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Identification of the C2-^{1}H histidine NMR resonances in chloramphenicol acetyltransferase by a ^{13}C-^{1}H heteronuclear multiple quantum coherence method

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Chloramphenicol acetyltransferase (CAT) was used to assess the feasibility of study of specific proton resonances in an enzyme of overall molecular mass 75000. [*ring* 2-^{13}C]Histidine was selectively incorporated into the type III chloramphenicol acetyltransferase (CAT_{III}) using a histidine auxotroph of *E. coli*. Heteronuclear multiple and single quantum experiments were used to select the C2 protons in the histidyl imidazole ring. One- and two-dimensional spectra revealed six signals out of a total of seven histidine residues in CAT_{III}. pH titration, chemical modification and ligand binding were used to demonstrate that the signal from H195, the histidine at the active site, is not among those observed. Nevertheless, this work demonstrates that selective isotopic enrichment and multiple quantum coherence techniques can be used to distinguish proton resonances in a protein of high molecular mass.

Chloramphenicol acetyltransferase; Heteronuclear multiple quantum coherence

1. INTRODUCTION

Chloramphenicol acetyltransferase (CAT; EC 2.3.1.28) is a homotrimer which catalyses the acetyl-CoA-dependent *O*-acetylation and inactivation of chloramphenicol, a modification which confers resistance to the antibiotic on host cells [1]. From the three-dimensional X-ray crystal structure of the type III variant of CAT (CAT_{III}), it is known that N3 of the imidazole ring of H195 is suitably placed to act as the proposed base catalyst in the reaction [2,3]. Study of the histidine C2-^{1}H resonances in CAT is complicated by its overall molecular mass incorporation in combination with single and multiple quantum coherence experiments has been particularly valuable in the simplification of ^{1}H spectra of smaller proteins by [4–7]. We sow here that these methods permit detection of histidine C2-^{1}H resonances even in proteins as large as CAT.

2. EXPERIMENTAL

[*ring*2-^{13}C]L-Histidine at 92% ^{13}C was obtained from Fluorochem (Old Glossop, Derbyshire, UK) and a 62% ^{13}C from Drs R. Gigg and J. Feeney (NIMR, Mill Hill, London). An *E. coli* histidine auxotroph (strainJM1100 from Dr R.A. Cooper [8]) was used to selectively incorporate labelled histidine into CAT. Transformation with the plasmid pUC18:IM3:ClaI, which produces high expression of CAT_{III} in *E. coli* [9], was conducted using a $CaCl_2$ procedure [10]. Optimal growth conditions for production of CAT_{III} were found to be in a medium containing (in g/l), Na_2HPO_4 6.0, KH_2PO_4 3.0, NaCl 0.5, NH_4Cl 1.0, $MgSO_4$ 0.49, thymine 0.1, glycerol 4.0, L-arginine, 0.28, L-asparatic acid 0.74, L-cysteine 0.20, L-glycine 0.20, L-glutamic acid 2.8, L-isoleucine 0.54, L-leucine 0.70, L-lysine 0.74, L-methionine 0.20, L-phenylalanine 0.14, L-proline 0.80, L-threonine 0.60, L-tryptophan 0.26, L-tyrosine 0.62, L-valine 0.82, L-alanine 0.35, L-serine 0.20, L-asparagine 0.23, L-glutamine 0.20. In addition, ampicillin and chloramphenicol were added to 100 μg/ml. [*ring* 2-^{13}C]L-Histidine was added at 40 μg/ml, which was sufficient to support growth to a final optical density of 4.5 at 600 nm. CAT production was highly variable; between 30 and 140 mg protein per litre of culture. Growth was initiated by inoculation of 500 ml of medium in a 2 litre baffled flask with a starter culture of 10 ml and grown overnight at 37°C with continuous shaking. Cells were then harvested and CAT purified as described previously [11]. For NMR analysis CAT was transferred into 50 mM Na_2HPO_4/NaH_2PO_4 in 99.9% D_2O by successive concentration and dilution over an Amicon YM10 membrane.

