Note

N^ε-Methylated Lysines from Formaldehyde-treated CaseinTadashi NAKAI, Takafumi OHTA
and Nobumasa HAYASEFaculty of Pharmaceutical Sciences,
Science University of Tokyo, Shinjuku-ku, Tokyo,
Japan

Received March 16, 1979

The reductive methylation of amino groups in protein by the use of formaldehyde is achieved usually in the presence of an additional reducing agent, *e.g.*, sodium borohydride¹⁾ or sodium cyanoborohydride.²⁾ By means of chromatographic analysis, Reis and Tunks³⁾ detected *N*^ε-monomethyllysine in acid hydrolyzates of formaldehyde-treated casein but not in those of untreated casein. This finding suggests that the ε-amino groups of lysine residues are methylated in part even when protein is treated with formaldehyde alone without any other reducing agent. The present paper describes actual isolation and identification of *N*^ε-dimethyllysine as well as *N*^ε-monomethyllysine from a sample of formaldehyde-treated casein, clearly showing that, under certain conditions, the groups in protein are not only monomethylated but also dimethylated on treatment with formaldehyde alone.

A mixture of 50 g of casein acc. to Hammarsten (E. Merck) and 500 ml of water was adjusted to *ca.* pH 7.2 with 0.2 *N* NaOH, mixed with 200 ml of a 9.4% (w/v) solution of HCHO, adjusted to pH 7.2 again, and finally made up to 1.5 liters with water. This mixture was heated in boiling water for 24 hr with stirring, left to stand in a refrigerator overnight and adjusted to *ca.* pH 4.6 with 0.2 *N* HCl. The precipitate formed was washed with ethanol until free HCHO was no longer detectable and then air-dried. This protein sample was hydrolyzed by boiling under reflux with 10 times its weight of 6 *N* HCl for 24 hr. The hydrolyzate was evaporated to dryness *in vacuo*. The residue was dissolved in water and mixed with a slight excess of phosphotungstic acid dissolved in 5% H₂SO₄. After standing at room temperature for 2 days, the precipitate formed was separated and treated with excess Ba(OH)₂, then CO₂ in the usual manner to give a solution of free basic amino acids. The solution was then subjected to Kossel-Kutscher fractionation using AgNO₃ and Ba(OH)₂.⁴⁾ The histidine and arginine fractions were discarded. From the lysine fraction which had been freed of Ba²⁺ and Ag⁺ the constituent amino acids were again precipitated with phosphotungstic acid and from

the precipitate obtained they were regenerated in the manner described above. Two ninhydrin- and also Dragendorff⁵⁾-positive substances, 'X' and 'Y', were detected in this fraction by means of paper chromatography on Toyo Roshi No. 50 paper with solvent systems I) isopropanol-29% aq. NH₃-water (8:1:1), II) chloroform-methanol-25% aq. NH₃ (4:1:1) and III) isopropanol-formic acid-water (4:1:1). 'X' behaved quite similarly to *N*^ε-dimethyllysine (provided by Dr. T. Nakajima), and 'Y' to *N*^ε-monomethyllysine (Calbiochem). *R_f* values (values of reference compounds in parentheses): 'X', 0.38 (0.39) in I) and 0.78 (0.78) in II); 'Y', 0.18 (0.18) in I), 0.62 (0.64) in II) and 0.51 (0.52) in III). This fraction was evaporated nearly to a sirup *in vacuo*. A 2-ml portion of the liquid was chromatographed on a 1.5 × 130 cm column of Whatman cellulose powder CF 11 equilibrated with the above-mentioned solvent system I), the same system being used as an eluent. The eluate was collected in 5.0 g fractions at a flow rate of 0.1 ml/min (Fig. 1).

FIG. 1. Paper Chromatograms of the Eluate Fractions Obtained on Cellulose Column Chromatography of the Lysine Fraction.

Solvent system, isopropanol-29% aq. NH₃-water (8:1:1); detection reagent, ninhydrin.

