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Interactions of Fluoroaniline with a Heme-octapeptide of Cytochrome *c*: a ^{19}F NMR Study*

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Abstract

Interactions of *ortho*-, *meta*- and *para*-fluoroaniline with a heme-octapeptide (H8PT) prepared from cytochrome *c* were studied by means of optical absorption and ^{19}F NMR. The binding constant (K_b) of *para*-fluoroaniline to H8PT was estimated from the Soret absorption spectral change to be 4.5×10^4 M^{-1}, which was larger than the K_b values of *ortho*- (1.8×10 M^{-1}) and *meta*-fluoroaniline (1.2×10 M^{-1}) by three orders. On adding H8PT to the fluoroaniline solutions, the ^{19}F NMR bands of *ortho*- and *meta*-fluoroaniline shifted to a lower magnetic field region by a chemical shift of nearly 100 Hz together with a small band-broadening, while that of *para*-fluoroaniline was shifted to a higher magnetic field by a chemical shift of more than 300 Hz together with heavy line-broadening up to nearly 300 Hz in half-band-width. The line-broadening and the low-field movement of the chemical shift of the ^{19}F NMR signal of *para*-fluoroaniline on adding H8PT were ascribed to the binding of *para*-fluoroaniline to the H8PT on the basis of the K_b values estimated from the optical absorption change. Assuming the fast chemical exchange regime, the exchange rate of *para*-fluoroaniline to H8PT and the residence time of *para*-fluoroaniline in the bound state were estimated to be 5.3×10^2 s^{-1} and 3.0×10^{-4} s, respectively.

Introduction

In a heme-octapeptide (H8PT) prepared from cytochrome *c* by trypsin digestion, the heme is linked to the octapeptide (one His, Thr, Ala, and Ile and two Glu and Cys) with two covalent bonds. The heme in H8PT has an internal (5th) imidazole axial ligand and a vacant 6th axial position and is not surrounded by an apoprotein like cytochrome *c*. Since the apoprotein part does not influence the binding behavior of the ligand in H8PT, this system is useful for studying ligand-heme interactions [1, 2, 3]. Aniline is a ligand of the heme iron complex and one of the substrates of a heme enzyme collectively named as cytochrome P-450 [4]. A study of the interaction of aniline with H8PT would provide significant and fundamental probes for further study of the interaction of the substrate with the hemoprotein.

In this report we discuss the optical absorption and ^{19}F NMR spectral behavior of *ortho*-, *meta*- and *para*-fluoroaniline in the presence of H8PT. We have estimated the binding constants (K_b) of these fluoroanilines to H8PT by using the Soret absorption change of H8PT. It was found that the ^{19}F NMR spectra of only *para*-fluoroaniline changed much on adding H8PT, in contrast to those of *ortho*- and *meta*-fluoroaniline. We have estimated the exchange parameters of the ligand on the basis of the ^{19}F NMR findings. Since the ^{19}F NMR spectrum of the axial ligand of the paramagnetic heme complexes is not hampered by the solvent and amino acid signals, ^{19}F NMR spectra are very useful for studying the interaction of the fluorinated substrate to the enzyme.

Experimental

The H8PT was prepared from *Candida krusei* cytochrome *c* by trypsin digestion, as previously described [1]. Amino acid analyses show His 1.11, Thr 1.00, Glu 2.15, Ala 1.03, Cys 1.60, Ile 0.99 and other amino acids below 0.06. The cysteine content was apparently lower than expected because of technical problems. *Ortho*- and *meta*-fluoroaniline were purchased from Aldrich and *para*-fluoroaniline from Wako Pharm. Co. (Osaka). Values of pH were kept at 7.2 in a 50% ethylene glycol–0.2 M potassium phosphate buffer. Ethylene glycol was added to avoid aggregation of the H8PT [3, 4]. For the ^{19}F NMR spectra we used a 50% ethylene glycol–0.2 M potassium phosphate buffer using D_2O (pD 7.2).

Optical absorption spectra were obtained at room temperature on a JASCO UVIDEC-510 digital-

*Abbreviations used are: H8PT, a heme-octapeptide prepared from cytochrome *c* by trypsin digestion; NMR, nuclear magnetic resonance; MCD, magnetic circular dichroism.

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recording spectrometer with cuvettes of 10 mm optical path. NMR spectra were recorded at 282.2 MHz on a Bruker CXP-300 FT NMR spectrometer. Typical spectra consisted of 100–10 000 transients using 8 k data points over 4000 Hz spectral band-widths. The signal/noise ratios were improved by exponential multiplication which introduces 1–10 Hz line broadening. Sample temperature was maintained at 298 ± 0.5 K. Other conditions were the same as those reported [5, 6]. Chemical shift values were obtained in Hz by reference to an internal standard of 2-fluoroethanol, thereby eliminating any susceptibility effects. In the Figures in this paper we normalized each of the original chemical shifts of *ortho*-, *meta*- and *para*-fluoroaniline to be zero.