All NMR spectra were obtained on a Bruker AM500 spectrometer at 313K unless stated otherwise. An 'inverse' probe was used to obtain all the spectra. Typical pulse lengths were: 90°(^{1}H) = 12 μs, 90°(^{13}C) = 10 μs, 90°(^{13}C for low power decoupling) = 70 μs. Low-power X-nucleus decoupling was afforded using a Bruker 5W BFX-5 linear amplifier. The pulse sequences used were chosen for their simplicity, thereby minimizing the loss of magnetization due to relaxation during the pulse sequence. The 1D heteronuclear multiple quantum pulse sequence is: 90°(^{1}H)-1/2J-90°(X)180°(^{1}H)90°(X)-1/2J-acq.-, and for the 2D heteronuclear multiple quantum experiment: 90°(^{1}H)-1/2J-90°(X)-t_1/2-180°(^{1}H)-t_1/2-90°(^{1}H)-1/2J-acq., both sequences having phase cycling adapted from [12]. Solvent suppression in both the 1D and 2D experiments was afforded by using a low-power pre-irradiation pulse during the relaxation period. Either the GARP [13] or the WALTZ-16 decoupling sequence was used for X-nucleus decoupling. Typically, for 1D datasets, 500 scans were obtained; for 2D datasets, 2K × 256 data matrices were accumulated, with 196 scans per t_1 value.

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Abbreviations: CAT, chloramphenicol acetyltransferase; HMQC, heteronuclear multiple quantum coherence

