the ring C-2 carbon (21). H-13C HMQC studies of both ubiquitin and UbH68N performed using 110 mM [1,1-13C2]acetic anhydride confirm the greater acetylation of a residue corresponding to chemical shifts of 1H and 13C of 2.06 and 174.26 ppm, respectively, which we have previously assigned to the N-terminal methionine amino group. This result is consistent with preliminary mass spectrometry data indicating a high level of acetylation of Met-1 by acetic anhydride (22). In addition, two small resonances are observed in the upper right-hand region of the spectrum (Fig. 3A) at δH, δ13C = 1.864, 174.375 and 1.838, 174.31 ppm that are found to disappear gradually over subsequent 3-h spectral accumulation periods (Fig. 3, B and C). Such time-dependent resonance intensities presumably correspond to labile acetyl histidine or acetyl tyrosine adducts. The presence of two closely spaced resonances (Δ13C = 0.055 ppm; ΔδH = 0.018 ppm) would be consistent with the formation of an acetyl histidine adduct acetylated at either Nε or Nτ (17). In principle, acetylation of histidine can also lead to more complex spectra because of the formation of the diacetylated species and of cis/trans isomers of each of the acetyl imidazole bonds (Δ13C = 0.04 ppm; ΔδH < 0.0005 ppm) (17). Hence, these spectra are most readily interpreted in terms of the formation of a labile acetyl histidine adduct. Further support for this conclusion is derived from studies of the acetyla-

**Fig. 4. Time dependence of peak volumes corresponding to the study shown in Fig. 3 (A) and to a similar study utilizing 0.66 mM H68N mutant in place of the wt ubiquitin (B). Reactions were performed at 25 °C with 0.22 M potassium phosphate buffer in 100% D2O and 110 mM [1,1-13C2]acetic anhydride. The NMR spectral parameters and processing were the same as described in Fig. 1. In the absence of His-68, the acetylation of Lys-6 no longer exhibits the slow increase seen with the wild type enzyme.**

**Scheme 2. Direct and histidine-mediated pathways for acetylation of Lys-6.**
tion of UbH68N by \([1,1\text{,}^{13}\text{C}]\) acetic anhydride, in which these adduct resonances are not observed (figure not shown).

Remarkably, although the acetylation of ubiquitin by acetic anhydride proceeds sufficiently rapidly so that the reaction is essentially complete by the first observation period (Fig. 4A), the time-dependent loss of intensity of the resonances assigned to acetyl-His-68 are paralleled by a time-dependent increase in the intensity of acetyl-Lys-6. In contrast, we observed no time-dependent increase in the acetyl-Lys-6 resonance of UbH68N after treatment with the same concentration of acetic anhydride (Fig. 4B). These results indicate a direct transfer of the \([1,1\text{,}^{13}\text{C}]\) acetyl moiety from acetyl-His-68 to Lys-6, as shown in Scheme 2. This behavior is consistent with the fact that acetyl imidazole is itself an acetylating agent that has been used in many studies (23–26). In addition, the apparent decrease in intensity of some of the acetyl lysine resonances in Fig. 4 probably results from minor precipitation of the acetylated ubiquitin, which is more significant for the more highly acetylated species.

Another interesting feature of these spectra is the non-symmetric appearance of the contour corresponding to acetyl-Lys-63, which becomes particularly apparent under conditions of high levels of acetylation of the protein by acetic anhydride (Fig. 5). Fig. 5 shows a two-dimensional \(^1\text{H}\text{-}^{13}\text{C}\) HMQC spectrum of 4 mM wt ubiquitin and 8 mM acetic anhydride obtained 5.5 h into the reaction time course. In this case, the intensity of the resonance previously assigned to acetyl-Met-1 is significantly greater than in the previous study using aspirin (17). Furthermore, the acetyl-Lys-63 resonance now appears as two overlapping signals. In the crystal, the N–S distance of 3.6 Å corresponding to the Lys-63 Nα and the Met-1 sulfur has been suggested to correspond to a hydrogen bonding interaction (18). Hence, the data suggest that under the conditions of a high level of acetylation of the N-terminal methionine residue, there is a structural perturbation, possibly mediated by a hydrogen bonding interaction, which leads to a small shift of the resonance for acetyl-Lys-63.

**DISCUSSION**

The above results provide strong support for the conclusion that surface catalysis can contribute to the formation of adducts of proteins and, presumably, other macromolecules. In contrast to catalysis at the active site of proteins, in which very precise structural relationships are maintained, the effects observed here occur in the presence of only an average proximity of flexible side chains. Thus, for example, in the crystal structure of ubiquitin the mean ratio of the B-factors for the ϵ-amino and α-amino groups of Lys-6 is 3.4; the ratio of the imidazole nuclei to the α-amino of His-68 is 4.6 (18). The lack of a well-defined interaction is also reflected in the fact that the acetyl-Lys-6 resonance shifts were nearly unaffected by the H68N residue substitution. In general, the catalytic effect observed could arise from a histidine-assisted deprotonation of the lysine side chain amino group or via the intermediacy of an acetyl histidine adduct. Support for the first alternative can be derived from the reduced \(pK_a\) value observed for dimethyl-Lys-6 in reductively methylated ubiquitin (17). Alternatively, acetyl imidazole is an established acetylating agent, so the formation of a reactive acetyl imidazole side chain provides a very reasonable explanation for these results (Fig. 4).

Based on ubiquitin amine surface accessibility alone (17), one would predict that the smaller, more reactive protein-modifying agent, acetic anhydride, would have a similar residue acetylation profile to aspirin. However, Lys-6 is the third most reactive at an acetic anhydride:ubiquitin molar ratio of 13:1 (Fig. 4). The basis for this difference in reactivity is unknown at present. At very high acetic anhydride:ubiquitin ratios, the intensities for the acetyl lysine resonances of the accessible residues tend to become more similar, indicating a more complete degree of modification (e.g. Fig. 5, obtained at a ratio of 2000:1).

Transient acetyl histidine adducts have previously been reported using other methods of detection (19, 20). The factors involved in the observation of transient acetyl histidine adducts by NMR are not completely clear at present. The data obtained demonstrate that the potential significance of a catalytic microenvironment extends beyond the example of the Amadori rearrangement of Schiff’s bases to other types of adducts formed with macromolecules. In fact, the most highly acetylated lysine residues by aspirin in albumin (27) and hemoglobin (28) are within several Ångstroms of a histidine resi-
idue, and transient acetyl histidine adducts may be responsible for the enhanced reactivity of certain lysine residues in these proteins as well. In addition to the role of histidine discussed above, other residues can presumably play analogous catalytic roles. Aspartyl and glutamyl carboxyl groups have also been found in the vicinity of highly glycated lysyl residues (6, 13). Similarly, tyrosyl side chains could be transiently acetylated and become involved in analogous transacetylation reactions (2). It seems likely that all of the catalytic effects observed at the active sites of enzymes may play a role in non-enzymatic protein modification, although the effects in general are expected to be considerably smaller because of the lack of defined surface residue stereochemical constraints (29).

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