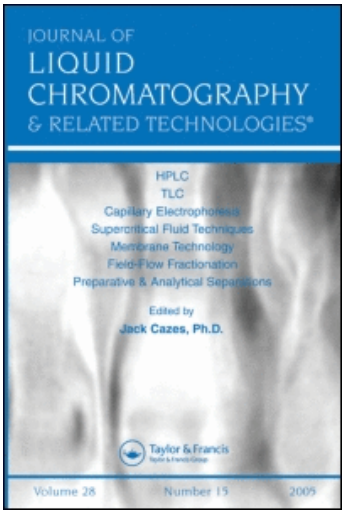

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Investigation of Interactions Between Drug Enantiomers and Flavoprotein as a Chiral Selector by Affinity Capillary Electrophoresis

N. Mano[a]; Y. Oda[a]; Y. Ishihama[a]; H. Katayama[a]; N. Asakawa[a]
[a] Analytical Chemistry Section Tsukuba Research Laboratories, Eisai Co., Ltd, Tsukuba Ibaraki, Japan

To cite this Article Mano, N. , Oda, Y. , Ishihama, Y. , Katayama, H. and Asakawa, N.(1998) 'Investigation of Interactions Between Drug Enantiomers and Flavoprotein as a Chiral Selector by Affinity Capillary Electrophoresis', Journal of Liquid Chromatography & Related Technologies, 21: 9, 1311 — 1332
To link to this Article: DOI: 10.1080/10826079808005880
URL: http://dx.doi.org/10.1080/10826079808005880

J. LIQ. CHROM. & REL. TECHNOL., 21(9), 1311–1332 (1998)

INVESTIGATION OF INTERACTIONS BETWEEN DRUG ENANTIOMERS AND FLAVOPROTEIN AS A CHIRAL SELECTOR BY AFFINITY CAPILLARY ELECTROPHORESIS

N. Mano,* Y. Oda, Y. Ishihama, H. Katayama, N. Asakawa

Analytical Chemistry Section
Tsukuba Research Laboratories, Eisai Co., Ltd.
Tokodai 5-1-3, Tsukuba
Ibaraki 300-26, Japan

ABSTRACT

Interactions between drug enantiomers and flavoprotein as a pseudo chiral stationary phase were investigated by using affinity capillary electrophoresis (CE) in order to avoid the effects of nonspecific interactions that occur in chiral HPLC. Circular dichroism (CD) measurement was used to monitor changes of the secondary structure of flavoprotein under various analysis conditions in affinity CE. The chiral discrimination region for ketoprofen on the flavoprotein surface was concluded to consist of α-helix structure, and the decrease of chiral separation ability with increase of methanol content in the electrophoretic buffer was directly related to conformational change of the α-helix. Studies with chemically modified flavoprotein indicated that two types of interaction at the chiral discrimination region are required for chiral separation: π-π interaction of a tryptophan residue with the aromatic ring of ketoprofen, and ionic interaction of the carboxyl group of ketoprofen with an amino group and a carboxyl group of the protein.

INTRODUCTION

High-performance liquid chromatography (HPLC) with a protein-conjugated chiral stationary phase (protein-CSP) and an aqueous mobile phase[1,2] can be used to separate a wide range of enantiomers,[3] and is often used for pharmacokinetic and toxicokinetic studies, and so on,[4-9] during pharmaceutical development. Many kinds of protein-CSPs have been reported.[10-17] We have shown that a flavoprotein-CSP can simultaneously separate individual drug enantiomers and their metabolites in the reversed-phase mode.[18] However, the chiral discrimination mechanism is expected to be complicated, and may involve ionic interaction, hydrophobic interaction, hydrogen bonding and π-π interaction.

Several researchers have investigated the interactions between protein-CSPs and solute. Wainer and co-workers[19-21] reported that $k'/k'+1$ showed a linear relation to protein-binding capacity when human serum albumin (HSA) was employed as a chiral selector, and allosteric effects were observed. Oda et al.[22] prepared an acyl-derivatized avidin column and investigated the retention and chiral separation of several drug enantiomers. They demonstrated that a carboxyl group at the binding site plays an important role in the chiral separation of basic drug enantiomers, while the chiral separation of acidic drug enantiomers is related to an interaction between an amino group of the binding site and a negative charge in the solute molecule. Kaliszan et al.[23-25] investigated the relationship between chiral separation and physicochemical properties of solutes such as hydrophobicity, molecular size, and electron excess charge on the nitrogen atom. They concluded that solutes bind to the chiral discrimination region through hydrophobic interaction and a nearby ionic region is also related to the chiral separation. Pinkerton et al.[26] found that only the third domain of turkey ovomucoid has chiral discrimination capacity, and they considered that ionic interaction, hydrophobic interaction, and hydrogen bonding all contribute to the chiral discrimination on the basis of NMR and computational studies.

We have developed four protein-CSPs[27-30] and established that mobile phase conditions such as pH and organic solvent content influence retention and chiral separation.[31] However, protein-CSPs show not only specific binding, but also non-specific interaction, e.g., at the silica gel surface, at unreacted aminopropyl groups, and in the linkage region, as we showed in the case of flavoprotein-CSP,[32] and it is very difficult to investigate only the specific interaction involved in chiral discrimination by using HPLC.

Capillary electrophoresis (CE) has a very high separation efficiency[33,34] and many drug enantiomers have been separated by using various chiral selectors.[35-41] Chiral separation by CE using protein as a pseudo stationary phase has been reported recently.[42-46]

In CE, the protein used as a chiral selector is in a nearly native situation in solution, so retention of the solute may reflect quite well the specific interaction between protein and solute in solution. Lloyd et al.[47] compared retention on HPLC and CE with HSA as a chiral selector, and found that the capacity factor (k') of benzoin on HPLC and CE showed a linear relationship in running buffer, containing various amounts of 1-propanol.

In this study, we investigated the relationship between the k' values on HPLC and CE for eight kinds of racemic compounds under the same conditions. The results suggested that affinity CE, using protein as a chiral selector, can accurately evaluate the specific interaction between proteins and solutes.

