Examination of the Aspirin Acetylation Site of Human Serum Albumin by ^{13}C NMR Spectroscopy

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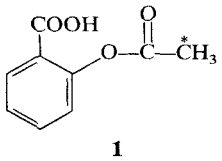
Human serum albumin has been specifically acetylated using aspirin in which the methyl carbon of the acetyl group was enriched to 90% ^{13}C. A single resonance at 23.13 ppm downfield from tetramethylsilane was observed in ^{13}C difference spectra obtained at both 25.2 and 45.3 MHz. Chemical shift studies of several model compounds suggest that this is the resonance position to be expected for an acetamide group exposed to solvent. The line width observed for the enriched methyl resonance is consistent with free rotation of the methyl group.

Pinckard, Hawkins and Farr have established that aspirin, a widely used analgesic and antipyretic, acetylates a large number of constituents of the human body including plasma proteins.[1] Albumin is the major protein component of plasma and these authors have demonstrated that this protein is modified with high specificity at a single lysine residue under physiological conditions.[2] More recently, Walker has determined that it is lysine-199 of human serum albumin which is acetylated.[3] There is evidence which suggests that the specificity toward acetylation of this amino acid is largely due to an abnormally low pK_a of the ε-amino group. The pK_a of this function may be in the region 8.0–8.7[4,5] so that, near neutral pH, it would have appreciable nucleophilic character while the other, more normal lysine side chains ($pK \sim 11$) would be essentially fully protonated and, therefore, less reactive under these conditions. An unusual molecular environment for lysine-199 of human albumin is thus suggested by these observations.

The goal of the present work was to explore the nature of the environment around lysine-199 of human albumin by ^{13}C NMR spectroscopy. Acetylation of the protein with aspirin which had been enriched with ^{13}C at the methyl carbon (**1**) provided a



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system in which the NMR signal for the enriched position could be detected reliably by ^{13}C difference spectroscopy. Our results indicate that lysine-199, at least after acetylation, lies in a milieu which substantially exposes the acetyl group to solvent.

EXPERIMENTAL

Materials

Acetyl[2-^{13}C]salicylic acid was synthesized from [2-^{13}C]acetic anhydride (Merck, Sharpe and Dohme, 90% enriched) and salicylic acid (Baker and Adamson). A mixture of 230 μl of the anhydride and 104 mg of salicylic acid was sealed in a glass tube and heated in a water bath at 70° for 24 h. The product was precipitated by adding 1.5 ml light petroleum and cooling. Recrystallization from ethyl ether/light petroleum followed and afforded 22 mg of product, m.p. 134–135° (Lit.[6] 135°).

Acetylsalicylic acid of normal isotopic content was purchased from Sigma and recrystallized by the procedure described above before use.

ε-Acetyl-L-lysine, L-lysine hydrochloride, and α-acetyl-L-lysineamide acetate were obtained from Research Plus Laboratories (Denville, New Jersey), Aldrich and Cyclo Chemical Co., respectively, and were used as received.

To prepare α,ε-diacetyl-L-lysine, ε-acetyl-L-lysine (0.48 g, 25 mmol), *p*-nitrophenylacetate (0.90 g, 5 mmol), sodium bicarbonate (0.74 g, 9 mmol), 10 ml of water and 3 ml of ethyl acetate were stirred together at room temperature for 21 h. The ethyl acetate layer was removed and, after extraction with ether, the aqueous layer was acidified to pH 1 with 6 N HCl. Another extraction with ether and then carbon tetrachloride followed, whereupon the aqueous phase was concentrated on a rotary evaporator (dry ice trap) until all volatile material was removed. Attempts to recrystallize the semisolid residue which remained

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were unsuccessful.[7] However, the ^1H NMR spectrum of the material in deuterium oxide solution was consistent with the expected structure, showing signals centered at 4.2 ppm (triplet, 1H), 3.2 ppm (triplet, 2H), 2.2 and 2.1 ppm (singlets, 3H each) and a broad resonance at 2.0 to 1.4 ppm (6H) from sodium 2,2-dimethyl-2-silapentane sulfonate. The carbon NMR spectrum was also consistent with the structure, as discussed below.

