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Quantitative Analysis of the Structure–Hydrophobicity Relationship for Di- and Tripeptides Based on Voltammetric Measurements with an Oil/Water Interface

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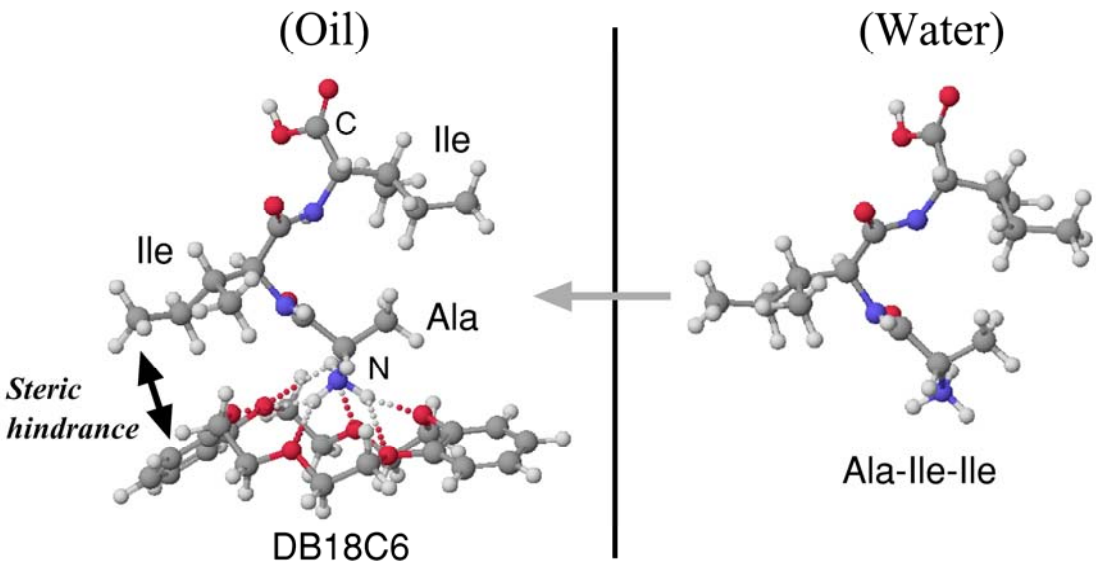
Graphical contents entry

Quantitative Analysis of the Structure–Hydrophobicity Relationship for Di- and Tripeptides Based on Voltammetric Measurements with an Oil/Water Interface

Toshiyuki Osakai,* Toshimi Hirai, Tateaki Wakamiya, and Shigeo Sawada

Ion transfer voltammetry with a polarized nitrobenzene/water interface has revealed that a notable steric hindrance exists between the benzene rings of dibenzo-18-crown-6 (DB18C6) and a bulky amino acid side chain at the central position of a tripeptide.



Summary

The transfer of 18 di- and 27 tripeptides with un-ionizable amino acid side chains at a nitrobenzene/water (NB/W) interface was studied by cyclic voltammetry. The reversible half-wave potential ($E^{r}_{1/2}$), *i.e.*, the midpoint potential could be accurately determined at pH 2 for both the facilitated and non-facilitated transfers, respectively, in the presence and absence of dibenzo-18-crown-6 (DB18C6) in NB. A multiple linear regression analysis was then performed for the $E^{r}_{1/2}$ using the 'corrected' Dubois steric parameter for amino acid side chain substitutents. The result shows that the hydrophobicity of the peptides is governed not only by the intrinsic hydrophobicity of the peptide backbone and side chains, but also by the steric effects of side chain substituents. For the non-facilitated transfer of peptides, the steric effect of a bulky side chain is more significant at the N-terminus than at the C-terminus (and central for tripeptides). The more bulky the side chain at the N-terminus, the less hydrophobic the peptide becomes due to inhibition of the solvation of a terminal $-NH_3^+$ group by organic solvents. For the facilitated transfer by DB18C6, however, the steric effect of a bulky side chain is the most significant at the central position of a tripeptide. A MOPAC calculation of optimized structures of DB18C6–peptide complexes has also shown that there is a notable steric hindrance between the central side chain and the benzene rings of DB18C6, which would reduce the 'apparent' hydrophobicity or transferability of the tripeptide.

Introduction

The hydrophobicity of component amino acids and peptide segments is one of the most important factors that determines the higher-dimensional structures (*i.e.*, folding processes) of proteins.[1] It is also essential for predicting the binding affinity of a membrane protein to a lipid bilayer.[2] Such structural characteristics of proteins, which are strongly influenced by the hydrophobicity of the components, are deeply related to their biological functions. Moreover, the hydrophobicity of peptides and analogues as potential therapeutic drugs[3] and agrochemicals[4] would be highly important in the study of quantitative structure–activity relationship (QSAR).[5]

In the pioneering work of Nozaki and Tanford[6] the relative free energies ($\Delta\mu^0$) of transfer of amino acid side chains from dioxane or ethanol to water were determined based on the solubility data and were proposed as the hydrophobicity scale of amino acid side chains. After that, Fauchère and Pliška[7] proposed a similar hydrophobicity scale based on the partition coefficient (log *P*) of amino acid derivatives in the 1-octanol/water system. Similarly, based on log *P* values, Rekker[8] proposed the hydrophobic fragmental constant (*f*-value), by which the hydrophobicity of various compounds including peptides can be evaluated. In recent years Akamatsu *et al.*[9–11] successfully obtained reliable log *P* values in the 1-octanol/water system for various di- to pentapeptides with un-ionizable side chains. The lop *P* value for di- and tripeptides was shown to be governed not only by the 'intrinsic' hydrophobicity of the peptide backbone and side chains but also by the steric effects of side chains on the relative solvation of backbone and terminal functional groups. Akamatsu and Fujita[10,11] then defined a new 'effective' hydrophobicity scale (π_α) for un-ionizable amino acid residues in addition to their intrinsic hydrophobicity scale (π). The π_α scale of an un-ionizable residue shows two different values for N-termini and for others (*i.e.*, central and C-termini). Besides the above scales proposed based on partitions in organic solvent/water

systems, some hydrophobicity scales have been presented based on different physicochemical parameters including hydration potential of RH (R being an amino acid residue), residue accessible surface area, *etc.*[2,12,13]

In a previous study[14] we employed a voltammetric technique with a polarized nitrobenzene/water (NB/W) interface to study the interfacial transfer of 13 dipeptides with un-ionizable side chains facilitated by dibenzo-18-crown-6 (DB18C6). This technique is often called 'ion transfer voltammetry' (see reviews[15–19]) and is very promising for determination of the Gibbs energy of transfer of ions as their hydrophobicity scale.[20,21] In this method the interfacial transfer of a 'single' ion can be observed as an electric current, while in conventional partition experiments, the distribution ratio of an ion should be influenced by distribution of the counterion and/or other coexisting ion(s). Therefore, in ion transfer voltammetry, more accurate and reliable determination of Gibbs transfer energies of ions can be achieved. So far, ion transfer voltammetry has been extensively used for the study of transfer of ionic drugs at oil/water (O/W) interfaces.[22–29] Scholz *et al.* have determined standard Gibbs transfer energies of amino-acid and peptide ions by means of three-phase electrodes.[30–33] In our previous study[14] the reversible potential of the transfer of protonated dipeptide cations facilitated by DB18C6 at pH 2.3 was found to show good correlations with the hydrophobicities of dipeptides, which were estimated by the hydrophobicity scales previously proposed based on partitions in organic solvent/water systems. Especially, the best correlation (with r = 0.991) was obtained for the π_α scale by Akamatsu and Fujita,[10,11] in which the steric effects of amino acid side chains were incorporated.

In this study the voltammeric study was extended to 27 tripeptides and additional 5 dipeptides with un-ionizable side chains (Gly, Ala, Val, Leu, Ile, Phe, Trp). For all the peptides, a well-defined reversible wave due to the transfer of a peptide cation facilitated by DB18C6 was obtained. Among the total 45 peptides including the previously studied 13 dipeptides, as many as 35 peptides gave a well-defined wave due to the simple (*i.e.*, non-

facilitated) transfer even in the absence of DB18C6 in NB. The reversible half-wave potentials of the facilitated and non-facilitated transfers of peptides were analyzed by multiple linear regression (MLR) in a similar manner to Akamatsu *et al.*[9–11] The results confirmed substantially the position-dependent steric effects of amino acid side chains on the hydrophobicity of peptides, however the steric effects were found to be altered by the complexation of a terminal $-NH_3^+$ group with DB18C6.

