# Catalytic Efficiency of Synthetic Micellar Catalysts Bearing a Mercapto Group as the Reaction Center†

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In order to obtain a clue to understanding the micro-environmental effect on the reactivity of a mercapto group placed in a reaction center of enzymes, micellar surfactants bearing a mercapto group were synthesized and their catalytic activity in the degradation of *p*-nitrophenyl carboxylates was studied. *N*-Hexadecyl-$N^{\alpha}$-glutaryl-L-cysteinamide (AM·Cys-1) has an ability to form anionic micelles in aqueous media. The catalytic activity of AM·Cys-1 was compared with that of another synthetic surfactant, *N*-hexadecanoyl-L-cysteine (AM·Cys-2). These surfactants below their critical micelle concentrations markedly accelerated the degradation of several *p*-nitrophenyl carboxylates. On the contrary, the concentration - rate profiles for the degradation of *p*-nitrophenyl dodecanoate (PNPL) as catalyzed by the surfactants indicate that the reactivity of the mercapto group is reduced upon formation of the anionic micelles. The large rate retardation is primarily due to the decrease in concentration of the active thiolate anion. This was supported by the fact that the $pK_a$ values for mercapto groups of the anionic micelles, for which the carboxyl group acts as an anionic head, were increased by 0.8—1.6 $pK_a$ unit over those of the corresponding monomeric surfactants in the bulk phase. These surfactants showed profound reactivity even in a neutral pH region when mixed with cationic CTAB micelle. The electrostatic field effect provided by the cationic head of CTAB micelle seems to enhance the nucleophilicity of the mercapto group in the mixed micelles.

The mercapto group is located at the active site of thiol proteases such as papain, ficin, and bromelain and playes an important role in enzymatic reactions.[1] The reactivity of the mercapto groups of these enzymes, however, drastically changes from one to another depending on the nature of micro-environment where they are located. Because of the complexity of protein molecules, the difference in reactivity among various types of mercapto groups has not yet been satisfactorily clarified. Only a few studies on the reactivity of mercapto groups of non-enzymatic systems in the degradation of *p*-nitrophenyl acetate have been reported so far.[2] Some studies were carried out in cationic micelles such as hexadecyl- and octadecyltrimethylammonium bromide as enzyme model systems.[3] Shinkai and Kunitake introduced the concept of hydrophobic ion pair for the reaction of coenzyme A (CoASH) and glutathione (GSH) with *p*-nitrophenyl acetate in a cationic micelle in which the hydrophobic environment has an important role for the development of catalysis.[4] Recently, Moss and his coworkers synthesized self-contained thiol-functionalized surfactants and investigated their catalytic activity in the degradation of *p*-nitrophenyl acetate.[5] In their system, the mercapto group seems to be placed in the cationic Stern layer judging from the molecular structure of surfactants.

In order to clarify the various features of reactivity of the mercapto group in enzymatic reactions and to create more elaborated models of thiol proteases, we prepared in this work a novel type of surfactants, *N*-hexadecyl-$N^{\alpha}$-glutaryl-L-cysteinamide (AM·Cys-1). The mercapto group is expected to be placed in a hydrophobic core of the micellar phase. The catalytic activity of AM·Cys-1 was compared with that of another synthetic surfactant, *N*-hexadecanoyl-L-cysteine (AM·Cys-2). The structural and micellar effects provided by the present cysteine-containing surfactants have been clarified in the deacylation of *p*-nitrophenyl carboxylates. The effective catalysis by mixed micelles composed of hexadecyltrimethylammonium bromide (CTAB) and either AM·Cys-1 or -2 in the deacylation has also been investigated here.

† Contribution No. 509 from this Department.

## Experimental

Spectroscopic data were taken on a JASCO DS-403G grating IR spectrophotometer, a Varian A60 NMR spectrometer, and a Hitachi 124 spectrophotometer. pH-Measurements were carried out with a TOA HM-9A pH meter equipped with a TOA GC-125 combined electrode after calibration with a combination of appropriate aqueous standard buffers.

*Materials.* Hexadecyltrimethylammonium bromide (CTAB) of Nakarai Chemicals was recrystallized from ethanol, mp 237—239 °C (dec). Sodium dodecyl sulfate (SDS) and α-hydro-ω-dodecyloxytricosa(oxyethylene) (Brij 35) were purchased from Nakarai Chemicals as extra pure grade and used without further purification. 1-Dodecanethiol ($C_{12}$-SH) was obtained from Ishizu Pharmaceutical Co. and distilled under nitrogen, bp 145—147 °C/16 mmHg. 2-Mercaptoethanol was purchased from Nakarai Chemicals and distilled under nitrogen, bp 54.5 °C/13 mmHg. Glutathione (GSH, Wako Pure Chemical Industries), L-cysteine hydrochloride (Cys·HCl, Ishizu Pharmaceutical Co.), and 2,2′-dinitro-5,5′-dithiodibenzoic acid (DTNB, Nakarai Chemicals) of bio-analytical grade were used without further purification. Rhodamine 6G was purchased from Daiwa Chemicals as extra pure grade. *p*-Nitrophenyl carboxylates were prepared by the reaction of the corresponding carbonyl chlorides with *p*-nitrophenol. The esters were identified by elemental analyses and spectroscopic measurements.[6]

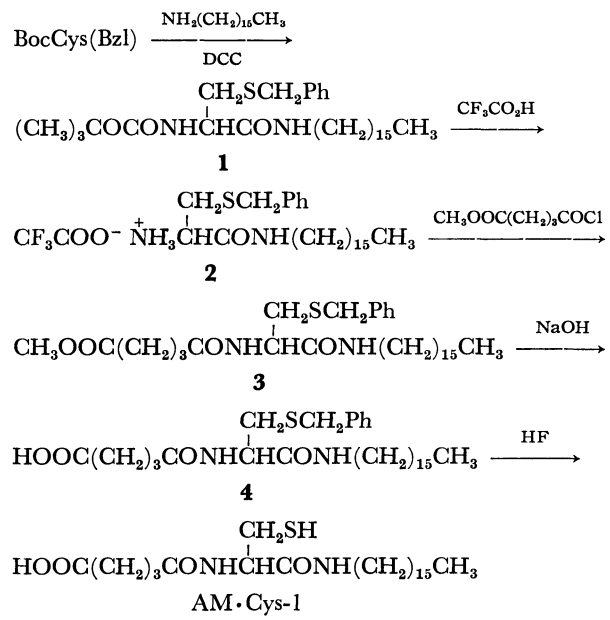
*N-Hexadecanoyl-L-cysteine (AM·Cys-2).* This was prepared by the condensation of hexadecanoic acid with *S*-benzyl-L-cysteine in the presence of ethyl chloroformate followed by elimination of the benzyl group in a manner reported by Heitmann.[7]

*N*-Hexadecanoyl-*S*-benzyl-L-cysteine: mp 79—81 °C. IR (KBr disc): 3320 (NH str.); 2920 and 2860 (CH str.); 1750 and 1650 (C=O str.); 825, 770, and 700 $cm^{-1}$ (CH bend. of benzene). NMR ($CDCl_3$, TMS): δ 0.87 (3H, t, $C\underline{H}_3(CH_2)_{13}$-$CH_2$–), 1.24 (26H, broad, $CH_3(C\underline{H}_2)_{13}CH_2$–), 2.20 (2H, t,

$CH_3(CH_2)_{13}C\underline{H}_2CO$–), 2.91 (2H, d, –NHCH($C\underline{H}_2SCH_2Ph$)-$CO_2H$), 3.68 (2H, s, –$SC\underline{H}_2Ph$), 4.80 (1H, t, –NHC$\underline{H}$-($CO_2H$)–), 6.37 (1H, d, –N$\underline{H}$–), and 7.51 (5H, s, phenyl H's).

