OBSERVATION OF ϵ-*N*-TRIMETHYLLYSINE RESIDUES OF PROTEINS BY NATURAL ABUNDANCE CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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1. Introduction

Numerous narrow individual-carbon resonances* (of nonprotonated carbons) are observed in the aromatic regions of natural-abundance ^{13}C NMR spectra of small native proteins [1–3]. In contrast, few, if any, of the aliphatic carbons yield resolved individual-carbon resonances, even at a magnetic field strength as high as 63.4 kg [4]. We show in this report that the trimethylammonium group of the single ϵ-*N*-trimethyllysine residue of *Candida krusei* cytochrome *c* [5,6] yields an identifiable narrow individual-carbon resonance in the region of the relatively broad α-carbon resonances.

2. Materials and methods

Candida krusei cytochrome *c* (Type VII) and horse-heart cytochrome *c* (Type III) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Protein samples were prepared essentially as described previously [7]. After completion of the NMR experiments, each sample was analyzed spectrophotometrically to determine the amounts of reduced and oxidized protein. The samples of ferro- and ferrocytochrome *c* from *Candida krusei* were about 7% oxidized and about 20% reduced, respectively. The sample of horse-heart ferrocytochrome *c* was about 5% oxidized.

Natural-abundance ^{13}C Fourier transform NMR spectra were recorded at 67.9 MHz, with the use of 10 mm sample tubes. The spectrometer has been described [8]. Conditions of spectral accumulation and processing were essentially as described [8], unless otherwise indicated, in the caption of fig.1. Chemical shifts, reported in parts per million downfield from Me_4Si, were measured digitally with respect to a trace of internal dioxane (not present in the samples used for fig.1), at 67.8 ppm. The nuclear Overhauser enhancement (NOE) was measured by the method of gated decoupling [9] as follows. The ^1H-irradiation was applied during each data acquisition interval (0.29 s) and it was turned off for 1.7 s (an interval more than 5-times the value of each pertinent spin-lattice relaxation time) before the start of each data acquisition interval.

3. Results and discussion

Figure 1A shows the aliphatic region of the fully proton-decoupled ^{13}C NMR spectrum of horse-heart ferrocytochrome *c*, a protein which does not contain any ϵ-*N*-trimethyllysine residues [10]. The downfield portion of the spectrum (about 50–75 ppm) contains the C^{α} resonances (except those of glycine residues) and the resonances of C^{β} of the threonine and serine residues [11,12]. The broad band in the range 50–60 ppm in fig.1A probably contains only the resonances

Abbreviations: Me_4Si tetramethylsilane, NOE nuclear Overhauser enhancement

* We use the term 'individual-carbon resonance' to designate a peak that arises either from a single carbon of a protein or from two or more equivalent carbons (such as those of a rapidly rotating trimethylammonium group of an ϵ-*N*-trimethyllysine residue)

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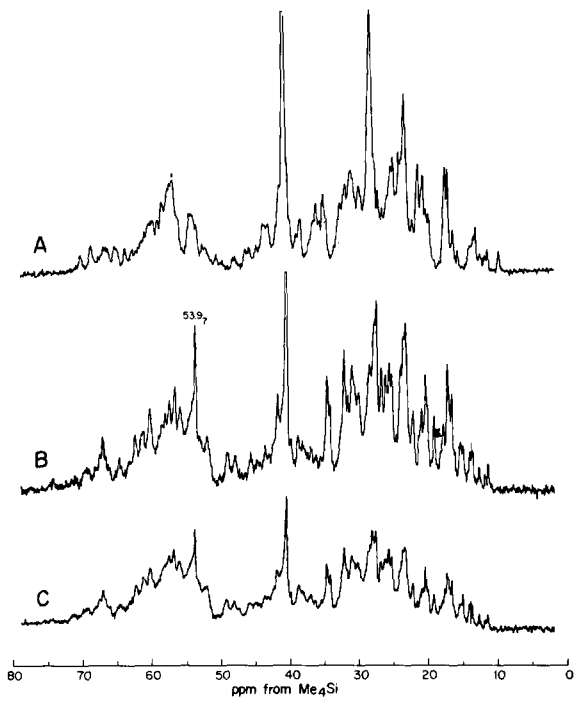