We then investigated the effect of varying the mobile phase conditions on the retention and chiral separation in affinity CE. We also examined the nature of the moieties involved in the chiral discrimination on the protein surface by using flavoprotein specifically modified at tyrosine residues, tryptophan residues or amino or carboxyl groups.

EXPERIMENTAL

Reagents and Materials

Ketoprofen (KE), ibuprofen (IB), flurbiprofen (FL), fenoprofen (FE), proglumide (PG), oxprenolol (OX), aminoglutethimide (AG), 2-hydroxy-5-nitrobenzylbromide (HNBB), and N-acetylimidazole were purchased from Sigma (St. Louis, MO, USA). Pranoprofen (PP) was extracted from pharmaceutical capsules (Mabul capsule). N,N-Disuccinimidylcarbonate was from Wako Pure Chemical (Osaka, Japan), and Nucleosil 5NH2 was from Macherey-Nagel (Düren, Germany). Sulfosuccinimidyl carbonate and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride were from Pierce (Rockford, IL, USA). Flavoprotein was purified from chicken egg-white.[48] Protein assay Kit II was from Bio-rad (Hercules, CA, USA). Other reagents were commercial products of high purity or HPLC grade.

Apparatus

The HPLC system was an LC-10A system equipped with an LC-10AD pump, an SCL-10A auto sampler and an SPD-10A UV detector (Shimadzu, Kyoto, Japan), and all components were controlled by CLASS LC-10 software. The flavoprotein (apoprotein) column (4.6 mm I.D. x 100 mm) was prepared by our reported method.[30] The CE system was equipped with an 890-CE high-voltage supply (Jasco, Tokyo, Japan) and an 870-CE UV detector (Jasco). An uncoated fused silica capillary (50 μm I.D., 375 μm O.D., 30 cm total length, 17 cm effective length, GL Science, Tokyo) was used for measurement of the electrophoretic mobility of drugs. The measurement of the electrophoretic mobility of apoprotein and the affinity CE analyses were carried out by using a polyethylene glycol coated capillary (μSIL, 50 μm I.D., 375 μm O.D., 40 cm total length, 25 cm or 15 cm effective length, J&W Scientific, Folsom, CA). The UV spectrophotometer was a model U-3500 (Hitachi, Tokyo) and the circular dichroism (CD) spectra were obtained with a J-720WI instrument (Jasco).

Purification of Apoprotein

Flavoprotein (2 g) was dissolved in 50 mM sodium acetate buffer (pH 4.0) containing 5% sodium chloride. Activated charcoal (Nacalai Tesque, Kyoto) was added to the flavoprotein solution, which was adjusted to pH 3.0 by adding acetic acid. After mixing, the activated charcoal was filtered off and the filtrate was adjusted to neutral pH with sodium hydroxide. This solution was subjected to gel chromatography (Hiload 26/60 Superdex 200 prep grade, 2.6 cm I.D. x 60 cm, Pharmacia Biotech, Uppsala, Sweden) using 50 mM tris-hydrochloride buffer containing 150 mM sodium chloride. A single peak at a position corresponding to nearly MW 30000 was collected, dialyzed using a semipermeable membrane (MW cut-off; 6000-8000, Spectrum Medical Industries, Inc., Los Angeles, CA), and then lyophilized (960 mg, white powder).

Chemical Modification of Apoprotein

Tryptophan residues

Apoprotein (29 mg) was dissolved in sodium acetate buffer (pH 5.0, ionic strength 0.05, 2.375 mL); and HNBB methanol solution (2.32 mg/0.125 mL) added. After reaction for 30 min at room temp. the mixture was dialyzed and lyophilized (HNBB-apoprotein, pale-yellowish powder).

Tyrosine residues

Apoprotein (29 mg) was dissolved in sodium acetate buffer (pH 5.0, ionic strength 0.05, 2.375 mL) and N-acetylimidazole methanol solution (6.6 mg/0.125 mL) was added. After reaction for 60 min at room temperature, the mixture was dialyzed and lyophilized (O-acetylated apoprotein, white powder).

Amino groups

Sulfosuccinimidyl acetate was dissolved in 50 mM sodium hydrogen carbonate (25 mg/0.5 mL) and added to the apoprotein solution (50 mM sodium hydrogen carbonate, 29 mg/2 mL). After reaction for 60 min at room temperature, the mixture was dialyzed and lyophilized (N-acetylated apoprotein, white powder).

Carboxyl groups

The Apoprotein (29 mg) was dissolved in a solution of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride in 50 mM potassium dihydrogen phosphate (pH 4.6, 50 mg/2 mL). Glycine ethyl ester hydrochloride in 50 mM potassium dihydrogen phosphate (10 mg/0.5 mL) was added. After reaction for 6 hours at room temperature, the mixture was dialyzed and lyophilized (ethylglycyl-apoprotein, white powder).

Theory of Affinity CE

The electrophoretic separation process in affinity CE can be explained in terms of a simple model.[46] In the running buffer, solute (S) and protein (P) are in a binding equilibrium

$$S + P \rightleftharpoons SP \qquad (1)$$

We assume that the interaction forms a monovalent complex (SP). Then the binding constant (K) is expressed as

$$K = \frac{[SP]}{[S][P]} \qquad (2)$$

In affinity CE using a protein as a chiral selector, the binding capacity of the solute to the protein is similar to that in electrokinetic chromatography.[46]

