Interaction of Fluoroaniline with Cytochrome P-450$_{scc}$ and Myoglobin: Temperature and pH Dependence Studies*

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Abstract

Bindings of fluoroaniline to cytochrome P-450$_{scc}$ (P-450$_{scc}$) and metmyoglobin (metMb) were studied in terms of optical absorption at various temperatures and pH values. The following results were obtained. (i) P-450$_{scc}$ formed a low-spin complex on addition of fluoroaniline, while metMb remained in the high-spin state. (ii) The affinities of fluoroaniline compounds to P-450$_{scc}$ are more marked than those to metMb. (iii) The affinity of *para*-fluoroaniline to P-450$_{scc}$ is more marked than that of *ortho*- and *meta*-fluoroaniline. (iv) The temperature dependences of K_b values of fluoroaniline were more prominent for P-450$_{scc}$ than those for metMb, suggesting that the heme environment of P-450$_{scc}$ is more labile to temperature compared to that of metMb. (v) Enthalpy–entropy compensation effects were observed with fluoroaniline bindings to P-450$_{scc}$ and metMb. The correlation coefficients (r) of 0.986 and 0.998 and isokinetic temperatures (β) of 420 K and 571 K were calculated for P-450$_{scc}$ and metMb, respectively. (vi) Increasing pH value enhances the affinity of fluoroaniline to both P-450$_{scc}$ and metMb, which is in contrast to the binding of cholesterol to P-450$_{scc}$.

Introduction

metMyoglobin (metMb) has a nitrogen atom of an imidazole of the proximal histidine as its 5th axial ligand and the 6th axial position of metMb is occupied by a water molecule or is vacant. On the other hand, cytochrome P-450 is a protoheme-containing protein having a thiolate ligand at its 5th axial position and the 6th axial position of cytochrome P-450 is occupied by a water molecule or is vacant [1, 2]. Since the spin state of the heme moiety is associated with the substitution of the 6th axial ligand, ligand substitution results in changes in optical and magnetic properties [2–6]. Changes in these properties of P-450 may also be influenced by changes in pH [7] and temperature [8, 9].

As cytochrome P-450$_{scc}$ (P-450$_{scc}$) catalyzes the side-chain cleavage of cholesterol, physicochemical studies have been focused mostly on the steroidal substrates [3, 8, 10–12]. P-450$_{scc}$ utilizes oxygen for the hydroxylation of substrates, whereas metMb acts as an oxygen carrier in physiological functions. Hence, the differences in heme environments between the two hemoproteins should be interesting. Aniline is a good ligand for heme iron and one of the substrates for cytochrome P-450 type enzymes [2]. In our previous report we described the bindings of aniline to model compounds of heme proteins [13]. The study suggested the importance of further study of the interactions of aniline with heme proteins. Data on the binding of fluoroaniline to P-450$_{scc}$ and metMb have not, to date, been reported in correlation with the temperature and pH dependence.

Here we report temperature and pH dependence studies of the binding of *ortho*-, *meta*- and *para*-fluoroaniline to P-450$_{scc}$ and metMb by using optical absorption spectra. Binding constants of fluoroaniline to P-450$_{scc}$ and metMb were estimated as described previously [13]. Derived thermodynamic values for the binding of fluoroaniline to P-450$_{scc}$ and metMb are also reported.

Experimental

The high-spin form of P-450$_{scc}$ was purified electrophoretically to homogeneity from bovine adrenal cortex mitochondria by the method described previously [3]. Horse heart ferric myoglobin (metMb) was purchased from Sigma. *ortho*- and *meta*-fluoroaniline were purchased from Aldrich and *para*-fluoroaniline from Wako Pharmaceutical Co. (Osaka). Other reagents were of the highest guaranteed grade and were used without further purification. The pH values were maintained by 100 mM potassium phos-

*Abbreviations used are: P-450$_{scc}$, bovine adrenal cytochrome P-450$_{scc}$; metMb, horse heart metmyoglobin; K_b, binding constant; r, correlation coefficient; β, isokinetic temperature; $\Delta H°$, standard enthalpy; $\Delta S°$, standard entropy; $\Delta G°$, standard free energy.