Fig.1. Region of aliphatic carbons in natural-abundance ^{13}C Fourier transform NMR spectra of cytochromes *c* (0.1 M NaCl, 50 mM phosphate buffer in H_2O, 25°C). Time-domain data were accumulated in 8192 addresses, with a recycle time of 2.016 s and 90° radio-frequency pulse excitation. A digital broadening of 2.2 Hz was applied. The ^1H-irradiation for proton-decoupling was centered 2 ppm downfield from the ^1H resonance of Me_4Si. (A) 13 mM Horse-heart ferrocytochrome *c*, at pH 7.0, after 32 768 accumulations with full proton-decoupling. (B) 15 mM *Candida krusei* ferrycytochrome *c*, at pH 6.8, after 16 384 accumulations with full proton-decoupling. (C) Same sample as in B, after 32 768 accumulations with gated proton-decoupling (see Materials and methods).

of methine α-carbons [11,12]. Each of the contributing C^{α} resonances is expected to have a natural linewidth of about 20 Hz at 67.9 MHz (see table 1 of ref. [2] and fig.2 of ref.[8]).

Figure 1B shows the aliphatic region of the fully proton-decoupled ^{13}C nmr spectrum of *Candida krusei* ferrocytochrome *c*, a protein which contains one ϵ-*N*-trimethyllysine residue [5,6] at position 72* in the sequence [6,13]. In the α-carbon region of this spectrum there is a prominent narrow resonance (natural linewidth $\lesssim$ 5 Hz) at 54.0 ppm (fig.1B), which is not observed in the spectrum of horse-heart ferrocytochrome *c* (fig.1A). The small linewidth of this resonance is inconsistent with an assignment to C^{α} resonances [2]. Furthermore, a spectrum recorded under conditions of gated decoupling (fig.1C) yields an NOE value of 2.8 ± 0.4 for the narrow peak at 54.0 ppm and an NOE value of 1.3 ± 0.2 for the α-carbon envelope. Here we define the NOE as the ratio of intensities observed with and without proton-decoupling. The large NOE of the peak at 54.0 ppm is evidence of fast internal motion (relative to the rate of overall rotation of the protein) of the C–H groups which give rise to this resonance [2,14].

The chemical shift of the trimethylammonium carbons of aqueous *N*-hexyltrimethylammonium bromide is 54.3 ppm [15]. Recently, Eakin et al. [16] reported the preparation of *Neurospora crassa* cytochrome *c* which was ^{13}C-enriched at the methyl groups of ϵ-*N*-trimethyllysine-72. The chemical shifts of these enriched carbons of the ferro- and ferricytochrome *c* were reported as 53.6 ppm and 54.3 ppm, respectively

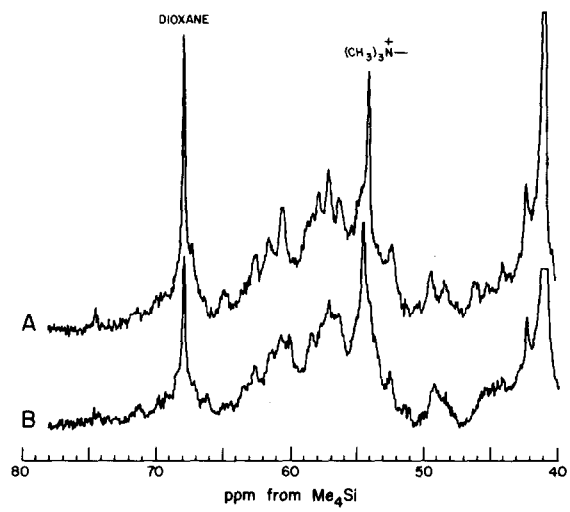

Fig.2. Downfield portion of the aliphatic region in natural-abundance ^{13}C Fourier transform NMR spectra of *Candida krusei* cytochrome *c* (0.1 M NaCl 50 mM phosphate buffer in H_2O, with trace of internal dioxane, at 25°C). Accumulation and processing conditions were those of fig.1B. (A) 15 mM Ferrous protein, at pH 6.8 (B) 13 mM Ferric protein, at pH 6.7.

