BIOORGANIC CHEMISTRY 20, 356–365 (1992)

The Effect of Long-Chain Alkyl Betainates on the Arylesterase-like Activity of Bovine Serum Albumin

RICHARD A. THOMPSON* AND STIG ALLENMARK†

*Laboratory of Microbiological Chemistry, Department of Clinical Bacteriology, University of Gothenburg, Guldhedsgatan 10A, S-413 46 Gothenburg, Sweden; and †Department of Organic Chemistry, University of Gothenburg, S-412 96 Gothenburg, Sweden

Received September 9, 1992

The effect of a hydrolyzable cationic surfactant, tetradecyl betainate (tetradecyloxycarbonyl-*N,N,N*-trimethylmethanaminium chloride), on the arylesterase-like activity of bovine serum albumin (BSA) was investigated. The rate of hydrolysis of *p*-nitrophenyl hexanoate in the presence of BSA and varying concentrations of the surfactant was followed. The rate was found to be dependent on the concentration of the cationic surfactant and a maximum was found in the curve at ca. 3 mM. The Michaelis–Menten constants (K_m/n, where n is the number of active sites) and the "catalytic" rate constants (k_2) were determined for the reactions, and were found to be 11 and 40 times larger, respectively, in the presence of the surfactant. The hydrolysis of radiolabeled tetradecyl betainate in the presence and absence of BSA was also followed. This study, together with a binding study based on gel permeation chromatography, showed that the surfactant binds to the protein, but that no hydrolysis of the betaine ester takes place while bound to BSA. It was thus concluded that the increased value of k_2 in the presence of a cationic surfactant was not, as has been previously suggested, due to an increased local hydroxide concentration resulting from the formation of a new pseudophase.

INTRODUCTION

It has been shown that both human serum albumin (HSA) and bovine serum albumin (BSA) show esterase-like activity toward arylesters (*1–4*). The reactions are biphasic, consisting of a burst phase followed by a slower stationary phase. Studies of the burst phase, using *p*-nitrophenyl acetate as the substrate, showed that the reaction is the result of a rapid and stable acetylation of a tyrosine residue in the albumin (*1, 2*). A rapid reversible binding of the substrate to the protein is the major contributor to the fast reaction rate, in both the burst (*2*) and the stationary phase (*4*).

Means and Bender (*2*) have shown that the reactions found in the stationary phase are the result of multiple reactive sites and that when an excess of substrate is used the equivalent of five acetate moieties are incorporated into the protein. The same type of mechanism has also been shown with *p*-nitrophenyl glycinate (*5*) and *p*-nitrophenyl 4-guanidinobenzoate (*6*). The observed reaction rate (Eq. [1]) is thus the result of the sum of a number of reactions taking place simultaneously at different sites on the protein, S_1, S_2, S_3, . . . , S_n, plus a spontaneous reaction taking place independent of the protein (k_0) (*2*).

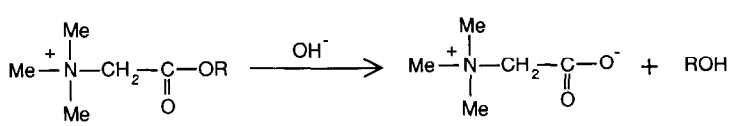
0045-2068/92 $5.00

$$k_{obs} = k_1[S_1] + k_2[S_2] + k_3[S_3] + \cdots + k_n[S_n] + k_0 \quad [1]$$

The effect of serum albumin on the hydrolysis of arylesters has been shown to be greatly influenced by the presence of surfactants. Anionic surfactants decrease the hydrolysis rates (*2, 3, 7*), whereas cationic surfactants increase the rates (*3, 8, 9*). Koga *et al.* have shown that the effect of cationic surfactants is concentration dependent and shows a maximum (*8*). They have suggested that the increased hydrolysis rate is due to the formation of a new pseudophase as a result of the protein–surfactant complex. The pseudophase was suggested to increase the substrate concentration in the complex and/or increase the cationic charge around the complex surface leading to an increased hydroxide ion concentration.

