Kinetic Study of Casein Micelle Formation Observed by a Stopped-Flow Apparatus

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The time dependent optical density changes were observed when aqueous solutions (Tris buffer) of sodium casein from milk were mixed with those of calcium chloride by a newly constructed stopped-flow apparatus which was controlled by an 8-bit microcomputer. Two approaches for single exponential decay curve analysis were used, i.e. the Guggenheim analysis and an analysis according to a linear relation between the time and the logarithm of the corresponding absorbance change, at a concentration range between 0.02—0.05 wt% of the casein and in that between 10—30 mmol dm^{-3} of calcium chloride at 25 °C. The same time constants and reaction amplitudes were obtained from both analyses of the decay curves. The obtained rate parameters were found to be concentration dependent. The result was interpreted by a stepwise reaction mechanism for the formation of a casein aggregate. The rate-determining step was speculated to be the first step of the larger molecular aggregate of the casein according to the concentration dependence of the apparent rate constant and the reaction amplitude. The rate constant for the casein micellization could be approximately estimated as being of the order of 10^4 mol^{-1} dm^3 s^{-1} at 25 °C. The effects of NaCl, $MgCl_2$, and $CaBr_2$ on casein micelle formation were also examined; it was kinetically confirmed that only calcium cations played an important role in the formation of the casein micelle.

Caseins are proteins comprising several polypeptides; the characteristic properties in aqueous media have been widely studied by various methods.[1—5] It is well known that the casein molecules form the micelle, the aggregation number of which is quite large when it is compared with those of surfactants in aqueous media. The micelle formation mechanism in aqueous solutions of surfactants seems to have been established kinetically by various chemical relaxation methods.[6—8] However, few investigations concerning the dynamic characters for the casein micelles have not been carried out,[9—14] although they are very important regarding the mechanism of protein conformational change. Most of the kinetic studies have been limited to those in the time range of more than few tens of seconds, their time courses seeming to be very complex. One of the attractive procedures used to analyze the aggregation reaction of casein involves an enzymatic model using a von Smoluchowski-type reaction.[12,14,15] However, the reaction mechanisms are still unconfirmed. We have speculated that the primary step in the formation of the casein micelles exists in a shorter time range, and might be observed in dilute solutions. For observing such process, a stopped-flow method involving the observation of the optical density change might be powerful owing to a large change in the optical density which is expected when casein micelles are formed. When stopped-flow equipment is used to follow reaction the process, it is necessary that the observed time courses (the time dependence of the optical density change) are precisely analyzed in order to clarify the reaction mechanism.[16]

Under these circumstances, the present study was undertaken in the hope that an elucidation of a simple time dependence for micelle formation could provide a fundamental understanding of any structural changes in the protein. This information is also important in relation to the dynamic stabilities and solution properties of various proteins, since the protein aggregation is well known to play an important role in both denaturation and renaturation.[17]

Experimental

Apparatus: We used a newly constructed stopped-flow equipment comprising a DC lamp (100 W), a mixer, a monochrometer (Shimadzu monochrometer Grating 1200), a photomultiplier, an offset electronic circuit, an operational DC amplifier, a wave memory (NF MODEL WM-841), a digital voltmeter (Advantest TR6845) and an 8-bit microcomputer (NEC 8800). The mixer was driven by pistons with compressed air; the dead time for the mixing was about 5 ms. The temperature of the mixer and the solution reservoirs was controlled by circulating the water maintained at a constant temperature. For each run, 0.252 cm^3 solutions were mixed; after more than five run drives the data were accumulated and averaged. The absorption spectra were recorded from 370 to 600 nm by Hitachi 323 Recording Spectrophotometer and JEOL Ubest-30 spectrometer.

Chemicals: Highly purified sodium casein was supplied from Technical Research Institute, Snow Brand Milk Products, Co., Ltd. and was used without further purification. The metal contents in the sodium casein samples were analyzed and found to be Na=1200 mg/100 g, Ca=4.3 mg/100 g, P =700 mg/100 g; all other metals were less than 1 mg/100 g. The purest grade calcium chloride, magnesium chloride, calcium bromide and tris(hydroxymethyl)aminomethane [Tris] were purchased from Wako Pure Chemicals. They were used without further purification. The solutions were prepared using a Tris–HCl buffer solvent from doubly distilled water which was degassed by ultrasound under reduced pressure. The desired pH values for the solutions were obtained by changing the ratio of the Tris and HCl concentrations. Most of the experiments were carried out at pH 7.20, unless stated otherwise.