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phate buffer solution. Optical absorption spectra were recorded on a Shimadzu UV-365 digital recording spectrophotometer equipped with a temperature controller, NESLAB RTE-9. Cuvettes of 10 mm optical path were used for the absorption measurements.

Results

Binding of Fluoroaniline to P-450$_{scc}$ and metMb

As observed in Fig. 1A, binding of *ortho*-fluoroaniline to P-450$_{scc}$ is associated with a decrease in the Soret absorption maximum and a shift of the peak from 393 nm to 417 nm with a clear isosbestic point at 417 nm. This is a characteristic spectral change of low-spin P-450$_{scc}$ complexes [2–4]. With the addition of excess *ortho*-fluoroaniline (more than 16.19 mM) the isosbestic point shifted (dotted line) with the formation of P-420$_{scc}$, a denatured form of P-450$_{scc}$. This denaturation was examined by the Soret absorption maximum of the CO-bound reduced denatured form of P-450$_{scc}$, P-420$_{scc}$, which appeared at 420 nm (not shown here) [2]. Addition of *meta*- and *para*-fluoroaniline to P-450$_{scc}$ solution caused essentially the same spectral change.

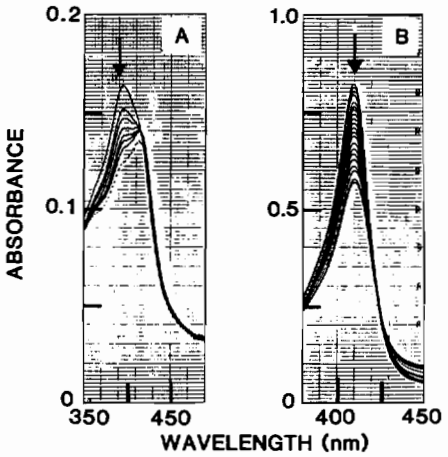

Fig. 1. Typical Soret absorption spectral change of: (A) P-450$_{scc}$ (1.50 μM) caused by addition of *ortho*-fluoroaniline (pH 7.2) at 20 °C) (the dotted line indicates the spectral deviation from the isosbestic point by adding more than 16.19 mM *ortho*-fluoroaniline); (B) metMb (5 μM) caused by addition of *meta*-fluoroaniline (pH 6.9) at 20 °C. Spectral conditions are described in 'Experimental'.

TABLE I. Binding Constants (K_b) (M^{-1}) of Fluoroaniline to P-450$_{scc}$ and metMb at Various Temperatures (pH 7.2)

Aniline Compounds	Temperature (°C)	K_b (M^{-1})	
		P-450$_{scc}$	metMb (×10)
ortho-Fluoroaniline	10	2.70 × 10	2.60
	20	1.42 × 10	1.40
	30	0.85 × 10	1.20
	37	0.68 × 10	0.95
meta-Fluoroaniline	10	1.50 × 10	1.35
	20	1.10 × 10	1.05
	30	0.70 × 10	1.00
	37	0.63 × 10	0.90
para-Fluoroaniline	10	2.90 × 10^2	3.00
	20	2.20 × 10^2	1.80
	30	6.25 × 10	1.30
	37	4.62 × 10	1.00

As presented in Fig. 1B, the binding of *meta*-fluoroaniline to metMb decreased the magnitude of the Soret absorption maxima (408 nm) with an isosbestic point at 424 nm. This spectral change does not indicate the formation of a low-spin fluoroaniline–metMb complex. The visible spectrum (not shown here) is also consistent with this conclusion. Spectral changes of *ortho*- and *para*-fluoroaniline with metMb were essentially the same as that observed for *meta*-fluoroaniline. Unlike P-450$_{scc}$, the spectral peak shift in metMb was not observed even on adding a large excess of fluoroaniline (up to 40.0 mM *ortho*-, 38.0 mM *meta*- and 42.0 mM *para*-fluoroaniline).

