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# Fluorine-19 Magnetic Resonance of Muscle Calcium Binding Parvalbumin: pH Dependency of Resonance Position and Spin–Lattice Relaxation Time

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The muscular parvalbumins are low molecular weight (mol. wt. ≅ 11,500), acidic proteins which bind two calcium ions with high affinity ($PK_q \cong 5$ to 7) [1, 2]. The crystal structure of one of the parvalbumin isotypes isolated from the common mirror carp (*Cyprinus carpio*) has been solved and refined to 1.9 Å resolution [3]. The specific function of muscular parvalbumins is not known; however, they appear to be calcium-modulated proteins [4], a class of high affinity calcium-binding proteins which also includes the calcium-binding component of troponin, the alkaline-extractable light chain of myosin and the cyclic-AMP phosphodiesterase activator protein [4]. It is of particular interest that Potter *et al.* [5] has recently shown that parvalbumins isolated from carp can activate rat brain phosphodiesterase in a calcium-dependent manner.

A complete understanding of the function of the muscular parvalbumin will be dependent on knowledge of the precise nature of calcium-induced conformational events. One approach to this problem involves the covalent attachment of sensitive magnetic resonance probes to functionally important sites in the protein molecule. In this communication we report on the covalent attachment of a trifluoroacetonyl group to the single sulfhydryl (Cys-18) of muscle calcium binding parvalbumin from carp. Cys-18 is a particularly interesting residue since it lies in close proximity to a potentionally important internal ionic bond formed between the carboxyl group of Glu-81 and the guanido group of Arg-75. The results of a number of investigations have suggested the cleavage of this linkage with the release of one of the two bound calcium ions [6, 7]. In this report, emphasis is placed on the procedure for labeling the muscular parvalbumins with 1,1,1-trifluoro-3-bromo propanone and on the interesting and unexpected pH-dependency of the resulting fluorine resonance position and the spin–lattice relaxation time ($T_1$).

## Experimental

### *Materials and Methods*

Carp muscle calcium binding parvalbumin was isolated from the white muscle of common mirror carp according to the method of Pechere *et al.* [8]. 1,1,1-Trifluoro-3-bromo propanone was purchased from Peninsular Chem-Research Inc.. 2-Chloromercuri-4-nitrophenol, used to quantitate the extent of the sulfhydryl group labeling, was obtained from Eastman Organic Chemicals. All other chemicals were high grade commercial products. Protein concentrations were determined by ultraviolet absorbance at the 259 nm pehnylalanine maximum ($\epsilon \cong 2000$) on a Cary 14 recording spectrophotometer. Amino acid composition analyses were performed on a Beckman Model 120 C amino acid analyzer. Circular dichroism analyses were performed on a Jasco J10B instrument.

Fluorine-19 magnetic resonance spectra were obtained on the JEOL PS-PFT-100 P/EC 100 Fourier transform spectrometer, operating at 94 MHz and 23 °C. For most $^{19}$F-NMR experiments a 5 kHz spectral window, 8K data points and a flip-angle of 90° was employed. All fluorine chemical shifts are reported from trifluoroacetic acid (TFA), which was used as an external reference. Protein concentrations in the NMR experiments were typically 7 m*M* in 0.8 ml sample volumes, employing 10 mm NMR tubes. The spectrometer was low field frequency locked on the deuterium resonance from solvent $D_2O$. The pH values reported are direct measurements uncorrected for the presence of deuterium in the solvent.