We have been working for a number of years with hydrolyzable surfactants. Our primary interest in this field is the development of what, in analogy with the soft drug concept, can be called soft antimicrobial agents. These are compounds that have antimicrobial activity, but which, due to deliberately introduced structural weaknesses, degrade at a controllable rate and in a predictable manner (*10*). Our efforts have been concentrated in the area of cationic surfactants. The traditional "hard" cationic surfactants have good antimicrobial activity due to their membrane disruptive nature (*11*), but can be damaging to mammalian cells upon long exposure. We have shown that long-chain alkyl betainates (alkoxycarbonyl-*N*,*N*,*N*-trimethylmethanaminium chlorides: type **I** compounds, Scheme 1) have good antimicrobial activity and can be degraded by alkaline hydrolysis to nontoxic compounds, betaine and a fatty alcohol (*12*). Kinetic studies of the hydrolysis rates of these esters showed that the rate is concentration dependent and is subject to micellar catalysis as a result of self aggregation (*13, 14*).

It was felt that this type of hydrolyzable amphiphile would be suitable for the study of the possible formation of a pseudophase on the surface of albumin. The purpose of the present study is twofold. First, we quantify the effect of BSA on the rate of hydrolysis of the long-chain alkyl betainates. Second, we use these substances as model compounds to study the mechanisms behind the effects of cationic surfactants on the arylesterase-like activity of BSA.



I

Ia: R = $C_{14}H_{29}$ **Ib:** R = $C_{12}H_{25}$ **Ic:** R = $C_{10}H_{21}$

SCHEME 1

MATERIALS AND METHODS

Materials

Synthesis of the dual-labeled tetradecyl betainate was described earlier (*15*), and ^{3}H-labeled tetradecyl betainate **(Ia)** was synthesized using this procedure.

The synthesis of ^{3}H-hexadecyltrimethylammonium bromide (CTAB) was performed in two steps. The first was a reductive methylation of hexadecylamine in the presence of formaldehyde and formic acid (Eschweiler–Clarke procedure) and the second step was a methylation of the product with ^{3}H-methyl iodide and methyl bromide.

The BSA was from Serva (11930) and was used without further treatment. The *p*-nitrophenyl hexanoate was from Sigma (St. Louis, MO).

Hydrolysis Studies

The hydrolysis procedure and radiochromatographic method for the determination of the reaction rate of **Ia** were as described earlier (*15*). The hydrolysis studies were performed in 10 mM phosphate buffer, pH 7.0, at 35°C. The BSA studies were done with a protein concentration of 1 mg/ml (15.1 μM).

The hydrolysis of *p*-nitrophenyl hexanoate **(II)** was followed spectrophotometrically by monitoring the formation of *p*-nitrophenolate ion at 400 nm. The measurements were made by the use of a Perkin–Elmer Lambda 2 spectrophotometer equipped with a thermostated cell holder. The reaction temperature was 35°C. To 2.225 ml of BSA solution (10 mM phosphate buffer, pH 7.0) was added 250 μl of either water or **Ia** dissolved in water. The reaction was started by the addition 25 μl of **II** in acetonitrile, giving 1% acetonitrile in the final solution.

In the first experimental series, in which the concentration of **Ia** was varied, the final concentrations of BSA and **II** were 12.8 and of **II** 25 μM, respectively. The observed first-order rate constants (k_{obs}) were obtained by subtracting the rate constant for the spontaneous hydrolysis (k_0) from the detected rate constant, k_{det} (Eq. [2]).

$$k_{obs} = k_{det} - k_0 \qquad [2]$$

The second experimental series, in which the concentration of **II** was varied, was performed with **[II]** $\gg$ [BSA] (the ratio was between 8 : 1 and 24 : 1). In the final solution, the concentration of BSA and **Ia** were 2.66 and 340 μM, respectively. The initial velocities were obtained by plotting the concentration of **II** against time. The concentrations were obtained from the measured absorbance at a given time (A_{det}) minus the absorbance from the *p*-nitrophenolate produced by the spontaneous hydrolysis, as shown by

$$A_t = A_{det} - \varepsilon\,[\mathbf{II}]_0(1 - \exp(-k_0 t)), \qquad [3]$$

where ε is the molar absorptivity of *p*-nitrophenol in the presence of BSA (8.8×10^3 M^{-1} cm^{-1}) and $[\mathbf{II}]_0$ is the initial concentration of *p*-nitrophenyl hexanoate.

Binding Studies

Binding studies of **Ia** to BSA were performed by gel permeation chromatography. The validity of the method was checked by comparing results of binding studies with both gel permeation chromatography and equilibrium dialysis for a stable cationic surfactant (CTAB).