All of the experiments were carried out at least within one day after preparing the casein solutions at 25 °C.

Results

The visible absorption spectra were recorded over the concentration range 0.01 to 0.05 wt% casein. A slight absorbance was observed in solutions of casein. This might mean that the solute molecules do not dissolve in their monomer forms in an aqueous media. When calcium chloride was added, the absorbance was found to increase remarkably at all wavelengths measured. The absorbance decreased monotonously with an increase of the wavelength. No characteristic absorption peak was observed over the range of the measured wavelength. Such a broad change of the absorbance may be due to solution turbidity caused by casein aggregates. Figure 1 shows the concentration dependence of the absorbance at 440 nm. The absorbance increased linearly with concentration, and it increased more steeply when $CaCl_2$ was added. It is important that the absorbance in solutions with $CaCl_2$ is much larger than those without $CaCl_2$.

Most of the stopped-flow experiments were carried out at 440 nm, since the sensitivity of our apparatus was relatively high at this wavelength. When casein solutions were mixed with a solvent buffer, or with the similar solution of casein, no optical density change was observed over a time range of more than 20.48 ms in full scale in the wave memory. Upon mixing with $CaCl_2$ solution, clear changes were observed over the appropriate time range for the apparatus.

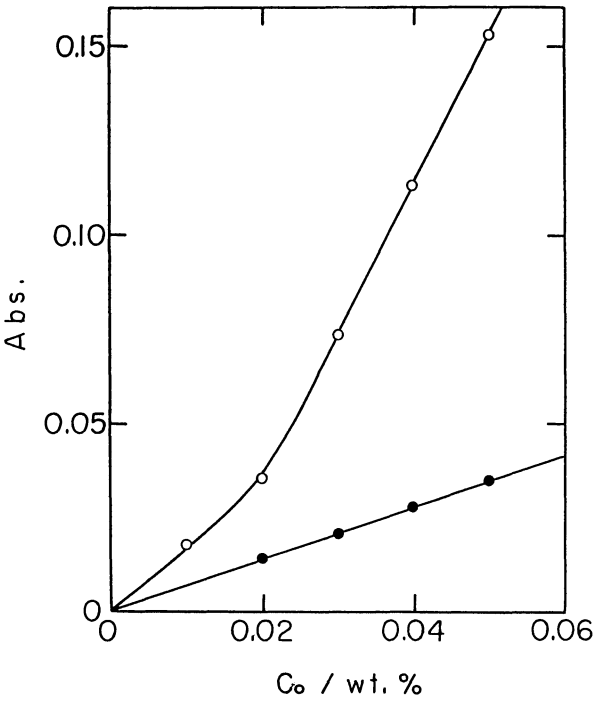

Fig. 1. Concentration dependence of absorbance for aqueous solutions of casein at 25 °C. ○: with 15 mmol dm^{-3} $CaCl_2$, ●: without $CaCl_2$.

The relation between the voltage of the signals from the photomultiplier and the product concentration, [P], is given by $\log\{(V_{100}-V_0)/(V_t-V_0)\}=\varepsilon l\,[P]$, where V_{100} is the signal voltage at the open current, V_0 that at the dark current, V_t the voltage at time t, l the effective observation cell length and ε a constant. We define $\varepsilon l\,[P_\infty]$ as the reaction amplitude where $[P_\infty]$ is the product concentration at infinite time of the reaction. The time dependence of the product is given as follows if it satisfies the first-order rate equation:

$$[P]=[P_\infty]\{1-\exp(-k_{app}t)\}, \quad (1)$$

where k_{app} is the apparent first-order rate constant. Using the relations between the observing voltage change in the signals and the concentration of the product at time t, Eq. 1 is expressed as

$$\log\{(V_t-V_0)/(V_\infty-V_0)\} = \log\{(V_{100}-V_0)/(V_\infty-V_0)\}\exp(-k_{app}t). \quad (2)$$

