Micellar Activation of Nucleophilic Reactivity of Coenzyme A and Glutathione toward *p*-Nitrophenyl Acetate*

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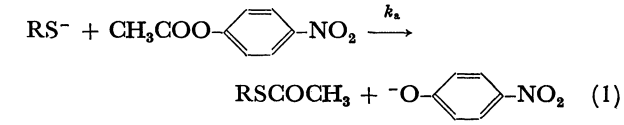
The reaction of coenzyme A(CoASH) and glutathione (GSH) with *p*-nitrophenyl acetate was studied in aqueous media at 30 °C. The reaction is highly accelerated in the presence of hydrophobic aggregates such as hydrophobic oligocations and CTABr micelles. Nonionic and anionic micelles were ineffective. The pK_a of CoASH is lowered by 0.5 pK_a unit in 2 mM of CTABr, while that of GSH is hardly affected. The second-order rate constant for CoASH is improved (290-fold) more than that of GSH(100-fold), relative to the nonmicellar system. Enhanced reactivities are efficiently suppressed by addition of KCl. These results are the first example of the marked activation of sulfhydryl coenzymes by the micellar microenvironment, and explicable by the concept of *hydrophobic ion pairs*: the coenzymes are bound onto the cationic micelle and the thiol anions form ion pairs with surfactant cations at the relatively hydrophobic environment of the micelle.

The reactivity of thiol groups has been of much concern in relation to functions of some sulfhydryl enzymes and enzymes requiring coenzyme A(CoASH) or glutathione (GSH).[1,2]

Nucleophilic reactions of thiol anions toward *p*-nitrophenyl acetate (NpAc: Eq. 1) have been evaluated by several investigators. Schonbaum and Bender[3] reported the hydrolysis of NpAc by *o*-mercaptobenzoic acid, and Jencks and Carriuolo[4] determined second-order rate constants (k_a) for the reaction of 2-mercaptoethanol and mercaptoacetate with NpAc. Subsequently, Whitaker[5] and Ogilvie and coworkers[6] reported the rate constants for the reaction of biologically important thiols such as cysteine and glutathione (GSH) with NpAc. However, the nucleophilic reactivity of thiol groups *in vivo* is incomparably enhanced relative to those observed *in vitro*. It is known that the active site of sulfhydryl enzymes is situated in the hydrophobic region,[7] but the essential role of the hydrophobic environment is not established. Heitmann[8] demonstrated that the nucleophilicity of *N*-dodecanoyl-DL-cysteinate mixed with a cationic micelle is appreciably improved (6—7 fold toward chloroacetamide, 100—200 fold toward NpAc). Tagaki and coworkers[9] also reported that a micellar solution of octadecyltrimethylammonium bromide markedly enhanced the reaction rate of alkanethiols and NpAc.

The possibility thus arises that the nucleophilic reactivity of a number of biological cofactors containing thiol groups is commonly affected by the micellar environment. One may anticipate that the reactions of coenzymes occurring in micelles are akin to those occurring in apoenzymes. That is, coenzymes may be activated by the micellar environment as done by apoenzymes. In this study, we examined the effect of micellar environments on the nucleophilic reactivity of coenzyme A(CoASH) and GSH toward NpAc. These coenzymes have additional anionic charges other than the thiol group and are expected to be easily bound onto the cationic micelles. We have discovered that the nucleophilicity of these biological cofactors toward NpAc (Eq. 1) is pronouncedly enhanced in the presence of a cationic micelle of hexadecyltrimethylammonium bromide (CTABr) and analogous hydrophobic aggregates (tetracationic detergents), and that the rate enhancement is attributed to the formation of *hydrophobic ion pairs*.[10-12] This is probably the first example that the nucleophilicity of coenzymes is markedly improved by the microenvironmental effect.