The eluate fractions containing 'X' as the main component were combined and evaporated to a sirup *in vacuo*. The sirup was dissolved in 10 ml of 70% (v/v) ethanol, mixed with 50 ml of a 28 mg/ml solution of picronic acid dissolved in boiling 70% (v/v) ethanol and allowed to stand overnight. The small amount of ammonium picrolonate that crystallized out was filtered off. The filtrate was evaporated to dryness. The residue was recrystallized several times from 30% (v/v) ethanol, yielding 80 mg of yellowish-brown needles, mp 106~108°C. This picrolonate was converted into a hydrochloride by heating with 6 *N* HCl, then extracting the liberated picronic acid with ether. The hydrochloride was then esterified by heating with abs. methanol saturated with dry HCl at 50°C for 3 hr; mp (dec.) 195°C (softening followed by gas evolution at *ca.* 80°C). NMR δ_{Me₄Si}^{D₂O}: *ca.* 2.20 (6H, broad multiplet, β-, γ- and δ-CH₂), 3.29 (6H, singlet, (CH₃)₂N^ε), 3.58 (2H, triplet, ε-CH₂), 4.26 (3H, singlet, COOCH₃), 4.60 (1H, triplet, α-CH). MS *m/e*: 188 (M⁺), 129,

112, 98, 84, 58. The M^+ peak and the one at m/e 129 were relatively small. The latter was ascribed to the so-called "amine fragment"⁶⁾ $[(CH_3)_2N-(CH_2)_4CHNH_2]^+$. The peak at m/e 58 was very intense and presumably ascribable to $[(CH_3)_2NCH_2]^+$. It can be presumed that, in the same manner as the "amine fragment" of N^ϵ -monomethyllysine ester,⁷⁾ the amine fragment of N^ϵ -dimethyllysine ester readily loses $(CH_3)_2NH$, CH_3NH_2 or NH_3 to yield a stable cyclized fragment of mass 84, 98 or 112. The present results with prominent peaks at m/e 84 and 98 are in line with this presumption.

The eluate fractions containing 'Y' were combined and evaporated to a sirup. The sirup was treated with picrolonic acid in the same manner as described above to yield a picrolonate (yellow needles), mp 212~215°C, which was converted into a hydrochloride in the manner described above. NMR $\delta_{Me_4Si}^{CF_3COOD}$: 1.98 (4H, γ -, δ - CH_2), 2.35 (2H, β - CH_2), 2.98 (3H, CH_3N^ϵ), 3.32 (2H, ϵ - CH_2), 4.50 (1H, α -CH). This resonance pattern was quite similar to that of N^ϵ -monomethyllysine hydrochloride (Calbiochem).

The possibility of the occurrence of dimethylation as well as monomethylation of the ϵ -amino groups on formaldehyde treatment of protein was thus ascertained by the isolation of the two compounds.

Acknowledgments. We are greatly indebted to Dr. S. Takitani for the kind discussion and to Dr. T. Ohmoto (Toho University) for providing facilities. Thanks are also due to Dr. T. Nakajima (Osaka University Medical School) for the generous gift of authentic specimens.

REFERENCES

- 1) G. E. Means and R. E. Feeney, *Biochemistry*, **7**, 2192 (1968).
- 2) D. Dottavio-Martin and J. M. Ravel, *Anal. Biochem.*, **87**, 562 (1978).
- 3) P. J. Reis and D. A. Tunks, *Aust. J. Biol. Sci.*, **26**, 1127 (1973).
- 4) M. Guggenheim, "Die biogenen Amine," 4th ed., S. Karger, Basel, 1951, pp. 52~55.
- 5) E. Tyihák and D. Vagujfalvi, *J. Chromatogr.*, **49**, 343 (1970); E. Tyihák, *J. Chromatogr.*, **14**, 125 (1964).
- 6) K. Biemann, J. Seibl and F. Gapp, *J. Am. Chem. Soc.*, **83**, 3795 (1961).
- 7) K. Murray, *Biochemistry*, **3**, 10 (1964).