*N*-Hexadecanoyl-L-cysteine: mp 83—85 °C, nitroprusside positive, Ellman positive, $[\alpha]_D^{21}$ −26.0° (*c* 1.00, $C_2H_5OH$); $R_f$ (silica gel IB of J. T. Baker, methanol), 0.80; $R_f$ (silica gel IB of J. T. Baker, butanol–water–acetic acid at 4:2:1 by volume), 0.87. IR (KBr disc): 3320 (NH str.); 2920 and 2860 (CH str.); 1750 and 1650 $cm^{-1}$ (C=O str.). NMR ($CDCl_3$, TMS): $\delta$ 0.86 (3H, t, $C\underline{H}_3(CH_2)_{13}CH_2$–), 1.25 (26H, broad, $CH_3(C\underline{H}_2)_{13}CH_2$–), 2.28 (2H, t, $CH_3(CH_2)_{13}C\underline{H}_2CO$–), 2.96 (2H, dd, –NHCH($C\underline{H}_2SH$)$CO_2H$), 4.80 (1H, t, –NHC$\underline{H}$-($CH_2SH$)$CO_2H$), 6.25 (1H, d, –N$\underline{H}$–), and 8.18 (1H, s, –$CO_2\underline{H}$). Found: C, 63.55; H, 10.16; N, 3.80%. Calcd for $C_{19}H_{37}NO_3S$: C, 63.47; H, 10.37; N, 3.90%.

The synthetic procedures for *N*-hexadecyl-$N^{\alpha}$-glutaryl-L-cysteinamide (AM·Cys-1) is outlined in Scheme 1.




(Boc, *t*-butoxycarbonyl; Bzl, benzyl; Ph, phenyl)

Scheme 1. Synthetic procedures for AM·Cys-1.

*N-Hexadecyl-N$^{\alpha}$-t-butoxycarbonyl-S-benzyl-L-cysteinamide (1).* *t*-Butoxycarbonyl-*S*-benzyl-L-cysteine (8.0 g, 23 mmol) and dicyclohexylcarbodiimide (4.7 g, 23 mmol) were dissolved in dichloromethane–acetonitrile (1:1 by volume, 40 ml), and the solution was cooled down to 0 °C. Then, 1-aminohexadecane (5.5 g, 23 mmol) was added to the solution and the mixture was stirred for 3 h at 0 °C. Precipitated white solid was recovered by filtration and recrystallized from hexane–ethyl acetate (1:1 by volume); yield 8.7 g (72%), mp 88—90 °C. IR (KBr disc): 3300 (NH str.); 2910 and 2850 (CH str.); 1695 and 1640 $cm^{-1}$ (C=O str.). NMR ($CDCl_3$, TMS): $\delta$ 0.88 (3H, broad t, $C\underline{H}_3(CH_2)_{15}$–), 1.27 (28H, s, $CH_3(C\underline{H}_2)_{14}$-$CH_2NH(CO)$–), 1.45 (9H, s, $(C\underline{H}_3)_3CO$–), 2.82 (2H, d, –$C\underline{H}_2SCH_2Ph$), 3.23 (2H, broad t, $CH_3(CH_2)_{14}C\underline{H}_2NH$-(CO)–), 3.75 (2H, s, –$CH_2SC\underline{H}_2Ph$), 4.57 (1H, broad t, –NHC$\underline{H}$($CH_2SCH_2Ph$)CO–), and 7.31 (5H, s, phenyl H's).

*N-Hexadecyl-N$^{\alpha}$-methylglutaryl-S-benzyl-L-cysteinamide (3).* Trifluoroacetic acid (5.7 g) was added to a dichloromethane solution (16 ml) of **1** (534 mg, 1.0 mmol), and the mixture was stirred for 1 h at room temperature. Evaporation of the excess trifluoroacetic acid *in vacuo* below 40 °C gave pale yellow crystals (**2**), mp 72 °C. Elimination of the *t*-butoxycarbonyl group was confirmed by the NMR spectrum. The crystals were used for the following reaction without further purification. Amine component **2** (210 mg, 0.38 mmol) and triethylamine (115 mg, 1.14 mmol) were dissolved in dichloromethane (5 ml) and cooled down to 5 °C. Methyl 4-chloroformylbutanoate (81 mg) dissolved in dichloromethane (2 ml) was added to the solution in 15 min at this temperature. The mixture was stirred for 1 h at 0 °C, for 1 h at room temperature, and refluxed for 10 min; and then washed with water (5 ml), 5% aqueous sodium hydrogencarbonate (5 ml ×2), water (5 ml×2), and 5% aqueous citric acid (5 ml×2) in this sequence. After being dried over anhydrous sodium sulfate, the mixture was evaporated *in vacuo* at 50 °C to give a white solid which was then purified by gel-filtration chromatography (Sephadex LH-20, 1:1 chloroform–methanol as an eluant); yield 180 mg (50%), mp 86—88 °C. IR (KBr disc): 3275 (NH str.); 2900 and 2830 (CH str.); 1740 and 1640 $cm^{-1}$ (C=O str.). NMR ($CDCl_3$, TMS): $\delta$ 0.88 (3H, broad t, $C\underline{H}_3(CH_2)_{15}$–), 1.27 (30H, s, $CH_3(C\underline{H}_2)_{14}CH_2NH$– and $CH_3O_2CCH_2C\underline{H}_2CH_2CO$–), 1.75—2.55 (4H, m, $CH_3O_2$-$CC\underline{H}_2CH_2C\underline{H}_2CO$–), 2.71 (2H, d, –$CHC\underline{H}_2SCH_2Ph$), 3.24 (2H, broad t, –NHC$\underline{H}_2(CH_2)_{14}CH_3$), 3.66 (3H, s, $C\underline{H}_3O_2C$–), 3.74 (2H, s, –$SC\underline{H}_2Ph$), 4.57 (1H, t, –NHC$\underline{H}$($CH_2SCH_2Ph$)-CO–), and 7.29 (5H, s, phenyl H's). Found: C, 67.96; H, 9.63; N, 4.87%. Calcd for $C_{32}H_{54}O_4N_2S$: C, 68.29; H, 9.67; N, 4.97%.