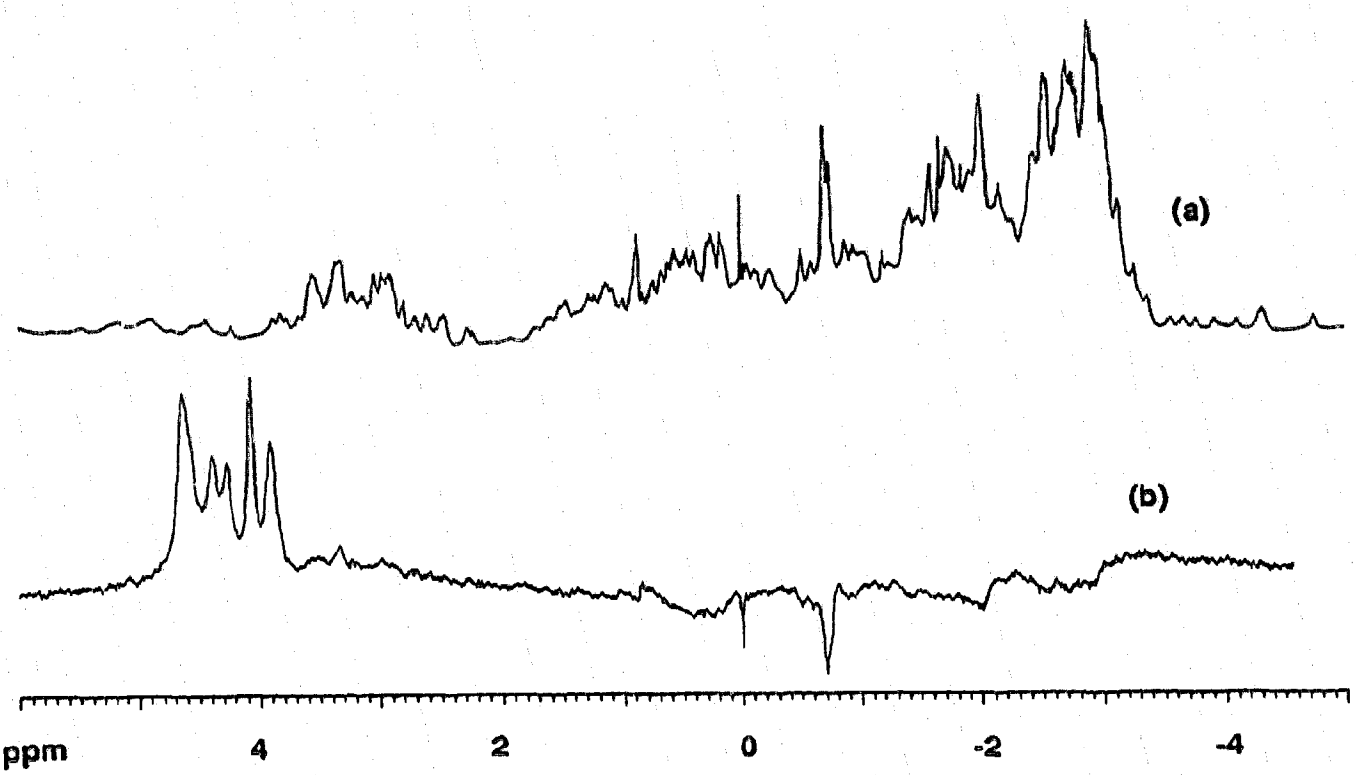

Fig. 1. 600 MHz ^{1}H NMR spectrum of [*ring* 2-^{13}C]histidine-labelled CAT (25 mg/ml) in 20 mM Na_2HPO_4/NaH_2PO_4, 0.1 M NH_4Cl (pH* = 7.5)/D_2O at 313 K. (a) Normal proton spectrum, obtained with presaturation of the HOD peak and referenced to dioxane at 0 ppm. (b) Proton detected ^{13}C-edited HMQC spectrum showing the filtered histidine C2 protons between −5 and 6 ppm. Other details are given in the experimental section.

3. RESULTS AND DISCUSSION

Previous attempts to identify the histidine C2-^{1}H resonances directly in the ^{1}H NMR spectrum of CAT_{III} met with only very limited success [14]. The results in Fig. 1 clearly demonstrate the utility of the labelling procedure and the heteronuclear ^{13}C-^{1}H multiple quantum coherence method (HMQC) in selecting the histidine C2-^{1}H signals. Five resonances are readily identified in the HMQC ^{13}C edited spectrum (Fig. 1b), out of a total of seven histidines in the translated nucleotide sequence of CAT_{III} [9]. The two-dimensional heteronuclear correlation spectrum of the sample further separated the histidine C2-^{1}H signals in the ^{13}C dimension, allowing six resonances to be distinguished (Fig. 2). The signal at F1 = 137.4 ppm and F2 = 4.60 ppm is broad and can only be observed at very low contour levels, or in 1D spectra at a lower pH* (Fig. 3). If the 2D experiment is carried out in NH_4Cl under the same conditions as Fig. 1, the signal is much sharper.

Examination of the sample at a lower pH* revealed that three of the signals had shifted (Fig. 2b). Complete titration curves for a range of pH* values from 6.0 to 8.0 were obtained using a series of one dimensional spectra (Fig. 3). A series of experiments were set up to attempt to assign H195, the active site histidine, to one of these. Addition of a small excess of chloramphenicol over the concentration of CAT subunits did not alter the ^{1}H or ^{13}C resonances of any of the signals (Fig. 2c). However, addition of a five fold excess of chloramphenicol did cause one resonance to change its ^{1}H and ^{13}C chemical shifts (Fig. 2d- the sharp signal at 3.87 and 138 ppm is from chloramphenicol). Assuming a dissociation constant for chloramphenicol of 3.7 μM [14], the active site of the enzyme should be greater than 90% saturated under both conditions. It is likely, therefore, that the effect of chloramphenicol at a higher concentration is caused by binding to a secondary lower affinity site. There is some evidence to support the existence of such a site from a crystallographic analysis of the binding of P-iodochloramphenicol [3].