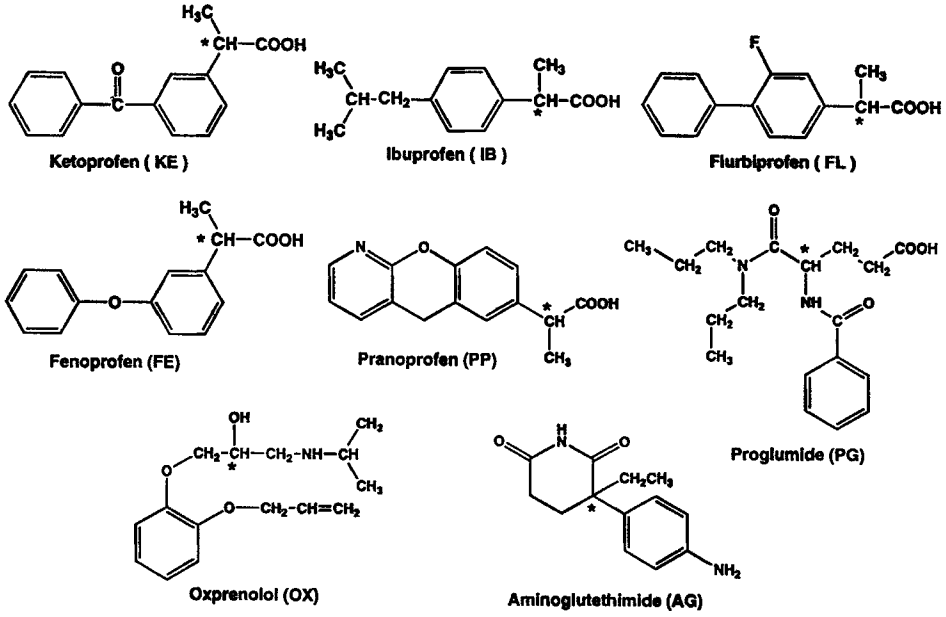

Figure 1. Molecular Structures of 8 Compounds Used in Comparison of Chiral HPLC and Affinity CE. The chiral centers are indicated by asterisks.

From in the viewpoint of chromatography, the k' value is given as the ratio of solute in the stationary phase to that in the mobile phase. Thus, the binding capacity of solute to protein as a pseudo stationary phase in affinity CE using a coated capillary with no electroosmotic flow can be chromatographically written as[46]

$$k' = K[P] = \frac{\mu s - \mu app}{\mu app - \mu com} \tag{3}$$

where μs is the electrophoretic mobility of the solute, μapp is the apparent electrophoretic mobility and μcom is the electrophoretic mobility of the protein-solute complex which is given by the Eq. (4),

$$\mu com = \mu pro \quad \frac{Z+z}{Z} \cdot \frac{M^{2/3}}{(M+m)^{2/3}} \tag{4}$$

where μpro is the electrophoretic mobility of the protein, Z and z are the charges of the protein and solute, respectively, and M and m are the molecular weights of the protein and solute, respectively. The charge of protein is calculated by using a semiempirical equation described by Rickard et al.[49]

RESULTS AND DISCUSSION

Comparison of HPLC and CE

Flavoprotein purified from chicken egg-white includes a considererable amount of riboflavin and is yellow in color. In the case of chiral HPLC, riboflavin can be washed out with an acidic solution. However, the protein as a chiral selector for CE is dissolved directly in the electrophoretic buffer, so we removed the riboflavin and further purified the flavoprotein by gel chromatography prior to use (see Experimental).

In this study, we used the eight model compounds shown in Fig. 1. KE, IB, FL, FE and PP are acidic α-aryl propionates having similar structures around the chiral center, while PG is an acidic compound containing a carboxyl group in the molecule, and OX and AG are basic compounds whose pKa values are appreciably different.

First we investigated the binding capacity and chiral separation of these model compounds by chiral HPLC and affinity CE using flavoprotein. Table 1 shows the k'1, k'2 and α values.

Acidic compounds were strongly retained on HPLC, and appreciably different values of k′ and α among the profens were obtained. Interestingly, OX and AG, which are basic compounds, showed good chiral separations although they had small k' values (less than 2).

In CE, however, the k' values of all the acidic compounds were around 2, and PP showed the smallest k' value among the profens, in spite of giving the largest k′ value on HPLC. The basic compound OX, which showed a smaller k′ value than AG on HPLC, was bound to flavoprotein more strongly than AG on CE. The k′ values of all the compounds on CE versus those on HPLC are plotted in Fig. 2. No relationship was apparent, in contrast to the findings of Lloyd et al. for benzoin.[47]

Table 1

Comparison of k′ and α Values of Model Compounds on HPLC and CE

Compound	HPLC k′1	HPLC k′2	HPLC α	CE k′1	CE k′2	CE α
KE	11.94	14.85	1.24	2.49	2.78	1.12
IB	8.52	9.09	1.07	2.66	2.75	1.03
FL	17.99	19.00	1.06	2.30	2.40	1.04
FE	10.10	10.10	1.00	2.70	2.70	1.00
PP	38.25	57.29	1.50	1.88	2.02	1.07
PG	5.61	21.27	3.79	1.85	1.94	1.05
OX	0.79	1.00	1.27	5.02	5.68	1.13
AG	1.01	1.54	1.52	0.94	0.98	1.04

k′1 = smaller capacity factor; k′2 = larger capacity factor; α = enantioselectivity. HPLC conditions: column, apoprotein column (100 mm x 4.6 mm I.D., 5 μm); mobile phase, acetate buffer (pH 5.0, I=0.05)/methanol (95:5); flow rate, 1.0 mL/min; detection, UV 214 nm; column temperature, 25±2°C; sample amount, 1 nmol (100 μM, 10μL injection). CE conditions: capillary, μSIL (polyethylene glycol coated capillary, 375 μm O.D., 50 μm I.D.); total length, 40 cm; effective length, 25 cm; electrophoretic buffer, acetate buffer (pH 5.0, I = 0.05)/methanol (95:5) containing 200 μM apoprotein; applied voltage, 10 kV; detection, UV 254 nm; sample amount, 1 mM.

It appears that the contribution of non-specific interaction to retention was different among the various compounds on HPLC. On the other hand, the rather similar k' values of the profens on CE, in spite of the considerably different k′ values on HPLC, suggested that these analogues bound to the same specific binding site on the protein surface with similar strength. Affinity CE should therefore be useful for the evaluation of specific interactions between solute and protein without the interference of non-specific interaction.