The voltage at infinite time, V_∞, is determined from the 2% increased values by averaging the last 10 digital signals in the wave memory and digital voltmeter. Plots of the logarithm of the left-hand side of Eq. 2 vs. time, (t), provide an apparent rate constant from the slope and the reaction amplitude from the intercept using a linear least mean-square method. The Guggenheim method can also be used to analyze the time dependence of the output signals, from which the apparent rate constant and reaction amplitude can also be obtained.[18] The appropriateness for these treatments was confirmed by numerical simulations for model calculations. The observed signals were analyzed by the two above-mentioned analytical procedures for the casein solutions in the concentration range from 0.02 to 0.05 wt% with various concentrations of $CaCl_2$. It was found that the time dependence of the optical density change is well fitted to Eq. 2. Figure 2 is one of the traces of the reactions when the casein solutions are mixed with that of $CaCl_2$. The rate constant and the reaction amplitude calculated from the above two procedures were the same. Also, the same apparent first-order rate constant, k_{app}, and the same reaction amplitude were obtained in an appropriate concentration range, even if a different sampling time in the wave memory was used. At concentrations less than 0.02 wt%, the optical density change was too small. On the other hand, upon increasing the casein concentration more than by 0.05 wt%, the reaction traces were not fitted to Eq. 2. This might indicate that more complex reactions proceeded at high concentration.

Figure 3 shows the casein concentration dependence of the apparent first-order rate constant at 10, 15, and 30 mmol dm^{-3} of $CaCl_2$. It can be seen that k_{app} at 10 and 15 mmol dm^{-3} $CaCl_2$ is apparently proportional to the casein concentration. However, at 30 mmol dm^{-3} it seems to be curved, and the rate constants obtained by the two procedures become different at high concentra-

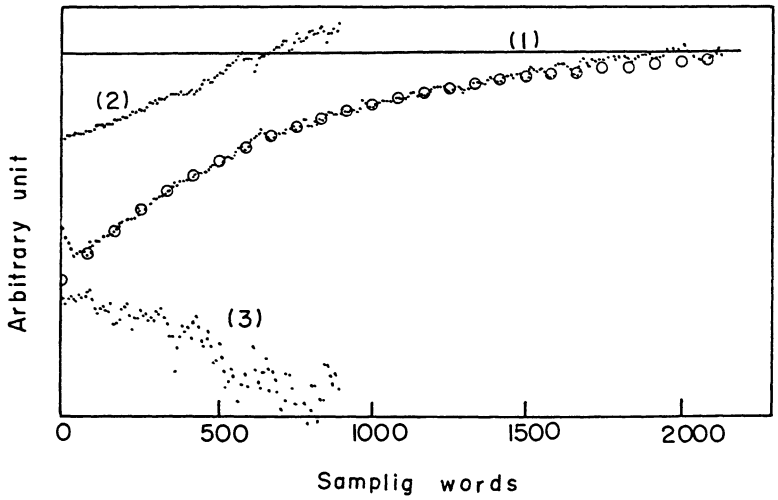

Fig. 2. Representative stopped-flow trace for an aqueous solution of casein with $CaCl_2$ (small dotted lines).
(1): Estimated infinite line.
(2): Plots of ln [log $\{(V_t-V_0)/(V_{100}-V_0)\}$] vs. time (**UA**),
(3): Guggenheim plots (**GA**).
The circles indicate the calculated values using the obtained rate parameters. In the following figure, we represent the results by the Guggenheim analytical procedure as **GA** and those by the plots of ln [log $\{(V_t-V_0)/(V_{100}-V_0)\}$] vs. t as **UA**.