*N-Hexadecyl-N$^{\alpha}$-glutaryl-S-benzyl-L-cysteinamide (4).* To a mixture of **3** (700 mg), dioxane (20 ml), and methanol (35 ml) was added 4% aqueous sodium hydroxide (9 ml). After being stirred for 6 h at room temperature, the mixture was evaporated *in vacuo* to remove methanol and then water (30 ml) was added to the residue. The solution was acidified to pH 5 by adding 10% aqueous citric acid. White precipitates were recovered by filtration and washed with water (5 ml× 10); yield 677 mg (99%), mp 101—102 °C. IR (KBr disc): 3250 (NH str.); 2900 and 2840 (CH str.); 1700, 1630, and 1525 $cm^{-1}$ (C=O str.). NMR ($CDCl_3$, TMS): $\delta$ 0.88 (3H, broad t, $C\underline{H}_3(CH_2)_{15}$–), 1.26 (30H, s, $CH_3(C\underline{H}_2)_{14}CH_2$– and $HO_2CCH_2C\underline{H}_2CH_2CO$–), 1.73—2.57 (4H, m, $HO_2CC\underline{H}_2CH_2$-$C\underline{H}_2CO$–), 2.71 (2H, d, –CH($C\underline{H}_2SCH_2Ph$)–), 3.24 (2H, broad t, –NHC$\underline{H}_2(CH_2)_{14}CH_3$), 3.74 (2H, s, –$SC\underline{H}_2Ph$), 4.57 (1H, t, –NHC$\underline{H}$($CH_2SCH_2Ph$)CO–), and 7.29 (5H, s, phenyl H's). Found: C, 67.23; H, 9.48; N, 5.06%. Calcd for $C_{31}H_{52}O_4N_2S$: C, 67.84; H, 9.55; N, 5.10%.

*N-Hexadecyl-N$^{\alpha}$-glutaryl-L-cysteinamide (AM·Cys-1).* L-Methionine (180 mg) and **4** (600 mg) were placed in a reaction vessel into which hydrogen fluoride (20 ml) was introduced. The mixture was stirred for 1 h at 0 °C, for 30 min at room temperature, and then evaporated *in vacuo* to remove hydrogen fluoride completely. Dichloromethane (30 ml) was added to the residue and white precipitates were recovered by filtration; this treatment was repeated four times. The white solid was dissolved in ethanol–chloroform (1:1 by volume, 5 ml) and purified by repeated gel-filtration chromatography (Sephadex LH-20, 1:1 ethanol–chloroform as an eluant); yield 135 mg (27%), mp 98—101 °C, nitroprusside positive. IR (KBr disc): 3250 (NH str.); 2910 and 2830 (CH str.); 1710, 1635, and 1535 $cm^{-1}$ (C=O str.). NMR ($CDCl_3$, TMS): $\delta$ 0.88 (3H, broad t, $C\underline{H}_3(CH_2)_{15}$–), 1.27 (30H, s, $CH_3(C\underline{H}_2)_{14}CH_2$– and $HO_2CCH_2C\underline{H}_2CH_2CO$–), 1.75—2.55 (4H, m, $HO_2CC\underline{H}_2$-$CH_2C\underline{H}_2CO$–), 2.82 (2H, dd, –CH($C\underline{H}_2SH$)–), 3.20 (2H, broad t, $CH_3(CH_2)_{14}C\underline{H}_2NH$–), and 4.60 (1H, broad t, –NH-C$\underline{H}$($CH_2SH$)–). Found: C, 63.63; H, 9.89; N, 5.93%. Calcd for $C_{24}H_{46}N_2O_4S$: C, 62.84; H, 10.10; N, 6.11%.

*Kinetic Measurements.* The concentration of free thiol for AM·Cys-1 and -2 was determined by using the Ellman's reagent (DTNB)[8] before kinetic runs. The reaction between a thiol compound and DTNB in 10% (v/v) aqueous ethanol

at pH 8.0 ($\mu$ 0.10 with KCl) and room temperature was followed at 412 nm to completion and the amount of free thiol was evaluated by using cysteine hydrochloride and/or 2-mercaptoethanol as a reference. The concentration of free thiol for AM·Cys-1 and -2 decreases upon oxidation to the corresponding disulfides at alkaline pH's, and the oxidation rate for the mercapto group of AM·Cys-2 was roughly estimated at several pH's by the Ellman's method. For example, the acyl transfer from an ester substrate to AM·Cys-2 was completed within 1 min at pH 11.82 while only a 5% amount of AM·Cys-2 was oxidized in 5 min. Under every conditions used in this work, the reaction between surfactant and ester species was fast enough to neglect oxidation of the mercapto group of the surfactants.

Rates of *p*-nitrophenol liberation from *p*-nitrophenyl esters were measured at 317 nm (pH<6) and 400 nm (pH>6) with a Hitachi 124 spectrophotometer. Each run was initiated by adding a dry dioxane solution (30 $\mu$l) of a substrate ester to a mixture of a reaction medium (3.0 ml) and a dry ethanol solution (30 $\mu$l) of a catalyst which was pre-equilibrated at 30.0±0.1 °C in a thermostatted cell set in the spectrophotometer. The reaction medium was prepared as follows: 10.0 ml of 1.0 M aqueous potassium chloride, 10.0 ml of an appropriate aqueous buffer, and 10.0 ml of dry ethanol were placed in a 100-ml volumetric flask; and subsequently the flask was filled with deionized and distilled water. Aqueous buffer solutions adopted in the present study were as follows: 1/10 M potassium dihydrogenphosphate–1/20 M sodium borate for pH 6—9, 1/20 M sodium borate–1/20 M sodium carbonate for pH 10—11, and 1/10 M sodium hydroxide–1/10 M sodium hydrogenphosphate for pH 11—12.

*Determination of* $pK_a$ *Values.* The $pK_a$ value for a mercapto group involved in AM·Cys-1 and -2 depends on their concentrations. The extent of dissociation of the mercapto group was evaluated by Eq. 1.

$$\alpha = \Delta\text{Abs(pH x)}/\Delta\text{Abs(pH 13.26)} \quad (1)$$

where $\Delta$Abs(pH x) refers to the difference between absorbances at 235 (maximum) and 265 nm (minimum) in 10.8% (v/v) aqueous ethanol at pH x. $\Delta$Abs for the completely ionized mercapto group of AM·Cys-1 was evaluated at pH 13.26. The modified Henderson-Hasselbalch equation as represented by Eq. 2 was adopted to evaluate $pK_a$ values.

$$\text{pH} = pK_a + n\log\{\alpha/(1-\alpha)\} \quad (2)$$

In order to determine the true $pK_a$ value in a micellar phase, the $pK_a$ value was plotted against the concentration of AM·Cys-1 as shown in Fig. 4.