Effect of Temperature on K_b

In order to obtain the binding constants (K_b) at various temperatures, spectral titrations of *ortho*-, *meta*- and *para*-fluoroaniline with P-450$_{scc}$ and

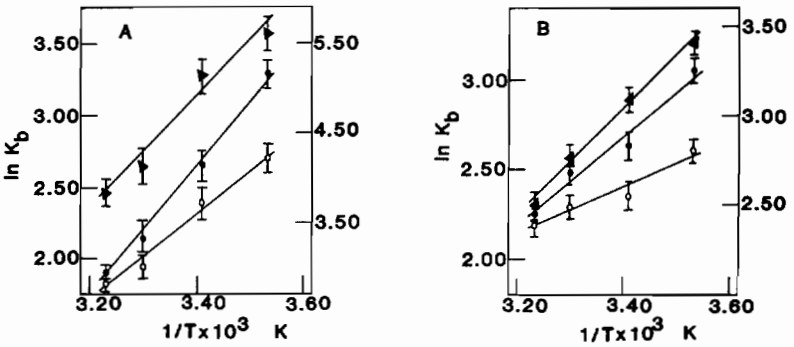

Fig. 2. Temperature-dependent bindings of (•) *ortho*-, (○) *meta*- and (▲) *para*-fluoroaniline to (A) P-450$_{scc}$ and (B) metMb at pH 7.2. For the binding of *para*-fluoroaniline to P-450$_{scc}$ and metMb, see the right-hand scale.

metMB were carried out at 10, 20, 30 and 37 °C and pH 7.2 (Table I). With increasing temperature the binding constants of *ortho-*, *meta-* and *para*-fluoroaniline to P-450$_{scc}$ and metMb decreased. The differences in K_b values of fluoroaniline to P-450$_{scc}$ between the higher temperature and lower temperature are more remarkable than those of metMb under identical conditions. These differences in K_b values between low temperature and high temperature were most striking for the binding of *para*-fluoroaniline to P-450$_{scc}$: namely, the K_b value of *para*-fluoroaniline to P-450$_{scc}$ at 37 °C is less than that at 10 °C by one-sixth, whereas the K_b value to metMb at 37 °C is less than that at 10 °C by one-third.

Thermodynamic Parameters

The dependence of binding constants on temperature was examined according to the van 't Hoff equation, $\ln K_b = -(\Delta H^\circ/RT) + (\Delta S^\circ/R)$. van 't Hoff plots of $\ln K_b$ against $1/T$ (Figs. 2A and 2B) indicate a relationship between the binding constants and temperature. ΔH° values were calculated from the slope of the straight lines where the slope = $-\Delta H^\circ/R$. Other thermodynamic parameters were derived with the equation $\Delta G^\circ = -RT \ln K_b = \Delta H^\circ - T\Delta S^\circ$ (Table II).

When the values of ΔH° *versus* ΔS° were plotted, as shown in Figs. 3A and 3B, a straight line was obtained, indicating an enthalpy–entropy compensation effect of different fluorine substituted anilines binding to P-450$_{scc}$ or metMb. The correlation coefficients (r) were estimated as 0.986 and 0.998 for P-450$_{scc}$ and metMb, respectively. Isokinetic temperatures (β) were calculated as 408 K and 571 K for P-450$_{scc}$ and metMb, respectively, from the slopes of the ΔH° *versus* ΔS° plots (Table III and Fig. 3) [14].