Chemical modification of the ^{13}C-labelled enzyme was carried out using 3-(bromoacetyl)chloramphenicol, which specifically alkylates H195 at the N3 position on the imidazole ring [15]. Subsequent incubation of the enzyme under mild alkaline conditions leads to hydrolysis of the oxy-ester and removal of the chloramphenicol moiety, to yield the N3 (H195) carboxymethylated derivative of CAT. Examination of the spectra of the modified enzyme at low and high pH* revealed that there were no significant changes in the ^{1}H and ^{13}C chemical shifts, although the two broad signals at 4.55 and 4.35 ppm were much better resolved (Fig. 2e

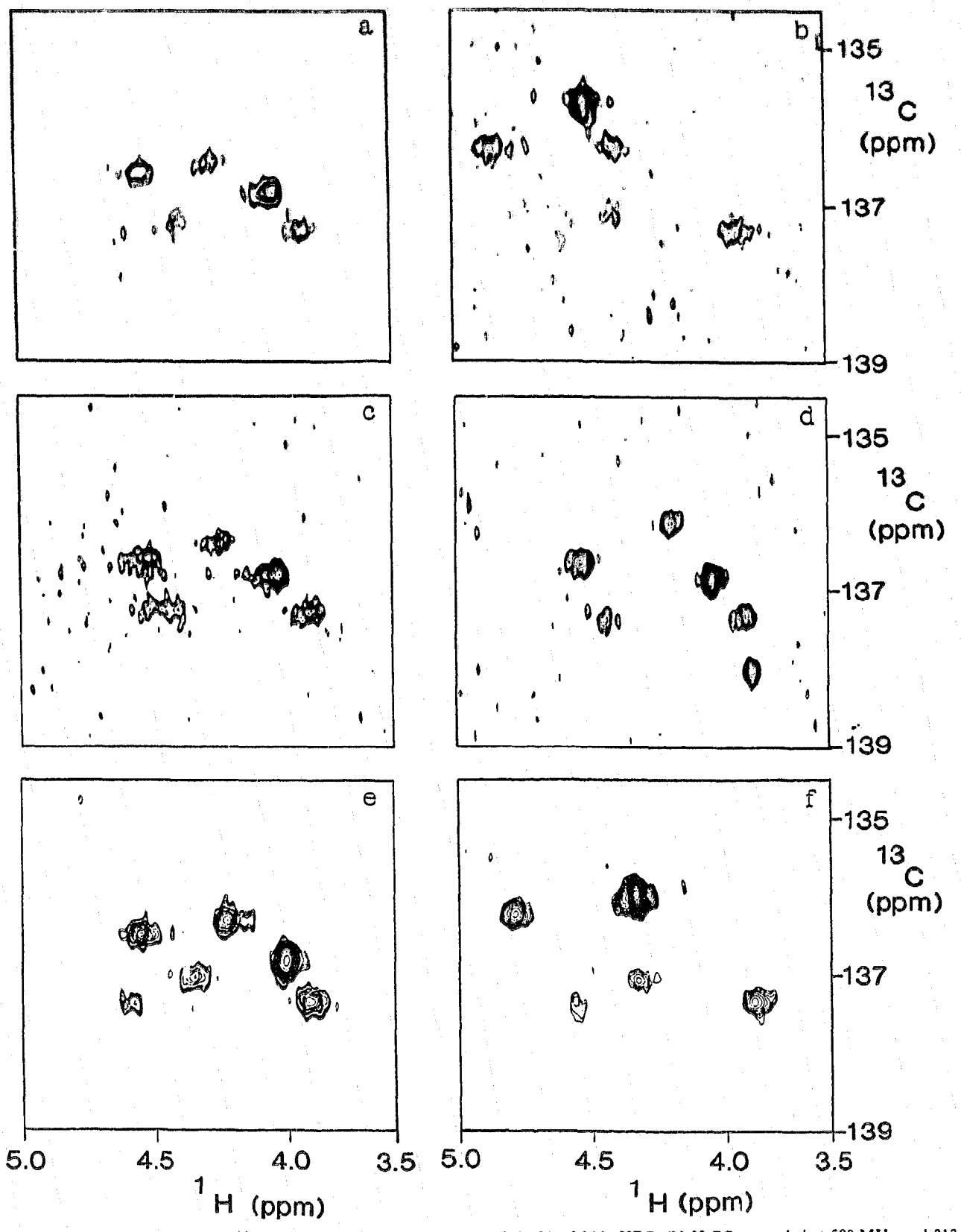

Fig. 2. 2D HMQC spectra of [*ring* 2-^{13}C]histidine-labelled CAT (25 mg/ml) in 50 mM Na_2HPO_4/NaH_2PO_4 recorded at 500 MHz and 313 K. (a) pH* = 7.50; (b) pH* = 6.24; (c) plus 1.3 mM chloramphenicol (pH* = 7.50); (d) plus 5.0 mM chloramphenicol (pH* = 7.50); (e) N3 (H195) carboxymethylated derivative (pH* = 7.49); (f) N3 (H195) carboxymethylated derivative (pH* = 6.46). Dioxane was used as a reference in both F1 (67.4 ppm) and F2 (0 ppm).