*CMC Measurements.* The critical micelle concentrations for AM·Cys-1 and -2 were determined at 30.0±0.1 °C, pH 9.30, and $\mu$ 0.10 (KCl) in 15.6%(v/v) and 11.5%(v/v) aqueous ethanol, respectively. Rhodamine 6G was used as a dye probe at $1.0\times10^{-5}$ M. The difference spectra between 520 (maximum) and 543 nm (minimum) were taken and the difference in absorption was plotted against the concentration of a surfactant in reference to the method used for the determination of CMC values for *N*-acyl-DL-cysteines.[7]

## Results and Discussion

The catalytic efficiency of the surfactants in the deacylation of *p*-nitrophenyl carboxylates has been investigated by the kinetic method in 10.8%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C and $\mu$ 0.10 (KCl). Apparent pseudo-first-order rate constants ($k_{obsd}$) were obtained by measuring the amount of liberated *p*-nitrophenol. The first-order kinetics was found to hold at least up to 80% conversion of the substrate for [C]≫[S]; C and S stand for catalyst and substrate species, respectively. Figure 1 shows the rate-concentration profile for the deacylation of *p*-nitrophenyl dodecanoate (PNPL) catalyzed by AM·Cys-1 and -2 at pH 9.3; $k_1$ refers to $k_{obsd}-k_{hyd}$. The apparent first-order rate constant ($k_1$) increases as the surfactant concentration is raised up to a certain concentration range which may be referred to the critical micelle concentration. The kinetic CMC values agree reasonably well with the corresponding values determined by the dye method as seen in Table 1. Beyond these concentration ranges, the $k_1$ values were nearly leveled off for both surfactant systems. The straight line portions below

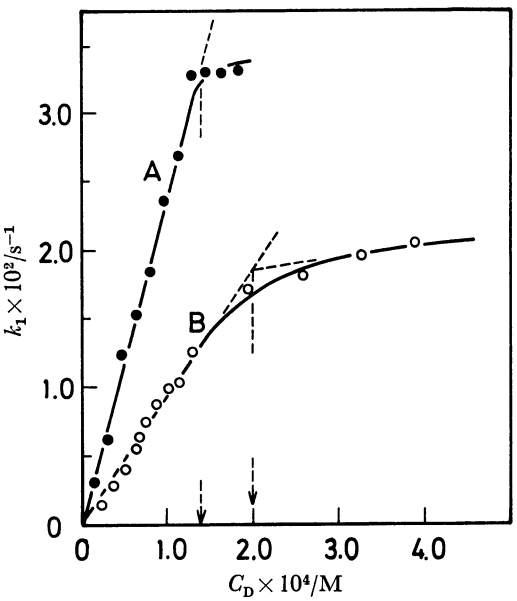



Fig. 1. Plots of first-order rate constant *vs.* surfactant concentration ($C_D$) for the deacylation of *p*-nitrophenyl dodecanoate in 10.8%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C, pH 9.3, and $\mu$ 0.10 (KCl): $k_1=k_{obsd}-k_{hyd}$; initial concentration of PNPL, $0.984\times10^{-5}$ M; A, AM·Cys-1; B, AM·Cys-2. The rate was not measured for the AM·Cys-1 system beyond this concentration range due to its limited solubility.

Table 1. Critical micelle concentrations for AM·Cys-1 and -2

| Surfactant | CMC×10⁴/M | |
|---|---|---|
| | Dye method | Kinetic method |
| AM·Cys-1 | 3.76[a] | 1.33[b] |
| AM·Cys-2 | 1.40[c] | 2.00[d] |

a) In 15.6%(v/v) aqueous ethanol at 30.0±0.1 °C, pH 9.30, and $\mu$ 0.10 (KCl). b) In 15.6%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C, pH 9.28, and $\mu$ 0.10 (KCl); initial concentration of PNPL, $0.984\times10^{-5}$ M. c) In 11.5%(v/v) aqueous ethanol at 30.0±0.1 °C, pH 9.30, and $\mu$ 0.10 (KCl). d) In 10.8%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C, pH 9.30, and $\mu$ 0.10 (KCl); initial concentration of PNPL, $0.984\times10^{-5}$ M. All the concentrations throughout this paper are given in M; 1 M=1 mol dm$^{-3}$.

Table 2. Kinetic parameters for the catalyzed deacylation of *p*-nitrophenyl dodecanoate and acetate[a)]

| Catalyst RSH | $\frac{[RSH]\times10^5}{M}$ | $pK_a^{app}$ | pH | $k_2/M^{-1}s^{-1}$ | |
|---|---|---|---|---|---|
| | | | | PNPL | PNPA |
| AM·Cys-1 | 9.82(<CMC) | 10.5[c)]; 10.5[d)] | 9.28 | 4180 | 0 |
| AM·Cys-2 | 10.5 (<CMC) | 10.3[c)]; 10.4[d)] | 9.30 | 1290 | 0 |
| $C_{12}$-SH[b)] | 5.01(<CMC) | 12.0[c)] | 12.41 | 1970 | — |
| GSH | 567 | 9.2[e)] | 9.41 | 0.011 | 1.92 |
| $HSCH_2CH_2OH$ | 98.3 | 9.4[e)] | 9.41 | — | 4.28 |
| | 9830 | | | 0.012 | — |
| $OH^-$[f)] | | | | 1.11 | 24.4 |

a) In 10.8%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C and μ 0.10 (KCl). Initial concentrations: PNPL, $0.984\times10^{-5}$ M; PNPA, $0.989\times10^{-5}$ M. The relative rate ($k_{obsd}/k_{hyd}$) in the presence of AM·Cys(Bzl)-1 ($39.9\times10^{-5}$ M > CMC) is 0.48 at pH 11.13. b) In 10.8%(v/v) aqueous dioxane at 40.0±0.1 °C and μ 0.10 (KCl). c) Determined by the kinetic method (Eq. 3) as a dissociation constant for a mercapto group. d) Determined by the photometric titration as a dissociation constant for a mercapto group. e) Cited from literature: J. W. Ogilvie, J. T. Tildon, and B. S. Strauch, *Biochemistry*, **3**, 754 (1964). f) Alkaline hydrolysis.

CMC are referred to the apparent second-order kinetics with rate constant $k_{2b}$ as catalyzed by the monomeric surfactants in the bulk phase, whereas those above CMC are due to the apparent second-order kinetics with rate constant $k_{2m}$ as catalyzed by the micellar surfactants. The leveling-off behavior suggests that the micelle formation results in the loss of thiol-reactivity for the present surfactants.