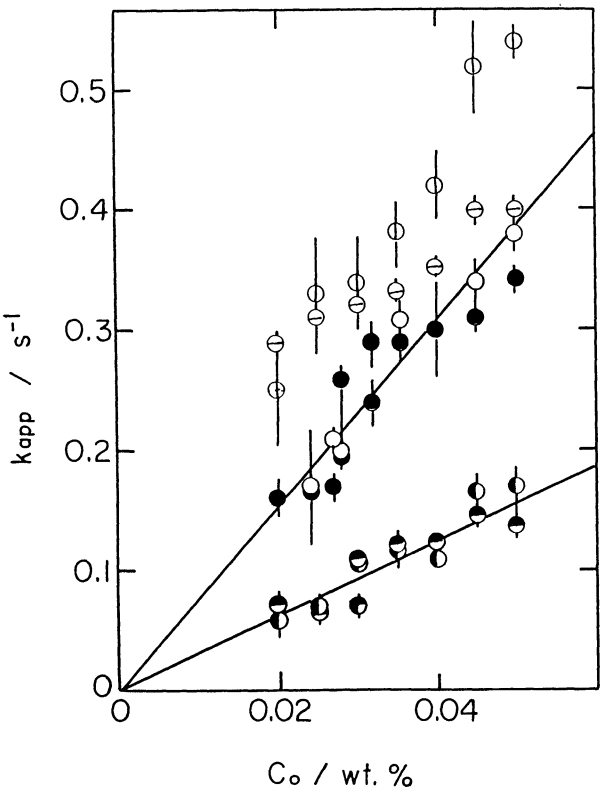

Fig. 3. Casein concentration dependence of the apparent first-order rate constant at 25 °C. ⊕: with 30 mmol dm^{-3} $CaCl_2$ by **GA**, ⊖: with 30 mmol dm^{-3} $CaCl_2$ by **UA**, ○: with 15 mmol dm^{-3} $CaCl_2$ by **GA**, ●: with 15 mmol dm^{-3} $CaCl_2$ by **UA**, ◐: with 10 mmol dm^{-3} $CaCl_2$ by **GA** and ◓: 10 mmol dm^{-3} $CaCl_2$ by **UA**.

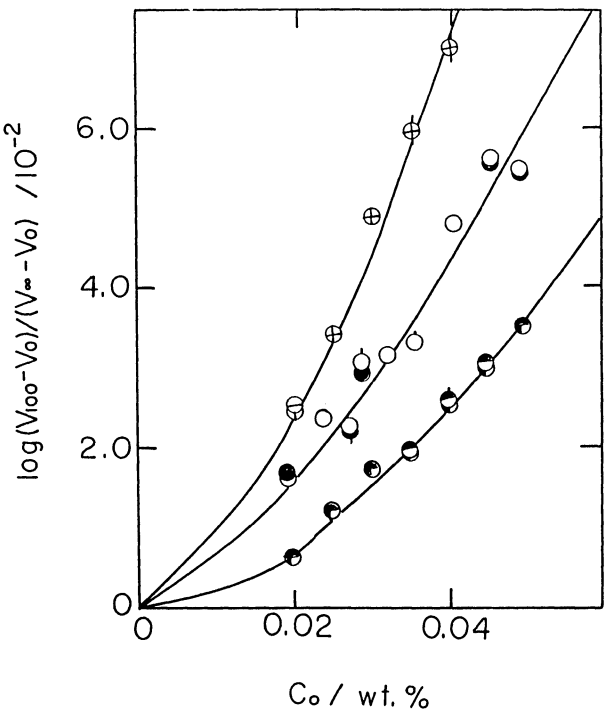

Fig. 4. Casein concentration dependence of the reaction amplitude at 25 °C. ⊕: with 30 mmol dm^{-3} $CaCl_2$ by **GA**, ⊖: with 30 mmol dm^{-3} $CaCl_2$ by **UA**, ○: with 15 mmol dm^{-3} $CaCl_2$ by **GA**, ●: with 15 mmol dm^{-3} $CaCl_2$ by **UA**, ◐: with 10 mmol dm^{-3} $CaCl_2$ by **GA** and ◓: with 10 mmol dm^{-3} $CaCl_2$ by **UA**.