$$RS^- + CH_3COO\text{-}C_6H_4\text{-}NO_2 \xrightarrow{k_a} RSCOCH_3 + {}^-O\text{-}C_6H_4\text{-}NO_2 \quad (1)$$

Surfactants[1]: CTABr, $CH_3(CH_2)_{15}N^+(CH_3)_3Br^-$

(N-R)$_4$Br$_4$, $H_3C\text{-}\overset{\overset{CH_3}{|}}{\underset{\underset{R}{|}}{N^+}}\text{-}(CH_2CH_2\overset{\overset{CH_3}{|}}{\underset{\underset{R}{|}}{N^+}})_3\text{-}CH_3\ 4Br^-$

SDS, $CH_3(CH_2)_{11}OSO_3Na$

POOA, $CH_3(CH_2)_7CH{=}CH(CH_2)_7CH_2\text{-}(OCH_2CH_2)_{10}OH$

Experimental

Materials. Coenzyme A and glutathione were purchased from Kojin and Wako Pure Chem. Ind., respectively, and used without further purification. These cofactors were assayed by the reaction with NpAc(see Results Section). Hexadecyltrimethylammonium bromide was the product of wako Pure Chem. Ind., and recrystallized from ethanol before use. Other surfactants (sodium dodecylsulfate, polyoxyethylene (n=10) oleyl alcohol, trioctylmethylammonium chloride) were used without further purification. Quaternized triethylenetetramine derivatives were prepared and characterized by Mr. Y. Okahata.

Kinetics. The kinetic measurements for the reaction of coenzyme A and glutathione with NpAc were carried out in 3 vol% aqueous ethanol at 30±0.1 °C at a calculated ionic strength (μ=0.01 with KCl) unless otherwise stated. The buffer solutions (0.02 M borate below pH 9.3 and ammonium above pH 9.3) were prepared in twice-distilled water under nitrogen. In order to avoid air oxidation of thiols, the kinetic runs were performed under anaerobic conditions(N_2). A typical example of the reaction procedure is as follows: 2.8 ml of a buffer solution containing an appropriate surfactant were placed in a modified Thunberg cuvette, and was degassed for 15 min by bubbling N_2. An aqueous solution (0.1 ml) of coenzyme was added, and 0.1 ml of an ethanol solution of NpAc was deposited in the side-arm of the cell. Both solutions

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were degassed for 15 min by passing N_2. The cell was closed and equilibrated at 30 °C, and the content of the side-arm was rapidly mixed with the buffer solution. The progress of the reaction was followed by monitoring the *p*-nitrophenolate ion released (401 nm) on a Hitachi 124 spectrophotometer equipped with a thermostated cell-housing. In runs in the presence of surfactants, excess substrates were employed in order to lower the concentration of thiols as far as possible, since addition of polyanionic species may significantly affect the catalysis of the cationic micelle. On the other hand, excess thiols were employed in the absence of the surfactant. The pseudo-first-order rate constants were calculated with a Sharp Compet 364 for up to 3—4 half-lives. The pH of the reaction mixture was confirmed not to vary before and after the reaction by pH measurements (TOA Digital pH Meter, model HM-15A).

Results

Burst Release of p-*Nitrophenolate and Stoichiometry.* The reaction under anaerobic conditions was initiated by mixing of a surfactant solution containing coenzymes and an ethanol solution of NpAc. Figure 1 gives the time course of the absorbance change at 401 nm for the reaction of GSH and NpAc in the presence of 2 mM of CTABr. The burst release of *p*-nitrophenolate at pH 9.20 almost concluded within 10 s, and the slow, linear increase in the absorbance ensued. Since the absorbance increase in the second stage of the reaction was parallel to (or, under the insufficiently anaerobic condition, slightly slower than) that observed in the absence of the coenzyme moiety, the decomposition of the thiol ester (turnover) is negligibly slow, and the second stage should correspond to the spontaneous decay of NpAc (k_{spont}).