Results and Discussion

The Soret absorption of H8PT was decreased by adding *ortho*-, *meta*- and *para*-fluoroaniline. In the absence of fluoroaniline H8PT showed an MCD spectrum typical of high-spin complexes, while in the presence of fluoroaniline the spectrum was typical of low-spin complexes [7] (*cf.* Fig. B in Supplementary Material). From the Soret spectral changes of H8PT, binding constants (K_b) of fluoroaniline to H8PT were estimated on the assumption that a 1:1 fluoroaniline: H8PT complex was formed (Table I). It was found that the K_b (4.5×10^4 M^{-1}) of *para*-fluoroaniline to H8PT is higher by three orders than those ($1.2–1.8 \times 10$ M^{-1}) of *ortho*- and *meta*-fluoroaniline.

TABLE I. Binding Constants (K_b) of Fluoroaniline to H8PT, Estimated from Optical Absorption Change

Species	K_b (M^{-1})
ortho-fluoroaniline	1.8×10
meta-fluoroaniline	1.2×10
para-fluoroaniline	4.5×10^4

Changes in ^{19}F NMR spectra of fluoroaniline in the presence of H8PT were studied. Figure 1 shows the ^{19}F NMR change caused by adding H8PT. On addition of H8PT to the fluoroaniline solutions, the ^{19}F NMR signals of *ortho*- and *meta*-fluoroaniline moved to lower magnetic fields by a chemical shift of nearly 100 Hz, together with small line-broadenings, while the ^{19}F NMR signal of *para*-fluoroaniline moved to a higher magnetic field by a chemical shift of nearly 360 Hz together with heavy line-broadening up to 300 Hz in half-band-width. Nuclear spin–lattice and spin–spin relaxation times of the *para*-fluoroaniline–H8PT complex are markedly shorter than those of free *para*-fluoroaniline. The changes in the ^{19}F NMR chemical shifts of the *para*-fluoroaniline–

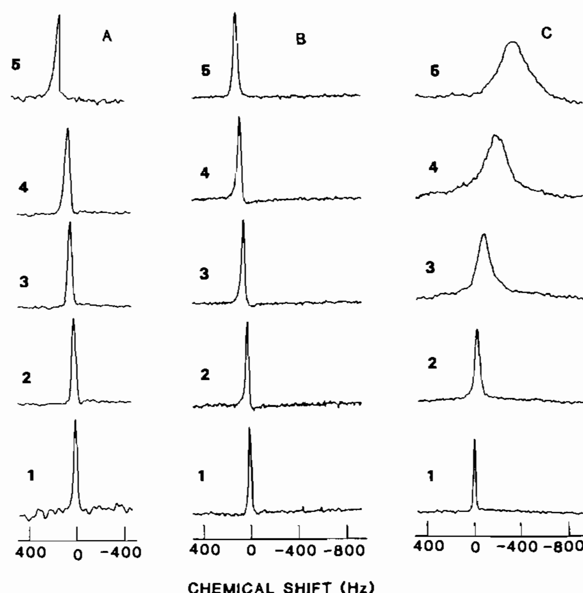

Fig. 1. ^{19}F NMR spectra of 5 mM *ortho*-, *meta*- and *para*-fluoroaniline in the absence (1) and in the presence of 0.31 mM (2), 0.94 mM (3), 1.88 mM (4) and 3.77 mM (5) of H8PT. Spectral conditions are described in the 'Experimental'.

H8PT complex represent a weight average of those of free and coordinated aniline, suggesting that ligand exchange is very fast on the NMR time scale. Taking account of the high binding ability of *para*-fluoroaniline to H8PT, nearly 99% of the added H8PT will be bound to *para*-fluoroaniline under our NMR conditions. Assuming the fast chemical exchange regime [8], the exchange rate of *para*-fluoroaniline to H8PT was estimated to be 5.3×10^2 s^{-1}. The residence time of *para*-fluoroaniline thus was evaluated to be 3.0×10^{-4} s. Due to the very low binding abilities of *ortho*- and *meta*-fluoroaniline to H8PT, no clear NMR parameters about the *ortho*- and *meta*-fluoroaniline interactions with H8PT were evaluated in this study.

It was very interesting to note that the binding and spectral behavior of *para*-fluoroaniline were different from those of *ortho*- and *meta*-fluoroaniline. The electronic character of *para*-substituted fluorine must be the same as that of *ortho*-substituted fluorine. The stereo-specific character of *ortho*-substituted fluorine may contribute to the abnormal binding behavior of *ortho*-fluoroaniline to H8PT. The spin imbalance reaching the *para*-fluorine of aniline through σ or π orbitals (hyperfine contact) may be relatively strong compared to that reaching the *ortho*- and *meta*-fluorines, while the order of the dipolar coupling between the paramagnetic iron and the ligands may be *ortho* > *meta* > *para* [1].

Since ^1H NMR signals of axial ligands of paramagnetic hemoproteins are hard to observe because of overlap of the solvent and protein signals, ^{19}F

NMR is very useful for investigating ligand exchange in the paramagnetic hemoprotein.

Supplementary Material

Figures showing (A) Soret absorption spectral titration of H8PT on adding fluoroaniline; (B) absorption and MCD spectra of H8PT in the absence and presence of fluoroaniline; (C) ^{19}F NMR change in half-band-width; (D) ^{19}F NMR changes in chemical shift (6 pages) are stored with the Editors-in-Chief in Padua.

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