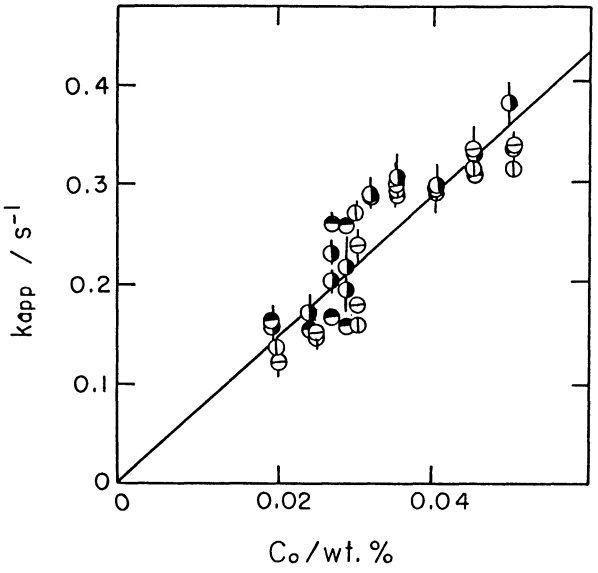

Fig. 5. Comparison of the effects of $CaCl_2$ and $CaBr_2$ on the first-order rate constant as a function of the casein concentration at 25 °C. The salt concentration was fixed at 15 mmol dm^{-3}. ◑: $CaCl_2$ solution by **GA**, ◓: $CaCl_2$ solution by **UA**, ⊖: $CaBr_2$ solution by **GA** and ⊕: $CaBr_2$ solution by **UA**.

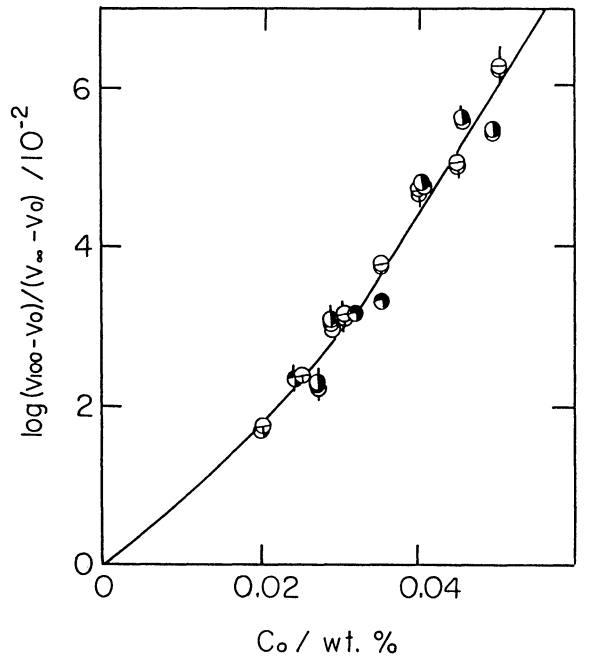

Fig. 6. Comparison of the effect of $CaCl_2$ and $CaBr_2$ on the reaction amplitude for casein solutions at 25 °C. ◑: $CaCl_2$ solution by **GA**, ◓: $CaCl_2$ solution by **UA**, ⊖: $CaBr_2$ solution by **GA** and ⊕: $CaBr_2$ solution by **UA**.

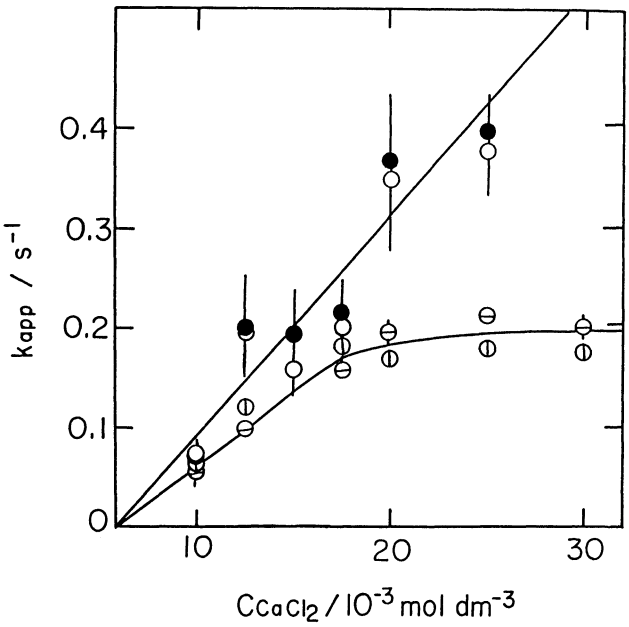

Fig. 7. Concentration dependence of $CaCl_2$ on the apparent first-order rate constant at 25 °C. ⊖: with 0.02 wt% casein by **GA**, ⊕: with 0.02 wt% casein by **UA**, ●: with 0.03 wt% casein by **GA** and ○: with 0.03 wt% casein by **UA**.