Effect of pH on K_b

Binding constants of *ortho-*, *meta-* and *para*-fluoroaniline to P-450$_{scc}$ and metMb were measured as a function of three pH values in the biological pH region. Both for P-450$_{scc}$ and metMb, the K_b values increased with increasing pH. Table IV shows the binding constants at pH 6.9, 7.2 and 7.5 and at 20 °C. A relationship between the binding constants and pH values is observed when $\log K_b$ *versus* pH values are plotted (Figs. 4A and 4B).

Discussion

Binding of Fluoroaniline to P-450$_{scc}$ and metMb

The characteristic Soret spectral change on adding fluoroaniline (Fig. 1A) indicates the formation of a low-spin P-450$_{scc}$–fluoroaniline complex having a nitrogen ligand *trans* to the thiolate [2–6]. The binding of *para*-fluoroaniline to P-450$_{scc}$ was higher than that of *ortho-* and *meta*-fluoroaniline to P-450$_{scc}$ by one order (Table I). The high affinity of *para*-fluoroaniline to a heme iron compared to *ortho-* and *meta*-fluoroaniline was observed for a heme octapeptide of cytochrome *c* [13]. The same tendency was also observed for the binding of fluoroaniline to metMb, as summarized in Table I. Probably some electronic characteristics such as spin imbalance reaching the *para*-fluorine atom in the fluorinated aniline molecule may contribute to this abnormally high affinity of *para*-fluoroaniline to P-450$_{scc}$ and metMb. Less stereospecific hindrance of *para*-fluoroaniline may also enhance its affinity to P-450$_{scc}$ and

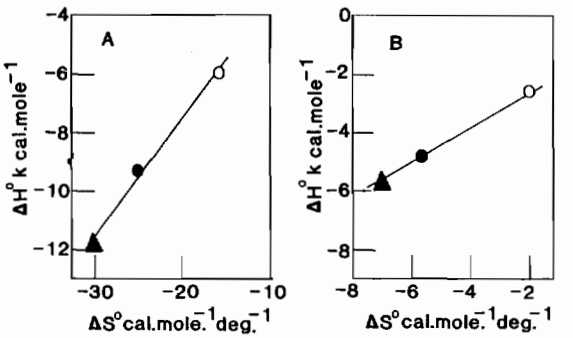

Fig. 3. ΔH° *vs.* ΔS° plots of the binding of (●) *ortho-*, (○) *meta-* and (▲) *para*-fluoroaniline to (A) P-450$_{scc}$ and (B) metMb.

TABLE II. Thermodynamic Parameters of the Binding of Fluoroaniline to P-450$_{scc}$ and metMb at 20 °C (pH 7.2)

Aniline compounds	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔS° (cal/mol deg)
(a) P-450$_{scc}$			
ortho-Fluoroaniline	−1.54	−9.15	−25.26
meta-Fluoroaniline	−1.40	−5.94	−15.51
para-Fluoroaniline	−3.14	−11.94	−30.01
(b) metMb			
ortho-Fluoroaniline	−1.54	−4.87	−5.71
meta-Fluoroaniline	−1.37	−2.57	−2.06
para-Fluoroaniline	−1.69	−5.81	−7.07

TABLE III. Enthalpy–Entropy Compensations of a Number of Biological Reactions

Enzyme	Reactions	K[a]	References
Cytochrome P-450$_{scc}$	fluoroaniline binding	408[b]	This work
Horse heart metMb	fluoroaniline binding	571[b]	This work
Cytochrome P-450$_{scc}$	cholesterol binding	420[c]	19
		403[d]	19
Horse, human and tubifex metMb	ligand binding	319[d]	21
Acetylcholinesterase	ligand binding	288[d]	22
Lysozyme	substrate binding	301[c]	23
α-Chymotrypsin	substrate hydrolysis	280–300[d]	24, 25
Mammalian erythrocyte	malonamide-induced hemolysis	308–318[d]	26
		304–320[d]	26
Cytochrome P-450	oxidation–reduction	433[b]	16
Cytochrome *c*	reduction	264[b]	27

[a]Calculated from the slopes of: [b]ΔH° *vs.* ΔS°; [c]$\Delta G^\ddagger$ *vs.* $\Delta H^\ddagger$; and [d]$\Delta H^\ddagger$ *vs.* $\Delta S^\ddagger$, where $\Delta G^\ddagger$ is the activated free energy, $\Delta H^\ddagger$ is the activated enthalpy, $\Delta S^\ddagger$ is the activated entropy.