Experimental

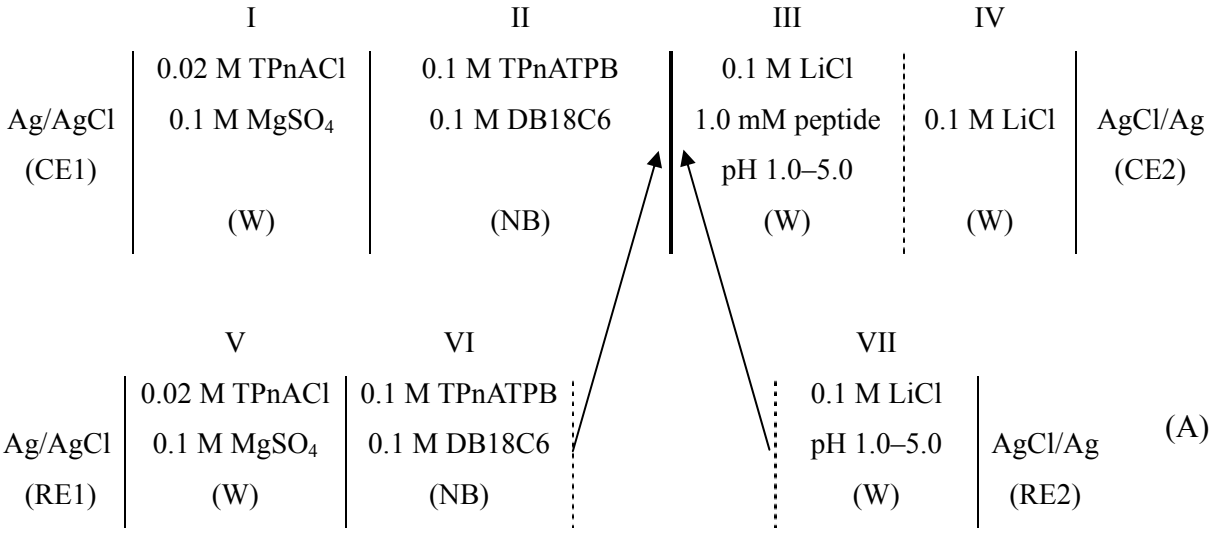
Reagents

All peptides used consist of L-amino acids and were obtained as described below. Ala-Leu-Leu, Phe-Leu-Leu, Trp-Leu-Leu (Kishida Chemical Co. Ltd, Japan); Leu-Leu-Ala, Leu-Leu-Val, Leu-Leu-Leu, Val-Val-Val (Watanabe Kagaku Kogyo K.K., Japan); and Phe-Phe-Phe, Val-Phe-Phe, Phe-Trp-Val, Val-Trp-Phe, Val-Phe-Leu, Leu-Phe-Phe, Phe-Phe-Leu (Kokusan Chemical Co. Ltd., Japan) were purchased and used as received. The other tripeptides were synthesized in a conventional manner[30] using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide[31] (EDC) as the coupling reagent. The Boc- (*tert*-butyloxycarbonyl)-amino acids and amino acid methyl esters used in the syntheses were purchased from Watanabe Kagaku Kogyo. The tripeptides synthesized were confirmed using FAB-MS (model JSM-700TKM, JEOL Co., Japan). IIe-IIe, IIe-Leu, Ile-Phe (Kokusan Chemical) and Gly-Phe, Trp-Gly (Sigma-Aldrich Co.) were purchased and used as received. The preparation and purification of tetrapentylammonium tetraphenylborate (TPnATPB), the preparation of an aqueous solution of tetrapentylammonium chloride (TPnACl), and the purification of NB were described previously.[32] 1,2-Dichloroethane (DCE) for HPLC was purchased from Wako Pure Chemical Industries, Ltd., Japan and used as received. All the other reagents were of the highest grade available and used as received.

Electrochemical measurements

Voltammetric measurements were performed using a computer-assisted measurement system.[33] Usually, a four-electrode electrolytic cell[33] was used, in which a reproducible flat NB/W interface (surface area, 0.062 cm^2) was formed. For the use of precious peptides, a three-electrode cell was occasionally used, in which a small glass tube (3-mm inner diameter; 7-cm long) with a rubber cap was used as the aqueous-phase container; by inserting the needle of a microsyringe into the rubber cap, the O/W interface formed around the end of the glass tube was adjusted to be flat. The test NB/W interfaces were polarized using a potentiostat (model HA1010mM1A, Hokuto Denko Co., Japan) equipped with a positive-feedback circuit for IR compensation.[33]

Unless noted otherwise, the electrochemical cell studied was



(A)

where || represents the test NB/W interface. The potential difference of the interface was controlled using the two reference electrodes (RE1 and RE2) immersed in the respective phases by means of Luggin capillaries whose tips were located near the test interface. The current flowing through the test interface was detected by means of the counter electrodes (CE1 and CE2). In the three-electrode system, an Ag/AgCl coil electrode was directly immersed in the aqueous phase (III) and used for both CE2 and RE2. The pH of the aqueous

phase was adjusted with sulfuric acid for pH 1–2.5, 0.05 M citrate–lithium citrate buffer for pH 3–3.5, and 0.05 M acetate–lithium acetate buffer for pH 4–5. The electrolytic cells were water-jacketed to maintain the temperature at 25 ± 0.1 °C.

The Galvani potential difference of the NB/W interface, $\Delta_{\mathrm{O}}^{\mathrm{W}}\phi$ ($\equiv \phi^{\mathrm{W}} - \phi^{\mathrm{O}}$), was estimated by referring the reversible half-wave potential (*i.e.*, the midpoint potential in cyclic voltammetry) for the transfer of tetramethylammonium ion (TMA^+), which is given by

$$E_{1/2,j}^{\mathrm{r}} = \Delta_{\mathrm{O}}^{\mathrm{W}}\phi^{\circ}{}_{j} + \frac{RT}{F}\ln\frac{\gamma_j^{\mathrm{O}}}{\gamma_j} + \frac{RT}{F}\ln\sqrt{\frac{D_j}{D_j^{\mathrm{O}}}} + \Delta E_{\mathrm{ref}} \quad (1)$$

where $\Delta_{\mathrm{O}}^{\mathrm{W}}\phi^{\circ}{}_{j}$ is the standard ion-transfer potential of ion *j* (here, TMA^+ with $\Delta_{\mathrm{O}}^{\mathrm{W}}\phi^{\circ}{}_{j}$ = +0.035 V [34]); γ_j and D_j are its activity coefficient and diffusion coefficient, respectively (in this equation and the following ones, the super- or subscript "O" represents the O phase and no super- or subscript the W phase); ΔE_{ref} ($= E - \Delta_{\mathrm{O}}^{\mathrm{W}}\phi$; E being the applied potential) is the constant which is determined only by the reference electrodes used; and R, T, and F have their usual meanings. By assuming $\gamma_j^{\mathrm{O}}/\gamma_j = 1$ and $D_j/D_j^{\mathrm{O}} = 2$ (*i.e.*, the reciprocal ratio of the viscosities of W and NB), ΔE_{ref} was then estimated to be +0.333 V for cell (A). It was confirmed that there was only a small difference (< 2 mV) in ΔE_{ref} between the four- and three-electrode systems.

Results and discussion

Voltammetric data

The voltammetric behaviors of the di- and tripeptides could likewise be understood in terms of the previously proposed reaction mechanism (see Fig. 1 in ref. 14), which involves the following equilibria in the W and O phases:

$$HA^+ \rightleftharpoons A^{\pm} + H^+ \text{ (in W):} \qquad K_1 = \frac{[A^{\pm}][H^+]}{[HA^+]} \quad (2)$$

$$HA^+ + L \rightleftharpoons HAL^+ \text{ (in O):} \qquad K_{\mathrm{c}}^{\mathrm{O}} = \frac{[HAL^+]_{\mathrm{O}}}{[HA^+]_{\mathrm{O}}[L]_{\mathrm{O}}} \quad (3)$$

$$A^{\pm} + L \rightleftharpoons AL^{\pm}\,(\text{in O}): \qquad K_c^{O\prime} = \frac{[AL^{\pm}]_O}{[A^{\pm}]_O[L]_O} \tag{4}$$

where HA^+ and $A^{\pm}$ denote the protonated form and zwitterion of a peptide; L is the neutral ionophore (here, DB18C6); and [ ] represents the concentration of each species in W or O. The distribution of neutral $A^{\pm}$ at the O/W interface is assumed to be in equilibrium:

$$A^{\pm}\,(W) \rightleftharpoons A^{\pm}\,(O): \qquad K_D = \frac{C^O_{A^{\pm}}(0,t)}{C_{A^{\pm}}(0,t)} \tag{5}$$

In this and the following equations, $C_j(0,t)$ represents the interfacial concentrations of ion j (here, $A^{\pm}$). The distribution of HA^+ having a charge of +1 at the O/W interface is assumed to obey the Nernst equation:

$$HA^+\,(W) \rightleftharpoons HA^+\,(O): \qquad \theta_{HA^+} = \frac{C^O_{HA^+}(0,t)}{C_{HA^+}(0,t)} = \exp\left[F(E - E^{\circ\prime})/RT\right] \tag{6}$$

with

$$E^{\circ\prime} = \Delta^W_O\phi^\circ_{HA^+} + \frac{RT}{F}\ln\frac{\gamma^O_{HA^+}}{\gamma_{HA^+}} + \Delta E_{ref} \tag{7}$$

Fig. 1 shows representative cyclic voltammograms for the facilitated transfer of a tripeptide (Phe-Leu-Leu) at the interface between the NB phase containing 0.1 M DB18C6 and the W phase (pH 2.0). The anodic (positive-current) peak corresponds to the transfer of the protonated form (HA^+) of the peptide from W to NB facilitated by DB18C6, while the cathodic (negative-current) peak corresponds to the back transfer to W. For this peptide, a wave for its non-facilitated transfer could be observed in the absence of DB18C6, but at a potential higher than about 0.22 V (see below). As seen in the inset of Fig. 1, the anodic peak current (I_{pa}) was proportional to the square root of the scan rate (v), showing that the ion transfer process was diffusion-controlled. The difference between the anodic and cathodic peak potentials (E_{pa} and E_{pc}) was about 60 mV, being close to the theoretical value (59 mV) for the reversible transfer of a monovalent ion. The midpoint potential, E_{mid} ($\equiv (E_{pa} + E_{pc})/2$), was then regarded as the reversible half-wave potential ($E^r_{1/2}$).