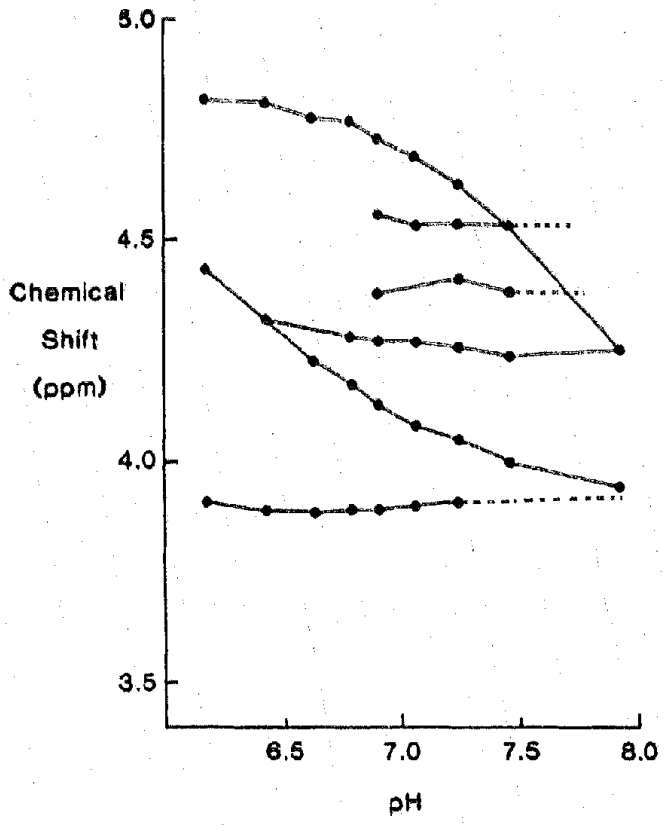

Fig. 3. pH* titration of the histidine C2 protons in CAT, taken from successive 1D HMQC spectra under the same conditions as Fig. 2. Precipitation precluded analysis below pH* 6.0. The signals at 4.55 ppm and 4.35 ppm were not detectable at low pH*.

and f). The titration behaviour was identical with that of the unmodified enzyme. These results indicate that the C2-^{1}H resonance of H195 is not among those observed, presumably because it is too broad to be detected. This could arise from exchange broadening, perhaps due to slow exchange between protonated and unprotonated imidazole [16]. Exchange of the C2-^{1}H with solvent was ruled out as a possible cause; the 1D HMQC spectra were identical in H_2O and D_2O. Nonetheless, it is clear that six of the histidine C2-^{1}H resonances in CAT can be clearly identified. Specific isotope labelling and multiple quantum coherence techniques can therefore be used to identify individual ^{1}H NMR signals in a protein of overall molecular mass 75 000.

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