Effect of pH of the Running Buffer on k′ and α Values in Affinity CE

In chiral protein HPLC, the pH of the mobile phase greatly influences the retention and chiral separation of drug enantiomers.[29-31,50] The effect of

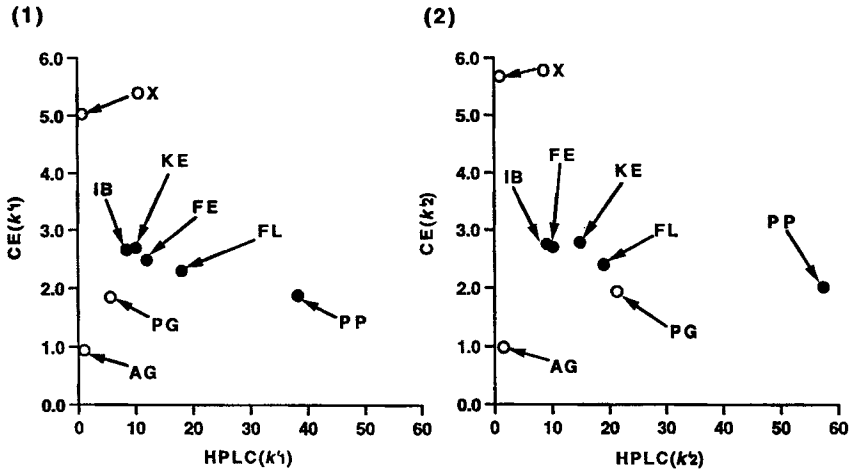

Figure 2. Plots of k' as Determined by CE vs k' as Determined by HPLC.

Table 2

Effect of pH of Running Buffer on Binding Capacity and Enantioselectivity

pH	k'1	k'2	α
4.4	3.01	3.38	1.12
5.0	2.12	2.27	1.07
5.6	1.94	1.94	1.00
6.2	1.64	1.64	1.00
6.8	1.22	1.22	1.00

k'1 = smaller capacity factor; k'2 = larger capacity factor; α = enantioselectivity. Conditions: capillary, μSIL (polyethylene glycol coated capillary, 375 μm O.D., 50 μm I.D.); total length, 40 cm;effective length, 25 cm; electrophoretic buffer, acetate or phosphate buffer (I=0.05) containing 200 μM apoprotein; applied voltage, 10 kV; detection, UV 254 nm; sample amount, 1 mM.

pH on the k′ and a values of KE in affinity CE is shown in Table 2. The binding and the chiral separation capacity decreased with increase of pH in the range of pH 4.4-6.8, as was found in the case of chiral flavoprotein HPLC.[30,31] However, there was no change of the CD spectra in the range of pH 3-7 (data not shown), so the change of pH of the running buffer did not influence the secondary structure of flavoprotein. Therefore, the effect of pH may be due to a change of the ionic state of amino acid residue(s) related to the interaction between KE and the protein binding site.

Further, KE is an acidic compound whose pKa value is 3.9, so neutral pH favors ionization of the carboxyl group. The non-ionic form of KE may bind stably to the chiral discrimination region on the flavoprotein surface in the range of pH 4.4-6.8, but binding of the negatively charged form may be disfavored by ionic interaction. This would explain why the binding capacity is lower at neutral pH than at acidic pH. On the other hand, in HPLC, KE is most strongly retained at pH 4.6 and more acidic pH of the mobile phase decreased both the retention and the chiral separation.[30] Therefore, both an acidic amino acid residue and a basic amino acid residue in the chiral discrimination may play important roles in the chiral separation by flavoprotein.

Effect of Methanol Content of the Running Buffer on k' and α Values in Affinity CE

The content of organic modifiers greatly affected the retention and the chiral separation in chiral protein HPLC,[29-31,50] implying that hydrophobic interaction between solute and protein binding site is very important. Table 3 shows the effect of the methanol content of the running buffer on binding and chiral separation in affinity CE. The k′ values slightly increased up to 20% methanol content and decreased at more than 25% methanol, whereas they decreased with increase of ethanol content in the range of 4-12% in HPLC.[30] On the other hand, the α values decreased with increase of methanol content, and the chiral separation of KE was not achieved at 25% methanol or more. When the flavoprotein as a solute was analyzed by CE, broadening of the flavoprotein peak was seen with increase of the methanol content of the running buffer (data not shown). This suggests that partial denaturation of flavoprotein resulted in increased adsorption on the capillary wall. Fig. 3 shows the change of molecular ellipticity of flavoprotein in relation to the methanol content of the running buffer. The increase of methanol content up to 20% caused a marked change of molecular ellipticity at 208 nm, reflecting conformational change of the secondary structure (α-helix) of flavoprotein, and this coincides with the change of α value in

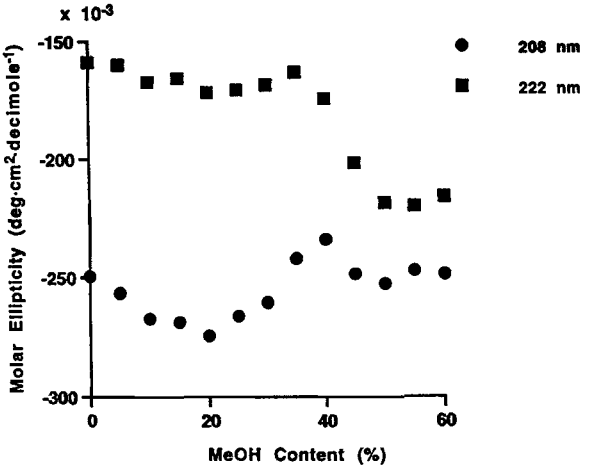

Figure 3. Effect of Methanol Content on the Molar Ellipticity at 245 nm of Flavoprotein. Conditions: sample concentration, 5 μM; solvent, acetate buffer (pH 5.0, I=0.05)/methanol mixture; cell length, 1 mm; scan range, 200-350 nm; scan speed, 50 nm/min; resolution, 0.1 nm; band width, 1.0 nm; accumulation, 5.

Table 3

Effect of Methanol Content in Running Buffer on Binding Capacity and Enantioselectivity

MeOH%	k′1	k′2	α
0	2.62	2.93	1.12
5	2.67	2.94	1.10
10	2.77	2.91	1.05
15	2.81	2.90	1.03
20	3.01	3.09	1.03
25	2.56	2.56	1.00
30	2.45	2.45	1.00

k′1 = smaller capacity factor; k′2 = larger capacity factor; α = enantioselectivity. Conditions: capillary, μSIL (polyethylene glycol coated capillary, 375 μm O.D., 50 μm I.D.); total length, 40 cm; effective length, 25 cm; electrophoretic buffer, acetate buffer (pH 5.0, I=0.05) containing 200 μM apoprotein; applied voltage, 10 kV; detection, UV 254 nm; sample amount, 1 mM.