α,ε-Diacetyl-L-lysinamide was prepared by a similar procedure using α-acetyl-L-lysinamide acetate as the starting material. The ^1H NMR and ^{13}C NMR spectra of the product were consonant with the structure expected.

N-Acetyl-ε-aminocaproic acid was obtained by treating ε-aminocaproic acid (Aldrich, 1 g), suspended in a mixture of benzene (10 ml) and pyridine (1 ml), with acetic anhydride (2 ml). After stirring overnight, the reaction mixture was concentrated with a rotary evaporator and the solution allowed to stand overnight at room temperature or at 5°. The crystals which formed were removed by filtration and recrystallized twice from water to give a white solid, m.p. 104–105° (Lit.[8] 104–105°).

N-Trideuterioacetyl-ε-aminocaproic acid was prepared in the same manner as the *N*-acetyl compound, as described above, except that acetic anhydride-d_6 (Stohler Isotope Chemicals) was used. The ^1H NMR and ^{13}C NMR spectra of the material were identical to those of the nondeuterated material save that the resonances (proton and carbon) of the acetyl methyl group were missing from the spectra.

Human serum albumin (Cohn fraction V) was obtained from Sigma (lots 81C-1302–8 and 62C-1301–8). The protein was dialyzed three times against 1 mM ethylenediaminetetraacetic acid, then three times against doubly distilled water and lyophilized.

Acetylated albumin was prepared according to the procedure of Pinckard, Hawkins and Farr;[2] 1 g of protein was treated with either aspirin of normal isotopic composition or ^{13}C enriched aspirin at pH 7.32 in phosphate buffer at the concentrations indicated by these authors. After 24 h the reaction mixture was cooled to 4° and dialyzed against 0.15 M NaCl containing 10 mM sodium salicylate. After extensive dialysis against water, the sample was lyophilized.

Procedures

Solution pH was read with a Radiometer Model PHM 63 or PHM 42 pH meter equipped with Metrohm combination electrodes. The values reported are the meter readings, uncorrected for the presence of deuterium in the solutions.

^1H NMR spectra were recorded on a Varian Associates T-60.

The ^{13}C NMR spectra were obtained with Varian CFT-20 and XL-100 instruments under conditions of complete proton decoupling. The sample temperature on the first instrument was 34 ± 1 °C while on the second the sample temperature was controlled at 25° with the Varian variable-temperature controller. Carbon spectra at 45.3 MHz were obtained with a Bruker WH-180 spectrometer; the sample temperature for these experiments was not recorded but was probably about 25°. Carbon spectra were referenced with an external capillary of tetramethylsilane or dioxane. The shift of external dioxane relative to external tetramethylsilane was determined to be 67.57 ppm. The variation of the carbon shifts of solutes in aqueous solution relative to these external references with temperature was determined to be approximately 0.006 ppm deg^{-1}. The accuracy of the chemical shifts is 0.05 ppm and was limited by the digital resolution of the spectrometers.

To obtain difference spectra solutions of the ^{13}C enriched acetylated protein and acetylated protein of normal isotopic composition were prepared which were as similar as possible as regards sample volume, protein concentration and pH. An equal number of transients were collected for each sample and both FIDs transformed in an identical manner. Difference spectra were then computed with the software provided with each instrument; small adjustments in the weighting factors used and the frequency offset between spectra were made until the base lines in the difference spectra were as flat as possible.