Some relatively hydrophilic peptides gave their transfer waves at rather positive potentials where the transfer of proton and/or electrolyte ions was prominent. In these cases,

however, the base current could be corrected for to obtain a well-defined wave for the peptide transfer, as shown in Fig. 2.

A typical pH-dependence of $E^{\mathrm{r}}_{1/2}$ for the facilitated transfer of a tripeptide is shown for Leu-Val-Phe in Fig. 2. As seen in the figure, $E^{\mathrm{r}}_{1/2}$ was not changed by pH in the range of pH ≤ 2, while it was shifted to higher potentials with increasing pH. Such pH dependence of $E^{\mathrm{r}}_{1/2}$ can be elucidated by the previously derived equation:[14]

$$E^{\mathrm{r}}_{1/2} = \Delta^{\mathrm{W}}_{\mathrm{O}}\phi^{\circ}_{\mathrm{HA}^+} - \frac{RT}{F}\ln\left(K^{\mathrm{O}}_{\mathrm{c}}[\mathrm{L}]_{\mathrm{O}}\right) + \frac{RT}{F}\ln\frac{\gamma^{\mathrm{O}}_{\mathrm{HA}^+}}{\gamma_{\mathrm{HA}^+}} + \frac{RT}{F}\ln\sqrt{\frac{\overline{D}}{D^{\mathrm{O}}_{\mathrm{HAL}^+}}} + \frac{RT}{F}\ln\left(1 + \frac{K_1}{[\mathrm{H}^+]} + \frac{K_1 K_{\mathrm{D,a}}}{[\mathrm{H}^+]}\sqrt{\frac{D^{\mathrm{O}}_{\mathrm{AL}^\pm}}{\overline{D}}}\right) + \Delta E_{\mathrm{ref}} \tag{8}$$

where $\overline{D}$ is the effective diffusion coefficient given by

$$\overline{D} = \frac{D_{\mathrm{HA}^+} + \left(K_1/[\mathrm{H}^+]\right)D_{\mathrm{A}^\pm}}{1 + \left(K_1/[\mathrm{H}^+]\right)} \tag{9}$$

and where $K_{\mathrm{D,a}}$ stands for the apparent distribution coefficient defined by

$$K_{\mathrm{D,a}} = \frac{C^{\mathrm{O}}_{\mathrm{AL}^\pm}(0,t)}{C_{\mathrm{A}^\pm}(0,t)} = K^{\mathrm{O}}_{\mathrm{c}}{}'K_{\mathrm{D}}[\mathrm{L}]_{\mathrm{O}} \tag{10}$$

The solid line in Fig. 2 represents the fitting curve obtained by using eqn. (8) with pK_1 = 3.55, $K_{\mathrm{D,a}}$ = 0.62, $D^{\mathrm{O}}_{\mathrm{AL}^\pm}/\overline{D}$ = 1/2 (assumed), and Σ(pH-independent terms) = 0.243 V. In the lower pH region, where the condition that pH « pK_1 – log(1 + $K_{\mathrm{D,a}}\sqrt{D^{\mathrm{O}}_{\mathrm{AL}^\pm}/\overline{D}}$) = 3.4 is fulfilled, $E^{\mathrm{r}}_{1/2}$ can be approximately given by

$$E^{\mathrm{r}}_{1/2} = \Delta^{\mathrm{W}}_{\mathrm{O}}\phi^{\circ}_{\mathrm{HA}^+} - \frac{RT}{F}\ln\left(K^{\mathrm{O}}_{\mathrm{c}}[\mathrm{L}]_{\mathrm{O}}\right) + \frac{RT}{F}\ln\frac{\gamma^{\mathrm{O}}_{\mathrm{HA}^+}}{\gamma_{\mathrm{HA}^+}} + \frac{RT}{F}\ln\sqrt{\frac{\overline{D}}{D^{\mathrm{O}}_{\mathrm{HAL}^+}}} + \Delta E_{\mathrm{ref}} \tag{11}$$

The reaction scheme in this case can be expressed more simply by using only eqns. (2), (3), and (6). According to eqn. (11), the voltammetric wave should shift to more negative potentials linearly with the logarithm of $[\mathrm{L}]_{\mathrm{O}}$. In practice, such ligand concentration dependence was observed as shown in Fig. 4; the $E^{\mathrm{r}}_{1/2}$ *vs.* log$[\mathrm{L}]_{\mathrm{O}}$ plot (not shown) showed a straight line with the slope close to the theoretical value of –0.059 V (= –2.303*RT*/*F*). These

voltammetric behaviors were common among the peptides studied as well as the previous dipeptides,[14] although there was somewhat difference in the $E^{\mathrm{r}}_{1/2}$ *vs.* pH curve between the peptides. However, for all the peptides, the values of $E^{\mathrm{r}}_{1/2}$ were constant in the pH range below at least 2. The $E^{\mathrm{r}}_{1/2}$ values at pH 2 thus determined are shown in Table 1.

As seen in Table 1, the $E^{\mathrm{r}}_{1/2}$ values at pH 2 for tripeptides are on the whole more negative than those for dipeptides, showing that the tripeptides are more hydrophobic than the dipeptides. For this the transfer waves for most tripeptides could be in the potential window even in the absence of DB18C6. A typical example is shown in Fig. 3. All the dipeptides studied in this study (Nos. 1–5 in Table 1) also gave a wave for their non-facilitated transfer, while many previous dipeptides[14] (Nos. 6–13) did not. The $E^{\mathrm{r}}_{1/2}$ values (at pH 2) for non-facilitated transfer of di- and tripeptides are also shown in Table 1. Theoretically, the $E^{\mathrm{r}}_{1/2}$ for the simple ion transfer is expressed as

$$E^{\mathrm{r}}_{1/2} = \Delta^{\mathrm{W}}_{\mathrm{O}}\phi^{\circ}_{\mathrm{HA}^+} + \frac{RT}{F}\ln\frac{\gamma^{\mathrm{O}}_{\mathrm{HA}^+}}{\gamma_{\mathrm{HA}^+}} + \frac{RT}{F}\ln\sqrt{\frac{D_{\mathrm{HA}^+}}{D^{\mathrm{O}}_{\mathrm{HA}^+}}} + \Delta E_{\mathrm{ref}} \tag{12}$$

By assuming $\gamma^{\mathrm{O}}_{\mathrm{HA}^+}/\gamma_{\mathrm{HA}^+} = 1$ and $D_{\mathrm{HA}^+}/D^{\mathrm{O}}_{\mathrm{HA}^+} = 2$ and using $\Delta E_{\mathrm{ref}} = +0.333$ V (see above), the standard ion transfer potentials ($\Delta^{\mathrm{W}}_{\mathrm{O}}\phi^{\circ}_{\mathrm{HA}^+}$) of the protonated peptides were determined as shown in Table 1.

The difference in $E^{\mathrm{r}}_{1/2}$ between the facilitated and non-facilitated transfers ($\Delta E^{\mathrm{r}}_{1/2}$), being also shown in Table 1, should be given from eqns. (11) and (12) as

$$\begin{aligned}\Delta E^{\mathrm{r}}_{1/2} &= \left(E^{\mathrm{r}}_{1/2}\right)_{\text{eqn. (12)}} - \left(E^{\mathrm{r}}_{1/2}\right)_{\text{eqn. (11)}} \\ &= \frac{RT}{F}\ln\left(K^{\mathrm{O}}_{\mathrm{c}}[\mathrm{L}]_{\mathrm{O}}\right) + \frac{RT}{F}\ln\sqrt{\frac{D_{\mathrm{HA}^+}D^{\mathrm{O}}_{\mathrm{HAL}^+}}{D^{\mathrm{O}}_{\mathrm{HA}^+}\overline{D}}} \approx \frac{RT}{F}\ln\left(K^{\mathrm{O}}_{\mathrm{c}}[\mathrm{L}]_{\mathrm{O}}\right)\end{aligned} \tag{13}$$

Using this relation, the values of $K^{\mathrm{O}}_{\mathrm{c}}$ could be determined from $\Delta E^{\mathrm{r}}_{1/2}$ for the peptides that gave the waves for both facilitated and non-facilitated transfers. As seen in Table 1, the $\log(K^{\mathrm{O}}_{\mathrm{c}}/\mathrm{M}^{-1})$ values were not very dependent on the nature of peptides, ranging from 4.4 to

5.0 (average 4.74 ± 0.15). As discussed previously,[14] this is because the $-NH_3^+$ group of a peptide is complexed with DB18C6 in such a way that it is anchored in the center and on the top of the DB18C6 macrocycle. It was suggested from a MOPAC calculation that the repulsive interactions between the amino acid side chains and the benzene rings of DB18C6 be not very serious. Roughly speaking, this is true, but the steric effects of bulky side chains on the complexation should exist as shown below.