The gel permeation measurements were made on a 10 × 100-mm desalting column packed with Sephadex G-25 Superfine (Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was equilibrated with the surfactant solution at a flow rate of 1.5 ml/min and 500 μl of a 1 mg/ml BSA solution was injected. The separation was followed by the use of a uv-absorbance detector (276-nm filter: Uvicord SII, Pharmacia LKB Biotechnology) in series with the radioactivity flow detector (Radiomatic A-250, Tampa, FL). The scintillation medium was Flo-Scint V (National Diagnostics, Manville, NJ) which had a flow rate of 6 ml/min.

For the equilibrium dialysis studies, 5 ml of 1 mg/ml BSA solutions in dialysis bags (SPECTRA/POR, Spectrum Medical Industries) was equilibrated with 15 ml [^{3}H]CTAB solution. The experiments were performed at pH 5 (100 mM sodium acetate buffer) and pH 7 (100 mM phosphate buffer). The samples were shaken for 200 h at 35°C. The free surfactant concentration was followed by taking 100-μl aliquots. Measurements were performed on a LKB 1217 (LKB Wallac, Finland) liquid scintillation counter. At equilibrium, surfactant concentrations were determined on both sides of the membrane and protein leakage was checked by absorbance measurements (260 and 280 nm).

RESULTS AND DISCUSSION

The effect of the concentration of the long-chain alkyl betainate **(Ia)** on the observed first-order rate constant (k_{obs}) of *p*-nitrophenyl hexanoate **(II)** in the presence respective absence of BSA is shown in Fig. 1. The results are in general agreement with those found earlier with tetradecyltrimethylammonium bromide (*8*). The bottom curve shows the hydrolysis rate in the absence of BSA. The rate constant increases approximately 40 times from 0.014×10^{-3} s^{-1} for the spontaneous reaction (k_0) in the absence of the surfactant, to 0.57×10^{-3} s^{-1} at a surfactant concentration of 1 mM. The increase in the rate of hydrolysis of **II** in the presence of the amphiphile is a result of micellar catalysis (*16–19*). The cationic surfactant **(Ia)** forms micelles, and the positive charge on the micelles is partially neutralized by an increased hydroxide concentration in the Stern layer. The substrate **(II)** is solubilized into the micelles and exposed to a more alkaline environment (*20*), thus leading to an increased rate of hydrolysis. Since **Ia** is also subject to micellar catalysis (*14*), there will be competition between the two reactions for the hydroxide ions.

The upper curve shows the hydrolysis rate of **II** in the presence of BSA and **Ia**. It can be seen that the rate in the absence of the surfactant, 0.58×10^{-3} s^{-1}, is about 40 times higher than that of the spontaneous hydrolysis. The hydrolysis rate increases as the betaine ester concentration is increased, reaching a maximum at a surfactant concentration of ca. 3 mM.

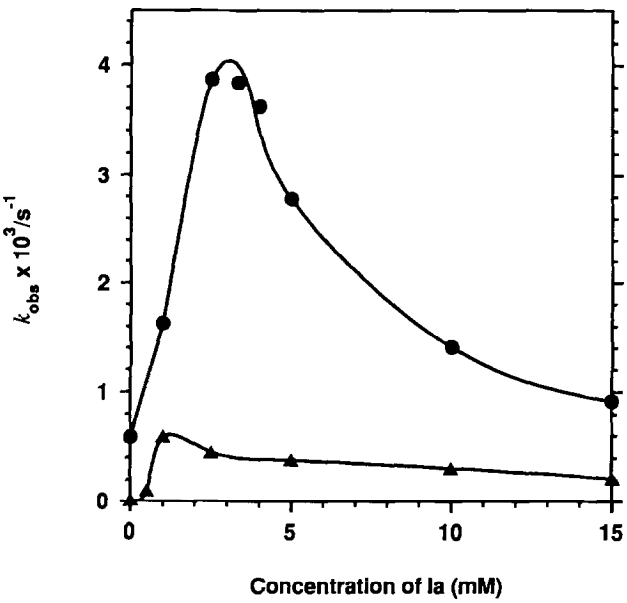
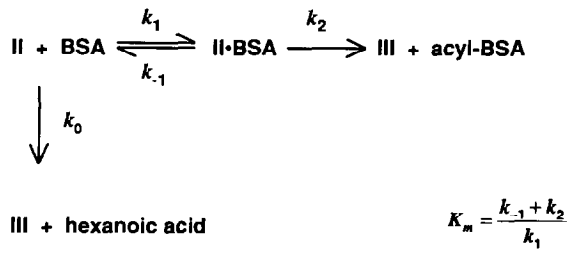


FIG. 1. Effect of **Ia** on the hydrolysis of **II**. Observed first-order rate constants in the presence (●) and absence (▲) of BSA.