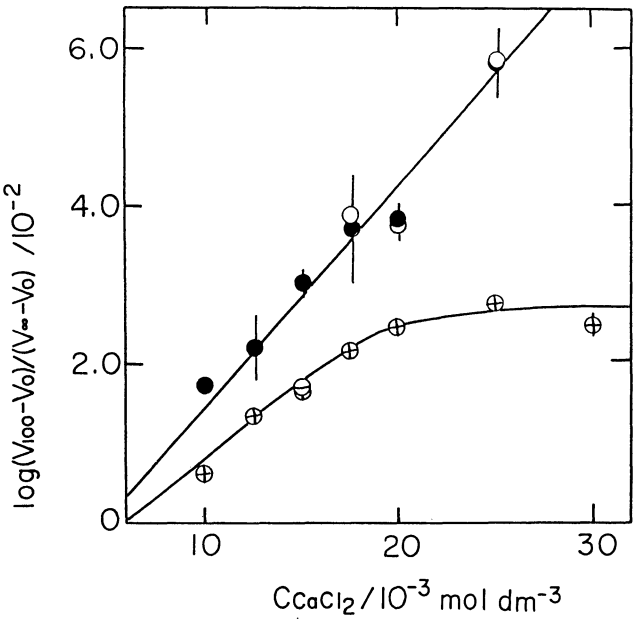

Fig. 8. Concentration dependence of $CaCl_2$ on the reaction amplitude at 25 °C. ⊖: with 0.02 wt% casein by **GA**, ⊕: with 0.02 wt% casein by **UA**, ●: with 0.03 wt% casein by **GA** and ○: with 0.03 wt% casein by **UA**.

tions of casein. Figure 4 represents the concentration dependence of the reaction amplitude. In order to observe the effect of an anion on the reaction, aqueous solutions of calcium bromide were mixed with casein solution. The results are shown in Figs. 5 and 6 together with those for solutions with $CaCl_2$. As can be seen in these figures, the dependences of the apparent first-order rate constant and the reaction amplitude on the casein concentration are very similar to those of $CaCl_2$. This means that only calcium cations play an

important role in changing the optical density.

The $CaCl_2$ concentration dependences of the apparent rate parameters are illustrated in Figs. 7 and 8. It can be seen that the apparent rate constant is also dependent on the $CaCl_2$ concentration and that the profiles depend considerably upon the casein concentration. In order to see the effects of other salts on this phenomenon, 50 mmol dm^{-3} sodium chloride and 30 mmol dm^{-3} magnesium chloride solutions were also mixed with the casein solutions. However, no optical density changes were observed. These results also indicate that the calcium cations bind specifically to the casein molecules and some conformational or structural changes of casein cause the optical density change for the casein solutions. The apparent rate constant and the reaction amplitude obtained at pH 7.2, 8.2, and 9.1 were almost independent of the solution pH.

Discussion

In kinetic experiments carried out using a stopped-flow apparatus, the method of distinguishing whether the reaction is first order or other is very important in order to consider precisely the reaction mechanisms in solutions. Only one analytical procedure of the reaction trace as a function of time might provide equivocal information. As shown above, in the present study the fact that the two analytical procedures of the time traces give the same rate constant and the same reaction amplitude shows that the observed time-dependent optical density change is due to a first-order rate process in the casein solutions, although the concentration range was restricted.

The effects of the calcium cation on the casein solution have been widely studied by various methods. It is said that the addition of the calcium ions to a casein solution promotes the formation of the casein micelle.[14,19] Not many kinetic studies, on the other hand, have been carried out regarding casein micelle formation. This may be because many complex reactions proceed quite rapidly in the concentrated solutions, even if the experiment is performed in solution with only a single polypeptide. Therefore, the time course of the reaction seems to be very complex.[9-12] It should be noteworthy that a single exponential decay phenomenon was observed in the low concentrations presently studied. It is certain that this phenomenon is associated with the casein aggregates, since the solution turbidity increases upon the addition of calcium cations. It is desired to clarify why such a simple time course is observed as in Fig. 2, and why the apparent rate constant and the reaction amplitude increase with the casein concentration, as can be seen in Figs. 3 and 4.