TABLE IV. Binding Constants (K_b) (M^{-1}) of Fluoroaniline to P-450$_{scc}$ and metMb at Various pH Values at 20 °C

Aniline compounds	pH	K_b (M^{-1})	
		P-450$_{scc}$	metMb (× 10)
ortho-Fluoroaniline	6.9	0.70 × 10	0.60
	7.2	1.42 × 10	1.40
	7.5	3.00 × 10	2.48
meta-Fluoroaniline	6.9	0.60 × 10	0.50
	7.2	1.10 × 10	1.05
	7.5	2.00 × 10	1.62
para-Fluoroaniline	6.9	1.75 × 10^2	0.90
	7.2	2.20 × 10^2	1.80
	7.5	2.60 × 10^2	3.00

metMb. The binding constant (2.2 × 10^2 M^{-1} at 20 °C) of *para*-fluoroaniline to P-450$_{scc}$ reported here (Table I) is much less than that (4.5 × 10^4 M^{-1} at 20 °C) to a heme octapeptide of cytochrome *c* [13]. Thus, the polypeptide chain around the heme plane in P-450$_{scc}$ may interfere in part with the ligand binding through steric hindrance [15].

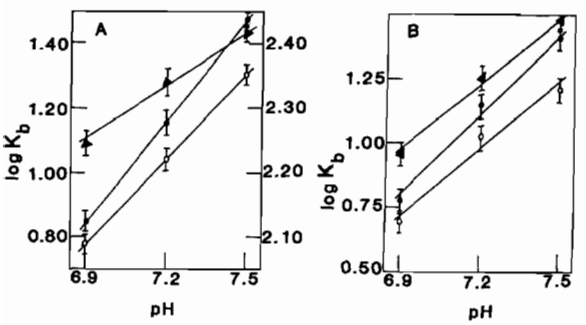

Fig. 4. The pH-dependent bindings of (•) *ortho*-, (○) *meta*-, and (▲) *para*-fluoroaniline to (A) P-450$_{scc}$ and (B) metMb at 20 °C. For the binding of *para*-fluoroaniline to P-450$_{scc}$, see the right-hand scale.

It is interesting to note that the affinity of *para*-fluoroaniline to P-450$_{scc}$ is higher than that to metMb. P-450$_{scc}$ has a hydrophobic heme environment while metMb has a rather hydrophilic heme environment compared to that of P-450$_{scc}$. Since *para*-fluoroaniline has hydrophobic character, the affinity of this compound to P-450$_{scc}$ will be higher than that to metMb. In addition, the heme cavity of metMb may be neither so large nor so flexible that metMb may not accept the binding of fluoroaniline to the heme cavity. Stereospecific hindrance of the binding of *ortho*- and *meta*-fluoroaniline to P-450$_{scc}$ and metMb may reduce the bindings of *ortho*- and *meta*-fluoroaniline to P-450$_{scc}$.

Effect of Temperature on K_b

It has been noted that the differences in the K_b values of fluoroaniline to P-450$_{scc}$ at the lower temperature and the higher temperature are more remarkable than those to metMb. These differences

were most striking for the binding of *para*-fluoroaniline to P-450$_{scc}$. Therefore, the conformational change of the heme environment in P-450$_{scc}$ may be more sensitive or labile to temperature than that of metMb. A very flexible substrate binding cavity of bacterial P-450$_{cam}$ was suggested from an X-ray crystallographic study [1]. It is noted here that P-450$_{scc}$ was quite stable in the presence of fluoroaniline over the range of the temperature studied [16].