RESULTS

Human serum albumin was treated with ^{13}C labeled aspirin under conditions which give predominant (~85%) acetylation at one lysine residue, lysine-199.[2,3] Figure 1 shows carbon spectra of the labeled protein obtained at 25.2 and 45.3 MHz [traces (a) and (b)]; the greater resolution and sensitivity of the higher field instrument is apparent. The spectra obtained are qualitatively similar to the carbon spectrum of bovine albumin reported by Bradbury and Norton.[9] Spectra were also collected using albumin acetylated with aspirin of normal isotopic composition; comparison of the two sets of data showed that the enriched carbon appeared as a spike on top of a broader mound of natural abundance carbon signals from the protein centered at ~25 ppm. Difference spectra generated with these data sets [traces (c) and (d)] made more clear the enriched methyl carbon resonance as a singlet 23.13 ± 0.15 ppm downfield from external TMS. A line width of approximately 30 Hz was observed at both frequencies.

Carbon chemical shifts can be influenced by aromatic ring current effects, structural distortions and changes of solvent.[10] In the absence of a known tertiary structure it is impossible to estimate how large the ring current effect could be in the present system. Shift changes due to distortions of bond angles in the labeled methyl group are probably negligible.[11] However, should the acetyl methyl group of acetylated albumin be in a strongly hydrophobic protein environment, its chemical shift could be changed from its value in pure water by a 'solvent' effect. To define the carbon chemical shift expected for the acetyl methyl in water and less polar solvents several model compounds containing structural elements of

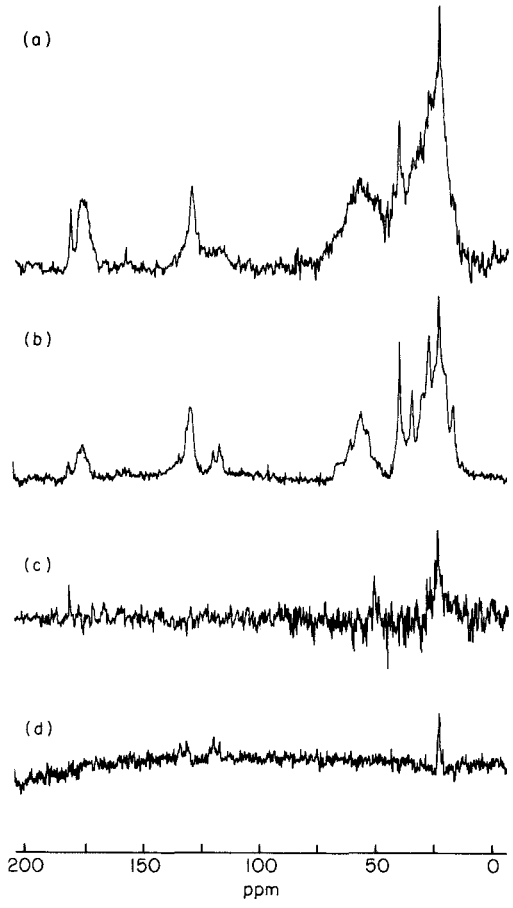

Figure 1. Carbon-13 spectra of [2-^{13}C]acetylated human albumin. Samples were approximately 1 mM in protein and contained 0.15 M NaCl; sample pH was 5.5. (a) Data obtained at 25.2 MHz, 30 000 accumulations taken over 10 h; (b) data obtained at 43.5 MHz, 6000 accumulations over 20 min; (c), (d) difference spectra obtained by subtracting from curves (a) and (b) the corresponding spectrum of natural abundance acetylated albumin. Spectra are reported using external tetramethylsilane as a reference and were obtained in all cases with complete proton decoupling. The spurious peaks in difference spectrum (c) are unexplained; the signals between 120 and 140 ppm in spectrum (d) correspond to the chemical shifts of salicylic acid which, apparently, was incompletely removed by dialysis.