The magnitude of the initial burst was plotted against the concentration of CoASH and GSH in Fig. 2. The slopes of Fig. 2 were evaluated by the least-squares method to be 15000 for CoASH and 16600 for GSH,

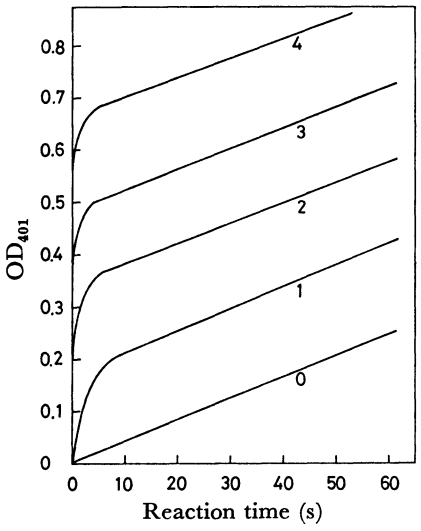


Fig. 1. Time-course of the absorption of *p*-nitrophenolate ion (401 nm) for the reaction of GSH and NpAc. pH 9.20, $[NpAc]=4.00\times10^{-4}$ M, $[CTABr]=2.0\times10^{-3}$ M. $[GSH]_T\times10^5$ M: (0) 0; (1) 1.00; (2) 2.00; (3) 3.00; (4) 4.00.

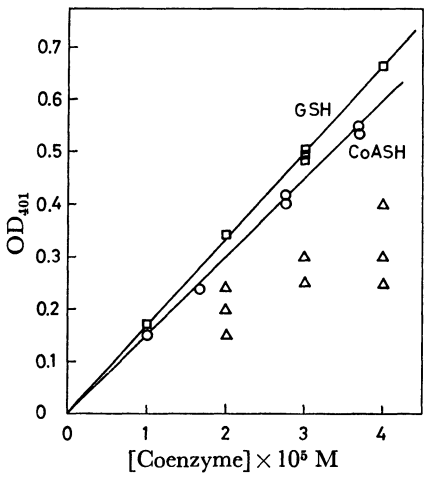


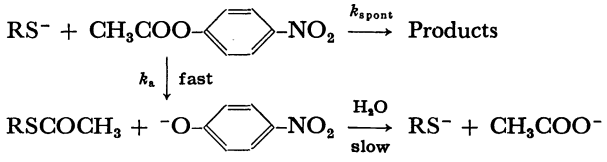
Fig. 2. $[CoASH]_T$ and $[GSH]_T$ *vs.* OD_{401} of the initial burst stage; △, N_2 was not bubbled prior to initiation of the reaction of GSH and NpAc.

respectively. These values are close to the molar absorption coefficient of the *p*-nitrophenolate anion ($\varepsilon=17800$) in the presence of 2 mM of CTABr. Thus, the coenzymes bound onto the CTABr micelle must react quantitatively with NpAc. The slight discrespancy observed would be due to the loss of the thiol function by air oxidation. When nitrogen was not bubbled prior to the reaction, the data lacked in reproducibility and a smaller slope (*ca.* 7500) resulted (Fig. 2). The influence of cationic micelles on the oxidation of thiols will be discussed elsewhere.

Results of Figs. 1 and 2 now enable us to depict reaction scheme 1 and the corresponding rate equation (Eq. 2),

$$v_{obsd}=k_{spont}[NpAc]+v_a \qquad (2)$$

where v_{obsd} is the reaction rate at the initial burst stage and v_a the reaction rate for the acyl transfer from NpAc to the coenzyme.



Scheme 1.