We would like to add that the $\Delta_O^W\phi°$ values of various peptide anions have been reported, which were determined using three-phase electrodes.[31,32] Although only few anions (Try-Gly$^-$, Leu-Leu$^-$, Gly-Phe$^-$, Leu-Leu-Ala$^-$, Leu-Leu-Leu$^-$) can be compared with the corresponding cations listed in Table 1, there is no distinct correlation between their $\Delta_O^W\phi°$ values. At present the reason is unknown; further study should be conducted on the difference in solvation state between anionic and cationic forms of a peptide.

Correlation study

Table 2 shows the previously reported hydrophobicity scales of un-ionizable amino acid residues. Correlations of the scales with $E_{1/2}^r$ for the facilitated and non-facilitated transfers of di- and tripeptides were studied and summarized in Table 3. In each of the four data groups, hydrophobicity scales, π, π_α, π(F.P.), $\Delta\mu^0$, and f, which were proposed based on partitions in organic solvent/water systems, gave good estimations for the hydrophobicities of peptides. In particular, the π_α scale of Akamatsu and Fujita[10,11] gave the best estimation, suggesting that the steric effects of amino acid side chains should be considered to evaluate accurately the hydrophobicity of peptides. This is in line with the previous results for the facilitated transfer of 13 dipeptides.[14] Then we performed a MLR analysis similar to the one presented by Akamatsu *et al.*,[9–11] and examined the steric effects of side chains in details.

MLR analysis

In the previous MLR analysis,[9–11] the 'intrinsic' hydrophobicity scales (π) of amino acid residues were estimated from log *P* values of the related compounds and used as a regressor in the MLR analysis. In this study we independently estimated intrinsic hydrophobicity scales of amino acids from the $E_{1/2}^{r}$ values for facilitated or non-facilitated transfer of amino acids, as described below.

Table 4 shows the $E_{1/2}^{r}$ values of some amino acids, which were likewise obtained using cell (A) at pH 2 in the presence and absence of 0.1 M DB18C6 in NB (in Table 4 are also shown the intrinsic hydrophobicity scales of amino acids and steric parameters[9,35] of their un-ionizable side chains). In the presence of DB18C6, only Gly gave no voltammetric wave in the potential window. However, by changing the organic solvent from NB to DCE, a well-developed wave could be obtained for the facilitated transfer of Gly as well as other amino acids. The $E_{1/2}^{r}$ values for the amino acids are in good relative agreement with recently reported values[36] ($r^2 = 0.991$). It was then found that the present $E_{1/2}^{r}$ values for Ala, Val, Leu, and Ile (including no benzene ring) at the DCE/W interface showed an excellent correlation with those at the NB/W interface:

$$E_{1/2}^{r}(\text{DCE/W}) = 1.044\, E_{1/2}^{r}(\text{NB/W}) - 0.028 \qquad (r^2 = 0.997) \qquad (14)$$

In this and the following equations, $E_{1/2}^{r}$ values are in V. When including the data of Phe and Trp into the regression analysis, the correlation became worse ($r^2 = 0.917$), most probably because of the specific interaction between the benzene rings of amino acid and NB. Using eqn. (14), the $E_{1/2}^{r}$ value of Gly at the NB/W interface was then estimated to be 0.400 V.

The $E_{1/2}^{r}$ values of Gly and Ala for their non-facilitated transfers at the NB/W interface were also estimated as follows: As seen in Table 4, the difference in $E_{1/2}^{r}$ between Leu and Val, *i.e.*, $\Delta_{O}^{W}\phi^{\circ}_{\text{Leu}} - \Delta_{O}^{W}\phi^{\circ}_{\text{Val}}$, was –0.025 V, being in good agreement with that between Ile and Val, *i.e.*, $\Delta_{O}^{W}\phi^{\circ}_{\text{Ile}} - \Delta_{O}^{W}\phi^{\circ}_{\text{Val}} = -0.023$ V. These values (–0.024 ± 0.001 V) should correspond to the contribution of a methylene group to the standard Gibbs energy of

ion transfer: $\Delta G^{\circ,\mathrm{O}\to\mathrm{W}}_{\mathrm{tr}}(\mathrm{–CH_2–}) = +2.3$ kJ mol^{-1}. This value was in fair agreement with the previous value (+2.5 kJ mol^{-1}) for the transfer of alkylammonium ions at the NB/W interface.[37] The $E^{\mathrm{r}}_{1/2}$ values of Ala and Gly were then estimated as

$$E^{\mathrm{r}}_{1/2}(\mathrm{Ala}) = E^{\mathrm{r}}_{1/2}(\mathrm{Val}) + 2\times 0.024 = 0.610\ (\mathrm{V}) \tag{15}$$

$$E^{\mathrm{r}}_{1/2}(\mathrm{Gly}) = E^{\mathrm{r}}_{1/2}(\mathrm{Ala}) + 0.024 = 0.634\ (\mathrm{V}) \tag{16}$$

Using the observed or estimated $E^{\mathrm{r}}_{1/2}$ values shown in Table 4, the intrinsic hydrophobicity scales of amino acids were defined as

$$\phi = E^{\mathrm{r}}_{1/2}(\mathrm{Gly}) - E^{\mathrm{r}}_{1/2}(\text{amino acid});\ \text{for facilitated transfer} \tag{17}$$

$$\phi' = E^{\mathrm{r}}_{1/2}(\mathrm{Gly}) - E^{\mathrm{r}}_{1/2}(\text{amino acid});\ \text{for non-facilitated transfer} \tag{18}$$

The values of ϕ and ϕ' are also shown in Table 4. Thus the intrinsic hydrophobicity scale was evaluated individually for the facilitated and non-facilitated transfers; ϕ or ϕ' was then used as a regressor in the MLR analysis for the respective data group. We would like to add that there is a very good correlation between ϕ and ϕ': $\phi' = 1.115\phi + 0.006$ ($r^2 = 0.987$).

To evaluate the steric effects of amino acid side chains, we used the steric parameter ($E'^{\mathrm{c}}_{\mathrm{S}}$) as another regressor. The $E'^{\mathrm{c}}_{\mathrm{S}}$ was the 'corrected' Dubois steric parameter related to the original Dubois E'_{S} as $E'^{\mathrm{c}}_{\mathrm{S}} = E'_{\mathrm{S}} + 0.306(n_{\mathrm{H}} - 3)$, where n_{H} is the number of α-hydrogen atoms in aliphatic substituents.[35] The E'_{S} is the steric parameter defined as being an 'improved' Taft E_{S} value.[38] Akamatsu *et al.*[9] reported that in their MLR analysis for log P values of oligopeptides, the $E'^{\mathrm{c}}_{\mathrm{S}}$ parameter worked best among various steric parameters.

In the present MLR analysis of $E^{\mathrm{r}}_{1/2}$ for di- and tripeptides, we employed the following regression equation:

$$E^{\mathrm{r}}_{1/2} = -a\sum\phi - b_{\mathrm{N}}E'^{\mathrm{c}}_{\mathrm{S}}(\mathrm{N}) - b_{\mathrm{M}}E'^{\mathrm{c}}_{\mathrm{S}}(\mathrm{M}) - b_{\mathrm{C}}E'^{\mathrm{c}}_{\mathrm{S}}(\mathrm{C}) + c \tag{19}$$

where $E'^{\mathrm{c}}_{\mathrm{S}}(\mathrm{N})$, $E'^{\mathrm{c}}_{\mathrm{S}}(\mathrm{M})$, and $E'^{\mathrm{c}}_{\mathrm{S}}(\mathrm{C})$ are the $E'^{\mathrm{c}}_{\mathrm{S}}$ values for the amino acid side chains, respectively, at the N-terminal, central, and C-terminal positions (for the dipeptides, the term of $E'^{\mathrm{c}}_{\mathrm{S}}(\mathrm{M})$ is omitted); a, b_{N}, b_{M}, b_{C}, and c are the coefficients that represent contributions of the respective terms. For the non-facilitated transfer, ϕ in eqn. (19) was replaced with ϕ'.

Table 5 shows the results of the MLR analysis. The coefficients of determination (r^2) show that satisfactory results are obtained in the regressions for all the data groups. The a values, however, are close to but somewhat larger than 1, suggesting that the intrinsic hydrophobicity scale, ϕ or ϕ', for amino acid side chains does not perfectly contribute to the total hydrophobicity of peptides even after the factors for steric effects are separated. Thus, there are still rooms for possible improvements in the regression equation. Nevertheless, it should be noted that there is a notable difference in the order of b_N, b_M, and b_C between the facilitated and non-facilitated transfers. For the non-facilitated transfers of di- and tripeptides, the coefficients of steric effects are increased in the order of ($b_M \approx$) $b_C < b_N$. This tendency is the same as that observed by Akamatsu *et al.*[9,10] for the log P values of di- and tripeptides in the 1-octanol/water system, and it means that a di- or tripeptide becomes less hydrophobic when it has a bulky side chain at the N-terminus than at the C-terminus (and central for tripeptides). This could be explained by assuming that the solvation of a terminal $-NH_3^+$ group by large organic solvents is inhibited by a bulky side chain substituent (we withdraw the previous suggestion[14] on an inhibition of the hydration of a terminal $-COO^-$ group). For the facilitated transfers, however, the order of steric-effect coefficients shows a different feature: *i.e.*, $b_C \approx b_N \ll b_M$ for tripeptides and $b_C \leq b_N$ for dipeptides. It should be noted that the b_M value for the facilitated transfer of tripeptides is significantly larger than that for the non-facilitated transfer. This means that the steric effect of a bulky side chain at the central position of a tripeptide works to make the $E_{1/2}^r$ value more positive, *i.e.*, to make the K_c^O value smaller (see eqn. (11)). Thus, the above-mentioned K_c^O's invariance to the structure of peptides is not true in a strict sense. It has been suggested that the bulky central side chain should considerably hinder the complexation of a tripeptide with DB18C6.