### *Preparation of Fluorine-Labeled Muscular Parvalbumin*

Approximately 120 mg of carp parvalbumin isotype 5 (MCBP-5) was dissolved in 3 ml of 8 *M* urea, 0.05 *M* EGTA (pH = 7.0). After two hours of gentle mixing, a two-fold molar excess of 1,1,1-trifluoro-3-bromo propanone was added. The pH was maintained at 7.0 with the addition of small amounts of 2 *M* NaOH. After 30 minutes of incubation, the reaction mixture was transferred to a dialysis sac, and the solution was exhaustively dialyzed, first against 0.05 *M* $CaCl_2$ and then against 0.1 *M* $NH_4HCO_3$(pH = 7.8), conditions which renature the protein, as indicated by circular dichroism analysis. The selectivity and extent of sulfhydryl group labeling was determined both by amino acid composition analyses and by reaction with the sulfhydryl reagent, 2-chloromercuri-4-nitrophenol. Under conditions that readily label native parvalbumin with the sulfhydryl chromophoric reagent (8 *M* urea, 0.05 *M* EGTA and 40 °C), only trace reaction occurs with $^{19}$F-labeled parvalbumin, indicating essentially quantitative reaction of the Cys-18 sulfhydryl group with the fluorine reagent. Since the fluorine reagent could possibly react with the exposed $\epsilon$-amino group of lysine residues, although much less favorably than with sulfhydryl groups under our experimental conditions, amino acid composition analyses were performed on

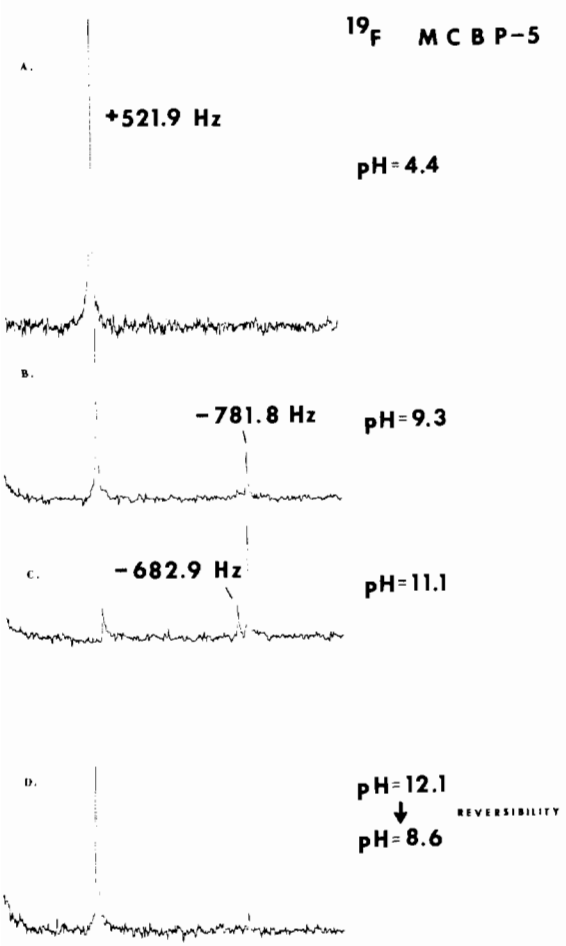


Figure 1. $^{19}$F-NMR spectra of trifluoroacetonylated parvalbumin as a function of pH. Experimental conditions: ~7 m*M* protein, 400 transients collected, external magnetic field increases from right to left. Spectrum "D" was obtained at pH = 8.6 after prior incubation of the sample at pH = 12.1. Further experimental details are given in the text.

both native and $^{19}$F-parvalbumin. These analyses confirmed that no side chains other than the single cysteine were labeled, within experimental error. Finally, in experiments with terbium(III)-substituted protein, it was established that sulfhydryl group labeling yields a protein with emission and circularly polarized emission spectra closely similar to those of native parvalbumin [9, 10].

## Results and Discussion

Fluorine-19 NMR spectra of trifluoroacetonylated muscle calcium binding parvalbumin at a number of pH values are shown in Figure 1. From pH = 4 to pH = 8 a single resonance is observed at about +522 Hz from TFA. Above pH = 8 a new, downfield peak at about −782 Hz emerges at the expense of the "low pH" resonance. A second, minor peak is also observed at slightly higher field (at about −683 Hz). The most interesting aspect of the resonance phenomena presented in Figure 1 is the apparent reversibility of the pH effects. Decreasing the pH from 12.1 to 8.6 results in an immediate loss of the major and minor "high pH" peaks and the re-emergence of the "low pH" resonance.