Determination of Second-Order Rate Constants. The rate constants for the initial burst reaction were determined at pH 8.78. The increase in absorbance at 401 nm satisfied the pseudo-first-order rate equation at least up to 3 half-lives, and the pseudo-first-order rate constants ($k_{a,obsd}$) thus evaluated were proportional to the concentration of NpAc (Fig. 3). Evidently, the reaction is first-order both in coenzyme and in substrate, giving the kinetic expressions of Eqs. 3 and 4,

$$v_a=k_{a,obs}[CoASH\text{ or }GSH]_T=k_a'[CoASH\text{ or }GSH]_T[NpAc] \qquad (3)$$

$$k_a'=k_a\left(\frac{K_a}{K_a+a_H}\right) \qquad (4)$$

where K_a is the acid dissociation constant of thiol groups

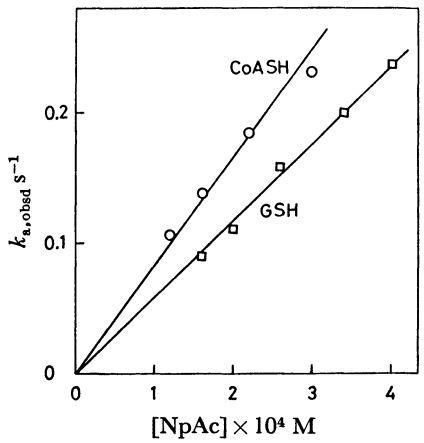


Fig. 3. [NpAc] *vs.* $k_{a,obsd}$. pH 8.78±0.03, [CTABr]= 2.0×10^{-3} M, $[CoASH]_T=2.00\times10^{-5}$ M, $[GSH]_T=2.19\times10^{-5}$ M.

Table 1. Apparent and true second-order rate constants

Surfactant	mM	k'_a at pH 8.78 $M^{-1}s^{-1}$	k_a $M^{-1}s^{-1}$	pK_a[c]
For CoASH:				
None		1.06	11	9.8
CTABr	2.0	863	3160	9.3
(N-OctDe)$_4$Br$_4$	0.52	885		
(N-Dod)$_4$Br$_4$	1.8	6.90		
(N-Oct)$_4$Br$_4$	1.9	2.21		
SDS	10	0.81		
POOA	0.11	1.11		
POOA	0.43	1.22		
POOA[a]	0.43	1.18		
POOA[b]	0.43	2.90		
For GSH:				
None		7.60	16	9.1
CTABr	2.0	530	1590	9.2
SDS	3.0	7.10		
	10	4.88		
POOA	0.11	9.61		
	0.32	10.1		

a) $[Oct_3MeNCl]=2.0\times10^{-4}$ M b) $[CTABr]=2.0\times10^{-4}$ M c) Estimated kinetically.

and $[CoASH]_T$ and $[GSH]_T$ are the total concentration of coenzymes. Typical examples of the apparent second-order rate constants (k_a') for the reaction of CoASH and GSH with NpAc in the presence and the absence of surfactants are recorded in Table 1.

Surfactant Concentration-Rate Profiles. In the non-micellar system, the apparent second-order rate constants at pH 8.78 were 1—8 $M^{-1}s^{-1}$ (Table 1). These values are not much different from those of alkane thiols,[5,6] indicating that the nucleophilic reactivity of biological cofactors toward NpAc is not anomalous in the nonenzymatic system.

In Figs. 4 and 5, the apparent second-order rate constants were plotted against the concentration of CTABr and quaternized triethylenetetramine derivatives. Plots of k_a' against the concentration of CTABr

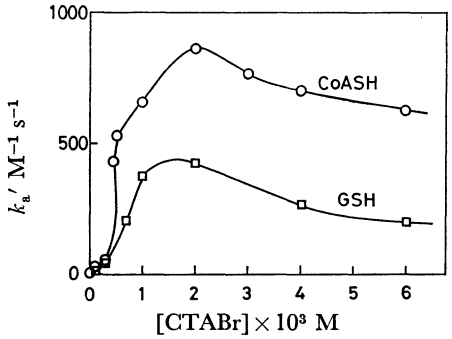


Fig. 4. Apparent second-order rate constants plotted against the concentration of CTABr. For CoASH+NpAc: $[CoASH]_T=2.00\times10^{-5}$ M, $[NpAc]=1.60\times10^{-4}$ M, pH 8.78±0.03. For GSH+NpAc: $[GSH]_T=2.05\times10^{-5}$ M, $[NpAc]=3.35\times10^{-4}$ M, pH 8.65±0.03.