The coefficient c corresponds to the $E_{1/2}^r$ values of Gly-Gly and Gly-Gly-Gly for dipeptides and tripeptides, respectively. As shown in Table 5, the $E_{1/2}^r$ value of Gly-Gly-Gly

is more positive than that of Gly-Gly for each of the facilitated and non-facilitated transfers, showing that Gly-Gly-Gly is more hydrophilic than Gly-Gly.

MOPAC calculation

In order to confirm the suggested steric hindrance from a central side chain, we have employed the PM3 method with CAChe MOPAC program[39] to calculate optimized structures of the complexes of DB18C6 with protonated forms of peptides or amino acids. In the calculation, no solvation was included; therefore the calculated structures are not necessarily the same as the real structures in the organic solvent. However, the neglecting of solvation would be rather convenient for focusing our attention on the steric effects of interest. Although the calculation results were somewhat affected by initial coordinates of the complexes, definite and reliable knowledge was obtained about the steric effects of side chains. Fig. 4(a) shows an optimized structure of the complex of DB18C6 with the smallest amino acid, Gly. As seen, the $-NH_3^+$ group of the protonated Gly is anchored in the center and on top of the DB18C6 macrocycle. Since there is no steric hindrance from the small Gly, the two benzene rings of DB18C6 are located by approaching the amino acid, so that the DB18C6 molecule has a 'bowl-like' structure. Such a structure was previously found for the complex with Phe–Ala[14] having a bulky side chain at the N-terminus. Also, the bowl-like structure of DB18C6, though slightly twisted, has been found in the complex with Ile–Ala–Ile, as shown in Fig. 4(b). However, it should be noted that introduction of a bulky amino acid, *e.g.*, Ile, to the central position induces a significant change in the DB18C6 structure. As seen in Fig. 4(c), the DB18C6 molecule in the complex with Ile–Ile–Ile takes an 'open' structure, suggesting a considerable repulsive interaction between the benzene rings of DB18C6 and the bulky side chain of Ile at the central position (as shown by a two-headed arrow). This view may be supported by the result that replacement of the N-terminal Ile with a smaller amino acid, Ala, does not change the open structure to the bowl-like one (see Fig. 4(d)). Thus, the

steric hindrance has been shown to be more serious for the central side chain than for the N-terminus. This is curious, but the less serious steric hindrance for the N-terminal side chain may be understood in terms of the sp^3-hybridized α-carbon, which can arrange even a bulky side chain adequately apart from the benzene rings of DB18C6. In contrast, the central side chain can access the benzene rings due to the free rotation of a peptide chain, so that the steric hindrance would be more serious.

The above conclusion has further been confirmed by a MLR analysis for the log K_c^O values of 25 tripeptides using the $E_S'^c$ values as regressors:

$$\log (K_c^O/M^{-1}) = \underset{}{5.026} - \underset{(0.236)}{0.242}\ E_S'^c(N) + \underset{(0.122)}{0.420}\ E_S'^c(M) + \underset{(0.155)}{0.084}\ E_S'^c(C) \quad (20)$$
$$\underset{}{} \qquad \underset{}{} \qquad \underset{}{} \qquad (r^2 = 0.703) \quad (s = 0.088) \qquad \underset{(0.104)}{}$$

where the figures in round brackets represent 95% confidence intervals of the corresponding term or coefficients. As seen in eqn. (20), the coefficient of $E_S'^c(M)$ is larger than that of $E_S'^c(N)$ or $E_S'^c(C)$, showing that a bulky side chain at the central position has a negative effect on log K_c^O (note that $E_S'^c$ has negative values; see Table 4). This is in line with the conclusion from the MOPAC calculation. It should also be noted that the coefficient of $E_S'^c(N)$ is minus and shows that a bulky side chain at the N-terminus facilitates the complexation with DB18C6. This reason is not clear but could be explained by an effective shielding of the charge of $-NH_3^+$ by the bulky substituent in organic media.

Concluding remarks

As mentioned in Introduction, ion transfer voltammetry is a useful tool for accurate determination of the transfer potential (or Gibbs transfer energy) of ions at the O/W interface. The use of this method has enabled us to assess the hydrophobicity of the di- and tripeptides based on reliable data for their ion transfer potentials.

For the simple transfer of the peptides in the absence of DB18C6, the MLR analysis for the transfer potentials has basically supported Akamatsu *et al.*'s conclusion[9,10] that the hydrophobicity of di- and tripeptides is determined mainly by the intrinsic hydrophobicity of the peptide backbone and side chains, but considerably by the steric effects of side chain substituents. The steric effect of a bulky side chain, which would be related to the inhibition of solvation in organic solvent, is more significant at the N-terminus than at the C-terminus (and central for tripeptides).

For the facilitated transfer by DB18C6, however, the MLR analysis has shown that the steric effects of side chains are altered by the complexation of a peptide with DB18C6 in organic solvent. The repulsive interaction of an amino acid side chain with the benzene rings of DB18C6 is the most significant at the central position of a tripeptide, and it makes the tripeptide apparently less hydrophobic. Thus, the 'apparent' hydrophobicity of peptides should be influenced by their complexation or dissolving conditions in solvent. This would offer valuable insight into the higher-dimensional structures of proteins.

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Table 1 The values of $E_{1/2}^{r}$ for the facilitated and non-facilitated transfers of di- and tripeptides at the NB (0.1 M DB18C6 or none)/W (pH 2) interface and the values of $\Delta_{O}^{W}\phi^{\circ}_{HA^{+}}$ and $\log K_{c}^{O}$

No.	Peptides	$E_{1/2}^{r}$/V			$\Delta_{O}^{W}\phi^{\circ}_{HA^{+}}$/V	$\log(K_{c}^{O}/M^{-1})$
		Facilitated	Non-facilitated	$\Delta E_{1/2}^{r}$		
1	Gly-Phe	0.307	0.531	0.224	0.189	4.8
2	Ile-Phe	0.219	0.457	0.238	0.115	5.0
3	Ile-Ile	0.259	0.491	0.232	0.149	4.9
4	Ile-Leu	0.258	0.487	0.229	0.145	4.9
5	Trp-Gly	0.306	0.522	0.216	0.180	4.7
6	Leu-Ala [a]	0.319	— [b]			
7	Val-Val [a]	0.310	— [b]			
8	Phe-Ala [a]	0.294	— [b]			
9	Leu-Val [a]	0.271	— [b]			
10	Val-Leu [a]	0.280	— [b]			
11	Ala-Phe [a]	0.281	— [b]			
12	Val-Phe [a]	0.252	— [b]			
13	Phe-Val [a]	0.251	— [b]			
14	Leu-Leu [a]	0.235	0.460	0.225	0.118 [c]	4.8
15	Leu-Phe [a]	0.215	0.430	0.215	0.088 [c]	4.6
16	Phe-Ile [a]	0.226	0.440	0.214	0.098 [c]	4.6
17	Phe-Leu [a]	0.210	0.426	0.216	0.084 [c]	4.7
18	Phe-Phe [a]	0.180	0.396	0.216	0.054 [c]	4.7
19	Ala-Leu-Leu	0.307	— [d]			
20	Phe-Leu-Leu	0.227	0.445	0.218	0.103	4.7
21	Trp-Leu-Leu	0.204	0.408	0.204	0.066	4.4
22	Leu-Leu-Ala	0.322	0.537	0.215	0.195	4.6
23	Leu-Leu-Val	0.277	0.493	0.216	0.151	4.7
24	Leu-Leu-Leu	0.249	0.471	0.222	0.129	4.8
25	Val-Val-Val	0.340	0.557	0.217	0.215	4.7
26	Phe-Phe-Phe	0.150	0.374	0.224	0.032	4.8
27	Val-Phe-Phe	0.214	0.441	0.227	0.099	4.8

28	Ala-Phe-Phe	0.252	0.473	0.221	0.131	4.7
29	Phe-Phe-Ala	0.262	0.492	0.230	0.150	4.9
30	Phe-Phe-Val	0.220	0.435	0.215	0.093	4.6
31	Phe-Val-Val	0.286	0.494	0.208	0.152	4.5
32	Val-Val-Phe	0.284	0.505	0.221	0.163	4.7
33	Leu-Val-Phe	0.243	0.474	0.231	0.132	4.9
34	Phe-Val-Phe	0.230	0.442	0.212	0.100	4.6
35	Phe-Val-Leu	0.258	0.467	0.209	0.125	4.5
36	Trp-Val-Phe	0.209	0.417	0.208	0.075	4.5
37	Trp-Val-Leu	0.221	0.433	0.212	0.091	4.6
38	Leu-Trp-Val	0.223	0.452	0.229	0.110	4.9
39	Val-Trp-Leu	0.214	0.444	0.230	0.102	4.9
40	Phe-Trp-Val	0.192	0.419	0.227	0.077	4.8
41	Val-Trp-Phe	0.190	0.426	0.236	0.084	5.0
42	Leu-Phe-Val	0.242	— [d]			
43	Val-Phe-Leu	0.247	0.470	0.223	0.128	4.8
44	Leu-Phe-Phe	0.178	0.412	0.234	0.070	5.0
45	Phe-Phe-Leu	0.190	0.406	0.216	0.064	4.7

[a] Ref. 14. [b] The voltammetric wave was not obtained in the potential window. [c] The previously reported values[14] have been slightly revised using ΔE_{ref} = +0.333 V (previously, +0.323 V). [d] Not determined because of deficiency of the reagent.