The rate increase in the presence of a cationic surfactant and serum albumin has been suggested to be due to the formation of a new pseudophase, resulting from the protein–surfactant complex (*3*, *8*). It was suggested that this pseudophase was a result of a micelle-like aggregation of the surfactant on the protein. The organized medium thus formed would lead to an increased hydroxide ion concentration, which could at least partially explain the increased rate of hydrolysis of *p*-nitrophenyl hexanoate. Further, the new pseudophase could lead to an increased substrate concentration in the protein–surfactant complex (*8*).

The hydrolysis of *p*-nitrophenyl hexanoate in the presence of BSA can be represented as shown in Scheme 2a. **II** · BSA is the Michaelis–Menten complex between **II** and BSA. **III** is the reaction product *p*-nitrophenol, and acyl-BSA is



SCHEME 2a

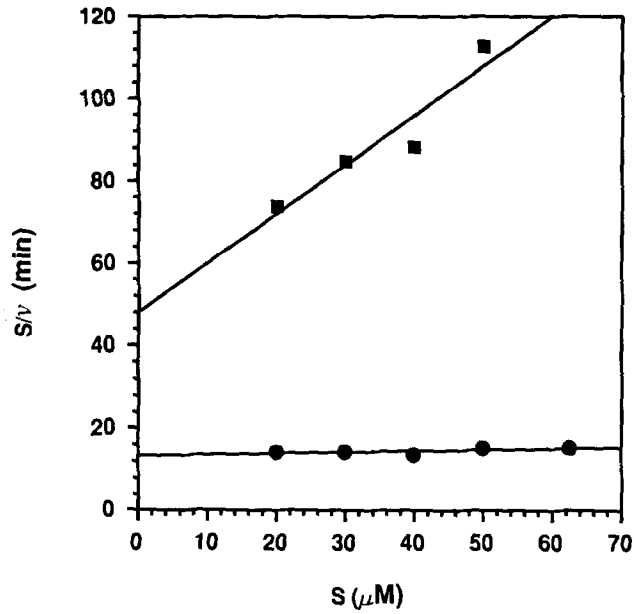
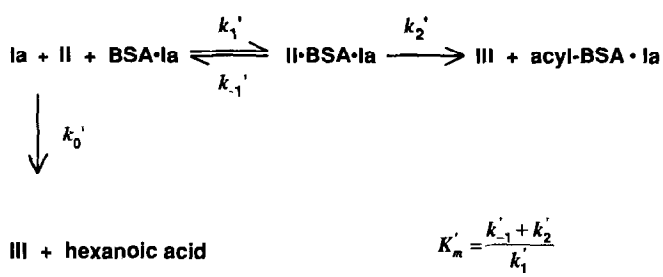


FIG. 2. Hanes plot of initial velocities of the hydrolysis of **II** in the presence of BSA with (●) and without (■) **Ia** added.

the acylated protein. A similar representation, but for the reaction of **II** in the presence of both BSA and **Ia**, is shown in Scheme 2b.

To determine the values of K_m, k_2, K'_m, and k'_2 a new series of experiments was performed. The reaction of **II** in the presence of BSA and in the absence and presence of **Ia**, respectively, was followed at various concentrations of **II** where **[II]** $\gg$ [BSA]. In the experiments with **Ia** present, the ratio of **Ia** to BSA was kept at the maximum in Fig. 1, i.e., 250 : 1. Due to the low concentrations of **Ia** it was assumed, for the purpose of these experiments, that $k'_0 \approx k_0$. The results of the experiments are shown in a Hanes plot in Fig. 2, where v is the initial velocity and S is the initial concentration of **II**.