Thermodynamic Parameters

A linear relationship was obtained when the values of ΔH° were plotted against ΔS° (Figs. 3A and 3B). This suggests that an extra thermodynamic relationship, *i.e.* an enthalpy–entropy compensation effect, is observed when fluorine-substituted aniline compounds bind to P-450$_{scc}$ or metMb. The correlation coefficients (r) of 0.986 and 0.998 were obtained for P-450$_{scc}$ and metMb, respectively. The isokinetic temperatures (β) were estimated as 408 K for P-450$_{scc}$ and 571 K for metMb. The β values in the literature for a number of biological reactions are given in Table III. For most enthalpy–entropy compensation effects in chemistry, β values lie in a relatively narrow range between 250–350 K [17], although higher β values ranging from 180–1320 K have also been reported [18]. A previous report [19] for the binding of cholesterol to P-450$_{scc}$ shows a correlation coefficient of 0.954 and an isokinetic temperature of 420 K. Our value for the binding of fluoroaniline to P-450$_{scc}$ is close to the value for cholesterol to P-450$_{scc}$ [19]. In the case of metMb, the β value is very high compared to the reported values (Table III). But we must emphasize here that the differences in free energy among bindings of various fluoroanilines to metMb are very small and β is neither zero nor infinity, hence enthalpy and entropy must compensate each other [17, 19]. A good enthalpy and entropy relationship for the binding of various fluoroanilines to P-450$_{scc}$ and metMb (Figs. 3A and 3B) suggests that these reactions may be caused by complex conformational changes of apoprotein and a direct participation of a water molecule in the reaction [17].

Effect of pH on K_b

Table IV shows that the binding constants of *ortho*-, *meta*- and *para*-fluoroaniline to P-450$_{scc}$ and metMb increase with increasing pH. A previous report [7] indicated that the affinity of cholesterol to P-450$_{scc}$ increases with decreasing pH (8 to 6). The pH effect on the binding of cholesterol to P-450$_{scc}$ was explained in terms of protonation of specific amino acids of P-450$_{scc}$, in that protonation facilitates the binding of the side chain of cholesterol rather than the rings of cholesterol [20]. As it is unlikely that a positively charged group on the protein surface provides the direct enhancement of binding of the saturated side chain in the substrate, an allosteric change of the active site structure of P-450$_{scc}$ was suggested for the binding of cholesterol to P-450$_{scc}$ [20]. In contrast to cholesterol, binding constants of *ortho*-, *meta*- and *para*-fluoroaniline to both P-450$_{scc}$ and metMb increased with pH (Table IV). This provides a possibility that the heme environments of P-450$_{scc}$ and metMb are so changed by increasing pH that the amino acid side chain enhances the binding of fluoroaniline to P-450$_{scc}$ and metMb. Another possibility for the enhancement of the binding of fluoroaniline to P-450$_{scc}$ and metMb at higher pH may be ascribed to deprotonation at the nitrogen atom of fluoroaniline compounds at higher pH, which may facilitate their binding to P-450$_{scc}$ and metMb. The latter possibility is more likely.

Conclusion

This study concludes that: (i) the affinity of fluoroaniline compounds to P-450$_{scc}$ is more marked than to metMb; (ii) the affinity of *para*-fluoroaniline to P-450$_{scc}$ is more marked than that of *ortho*- and *meta*-fluoroaniline; (iii) the heme environment of P-450$_{scc}$ is more sensitive to temperature than that of metMb; (iv) an enthalpy–entropy compensation effect is observed when various fluoroaniline compounds bind to P-450$_{scc}$ and metMb; (v) increasing pH enhances the binding of fluoroaniline to both P-450$_{scc}$ and metMb, which is in contrast to the binding of cholesterol.

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