Table 2 Hydrophobicity scales of un-ionizable amino acid residues

Amino acid	π [a]	π_α [b]		HI [c]	HP [d]	HS [e]	π(F.P.) [f]	$\Delta\mu^0$ [g]	f [h]
		N	MC						
Gly	0	0	0	0	0	0	0	0	0
Ala	0.32	0.19	0.24	2.2	–0.45	0.02	0.31	0.5	0.53
Val	1.27	0.49	0.82	4.6	–0.40	0.18	1.22	1.5	1.46
Leu	1.81	0.92	1.28	4.2	–0.11	0.10	1.70	1.8	1.99
Ile	1.81	0.72	1.17	4.9	–0.24	0.22	1.80	—	1.99
Phe	1.95	1.35	1.57	3.2	–3.15	0.14	1.79	2.5	2.24
Trp	1.92	1.72	1.93	–0.5	–8.28	–0.12	2.25	3.4	2.31

[a] Intrinsic hydrophobicity scale estimated from $\log P$ values of related compounds.[9] [b] Effective hydrophobicity scale of Akamatsu–Fujita[8] (N: for N-terminal residues; MC: for central and C-terminal residues). [c] Hydropathy index of Kyte–Doolittle.[2] [d] Hydration potential of Wolfenden *et al.*[12] [e] Logarithm of the hydrophobic scale of Chothia.[13] [f] π value of Fauchère–Pliška.[7] [g] Hydrophobicity scale of Nozaki–Tanford.[6] [h] f value of Rekker.[8] All the hydrophobicity scales are referred to Gly.

Table 3 Squared correlation coefficients (r^2) between the $E_{1/2}^{r}$ values for the facilitated and non-facilitated transfers of di- and tripeptides and their hydrophobicities evaluated as the sum of hydrophobicity scales of amino acid residues and the number (n) of peptides given in round brackets

Σ (hydrophobicity scale)	Tripeptides		Dipeptides	
	Facilitated	Non-facilitated	Facilitated	Non-facilitated
Σ π	0.755 (27)	0.763 (25)	0.811 (18)	0.675 (10)
Σ π_α	0.939 (27)	0.980 (25)	0.944 (18)	0.986 (10)
Σ HI	0.314 (27)	0.326 (25)	0.130 (18)	0.238 (10)
Σ HP	0.590 (27)	0.542 (25)	0.040 (18)	0.003 (10)
Σ HS	0.067 (27)	0.112 (25)	0.199 (18)	0.192 (10)
Σ π(F.P.)	0.766 (27)	0.777 (25)	0.746 (18)	0.570 (10)
Σ $\Delta\mu^0$	0.877 (27)	0.878 (25)	0.866 (14)	0.892 (6)
Σ f	0.863 (27)	0.877 (25)	0.857 (18)	0.739 (10)

Table 4 The $E_{1/2}^{r}$ values for the facilitated and non-facilitated transfers of amino acids at the NB (0.1 M DB18C6 or none)/W (pH 2) interface, and their intrinsic hydrophobicity scales (ϕ and ϕ') and the steric parameters of their un-ionizable side chains ($E_S'^{c}$)

Amino acid	$E_{1/2}^{r}$/V			Intrinsic hydrophobicity scale		
	Facilitated		Non-facilitated	ϕ	ϕ'	$E_S'^{c}$ [a]
Gly	0.400 [b]	(0.390) [c]	0.634 [b]	0	0	0
Ala	0.388	(0.376) [c]	0.610 [b]	0.012	0.024	–0.202
Val	0.346 [d]	(0.336) [c]	0.562	0.054	0.072	–1.294
Leu	0.318 [d]	(0.302) [c]	0.537	0.082	0.097	–1.438
Ile	0.312 [d]	(0.298) [c]	0.539	0.088	0.095	–1.814
Phe	0.307 [d]	(0.296) [c]	0.520	0.093	0.114	–0.898
Trp	0.281	(0.295) [c]	0.495	0.119	0.139	–0.858

[a] Calculated based on refs. 9 and 35; the reference point is shifted so that $E_S'^{c}$(H) = 0 for Gly. [b] Estimated as described in the text. [c] Obtained for the corresponding DCE/W interface. [d] These values were wrongly reported in the previous paper.[14]

Table 5 Coefficients (a, b_N, b_M, b_C, c) and their 95% confidence intervals (figures in round brackets), coefficient of determination (r^2), and standard errors (s) for the MLR analysis with eqn. (19)

	a	b_N	b_M	b_C	c	r^2	s
Facilitated:							
27 tripeptides	1.215	0.017	0.046	0.017	0.434	0.977	0.007
	(0.102)	(0.009)	(0.013)	(0.009)	(0.032)		
18 dipeptides	1.378	0.024	—	0.012	0.418	0.911	0.013
	(0.275)	(0.015)		(0.016)	(0.030)		
Non-facilitated:							
25 tripeptides	1.182	0.024	0.011	0.012	0.749	0.980	0.007
	(0.094)	(0.009)	(0.013)	(0.009)	(0.035)		
10 dipeptides	1.473	0.033	—	0.010	0.694	0.985	0.007
	(0.192)	(0.011)		(0.011)	(0.030)		

Figure captions

Fig. 1 Cyclic voltammograms for the transfer of 1.0 mM Phe-Leu-Leu at the NB/W (pH 2.0) interface in the presence of 0.1 M DB18C6 in NB. Scan rate (v): 0.01, 0.02, 0.05, 0.1, 0.2 V s^{-1}. The inset shows the dependence of the anodic peak current (I_{pa}; corrected for the base current) on the square root of v.

Fig. 2 Cyclic voltammograms obtained for the NB/W (pH 2.0) interface in the (a) presence and (b) absence of 1.0 mM Gly-Phe. Curve (c) represents the current corrected for the base current. Scan rate: 0.1 V s^{-1}.

Fig. 3 The pH dependence of $E^{r}_{1/2}$ for the facilitated transfer of Leu-Val-Phe at the NB/W interface in the presence of 0.1 M DB18C6 in NB. The solid line represents the fitting curve obtained by using eqn. (8).

Fig. 4 The ligand concentration dependence of the cyclic voltammogram for the transfer of 1.0 mM Trp-Leu-Leu at the NB/W (pH 2.0) interface. $\log([L]_O/M)$: (1) –2.25, (2) –2.0, (3) –1.75, (4) –1.5 (5) –1.25, (6) –1.0. Curve (0) represents the voltammogram for the simple transfer, *i.e.*, for $[L]_O = 0$ M. Scan rate: 0.1 V s^{-1}.

Fig. 5 Cyclic voltammograms for the transfer of 1.0 mM Trp-Leu-Leu at the NB/W (pH 2.0) interface in the (a) absence and (b) presence of 0.1 M DB18C6 in NB. Curve (c) represents the base current in the absence of DB18C6. Scan rate: 0.1 V s^{-1}.

Fig. 6 Optimized structures of the complexes of DB18C6 with protonated forms of (a) Gly, (b) Ile–Ala–Ile, (c) Ile–Ile–Ile, and (d) Ala–Ile–Ile. 'N' and 'C' show $-NH_3^+$ and –COOH groups, respectively.