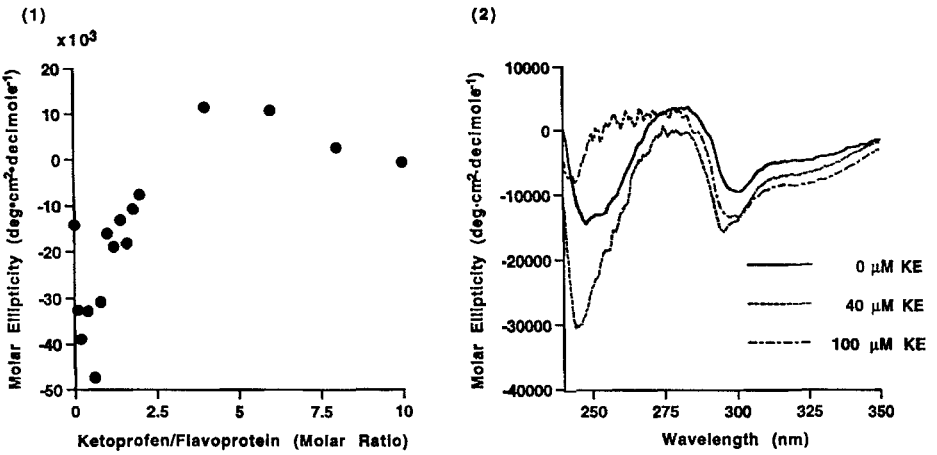

Figure 4. Variation of CD Spectra of Flavoprotein Induced by Binding of Ketoprofen. 1) Molar ellipticity of flavoprotein at 245 nm, 2) Representative CD spectra. Conditions: sample concentration, 50 μM; solvent, acetate buffer (pH 5.0, I=0.05)/methanol (95:5); cell length, 10 mm; scan range, 240-350 nm; scan speed, 50 nm/min; resolution, 0.1 nm; band width, 1.0 nm; accumulation, 5.

affinity CE. The decrease of the chiral separation of KE with increase of methanol content seems to be closely related to conformational change of flavoprotein, and the behavior of the k' values indicated that electrostatic repulsion between negative charges of the carboxyl group in KE and a component of the specific binding was reduced by the methanol-induced conformational change of flavoprotein, whereas the hydrophobic interaction was unaffected. In addition, the decrease of binding capacity at more than 25% of methanol may be due to be weakening of the interaction between the carboxyl group of KE and an amino group in the binding site.

CD Spectra of Flavoprotein-KE Complex

In order to investigate the nature of the amino acid residue in the chiral discrimination region that interacts with KE, the molecular ellipticity of flavoprotein was measured when various concentrations of racemic KE were added to a constant concentration of flavoprotein solution. Fig. 4(1) shows the change of the molecular ellipticity of flavoprotein at 245 nm, and representative CD spectra are shown in Fig. 4(2). Flavoprotein has a weak negative Cotton effect at 245 nm in methanol-acetic acid buffer (pH 5.0, I=0.05) mixed solution (5:95). When the molar ratio of KE and flavoprotein

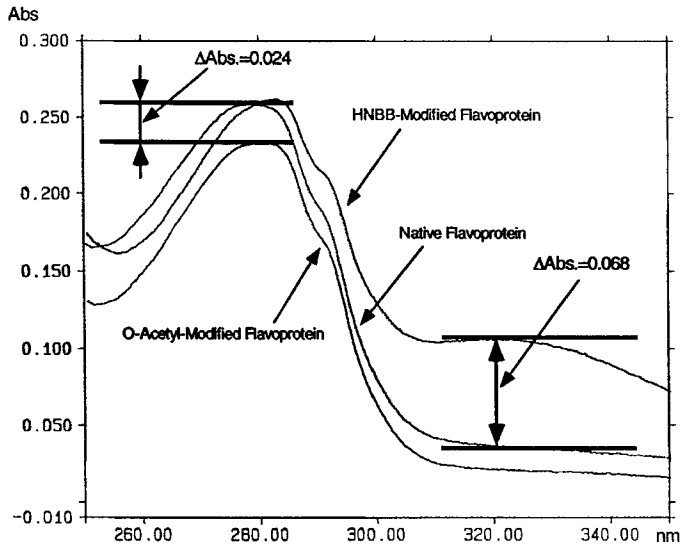

Figure 5. UV Spectra of Modified Flavoproteins. Conditions: sample concentration, 5 μM; dissolving solvent, acetate buffer (pH 5.0, I=0.05)/methanol (95:5); cell length, 10 mm; scan range, 250-350 nm; scan speed, 60 nm/min; slit, 2.0 nm.

was 1:1, the Cotton effect at 245 nm was enhanced, while it decreased at a molar ratio of more than 2. These results suggest a strong interaction of KE with the side chain of the amino acid residue which contributed to the Cotton effect at 245 nm. A molar ratio of more than 2 may result in racemization of this amino acid residue.

Chemical Modification of Flavoprotein

Tryptophan, tyrosine, phenylalanine, and cystine residues influence the long-wavelength CD of proteins.[51,52] Among them, tryptophan can be easily and specifically modified with HNBB under aqueous condition[53,54] and tyrosine is O-acetylated specifically by N-acetylimidazole.[53,55]

Figure 5 shows the UV spectra of unmodified, HNBB-modified and O-acetyl-modified flavoprotein. A new absorption maximum appeared at 320 nm due to the modified tryptophan residue and the molar number of residues involved was calculated from the difference absorbance versus the native