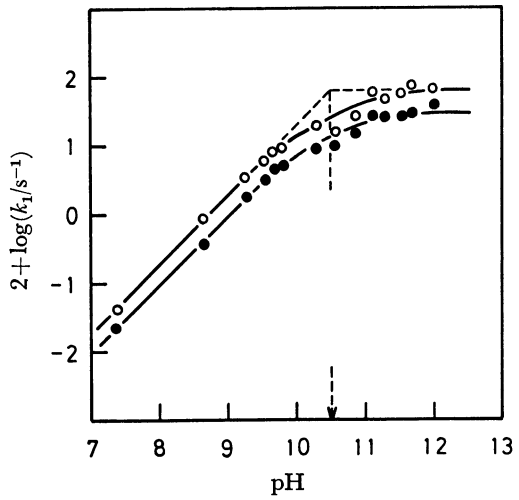
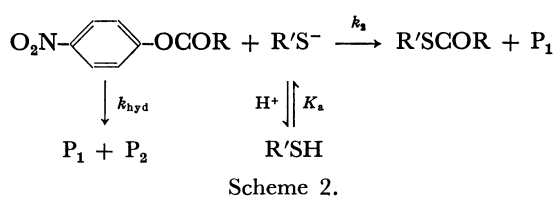



Fig. 2. pH-rate profiles for the deacylation of *p*-nitrophenyl dodecanoate as catalyzed by AM·Cys-1 above (○) and below (●) its CMC in 10.8%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C and μ 0.10 (KCl). Initial concentrations: AM·Cys-1, $8.20\times10^{-5}$ M (<CMC) and $1.63\times10^{-4}$ M (>CMC); PNPL, $0.984\times10^{-5}$ M.

The pH-rate profiles for the decomposition of *p*-nitrophenyl dodecanoate (PNPL) as catalyzed by AM·Cys-1 below and above its CMC are shown in Fig. 2. A plot of log $k_1$ against pH exhibited a straight line of slope 1.0 below pH 10.5, and log $k_1$ is leveled off beyond this pH. The behavior indicates that the active group of the surfactant for the degradation of PNPL is the thiolate anion. The apparent $pK_a$ value evaluated from Fig. 2 agrees satisfactorily with that determined by the photometric titration as seen in Table 2. The pH-rate profile for the surfactant-catalyzed degradation of PNPL above its CMC is almost identical with that observed below CMC. The result again indicates that the reactivity of AM·Cys-1 above its CMC is primarily due to the monomeric species. The similar behavior was observed for AM·Cys-2.

*Reactivity of the Mercapto Group Below CMC's.* The initial rapid release of *p*-nitrophenol from PNPL was followed by the slow process at pH 9.28 for [S]>[C]. The absorbance increase due to *p*-nitrophenol at 400 nm in the slow rate region is almost identical with that observed in the absence of the surfactants. The result indicates that the deacylation of the thiol ester yielded by the reaction between PNPL and surfactants can not proceed to any detectable extent under present conditions. Thus, the net reaction pathway below CMC is given by Scheme 2, where $P_1$ and $P_2$ stand for phenol and carboxylic moieties, respectively; $k_2$ refers to the true second-order rate constant for the acylation step and $k_{hyd}$ to the rate constant for the alkaline hydrolysis of PNPL.




Scheme 2.

The true second-order rate constant for the below-CMC region was determined by the aid of Eq. 3, where $k_2'$ is the apparent second-order rate constant at a given pH; $k_2'=k_1/[R'SH]_T$. A plot of $1/k_2'$ against $[H^+]$ is shown in Fig. 3 for the PNPL–AM·Cys-1 system.

$$\frac{1}{k_2'}=\frac{1}{k_2}+\frac{[H^+]}{k_2K_a^{app}} \quad (3)$$

The $k_2$ values thus obtained are summarized in Table 2. The reactivity of the thiolate anion of the surfactants

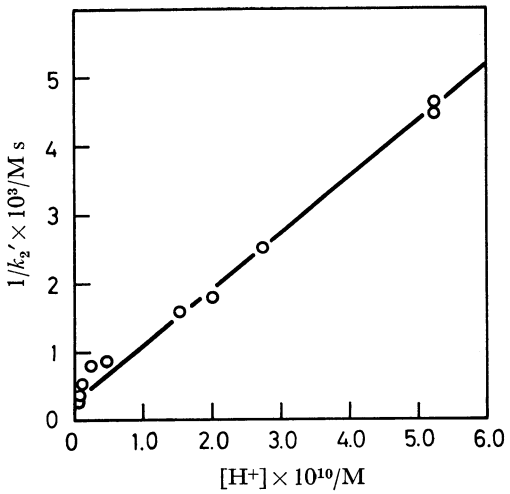



Fig. 3. A plot of $1/k_2'$ *vs.* $[H^+]$ for the deacylation of *p*-nitrophenyl dodecanoate as catalyzed by AM·Cys-1 in 10.8%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C and μ 0.10 (KCl). Initial concentrations: AM·Cys-1, $8.20 \times 10^{-5}$ M; PNPL, $0.984 \times 10^{-5}$ M.

on a carboxylic ester which bears a long alkyl chain (PNPL) in the aqueous phase is remarkably larger than that of the hydroxide ion as seen in Table 2, but not so on PNPA. The result also indicates that the catalytic activity of AM·Cys-1 in the decomposition of *p*-nitrophenyl dodecanoate is greater than that of AM·Cys-2 by 3.3 fold. A carboxylate anion as the surfactant-head would destabilize the anionic tetrahedral intermediate formed from the thiolate anion and the substrate in a manner as observed for the paracyclophane catalysis.[9] Therefore, the distance between the carboxyl group and the mercapto group in the surfactant molecules must control the reactivity toward ester substrates. The difference in reactivity between AM·Cys-1 and -2 would be attributed to the greater hydrophobic interaction of the former with PNPL.

*N*-Hexadecyl-$N^{\alpha}$-glutaryl-*S*-benzyl-L-cysteinamide [AM·Cys(Bzl)-1], in which the mercapto group is protected with a benzyl group, did not show any meaningful effect on the hydrolysis of PNPL below its CMC. On the other hand, AM·Cys(Bzl)-1 retarded the alkaline hydrolysis above its CMC. The result indicates that the anionic micelle may incorporate PNPL into its hydrophobic core and the ester bond of PNPL is masked from the hydroxide attack. Anionic micelles of sodium dodecanoate were found to behave similarly for the hydrolysis of several *p*-nitrophenyl esters as reported recently by Menger *et al.*[10] Thiol catalysts bearing a long alkyl chain such as AM·Cys-1, -2, and 1-dodecanethiol ($C_{12}$-SH) show catalytic activity in the decomposition of PNPL, but smaller thiols such as glutathione (GSH) and 2-mercaptoethanol give out little catalytic activity (Table 2). These smaller thiols, however, catalyzed the decomposition of *p*-nitrophenyl acetate (PNPA) by using them in a large amount. AM·Cys-1, -2, and $C_{12}$-SH were not used in higher concentrations for the reaction with PNPA because of their limited solubility. As we clarified previously,[11] the reactivity of monomeric esters decreases with increasing the alkyl chain length due to their self-coiling behavior so as to mask the ester bond from the hydroxide attack. Thus, the hydrophobic interaction between the catalyst and the substrate may cause not only the access of both reactants (proximity effect) but also the decoiling of the alkyl chain of PNPL.