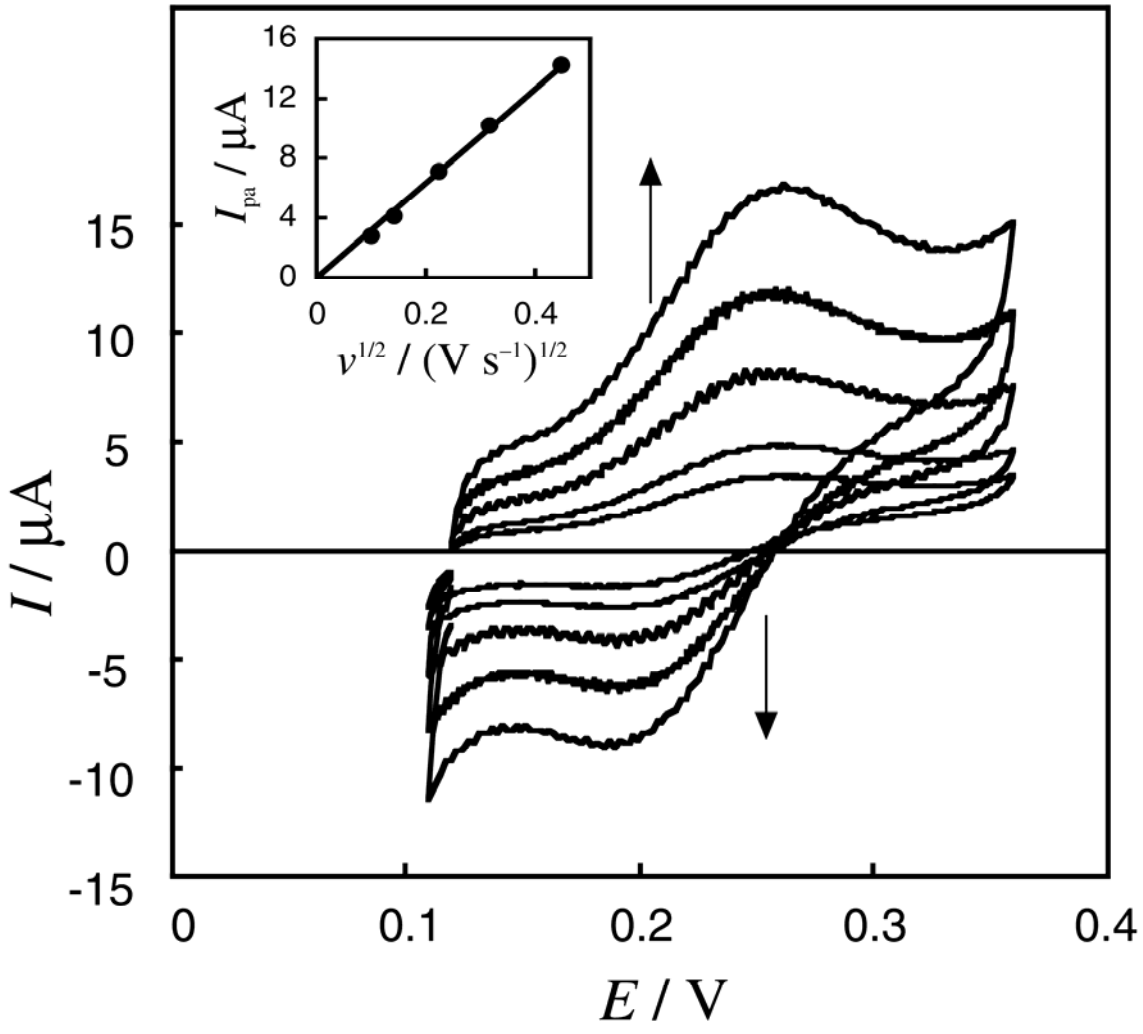
16
12
8
4
0
I_{pa} / μA
0
0.2
0.4
$v^{1/2}$ / (V s^{-1})$^{1/2}$
15
10
5
0
-5
-10
-15
I / μA
0
0.1
0.2
0.3
0.4
E / V