ε-acetyllysine were examined. The ^{13}C shift data obtained for these materials are given in Table 1; assignments of signals are based on the data of Haworth and Lilley.[12] In the case of *N*-acetyl-ε-aminocaproic acid, the assignment of the acetyl methyl signal was confirmed by specific deuteration of this group. Using the data in Table 1, the methyl carbon shift expected for ε-acetylated lysine was found to be 22.94 ± 0.05 ppm from external TMS at 34 °C. With the experimental temperature dependence of chemical shifts relative to this reference (cf. Methods), this shift should be 23.04 ± 0.05 ppm at 25 °C. In dimethyl sulphoxide, a solvent less polar than water, the acetyl methyl resonance appears at 24.0 ppm, a shift consistent with the magnitude of the effects of this solvent on the shifts of lysine[12] and with organic solvent effects on other methyl carbon shifts.[13]

DISCUSSION

Changes of solvent from water to less polar solvents shift the methyl carbon signals 1–2 ppm downfield,[13] a result confirmed by the observations with the model compound *N*-acetyl-ε-aminocaproic acid recorded in Table 1. The data reported there also define the expected chemical shift, in aqueous solution, for an acetyl methyl located at the ε-position on lysine. Acetylated human albumin exhibits a methyl carbon shift 0.1 ± 0.2 ppm to the low field side of this expected resonance position. Thus, there appears to be a possible small downfield shift of the signal due to the protein environment of the observed group, but this shift is obscured by experimental errors. The effect is much smaller than would be anticipated if the methyl group were in a nonpolar environment, such as that provided by a typical organic solvent. On the basis of this chemical shift evidence we conclude that, after acetylation, lysine-199 of human albumin is so oriented in the protein structure that the acetyl methyl is well exposed to an environment which is very similar to bulk water.

The rotational correlation time (τ_c) for monomeric human albumin is about 20 ns.[15] However, τ_c for bovine albumin is dependent on protein concentration[16] and one can estimate from the data for bovine albumin that τ_c for the human protein at the concentration used in the present work (~1 mM) will be approximately 70 ns. Given this value for τ_c and assuming that the enriched carbon atom is relaxed exclusively by proton–carbon dipolar interactions, one can show that the observed line width at 25 and 45 MHz is consistent with correlation times for internal rotation (τ_i) of the methyl group smaller than 0.1 ns.[17]

Table 1. Chemical shifts of model compounds

Compound	Solvent	Chemical shift (ppm)[a] C-α	C-β	C-γ	C-δ	C-ε	α-CH_3	ε-CH_3
Lysine (peptide)[b]	D_2O	55.0	31.6	23.2	32.3	41.3	—	—
α-Acetyl-L-lysineamide	D_2O	54.6	31.5	24.5	27.4	40.3	23.2	—
ε-Acetyllysine	D_2O	55.8	31.2	23.0	29.1	40.1	—	22.9
α,ε-Diacetyllysineamide	D_2O	54.0	28.2	28.0	31.1	40.2	23.2	22.9
N-Acetyl-ε-aminocaproic acid	D_2O	40.5	29.1	26.5	27.1	38.6	—	23.0
Lysine (peptide)[b]	DMSO[c]	51.8	31.5	22.1	26.6	38.6	—	—
N-Acetyl-ε-aminocaproic acid	DMSO	38.8	30.3	25.7	27.4	35.1	—	24.0

[a] Carbon chemical shifts relative to external TMS. Sample temperature was 34° and the determinations were made at 20 MHz.
[b] Data from Ref. 12 for lysine in a peptide structure.
[c] Bulk susceptibility corrections were not made but should not exceed 0.2 ppm.[14]

These values indicate essentially free rotation of the methyl group,[18] although confirmation of these conclusions by spin–lattice relaxation and Overhauser effect data is desirable.

Our results prove the feasibility of using ^{13}C enrichment to explore specific reactive sites on a protein as large as albumin, but do not provide any strong indication through chemical shift effects or nuclear relaxation of unusual protein structural features at lysine-199 after it has been acetylated. The special reactivity of the terminal amino group of this residue, thus, most likely arises from electrostatic interactions between this function and its immediate environment.

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