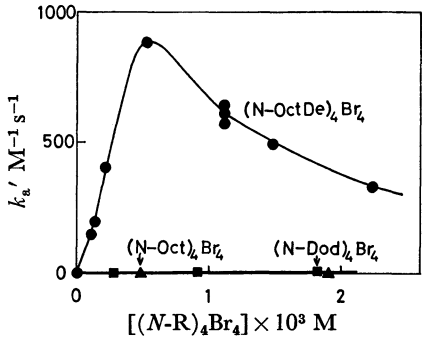


Fig. 5. Apparent second-order rate constants for the reaction of CoASH and NpAc plotted against the concentration of (N-R)$_4$Br$_4$. $[CoASH]_T=2.00\times10^{-5}$ M, $[NpAc]=2.00\times10^{-4}$ M, pH 8.78±0.03.

(Fig. 4) give multiphasic curves: around the CMC (9.2×10^{-4} M at 25 °C[13]: 8.0×10^{-4} M under the kinetic condition; Okahata, unpublished) the rate constants rise rapidly with increasing concentration of CTABr. Further increase in the CTABr concentration rather suppresses the rate constants, resulting in rate maxima at 2×10^{-3} M of CTABr. At the optimal CTABr concentration (2 mM), rate augmentations of 810-fold for CoASH and 75-fold for GSH are observed. Plots of k_a' against the concentration of (N-OctDe)$_4$ showed a similar concentration-rate profile with a rate maximum at 5×10^{-3} M, except that the reaction rates are significantly improved at very low concentration of (N-OctDe)$_4$Br$_4$ (Fig. 5). The rate augmentation in the presence of (N-OctDe)$_4$Br$_4$ for the reaction of CoASH and NpAc was 840-fold at the optimal concentration as compared with the typical non-micellar system. In contrast, the aqueous solutions of (N-Dod)$_4$ and (N-Oct)$_4$ did not accelerate this reaction (Fig. 5). Addition of anionic (SDS) and nonionic (POOA) surfactants above their CMC was also ineffective (Table 1). Addition of hydrophobic ammonium ions such as CTABr (below the CMC) and Oct_3MeNCl to the nonionic micellar system did not increase the rate, either (Table 1).

Effects of Ionic Strength and pH. The rate of micelle-catalyzed reaction is influenced by the ionic strength of the medium.[14,15] Apparent second-order rate constants (k_a') for the reaction of CoASH and GSH

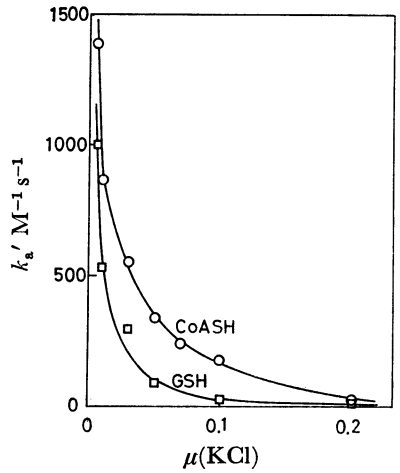


Fig. 6. Effect of ionic strength. [CTABr]$=2.0\times10^{-3}$ M, pH 8.78 ± 0.03. Other reaction conditions are recorded under Fig. 4.

bound to the CTABr micelle were evaluated as a function of ionic strength (μ(KCl)$=0.006-0.2$; Fig. 6). The reaction rates for both coenzymes are markedly suppressed with increasing salt concentration. Fig. 6 proves that the reactivity of GSH is more susceptible to ionic strength than that of CoASH, and the rate difference amounts to 54-fold for CoASH and 110-fold for GSH. At $\mu=0.2$, the $k_{a'}$ value for the reaction of GSH and NpAc is 9.05 M^{-1} s^{-1}, which is almost equal to the non-micellar value (7.60 M^{-1} s^{-1}).