Values for K_m and k_2 can be calculated from the Hanes plot. However, since



SCHEME 2b

TABLE 1

Kinetic Parameters for the Hydrolysis of **II** in the Presence of BSA

Added surfactant	$k_2 \times 10^3$ (s^{-1})	$k_2' \times 10^3$ (s^{-1})	K_m/n (mM)	K_m'/n (mM)	nk_2/K_m (s^{-1} M^{-1})	nk_2'/K_m' (s^{-1} M^{-1})
None	5.2		0.040		130	
Ia		202		0.425		475

there is more than one active site (Eq. [1]), the value obtained for K_m will be a combined value for all such sites. It has been shown that the active sites in the stationary phase show similar reactivity (*5*), thus the value obtained will be equal to K_m/n, where n is the number of sites.

The kinetic parameters for the hydrolysis of **II** are shown in Table 1. As shown earlier for *p*-nitrophenylacetate, the parameters in the absence of **Ia** indicate that the major contribution to the fast reaction rate is the rapid reversible binding of the substrate to the protein (*4*). The K_m/n value of 0.040 mM indicates a strong binding, whereas k_2 is only 5.2×10^{-3} s^{-1}.

It can be seen from Table 1 that K_m'/n is almost 11 times larger than K_m/n. It would appear that the addition of **Ia** to the system has a large negative effect on the binding of the substrate to the protein. On the other hand, k_2' is almost 40 times larger than k_2. The cationic surfactant has such a large positive effect on the rate constant in the acylation step that this more than compensates for the poorer binding. The results of $k_2' > k_2$ fit well with the concept of the formation of an organized medium through a new pseudophase. In the case of *p*-nitrophenyl glycinate, it has been postulated that the predominant reaction in the last step is not a nucleophilic catalysis, but a general acid or base catalysis (*5*). An increased hydroxide ion concentration resulting from an organized medium would thus have a large effect on the value of k_2'.

If this type of pseudophase is formed, then one would expect to see an increased rate of hydrolysis of **Ia** in the presence of BSA since the catalytic-like mechanism would be similar to the earlier observed effect of micelle formation on the rates of hydrolysis of the long-chain alkyl betainates (*13, 14*). Using a dual-labeled compound, we have shown that the hydrolysis of **Ia** can be followed in the presence of BSA (*15*). The results from that study, however, indicate that the rate of hydrolysis of the ester decreases in the presence of BSA. Since the study was performed at only one betaine ester concentration, a wider concentration range was now examined. The hydrolysis rates of **Ia** were followed in both the presence and the absence of BSA. The results are shown in Fig. 3. The catalytic effect on the hydrolysis rate of long-chain alkyl betainates in the absence of BSA is due to micellar catalysis resulting from the self aggregation of the amphiphiles, and has been discussed earlier (*13, 14*). As can be seen in Fig. 3, the observed first-order rate constants decrease in the presence of BSA. This is contrary to what would be expected if an organized medium had been formed on the surface of the protein.

It is known that cationic surfactants bind to BSA through noncooperative binding

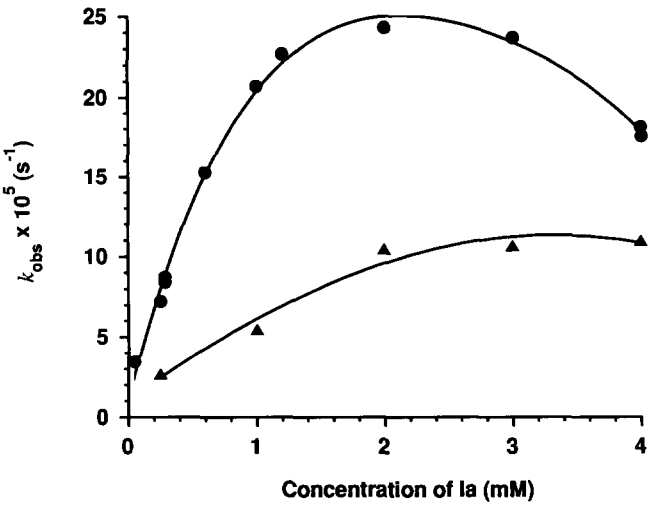


FIG. 3. Hydrolysis of **Ia** in the presence (▲) and absence (●) of BSA.

to specific sites at low surfactant concentrations and through cooperative binding at higher concentrations (*21, 22*). This binding would decrease the free concentration of **Ia**, and thus one would expect to see a slower rate of hydrolysis if the bound ester is not hydrolyzed. The decrease in k_{obs} as seen in Fig. 3 could, however, be masking a small positive contribution due to a catalytic-like effect from the surfactant–protein complex. To ascertain whether or not this was the case, we made the following assumption. We assumed that no, or insignificant, hydrolysis of betaine ester takes place while bound to BSA. Making this assumption allows us to use Fig. 3 to make a binding isotherm for **Ia** to BSA. By taking the pseudo-first-order rate constant in the presence of BSA at a given surfactant concentration (total ester concentration) and extrapolating this rate back onto the curve in the absence of BSA, the free ester concentration can be obtained. The number of bound moles of **Ia** per mole BSA can then be calculated.