Fig. 1

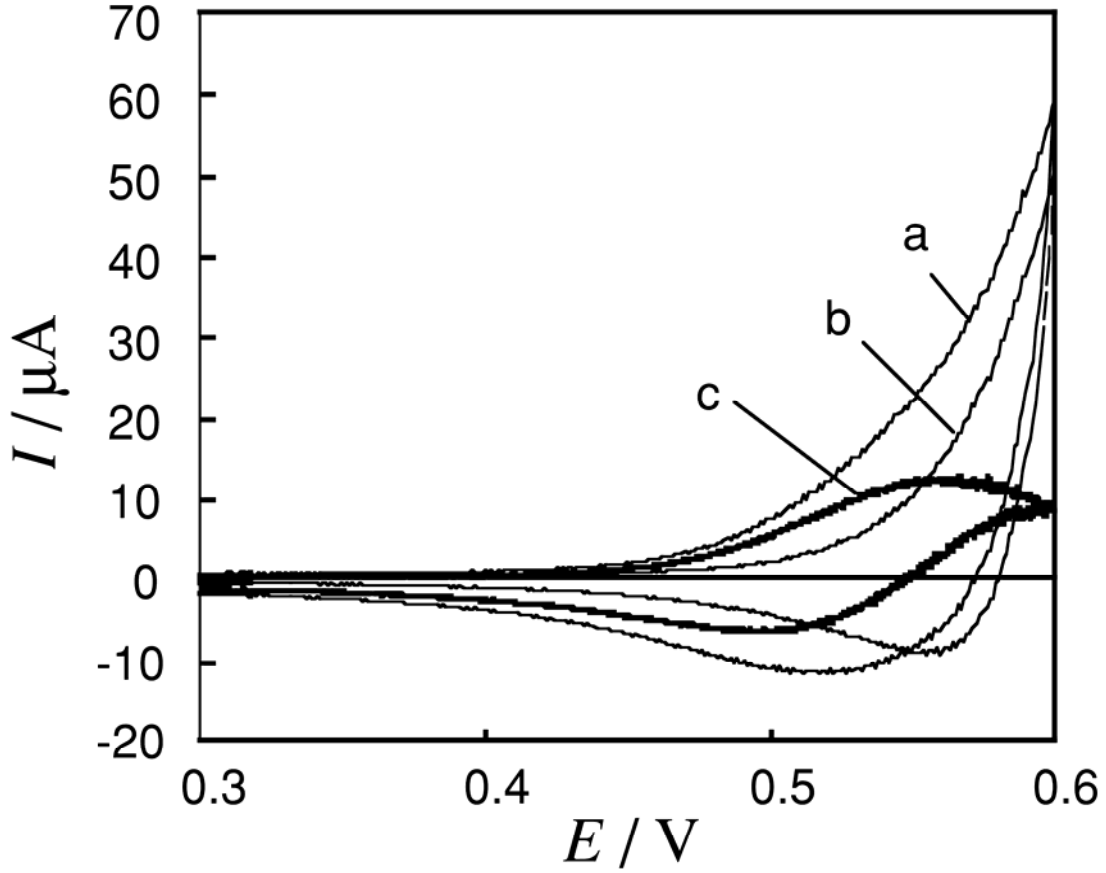

Fig. 2

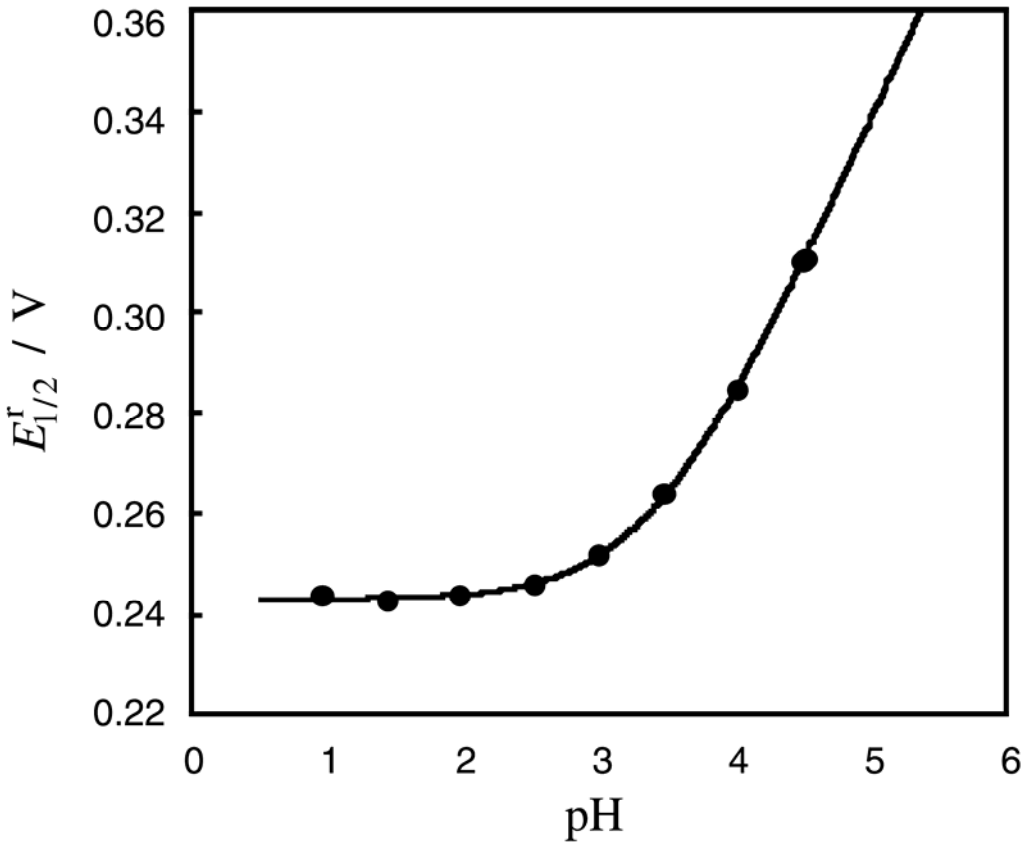

Fig. 3

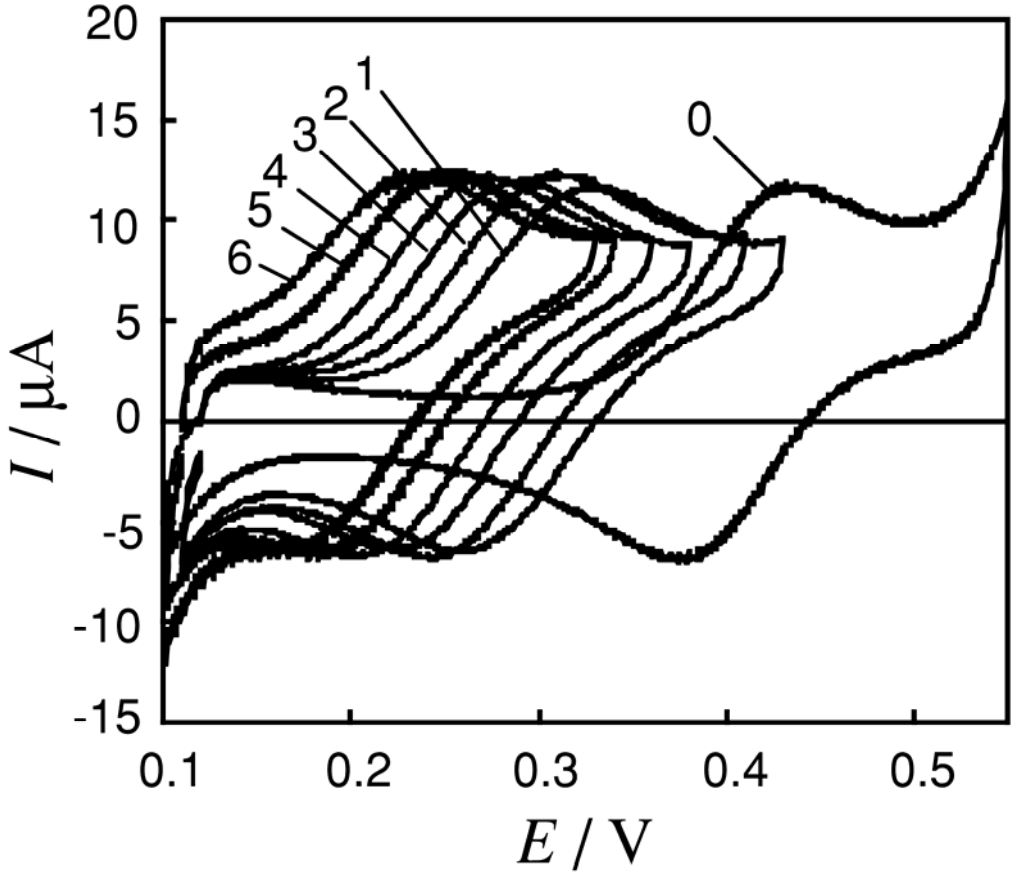

Fig. 4

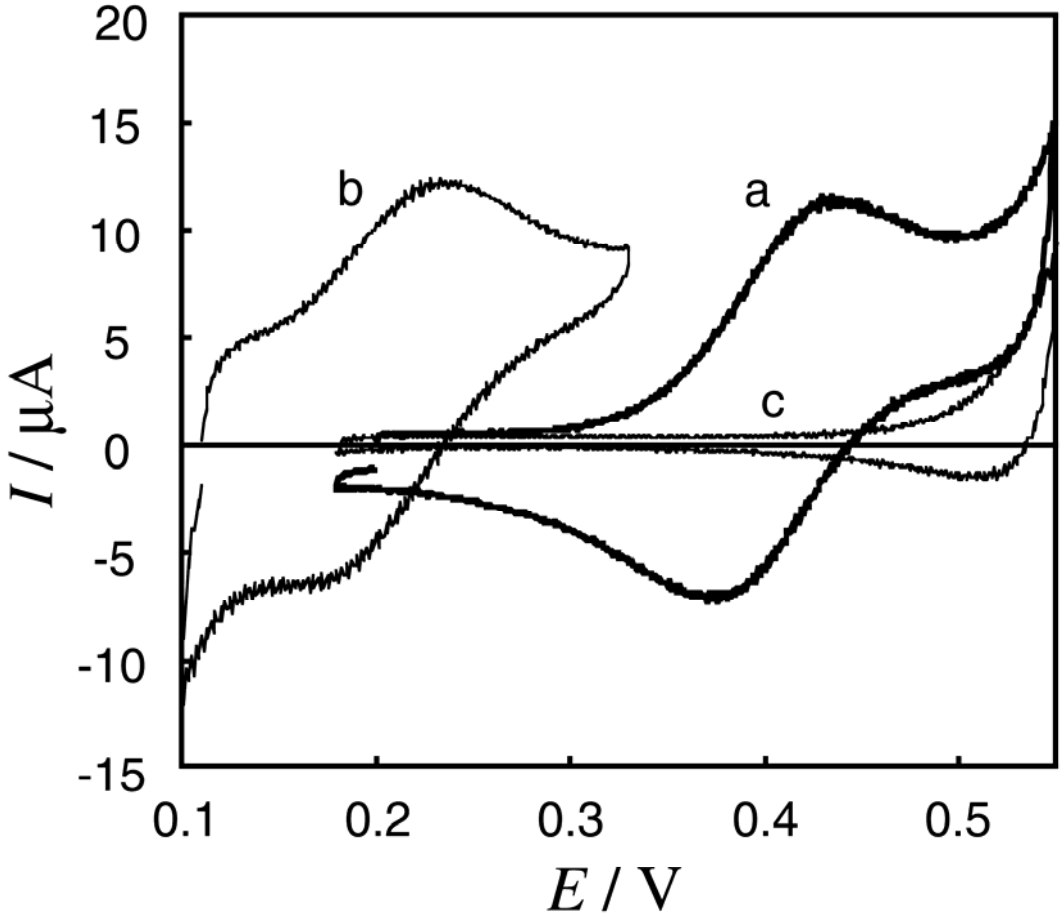

Fig. 5

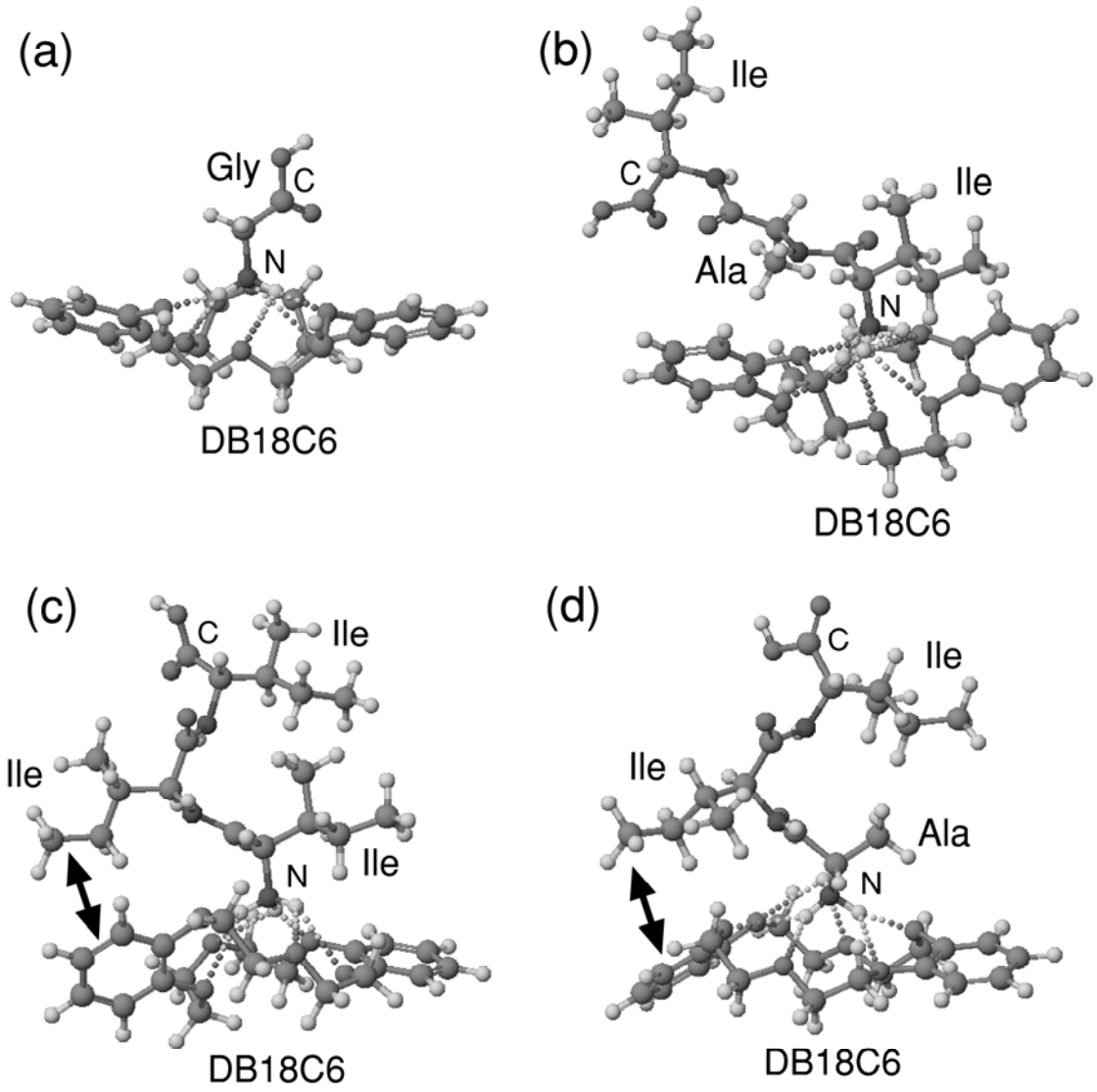
(a)
Gly
C
N
DB18C6
(b)
Ile
C
Ile
Ala
N
DB18C6
(c)
C
Ile
Ile
N
Ile
DB18C6
(d)
C
Ile
Ile
Ala
N
DB18C6

Fig. 6