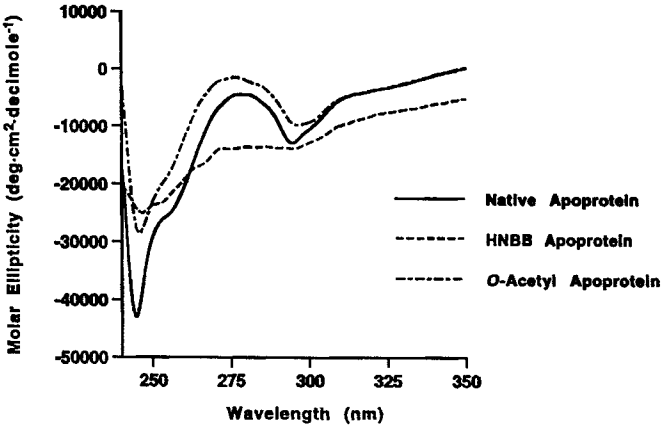

Figure 6. CD Spectra of Modified Flavoproteins. Conditions: sample concentration, 50 μM; solvent, acetate buffer (pH 5.0, I=0.05)/methanol (95:5); cell length, 10 mm; scan range, 240-350 nm; scan speed, 50 nm/ min; resolution, 0.1 nm; band width, 1.0 nm; accumulation, 5.

flavoprotein spectrum.[53] It amounted to 0.8 mole of tryptophan residue per mole of flavoprotein. On the other hand, O-acetyl modification is known to reduce the absorbance at 278 nm,[53] and the mole number of O-acetylated tyrosine residues per mole of flavoprotein was 4.0. Flavoprotein has 6 mole of tryptophan residue and 9 mole of tyrosine residue,[56] and the modified residues are presumably among those existing at the flavoprotein surface.

Figure 6 shows the CD spectra of native, HNBB-modified and O-acetyl-modified flavoprotein. The CD spectrum of O-acetyl-modified flavoprotein was similar in shape to that of native flavoprotein. On the other hand, HNBB-modified flavoprotein lacked both the negative Cotton effect at 245 nm and the positive Cotton effect at around 280 nm. The CD spectra of these modified flavoproteins at less than 230 nm (related to secondary structure) were almost the same as that of the native flavoprotein (data not shown). These results showed that a tryptophan residue on the flavoprotein surface is related to the flavoprotein-KE interaction.

The chiral discrimination by flavoprotein also appears to be influenced by ionic interaction between the carboxyl group in KE and ionic group(s) at the binding site. Therefore, carboxyl groups of glutamic acid and aspartic acid residues on the flavoprotein, whose pI value is 3.9-4.1,[57] were modified

Table 4

Electrophoretic Mobilities and Charge of Amino- and Carboxy-Modified Flavoprotein

	μeof ($cm^2/V \cdot s$)	μapp ($cm^2/V \cdot s$)	μpro ($cm^2/V \cdot s$)	Z
Native	3.457×10^{-4}	1.082×10^{-4}	-2.375×10^{-4}	30.9
Amino-modified	3.511×10^{-4}	6.590×10^{-5}	-2.852×10^{-4}	37.8
Carboxy-modified	2.855×10^{-4}	1.857×10^{-4}	-9.980×10^{-5}	10.9

*μeof = electrophoretic mobility of electroosmotic flow; μaap = apparent electrophoretic mobility of proteins; μpro = electrophoretic mobility of proteins; Z = charge. Conditions: capillary, dextran sulfate coated capillary (375 μm O.D., 50 μm I.D.); total length, 19.1 cm; effective length, 9.1 cm; electrophoretic buffer, phosphate buffer (pH 7.4, I = 0.05); applied voltage, 5 kV; detection, UV 214 nm; sample amount, 5 μM.

by coupling with glycine ethyl ester in the presence of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide, and amino groups of lysine and arginine residues were modified by acylation using sulfosuccinimidyl acetate.

The results of analysis of native, amino-modified and carboxy-modified flavoproteins by CE using a dextran-sulfate coated capillary[58] are shown in Table 4. The electrophoretic mobility of the amino-modified flavoprotein increased to 1.2 times and that of carboxyl-modified flavoprotein decreased to 0.42 times that of the unmodified protein. The charges of the three proteins were calculated by using a semiempirical equation,[49] and were 30.9 for the native protein, 37.8 for the amino-modified protein, and 10.9 for the carboxy-modified protein. These results showed that seven amino groups were modified in the amino-modified flavoprotein and twenty carboxyl groups were modified in the carboxy-modified flavoprotein.

Affinity CE Using Modified Flavoproteins

Figure 7 shows electrophoregrams of KE obtained with native, HNBB-modified and O-acetyl-modified flavoprotein as chiral selectors. With O-acetyl-modified flavoprotein, the migration and chiral separation of KE were almost the same as with the native flavoprotein. However, the HNBB-modified flavoprotein showed diminished chiral discrimination though the

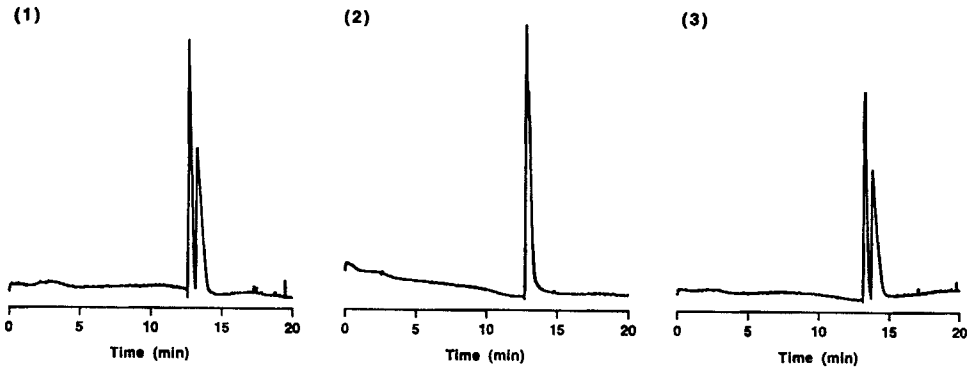

Figure 7. Electrophoregrams of Ketoprofen by affinity CE using Tryptophan- and Tyrosine-modified flavoproteins as chiral selectors. 1) Native flavoprotein, 2) HNBB flavoprotein, 3) *O*-Acetyl flavoprotein. Conditions: capillary, μSIL (linear polyacrylamide coated capillary, 375 μm O.D., 50 μm I.D.); total length, 40 cm; effective length, 25 cm; electrophoretic buffer, acetate buffer (pH 5.0, I=0.05)/methanol (95:5) containing 200 μM apoprotein or modified apoprotein; applied voltage, 10 kV; detection, UV 254 nm; sample amount, 1 mM.

migration time was almost unchanged. These results indicated that a tryptophan residue is involved in chiral discrimination of KE by flavoprotein, and π-π interaction between the aromatic ring in KE and the indole side chain in the tryptophan residue presumably plays an important part in the chiral separation of KE.