The data presented above could be accommodated by invoking the formation of a reversible Schiff base, involving the carbonyl function of the trifluoroacetonyl label and an available protein donor nucleophile. A covalent bonding event (*i.e.*, Schiff base formation) would be necessary to account for the >1000 Hz separation of the two major peaks present at intermediate pH values. It seems very unlikely that a shift of this magnitude could result from either local ring current effects or van der Waals interactions with neighboring residues, since neither of these effects is likely to produce shifts greater than 100 to 200 Hz [11, 12]. The apparent $pK_a \cong 10.2$ for the development of the high pH signal immediately suggests the involvement of a lysine $\epsilon$-amino function (*i.e.*, $pK_{a,Lys} \cong 10.4$) for the protein donor nucleophile. An examination of the crystal structure reveals that one and only one lysine side chain is in close proximity to the Cys-18 side chain [3] and hence to the attached trifluoroacetonyl group: lysine-32. In fact, by suitable rotations about the side chain bonds of Lys-32, the $\epsilon$-amino nitrogen can easily approach to within bonding distance of the carbonyl function of the trifluoroacetonyl probe attached to the Cys-18 sulfur atom, without appreciable distortion of the protein structure. The two "high pH" resonances could be accommodated by assuming a significant population of two products formed from the alternative tetrahedral intermediates which can result from donor nucleophile attack at the carbonyl function of the covalently attached fluorine probe.

It is possible to further examine the hypothesis of Schiff base formation, since such a linkage could be converted to an irreversible covalent bond by an appropriate reducing agent [12]. The fluorine-19 resonance from such a reduced species would be relatively pH-independent. Figure 2 shows $^{19}$F-NMR spectra at two pH values following the reduction (at pH = 9.5) of trifluoroacetonylated parvalbumin with a slight excess of $NaBH_4$. The results seem to support the hypothesis of Schiff base formation. A resonance appears at a new position and at roughly the same frequency for both the high and low pH cases (*i.e.*, the pH = 7.35 and pH = 10.10 resonances occur at +156.3 Hz and +146.5 Hz, respectively).

Figure 3 presents the results of fluorine-19 spin–lattice relaxation time ($T_1$) measurements on trifluoroacetonylated parvalbumin. The experiments were performed at an intermediate pH (pH = 9.3), so that both low and high pH species would be signifi-

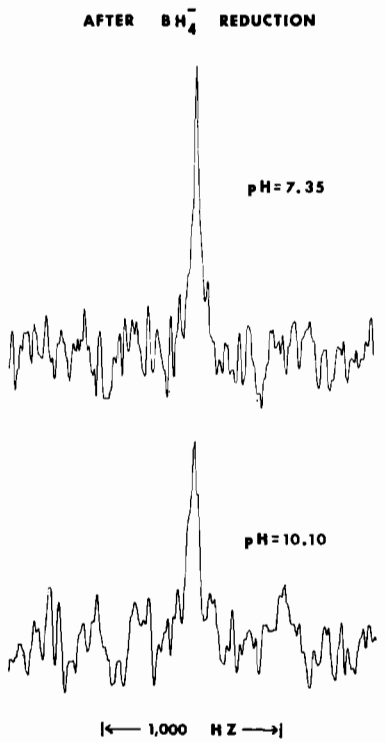


Figure 2. $^{19}$F-NMR spectra of trifluoroacetonylated parvalbumin at two pH values following reduction of the labeled protein with a slight excess of sodium borohydride at pH = 9.5. Experimental conditions: ~0.7 m*M* protein, 2000 transients collected.