Both AM·Cys-1 and -2 showed a marked catalytic activity in the deacylation of PNPL at pH 9.30 below

Table 3. Relative catalytic activity of AM·Cys-1 and -2 for the deacylation of *p*-nitrophenyl carboxylates[a]

| Substrate R[b] | pH 9.30 | | | pH 12.00 | | |
|---|---|---|---|---|---|---|
| | $k_{hyd} \times 10^5$/s$^{-1}$ | $k_{rel}$[c] | | $k_{hyd} \times 10^4$/s$^{-1}$ | $k_{rel}$[c] | |
| | | AM·Cys-1 | AM·Cys-2 | | AM·Cys-1 | AM·Cys-2 |
| $CH_3$– | 72.2 | 1 | 1 | | | |
| $CH_3(CH_2)_4$– | 32.0 | 9.8 | 4.4 | 587 | 3.0 | 1.9 |
| $CH_3(CH_2)_8$– | 5.23 | 575 | 199 | 65.7 | 43.8 | 19.9 |
| $CH_3(CH_2)_{10}$– | 1.82 | 1509 | 441 | 8.08 | 267 | 138 |
| $CH_3(CH_2)_{14}$– | 0.50 | 2397 | 801 | 0.88 | 1432 | 292 |
|  | 11.3 | 13.9 | 5.2 | | | |
|  | 13.6 | 11.4 | 3.6 | | | |
|  | 1.75 | 13.0 | 5.7 | | | |
|  | 140 | | 3.5 | | | |
|  | 530 | 18.0 | 6.6 | | | |

a) In 10.8%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C and μ 0.10 (KCl). Initial concentrations: substrate, $0.984—1.00 \times 10^{-5}$ M; AM·Cys-1 and -2, $7.90 \times 10^{-5}$ M. b) R for *p*-$NO_2C_6H_4OC(O)R$. c) $k_{rel}=k_{obsd}/k_{hyd}$: $k_{obsd}$, apparent first-order rate constant; $k_{hyd}$, hydrolysis rate constant in the absence of a catalyst.

their CMC's. These surfactants catalyzed not only the deacylation of *p*-nitrophenyl carboxylates bearing a long alkyl chain but also that of *p*-nitrophenyl esters of cyclohexane derivatives and *N*-benzyloxycarbonylglutarate as shown in Table 3. The present thiol catalysts exercise a significant activity toward hydrophobic substrates in their deacylation and the profound efficiency is observed selectively in the decomposition of esters bearing a longer alkyl chain.

Table 4. Effects of metal ions in the surfactant-catalyzed deacylation of *p*-nitrophenyl dodecanoate[a)]

| Metal ion/M | $V_0 \times 10^8$/M s$^{-1}$ [b)] | |
|---|---|---|
| | AM·Cys-1 | AM·Cys-2 |
| None | 10.9 | 8.27 |
| $Cu^{2+}$ : $1.02\times10^{-6}$ | 10.4 | 7.20 |
| $1.02\times10^{-5}$ | 6.85 | 4.68 |
| $1.02\times10^{-4}$ | 0 | 0 |
| $Zn^{2+}$ : $1.01\times10^{-6}$ | 10.6 | 7.55 |
| $1.01\times10^{-5}$ | 7.38 | 6.60 |
| $1.01\times10^{-4}$ | 0 | 0 |
| $Mg^{2+}$: $1.01\times10^{-6}$ | 10.9 | 8.27 |
| $1.01\times10^{-5}$ | 10.9 | 8.27 |
| $1.01\times10^{-4}$ | 10.9 | 8.27 |

a) In 10.8%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C, pH 9.27, and μ 0.10 (KCl). Initial concentrations: AM·Cys-1, $3.33\times10^{-4}$ M; AM·Cys-2, $3.92\times10^{-4}$ M; PNPL, $0.984\times10^{-5}$ M. All the metal ions were used as nitrates. b) $V_0$ stands for the initial velocity of reaction.

*Effects of Metal Ions on the Catalysis.* It is expected that an appropriate metal ion would show somewhat a unique effect on the reactivity of surfactants which involve effective donor groups. The metal ions used here ($Cu^{2+}$, $Zn^{2+}$, and $Mg^{2+}$) did not give out any effect on the alkaline hydrolysis of PNPL, while the surfactant-catalyzed deacylation of the ester was markedly retarded by $Cu^{2+}$ and $Zn^{2+}$ as shown in Table 4. Such a behavior of the synthetic surfactants bears a resemblance to those of thiol proteases. Thiol proteases such as papain are extremely sensitive to the presence of heavy metal ions, and metal ions of groups IB and IIB such as $Cu^{2+}$, $Zn^{2+}$, and $Hg^{2+}$ cause reversible inhibition of the enzyme catalysis. The inhibition seems to occur through formation of the thiolate complexes. Since the thiolate anion is one of the soft bases, the hard acid ($Mg^{2+}$) does not form the corresponding complex and consequently show no effect on the surfactant-catalyzed reaction.

*Evaluation of True* p$K_a$ *Values for the Mercapto Group in Micellar Phase.* In order to clarify the reason why the catalytic activity of the surfactants markedly decreases above CMC for the deacylation of *p*-nitrophenyl esters, we evaluated true p$K_a$ values for the mercapto group in micellar phase from the p$K_a$-concentration profiles. The p$K_a$- and *n*-values determined by Eq. 2 were plotted against the concentration of the surfactants as shown in Fig. 4. The p$K_a$-value of each surfactant increases as the concentration increases

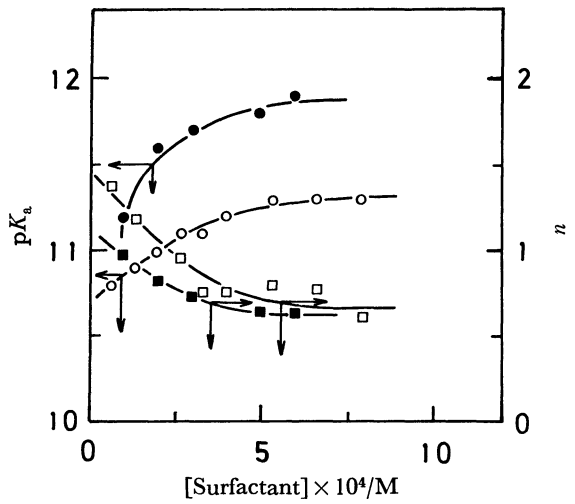



Fig. 4. Plots of p$K_a$- and *n*-values against concentration of the anionic surfactants. p$K_a$- and *n*-values were evaluated by the modified Henderson-Hasselbalch equation. For AM·Cys-1: ○, p$K_a$; □, *n*. For AM·Cys-2: ●, p$K_a$; ■, *n*.