Fig. 8 shows electrophoregrams of KE obtained with native, amino-modified and carboxy-modified flavoprotein as chiral selectors. The modification of amino groups quenched chiral separation capacity, and the peak shape was sharper than that with native flavoprotein though the migration time was almost the same. This phenomenon is considered to be due to loss of ionic interaction between the carboxyl group in KE and an amino group in the chiral discrimination region. On the other hand, the carboxyl-modified flavoprotein achieved chiral separation of KE, although the migration time was longer because of the reduction of electrophoretic mobility of the protein based on the change of negative charge. These results indicated that the ionic interaction between a carboxyl group in KE and an amino group in the chiral discrimination region is important for chiral separation. However, a repulsive interaction between the carboxyl group of KE and a carboxyl group of the protein destabilizes the interaction.

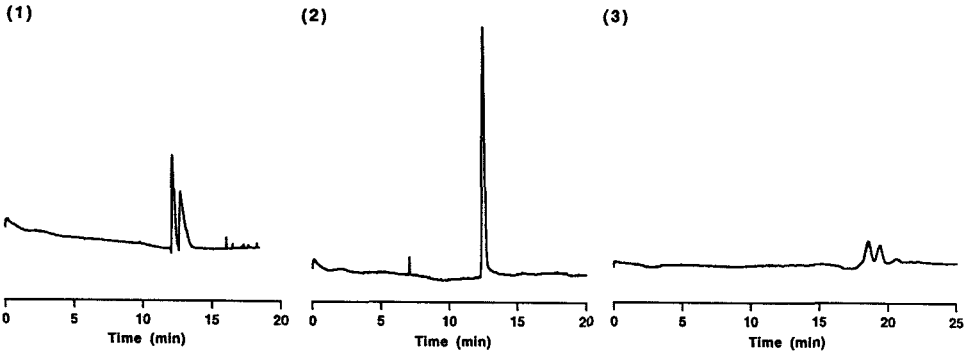

Figure 8. Electrophoregrams of Ketoprofen by affinity CE using carboxyl and amino group modified flavoproteins as chiral selectors. 1) Native flavoprotein, 2) Amino-modified flavoprotein, 3) Carboxy-modified flavoprotein. Conditions: capillary, μSIL (linear polyacrylamide coated capillary, 375 μm O.D., 50 μm I.D.); total length, 40 cm; effective length, 25 cm; electrophoretic buffer, acetate buffer (pH 5.0, I=0.05)/methanol (95:5) containing 200 μM apoprotein or modified apoprotein; applied voltage, 10 kV; detection, UV 254 nm; sample amount, 1 mM.

CONCLUSION

We have established that the chiral recognition region of flavoprotein for KE in affinity CE consists of an α-helix structure, and the critical groups involved are a tryptophan residue, an amino group, and a carboxyl group of the protein.

As the pKa of KE is 3.9, variation of the pH of the running buffer markedly influenced the retention and the chiral separation capacity. The observed changes were consistent with those reported in HPLC with a flavoprotein-CSP column (strongest retention at pH 4.0 and best chiral separation at pH 4.6).[30]

Increase of the methanol content in the running buffer resulted in changes of the α-helical structure of the chiral recognition region, decreasing chiral discrimination capacity. At more than 25% methanol, the putative π-π interaction between the aromatic ring of KE and the tryptophan residue is retained, so that binding capacity is largely maintained, but the α-helical structure is considered to be substantially denatured, abrogating the ionic interaction, and, consequently, the chiral discrimination. This

interaction, that the chiral discrimination requires a dual interaction (π-π and ionic), whereas the π-π interaction alone is sufficient for retention, is consistent with the observed effects of pH on affinity CE, and with the effects of organic modifier in chiral HPLC.[30] However, the retention behavior in HPLC was not the same as in affinity CE, because non-specific interaction of the solute with the silica gel surface, unreacted aminopropyl groups and spacer linkages contribute to the retention changes in HPLC.

Thus, affinity CE is particularly effective for investigating the mechanism of chiral discrimination by proteins.

ACKNOWLEDGMENT

The authors are grateful to Mr. H. Inoue, Kawashima Factory, Eisai Co., Ltd., for providing and purifying the flavoprotein.