The pH dependence of the rate is illustrated in Fig. 7. The acid dissociation constants (K_a) and the true second-order rate constants (k_a) were determined by fitting of the theoretical curves (Eq. 4) to the experimental plots. The solid curves in Fig. 7 were obtained

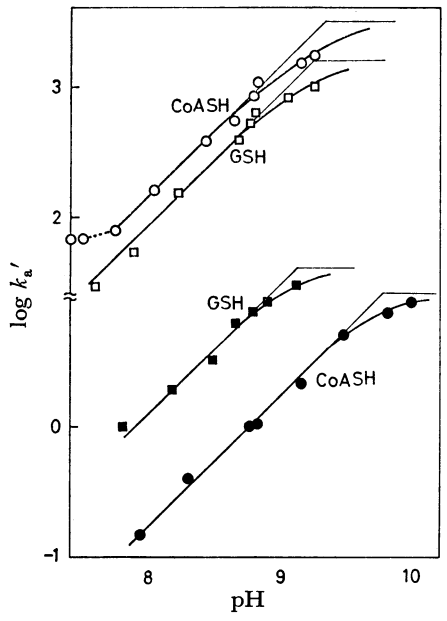


Fig. 7. pH Dependence. [CTABr]$=2.0\times10^{-3}$ M for ○ and □; other reaction conditions are recorded under Fig. 4. CTABr are not added for ● and ■; $[CoASH]_T=2.00\times10^{-4}$ M, $[GSH]_T=4.02\times10^{-4}$ M, $[NpAc]=2.00\times10^{-5}$ M.

by using Eq. 4 for k_a and pK_a values recorded in Table 1. The reactive species in this acyl transfer reaction is undoutedly the dissociated fraction of the thiol groups. The true second-order constants (k_a) in the presence of 2 mM of CTABr are thus estimated to be $(1-3)\times10^3$ M^{-1} s^{-1} (Table 1). Therefore, the cationic micelle of CTABr enhances the nucleophilicity of CoASH and GSH anions by 290-fold and 100-fold respectively. It is also worth while mentioning that the pK_a value of CoASH is considerably lowered (0.5 pK_a unit), while that of GSH is not.

As shown in Fig. 7, the rate constant for the reaction of CoASH and NpAc in the presence of CTABr deviates upwards at pH 7.4—7.6. A similar trend has been noticed in the polysoap-catalyzed reaction of CoASH with NpAc.[16] We consider that the adenine moiety of CoASH is responsible for the deviation, but the detail is not clear at present.

Discussion

It is frequently said that the micellar catalyses provide appropriate model systems of the enzymatic catalyses.[15,17] For instance, micelles significantly influence rates and equilibria of biologically important reactions. One of the recent, novel findings in the micellar catalysis is the fact that the nucleophilicity of a variety of anionic nucleophiles is unusually enhanced in cationic micelles (Ref. 10 and references cited therein) as well as in cationic polysoap.[18-20] Since this phenomenon is not limited to the conventional cationic micelle, it was proposed that the rate acceleration is largely derived from the formation of hydrophobic ion pairs between anionic species and cationic surfactant in a micellar, hydrophobic environment.[10-12] The anion included in a hydrophobic ion pair will attain its high reactivity from desolvation (dehydration), as already known for the ionic reactions in organic solvents.[21]