It was desirable to compare the binding isotherm obtained by the hydrolysis studies with that obtained by another method. The commonly used equilibrium dialysis technique, however, has equilibrium times too long to be used with the betaine esters (*21*). The technique that was found to be the most appropriate for this study was gel permeation chromatography. A comparison of the two techniques was performed with ^{3}H-CTAB and the results were found to be in good agreement. However, due to the limited capacity of the gel permeation column, free surfactant concentrations of only up to 0.3–0.4 mM were studied. The binding isotherm obtained for CTAB with gel permeation chromatography was in agreement, within the range studied, with that obtained by equilibrium dialysis (*23*).

The binding isotherms for **Ia** as obtained by the hydrolysis studies and gel permeation chromatography are shown in Fig. 4. The agreement between the two is exceptionally good, indicating that betaine ester bound to BSA is not hydrolyzed. This would suggest that no new organized pseudophase is formed. Thus, the

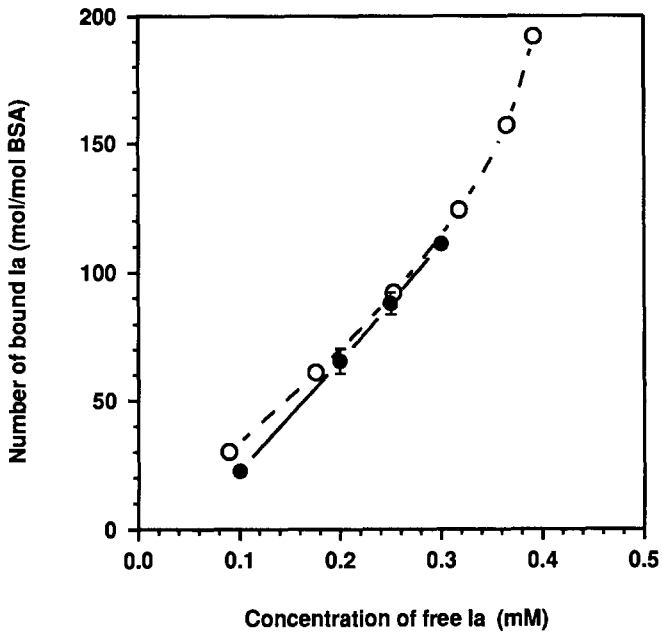


FIG. 4. Binding isotherm of **Ia** to BSA, as determined by hydrolysis studies (○) and gel permeation chromatography (●).

increase of k_2 in the presence of the cationic surfactant is not due to an increased hydroxide ion or substrate concentration.

What type of mechanism could be envisioned which would lead to $k_2' > k_2$? This type of effect could be the result of either a stabilization of the transition state or a destabilization of the ground state of one of the reactants by the surfactant. It has been noted earlier that the reaction is considered to be an acetylation of a tyrosine residue in the protein (*2*). A binding of the cationic surfactants in the active sites on albumin would lead to an increased hydrophobicity and an expulsion of water from the sites. The resulting desolvation of the tyrosine environment would lead to an increased nucleophilic reactivity and thus to a larger k_2 value. This same binding of surfactants in and around the active site, could possibly also obstruct the binding of the substrate to the protein leading to a larger K_m value.

In conclusion, the increased rate of hydrolysis of *p*-nitrophenyl hexanoate by BSA in the presence of cationic surfactants is due to a positive effect of the surfactants on k_2. This effect, however, is not a result of an increased hydroxide ion concentration following the formation of a new pseudophase, since the rate of hydrolysis of **Ia** is decreased in the presence of BSA.

ACKNOWLEDGMENTS

The authors gratefully acknowledge financial support by The Swedish Research Council for Engineering Sciences (Grant 91-458) and by Berol Nobel AB.

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