to a certain extent which is considered to be the true p$K_a$ in micellar phase: AM·Cys-1, 11.3; AM·Cys-2, 11.9. These values are larger than those for the corresponding monomeric surfactants. On the other hand, the *n*-value of each surfactant decreases as the surfactant concentration increases and levels off beyond a certain range: AM·Cys-1, 0.58; AM·Cys-2, 0.56. p$K_a$-Values are generally affected by electrostatic and hydrophobic fields. The result suggests that the electrostatic field effect overcomes the hydrophobic effect for the present surfactant systems since the evaluated p$K_a$-value for AM·Cys-2 is larger than that for AM·Cys-1 by 0.6 p$K_a$ unit. The $\alpha$-values for AM·Cys-1 and -2 are $3.57\times10^{-4}$ and $2.30\times10^{-5}$, respectively, at pH 9.30. As a whole, the diminished reactivity observed for the micellar systems of AM·Cys-1 and -2 is primarily attributed to the decrease in p$K_a$ of the mercapto group. The modified Henderson-Hasselbalch equation was applied to a micellar system of hexadecyl(imidazolylmethyl)-dimethylammonium chloride and a low *n*-value (0.8) was obtained.[12)]

*Effects of CTAB Micelle on the Catalysis.* The rate constant ($k_{obsd}$) for the deacylation of PNPL was measured in several micellar systems. Sodium dodecyl sulfate and $\alpha$-hydro-$\omega$-dodecyloxytricosa(oxyethylene) did not appreciably alter the rate relative to that for the alkaline hydrolysis, while a greater rate was observed in the presence of CTAB. Figure 5 shows the rate-concentration profiles for the decomposition of PNPA and *p*-nitrophenyl hexanoate (PNPH) as catalyzed by mixed CTAB–AM·Cys-1 micelle. A pronounced catalytic effect was observed with the mixed micelle and the profile is typical for the micellar catalysis. The reaction pathway for the decomposition of *p*-nitrophenyl esters as catalyzed by the mixed micelle is given by Scheme 3, where S stands for an ester substrate (PNPA or PNPH), CM for the mixed micelle formed with CTAB and a thiol surfactant, CM·S for a complex formed with the mixed micelle and a substrate, and P

Table 5. Kinetic parameters for the deacylation of *p*-nitrophenyl dodecanoate and acetate catalyzed by mixed micelles[a)]

| Catalyst | [Catalyst]/[CTAB] | PNPH | | | PNPA | | |
|---|---|---|---|---|---|---|---|
| | | $k_m \times 10^2$ / $s^{-1}$ | $K_b$ / $M^{-1}$ | $k_m/k_{hyd}$ | $k_m \times 10^2$ / $s^{-1}$ | $K_b$ / $M^{-1}$ | $k_m/k_{hyd}$ |
| AM·Cys-1 | 1/20 | 4.41 | 885 | 3080 | 4.75 | 33.3 | 3320 |
| AM·Cys-2 | 1/16 | 1.95 | 599 | 1360 | 0.874 | 71.4 | 611 |
| | 1/20 | 1.59 | 633 | 1110 | 0.526 | 117 | 368 |
| $C_{12}$-SH | 1/20 | 0.168 | 667 | 117 | 0.557 | 15.5 | 390 |

a) In 10.8%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C, pH 7.60, and $\mu$ 0.10 (KCl). Apparent first-order rate constants for the hydrolyses of PNPH and PNPA in the absence of the mixed micelles are $1.43 \times 10^{-5}$ $s^{-1}$ at pH 7.60.

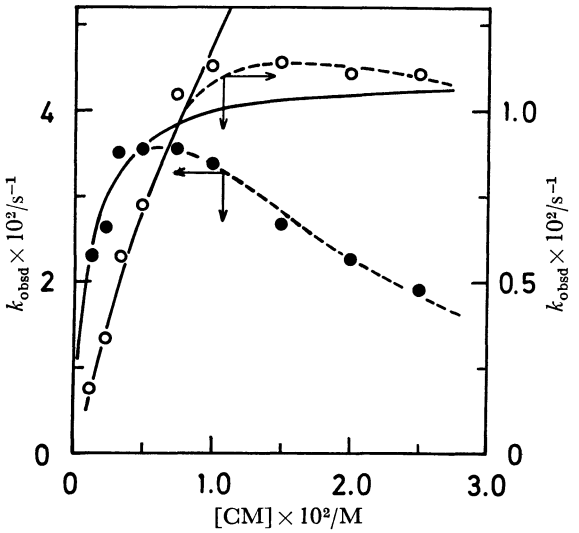



Fig. 5. Rate-concentration profiles for the deacylation of *p*-nitrophenyl hexanoate (●) and acetate (○) as catalyzed by CTAB–AM·Cys-1 mixed micelle in 10.8 %(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0± 0.1 °C, pH 7.63, and $\mu$ 0.10 (KCl). Initial concentrations: PNPA, $0.989 \times 10^{-5}$ M; PNPH, $0.983 \times 10^{-5}$ M. [CM]=[CTAB]+[AM·Cys-1]; [AM·Cys-1]/[CTAB] =1/20. Solid lines are the theoretical curves calculated by Eq. 5. The maximum rate constants for the hydrolysis of PNPA and PNPH in the CTAB micelle (0.125—$2.50 \times 10^{-2}$ M) are $3.75 \times 10^{-5}$ and $2.73 \times 10^{-5}$ $s^{-1}$, respectively, under the same kinetic conditions.

and P′ for reaction products; $k_{hyd}$ and $k_m$ refer to rate constants for product formation in the bulk phase and in the mixed micelle, respectively; $K_b$ represents a binding constant for formation of the micelle-substrate complex.

$$\mathrm{CM} + \mathrm{S} \underset{}{\overset{K_b}{\rightleftharpoons}} \mathrm{CM{\cdot}S} \xrightarrow{k_m} \mathrm{P}$$
$$\downarrow k_{hyd}$$
$$\mathrm{P'}$$

Scheme 3.

Under the present experimental conditions the reaction in the micellar phase resulted in the formation of the acylated micelle and any further reaction was not detected. For [CTAB]>CMC and [CTAB]>[Thiol surfactant]≫[Substrate], the pseudo-first-order rate constant ($k_{obsd}$) is given by Eq. 4[13] on the basis of Scheme 3.

$$k_{obsd} = \frac{k_{hyd} + k_m K_b[\mathrm{CM}]}{1 + K_b[\mathrm{CM}]} \quad (4)$$

Rearrangement gives Eq. 5.

$$\frac{1}{k_{obsd} - k_{hyd}} = \frac{1}{k_m - k_{hyd}} + \frac{1}{(k_m - k_{hyd})K_b[\mathrm{CM}]} \quad (5)$$

where [CM] refers to the sum of the initial concentrations of CTAB and a thiol surfactant. A plot of $1/(k_{obsd} - k_{hyd})$ *vs.* 1/[CM] allows to evaluate $k_m$ and $K_b$. The kinetic parameters thus evaluated are summarized in Table 5 for the reactions catalyzed by mixed micelles; CTAB–AM·Cys-1, CTAB–AM·Cys-2, and CTAB–$C_{12}$-SH.