* In this report, the amino-acid sequence positions in cytochromes *c* from all species are designated by the corresponding positions in the sequence of mammalian-type cytochromes *c*

[16]. We have observed that the sharp resonance at 54.0 ppm in the natural-abundance ^{13}C NMR spectrum of *Candida krusei* ferrocytochrome *c* (fig. 1B) also moves downfield (by about 0.4 ppm) when going to the ferric protein (see fig.2). This result indicates that this peak does not arise from a contaminant.

The chemical shift of the resonance under consideration is characteristic of a trimethylammonium group or an α-carbon. The small linewidth and large NOE indicate rapid internal rotation, inconsistent with an assignment to an α-carbon.The change in chemical shift upon oxidation of the iron (fig.2) is the expected behavior for the methyl carbon resonance of ϵ-*N*-trimethyllysine-72 [16]. On the basis of the above evidence we assign the peak at 54.0 ppm in fig.1B to the methyl carbons of ϵ-*N*-trimethyllysine-72 of *Candida krusei* ferrocytochrome *c*. Residue 72 lies in the evolutionary invariant region of residues 70–80 [10,17]. This region of the polypeptide chain has been suggested as the cytochrome *c* reductase binding site [18–20]. The relatively resolved resonance of the trimethylammonium carbons of ϵ-*N*-trimethyllysine-72 may be a convenient probe of the function of the methylated residue. ^{13}C NMR spectroscopy may also be used be used to determine the number of ϵ-*N*-trimethyllysine residues in a small protein.

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References

[1] Allerhand, A., Childers, R. F. and Oldfield, E. (1973) Biochemistry 12, 1335–1341.
[2] Oldfield, E., Norton, R. S., and Allerhand, A. (1975) J. Biol. Chem. 250, 6368–6380.
[3] Oldfield, E., Norton, R. S. and Allerhand, A. (1975) J. Biol. Chem. 250, 6381–6402.
[4] Norton, R. S., Clouse, A. O., Addleman, R. and Allerhand, A. (1977) J. Am. Chem. Soc., in the press.
[5] Narita, K., and Titani, K. (1968) J. Biochem. (Tokyo) 63, 226–241.
[6] DeLange, R. J., Glazer, A. N. and Smith, E. L. (1970) J. Biol. Chem. 245, 3325–3327.
[7] Olfield, E. and Allerhand, A. (1973) Proc. Natl. Acad. Sci. USA 70, 3531–3535.
[8] Wilbur, D. J., Norton, R. S., Clouse, A. O., Addleman, R. and Allerhand, A. (1976) J. Am. Chem. Soc. 98, 8250–8254.
[9] Freeman, R., Hill, H. D. W. and Kaptein, R. (1972) J. Magn. Reson. 7, 327–329.
[10] Dayhoff, M. O. (ed) (1972) Atlas of Protein Sequence and Structure, Vol 5, The National Biomedical Research Foundation, Silver Spring, Maryland, USA.
[11] Glushko, V., Lawson, P. J. and Gurd, F. R. N. (1972) J. Biol. Chem. 247, 3176–3185.
[12] Keim, P., Vigna, R. A., Morrow, J. S., Marshall, R. C. and Gurd, F. R. N. (1973) J. Biol. Chem. 248, 7811–7818.
[13] Narita, K., private communication.
[14] Doddrell, D., Glushko, V. and Allerhand, A. (1972) J. Chem. Phys. 56, 3683–3689.
[15] Williams, E., Sears, B., Allerhand, A. and Cordes, E. H. (1973) J. Am. Chem. Soc. 95, 4871–4873.
[16] Eakin, R. T., Morgan, L. O. and Matwiyoff, N. A. (1975) Biochem. J. 152, 529–535.
[17] Nolan, C. and Margoliash, E. (1968) Annu. Rev. Biochem. 37, 727–790.
[18] Takano, T., Kallai, O. B., Swanson, R. and Dickerson, R. E. (1973) J. Biol. Chem. 248, 5234–5255.
[19] Smith, L., Davies, H. C., Reichlin, M. and Margoliash, E. (1973) J. Biol. Chem. 248, 237–243.
[20] Margoliash, E., Ferguson-Miller, S., Tulloss, J., Kang, C. H., Feinberg, B. A., Brautigan, D. L. and Morrison, M. (1973) Proc. Natl. Acad. Sci. USA 70, 3245–3249.