cantly represented. The first point of interest concerns the magnitudes of the relaxation times, which are relatively short for both populations of fluorine nuclei. This result suggests that both signals derive from fluorine nuclei tightly associated with a macromolecule (*i.e.*, the high pH resonance does not derive from a cleavage product). The fluorine-19 $T_1$ for the label in the absence of macromolecule under similar experimental conditions is about 2.3 sec, an order of magnitude higher. Secondly, although the spin–lattice relaxation times are of the same order of magnitude for the two populations of protein-bound fluorines, there is a small but significant difference in their values (*i.e.*, $T_1$ (low pH resonance) $\cong$ 0.13 sec and $T_1$ (high pH resonance) $< 0.20$ sec). The lower efficiency of the dipolar relaxation process at higher pH is most likely attributable to the expected expansion of the protein, and concomitant increase in the degree of motional freedom, as the net negative charge on the molecule increases substantially with the deprotonation of the thirteen lysine side chains. This interpretation is dependent on the dipole-dipole mechanism dominating overall spin relaxation. This is a reasonable assumption, since $T_1$-temperature dependency studies indicate that the dipolar interaction predominates even for the free label in solution (*i.e.*, $T_1$ decreases linearly with temperature over the range 5 °C to 60 °C [13]). There is no detectable contribution from the alternative spin–rotation mechanism, which would give rise to a positive $T_1$-temperature dependency.

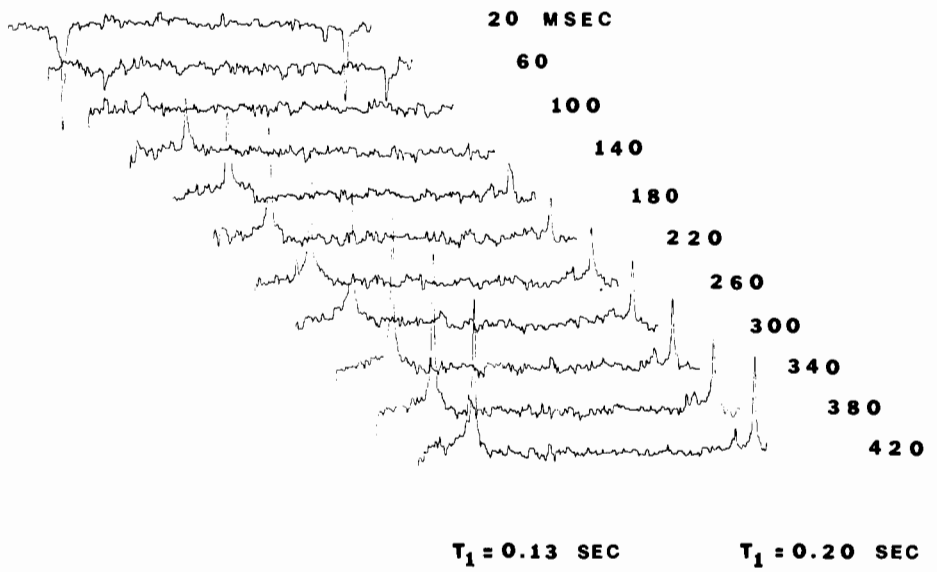


Figure 3. $^{19}$F-spin lattice relaxation time measurements. Experimental conditions: ~7 m*M* protein, 200 transients collected per spectrum, pH = 9.3.

The results presented in this communication show that it is possible to covalently label the single sulfhydryl group of carp muscle calcium binding parvalbumin with a fluorine-19 magnetic resonance probe. The labeled protein shows similar α-helical content and metal binding properties to the native parvalbumin. It is now possible to employ this $^{19}F$-labeled protein in static and dynamic studies of calcium ion induced conformational events. In addition, an interesting and unexpected result of the present $^{19}F$-NMR study is the apparent formation of a Schiff base between the carbonyl function of the fluorine label and the ε-amino group of a nearby lysine residue which, to the best of my knowledge, has not been reported previously for any protein system. Spin–lattice relaxation time measurements indicate (i) that the new resonance emerging at high pH (*i.e.*, that due to Schiff base species) derives from protein bound fluorine nuclei and (ii) that such $T_1$ measurements can be used to monitor local motions in various protein states, which will have utility in future dynamic studies.

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