The binding constant ($K_b$) for the mixed micelles with PNPA changes depending upon the nature of the thiol surfactants, whereas $K_b$ is constant with PNPH regardless of the surfactants. This seems to suggest that PNPH is incorporated into the hydrophobic core of the mixed micelles while PNPA into the hydrophilic Stern layer. The value of $k_m$ stands for the acyl transfer from the substrates to the mercapto group of the thiol surfactants, and the hydrolysis rate ($k_{CM}^{OH}$) for the esters with the concentrated hydroxide ion in the Stern layer of the mixed micelles** is negligibly small relative to the acyl transfer rate for a neutral pH region. The most effective micellar catalyst was constructed with AM·Cys-1 and CTAB and the $k_m$ value for the deacylation of PNPH is 3080 times as large as the corresponding $k_{hyd}$ value as shown in Table 5. Some plausible explanations for the difference in reactivity among the mercapto groups of AM·Cys-1, -2, and $C_{12}$-SH placed in micellar CTAB are as follows.

(1) If the $pK_a$ value for the mercapto group of AM·Cys-1 in micellar CTAB decreases more than those for AM·Cys-2 and $C_{12}$-SH in micellar CTAB, the difference in catalytic activity is attributed to variation in concentration of the active thiolate anion. This may not be the case since the mercapto group of AM·Cys-1 must be located in a more hydrophobic region than those of AM·Cys-2 and $C_{12}$-SH. In other words, the mercapto groups of AM·Cys-2 and $C_{12}$-SH are most likely

** The $k_{CM}^{OH}$ values were evaluated from the hydrolysis of *p*-nitrophenyl carboxylates as catalyzed by micellar CTAB under the same conditions.

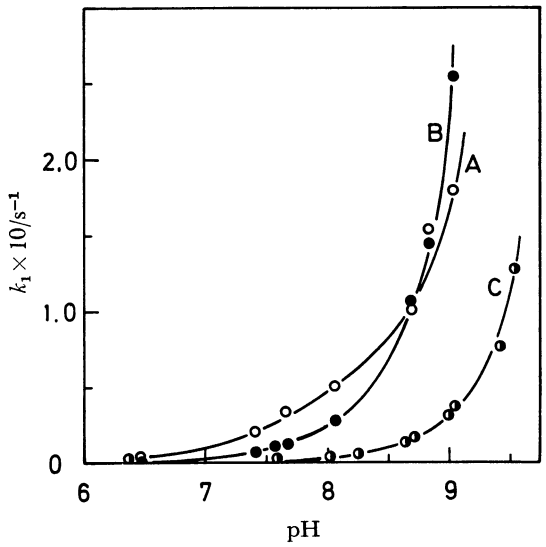



Fig. 6. pH-Rate profiles for the deacylation of PNPH as catalyzed by CTAB–AM·Cys-1 (A), CTAB–AM·Cys-2 (B), and CTAB–$C_{12}$-SH (C) mixed micelles in 10.8%(v/v) ethanol–1.0%(v/v) dioxane–water at 30.0±0.1 °C and μ 0.10 (KCl): $k_1=k_{obsd}-k_{CTAB}$; $k_{CTAB}$, apparent first-order rate constant for the hydrolysis of PNPH as catalyzed by hexadecyltrimethylammonium bromide. Initial concentrations: PNPH, $0.983\times10^{-5}$ M; [CM]=[CTAB]+[Thiol surfactant], $0.50\times10^{-2}$ M; [Thiol surfactant]/[CTAB]=1/20.

placed in the cationic Stern layer and, therefore, the p$K_a$ values for these mercapto groups are expected to decrease more than that for the mercapto group of AM·Cys-1 in micellar CTAB. Figure 6 shows the pH-rate profiles for the deacylation of PNPH as catalyzed by the mixed micelles. These profiles indicate that the p$K_a$ value for each thiol surfactant in the mixed micelle decreases by 1—2 p$K_a$ unit relative to that for each surfactant monomer, and the greater reactivity of the thiol surfactants in mixed micelles is partly due to the p$K_a$ effect. The p$K_a$ value for AM·Cys-1 is almost comparable to that for AM·Cys-2 and *smaller than that for $C_{12}$-SH*.

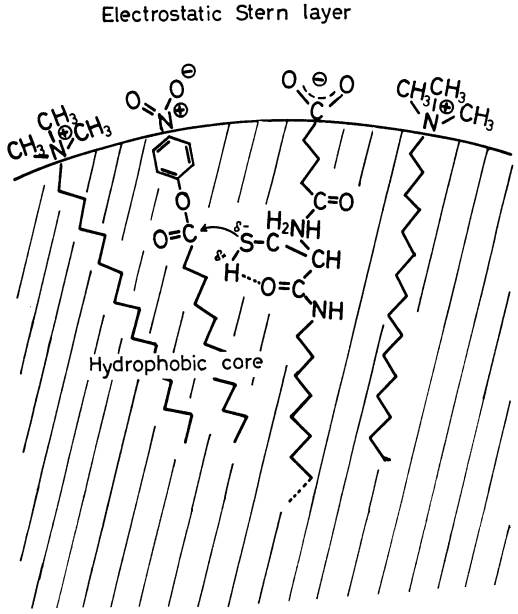



Fig. 7. Schematic representation of a plausible reaction mechanism for the deacylation of PNPH by CTAB–AM·Cys-1.

(2) The orientation of the ester bond of PNPH would be so arranged that an attack of the mercapto group on it is facilitated in the hydrophobic core.

(3) The intramolecular hydrogen bonding between the thiol and peptide-carbonyl groups as shown in Fig. 7 for the AM·Cys-1–CTAB system would enhance the nucleophilicity of the mercapto group in the effective hydrophobic core. Such development of a negative charge on the sulfur atom of the mercapto group in the hydrophobic core would be facilitated by the electrostatic field effect provided by the cationic micelle head in the transition state of acylation. Meanwhile, the mercapto groups of AM·Cys-2 and $C_{12}$-SH in the mixed micelle would not form an effective hydrogen bond since these may be placed in the more hydrophilic region. The reaction mechanism is analogous to that for papain catalysis proposed by Lowe[14] in which the nucleophilicity of a catalytic mercapto group is enhanced by its hydrogen bonding with an imidazolyl group.

*Conclusion.* The reactivity of mercapto groups drastically changes upon variation of their micro-environment. An electrostatic field provided by the Stern layer of an anionic micelle tends to increase p$K_a$ of a mercapto group placed in such a micelle, so that the mercapto group is masked to reduce its reactivity in a neutral pH region against an ester substrate. However, p$K_a$ of a mercapto group decreases when it is placed in a cationic micelle due to the effective electrostatic field effect provided by cationic heads. Furthermore, the nucleophilicity of the mercapto group placed in a hydrophobic core is enhanced by the intramolecular hydrogen bonding (Fig. 7) as well as by the electrostatic field effect transmitted into the hydrophobic core. These effects were demonstrated here with the mixed micelle composed of CTAB and AM·Cys-1 for the deacylation of ester substrates. The present results suggest that there are two kinds of mercapto groups; masked and super-reactive ones depending on their micro-environment and should be noted in connection with enzyme catalysis. In order to make a mercapto group more active in the hydrophobic region of micelles, it is required to introduce the second functional group at the juxtaposition of the mercapto group so that its nucleophilicity is much enhanced.

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