REFERENCES

1. S. Allenmark, B. Bomgren, H. Borén, J. Chromatogr., **264**, 297-300 (1983).

2. J. Hermansson, J. Chromatogr., **269**, 71-80 (1983).

3. K. M. Kirkland, K. L. Neilson, D. A. McCombs, J. Chromatogr., **545**, 43-58 (1991).

4. J. Haginaka, T. Murashima, H. Fujima, H. Wada, J. Chromatogr., **620**, 199-204 (1993).

5. G. Rosell, A. Camacho, P. Parra, J. Chromatogr., **619**, 87-92 (1993).

6. J. W. Kelly, J. T. Stewart, C. D. Blanton, Biomed. Chromatogr., **8**, 255-257 (1994).

7. A. Suzuki, S. Takagaki, H. Suzuki, K. Noda, J. Chromatogr., **617**, 279-284 (1993).

8. A. Doroudian, K. Yeleswaram, D. W. Rurak, F. S. Abbott, J. E. Axelson, J. Chromatogr., **619**, 79-86 (1993).

9. V. Chapeau, A. Moulin, M. Caude, A. Dufour, J. Chromatogr. B, **660**, 341-346 (1994).

10. S. G. Allenmark, S. Andersson, J. Chromatogr. A, **666**, 167-179 (1994).

11. S. R. Narayanan, J. Pharm. Biomed. Anal., **10**, 251-262 (1992).

12. I. Marle, A. Karlsson, C. Pettersson, J. Chromatogr., **604**, 185-196 (1992).

13. I. Marle, S. Jönsson, R. Isaksson, C. Pettersson, G. Pettersson, J. Chromatogr., **648**, 333-347 (1993).

14. J. Haginaka, T. Murashima, C. Seyama, J. Chromatogr. A, **666**, 203-210 (1994).

15. A. -F. Aubry, N. Markoglou, V. Descorps, I. W. Wainer, G. Félix, J. Chromatogr. A, **685**, 1-6 (1994).

16. J. Hermansson, A. Grahn, J. Chromatogr. A, **687**, 45-59 (1994).

17. J. Haginaka, Y. Miyano, Y. Saizen, C. Seyama, T. Murashima, J. Chromatogr. A, **708**, 161-168 (1995).

18. N. Mano, Y. Oda, S. Takakuwa, S. Chiku, H. Nakata, N. Asakawa, J. Pharm. Sci., **85**, 903-907 (1996).

19. E. Domenici, C. Bertucci, P. Salvadori, I. W. Wainer, J. Pharm. Sci., **80**, 164-166 (1991).

20. T. A. G. Noctor and I. W. Wainer, J. Liq. Chromatogr., **16**, 783-800 (1993).

21. G. Massolini, A.-F. Aubry, A. McGann, I. W. Wainer, Biochem. Pharmacol., **46**, 1285-1293 (1993).

22. Y. Oda, N. Mano, N. Asakawa, Y. Yoshida, T. Sato, J. Liq. Chromatogr., **17**, 3393-3409 (1994).

23. R. Kaliszan, T. A. G. Noctor, I. W. Wainer, Mol. Pharmacol., **42**, 512-517 (1992).

24. A. Nasal, A. Radwanska, K. Osmialowski, A. Bucinski, R. Kaliszan, G. E. Barker, P. Sun, R. A. Harwick, Biomed. Chromatogr., **8**, 125-129 (1994).

25. R. Kaliszan, A. Nasal, M. Turowski, J. Chromatogr. A, **722**, 25-32 (1996).

26. T. C. Pinkerton, W. J. Howe, E. L. Ulrich, J. P. Comiskey, J. Haginaka, T. Murashima, W. F. Walkenhorst, W. M. Westler, J. L. Markley, Anal. Chem., **67**, 2354-2367 (1995).

27. T. Miwa, T. Miyakawa, Y. Miyake, J. Chromatogr., **457**, 227-233 (1988).

28. Y. Oda, N. Asakawa, S. Abe, Y. Yoshida, T. Sato, J. Chromatogr., **572**, 133-141 (1991).

29. N. Mano, Y. Oda, T. Miwa, N. Asakawa, Y. Yoshida, T. Sato, J. Chromatogr., **603**, 105-109 (1992).

30. N. Mano, Y. Oda, N. Asakawa, Y. Yoshida, T. Sato, T. Miwa, J. Chromatogr., **623**, 221-228 (1992).

31. N. Mano, Y. Oda, N. Asakawa Y. Yoshida, T. Sato, T. Miwa, J. Chromatogr. A, **687**, 223-232 (1994).

32. N. Mano, Y. Ishihama, Y. Oda, N. Asakawa, Anal. Sci., **11**, 983-987 (1995).

33. J. W. Jorgenson, K. D. Lukacs, Anal. Chem., **53**, 1298-1302 (1981).

34. S. Hjertén, J. Chromatogr., **270**, 1-6 (1983).

35. E. Gassmann, J. E. Kuo, R. N. Zare, Science, **230**, 813-814 (1985).

36. S. Fanali, J. Chromatogr., **474**, 441-446 (1989).

37. H. Nishi, T. Fukuyama, S. Terabe, J. Chromatogr., **553**, 503-516 (1991).

38. S. Terabe, M. Shibata, Y. Miyashita, J. Chromatogr., **480**, 403-411 (1989).

39. H. Nishi, T. Fukuyama, M. Matsuo, S. Terabe, J. Chromatogr., **515**, 233-243 (1990).

40. A.Dobashi, T. Ono, S. Hara, J. Yamaguchi, Anal. Chem., **61**, 1984-1986 (1989).

41. K. Otsuka, S. Terabe, J. Chromatogr., **515**, 221-226 (1990).

42. S. Birnbaum, S. Nilsson, Anal. Chem., **64**, 2872-2874 (1992).

43. G. E. Barker, P. Russo, R. A. Hartwick, Anal. Chem., **64**, 3024-3028 (1992).

44. Y. Ishihama, Y. Oda, N. Asakawa, Y. Yoshida, T. Sato, J. Chromatogr. A, **666**, 193-201 (1994).

45. Y. Tanaka, S. Terabe, J. Chromatogr. A, **694**, 277-284 (1995).

46. Y. Tanaka, N. Matsubara, S. Terabe, Electrophoresis, **15**, 848-853 (1994).

47. A. Aahmed, H. Ibahim, F. Pastoé, D. K. Lloyd, Anal. Chem., **68**, 3270-3273 (1996).

48. M. B. Rhodes, N. Bennett, R. E. Feeney, J. Biol. Chem., **234**, 2054-2060 (1959).

49. E. C. Rickard, M. M. Strohl, R. G. Nielsen, Anal. Biochem., **197**, 197-207 (1991).

50. Y. Oda, N. Mano, N. Asakawa, Y. Yoshida, T. Sato, T. Nakagawa, Anal. Sci., **9**, 221-228 (1993).

51. D. B. Wetlaufer, Adv. Protein Chem., **17**, 303-390 (1962).

52. M. Legrand, R. Viennet, Bull. Soc. Chim. France, **679** (1965).

53. R. L. Lundblad, **Techniques in Protein Modification**, CRC press, Inc., Boca Raton, FL, USA, 1995.

54. D.E. Koshland Jr., Y. D. Karkhanis, H. G. Latham, J. Am. Chem. Soc., **86**, 1448-1450 (1964).

55. R. T. Simpson, J. F. Riordan, B. Vallee, Biochem., **2**, 616-622 (1963).

56. N. Norioka, T. Okada, Y. Hamazume, T. Mega, T. Ikenaka, J. Biochem., **97**, 19-28 (1985).

57. Y. Sato, K. Watanabe, Proteins Nucleic Acids Enzymes, **23**, 54-65 (1978).

58. H. Katayama, Y. Ishihama, N. Asakawa, in submitting.

Received June 10, 1997
Accepted August 25, 1997
Manuscript 4501