The apparent second-order rate constants ($k_{a'}$) for CoASH and GSH at pH 8.78 were evaluated to be 863 and 530 M^{-1} s^{-1}, respectively (Table 1). On the other hand, the $k_{a'}$ values for the reaction of dodecanethiol and NpAc are 417 M^{-1} s^{-1} ([octadecyltrimethylammonium bromide]$=ca.$ 10^{-3} M, pH 9.0)[9] and 462 M^{-1} s^{-1} ([partially laurylated poly(2-ethyl-1-vinylimidazole)]$=2.25\times10^{-3}$ M, pH 8.93).[20] The nucleophilicity of the micellar GSH is comparable to that of the hydrophobic ion pair of dodecanethiol, and that of CoASH is still higher. In the previous examples, some hydrophobic functions such as long alkyl chain, aromatic ring, and/or polymer backbone were indispensable for forming highly nucleophilic ion pairs.[18,22-24] In contrast, CoASH and GSH are polyanions with phosphate and carboxylate groups, and expected to be bound onto the cationic micellar surface mainly due to the electrostatic interaction. It is unlikely, by considering the poor solubility of these coenzymes in organic solvents, that the thiol groups are buried deeply in the hydrophobic core of the micelle. Therefore, the formation of reactive hydrophobic ion pairs is possible in a region fairly close to the surface of the micelle.

We previously showed that the nucleophilicity of

hydrophobic hydroxamate anions in the presence of nonionic micelles is markedly enhanced by the addition of hydrophobic ammonium salts.[10-12] As listed in Table 1, rate increases could not be observed by addition of CTABr below the CMC and Oct_3MeNCl. Supposedly, the hydrophobic interaction between these hydrophobic ammonium salts and the biological cofactors is not strong enough to bring the latter to the micellar phase of the nonionic micelle.

It is probable that the higher reactivity of CoASH relative to that of GSH is associated with the efficient binding of CoASH to the micellar phase. According to Cordes and Dunlap,[14] typical surfactant concentration-rate profiles such as Figs. 4 and 5 can be rationalized on the basis of (1) adsorption of progressively greater fractions of the nucleophile to the micellar phase until that fraction approaches unity (in the CTABr micellar system, NpAc is hardly concentrated in the micellar phase)[25] and (2) inhibition of the micellar catalysis by the counterions of the surfactant itself. Thus, Cordes and Dunlap[14] state that, when 50% of the nucleophile is associated with the micellar phase, about 50% of the maximum catalysis experienced. Figure 4 shows that the concentration of CTABr at the 50% reactivity is 0.9 mM for CoASH and 1.5 mM for GSH, so that CoASH possesses association constant several times larger than that of GSH. In a separate study, we estimated the binding constants of CoASH and GSH with 29 mol % laurylated poly(2-ethyl-1-vinylimidazole) to be 1100 and 240 M^{-1}, respectively.[16]

A similar trend is found for the effect of ionic strength (Fig. 6). The salt effect in the micellar system primarily arises from competitive occupation of the counterion site.[26] Figure 6 suggests that GSH is expelled out of the micellar phase more easily than CoASH by the counteranion binding.

In conclusion, the reactivity of sulfhydryl coenzymes can be enhanced remarkably in the presence of cationic micelles. This is explained by the formation of reactive ion pairs between CoASH and GSH anions and cationic CTABr molecules in the micellar phase. Since the coenzyme binding site of apoenzymes is said to be situated in relatively hydrophobic regions,[7] the CTABr micelle may serve as a model of apoenzymes.

References

1) Abbreviations employed are: coenzyme A, CoASH; glutathione, GSH; *p*-nitrophenyl acetate, NpAc; hexadecyltrimethylammonium bromide, CTABr; sodium dodecylsulfate, SDS; polyoxyethylene ($n=10$) oleyl alcohol, POOA; hexamethyl-N^1,N^2,N^3,N^4-tetraalkyl-triethylenetetramine tetrabromide, $(N\text{-R})_4Br_4$, where alkyl groups are octyl(Oct), dodecyl(Dod), and Octadecyl(OctDe).

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