It has been considered that small aggregates or subunits comprising mainly α_s-, β- and κ-casein held together by hydrophobic interaction, exist in dilute solutions of casein.[4,12] Also, a sufficiently high local protein concentration is also speculated when aggregates of protein are formed.[17] As can be seen in Fig. 1, a small absorbance is observed in the solution of casein without adding $CaCl_2$. This result implies the existence of small aggregates. The absorbance increases dramatically when calcium cations are added. On the basis of the present dynamic and static experimental situations and the previous examinations conducted so far, the aggregation reaction of casein may generally proceed in stepwise processes as follows:

$$A_s + A \overset{k_1}{\rightarrow} A_{s+1} \overset{k_2}{\underset{A}{\rightarrow}} A_{s+2} \overset{k_3}{\underset{A}{\rightarrow}} \cdots\cdots \overset{k_n}{\underset{A}{\rightarrow}} A_n. \quad (3)$$

Here k_i is the rate constant for each process and A_s indicates the small aggregate which might act as a nucleus for larger aggregate formation. When the calcium cations bind specifically to free casein or small aggregate, the repulsive forces between them may be weakened, and then larger aggregates may be formed. The reaction associated with ion binding is so fast that it is far beyond the time stale of the stopped-flow method,[20] since the ion binding sites are expected to be either phosphate or carboxylic groups.[21,22] Also, it may be natural to consider that the aggregates actually consist of various molecules with different aggregation numbers, that is, they exist under a proper distribution.[4] However, we simply analyze the observed phenomenon by a conventional reaction kinetics, which may give us a legible feature of the reaction. It is very hard to decide definitely the rate determining step for such an aggregation reaction. We assume here that the rates of steps beyond the the second are so rapid that the stationary states are established. This assumption is widely used to interpret the aggregation process for relatively small molecules,[23] and may also be reasonably accepted, since such an aggregation process proceeds cooperatively once the reaction starts. Then, the reaction could be simplified as follows:

$$A_s + A \overset{k_f}{\underset{nA}{\rightarrow}} A_{s+n+1}, \quad (4)$$

where s is the mean aggregation number for small aggregate which may grow to larger aggregate, A_{s+n+1}. For the second step in Eq. 4, we define the equilibrium constant, K, as $K=[A_{s+n+1}]/[A_{s+1}][A]^n$, which arises from the stationary state assumption. The rate equation for formation of A_{s+1} is expressed by

$$d[A_{s+1}]/dt = k_f (A_{s0} - [A_{s+1}])(A_0 - [A_{s+1}]), \quad (5)$$

where A_{s0} and A_0 bare the initial concentrations of the reactants, A_s and A, respectively. The solution of the above equation gives the time course of the reactants as

$$[A] = A_0(A_0 - A_{s0})/\{A_0 - A_{s0}\exp -(A_0 - A_{s0})k_f t\}$$

and

$$[A_{s+1}] = [A_0 A_{s0}\{\exp(A_0 - A_{s0})k_f t - 1\}]/ [A_0\exp(A_0 - A_{s0})k_f t - A_{s0}]. \quad (6)$$

It is expected that the concentration of small aggregate, A_{s0}, is smaller than that of a free or monomer casein, A_0,

because the analytical concentration is not so high. Therfore, the condition $(A_0/A_{s0})\exp(A_0-A_{s0})k_f t \gg 1$ may be well satisfied. Using the stationary state assumption for the second step in Eq. 4, we obtain the time dependence of larger aggregate, as follows:

$$[A_{s+n+1}] = K(A_0-A_{s0})^n A_{s0}\{1-\exp -(A_0-A_{s0})k_f t\} = [\overline{A_{s+n+1}}]\{1-\exp -(A_0-A_{s0})k_f t\}, \quad (7)$$

where $[\overline{A_{s+n+1}}]$ is the concentration of the larger aggregate at an infinite time of the reaction. This equation indicates that the appearance of larger aggregates may be approximated by a single exponential factor and that the rate of the appearance of larger aggregates depends on the initial concentrations of the monomer and small aggregate casein molecules. It might be possible that the aggregate formation reaction by self-association of the small aggregates, such as $nA_s \rightarrow (A_s)_n$, causes the observed optical density change. However, the concentration dependences of the apparent first-order reaction and the reaction amplitude could not be well explained by such a reaction mechanism. The aggregation reaction associated with only free or monomer casein molecules was also considered to be the cause of the phenomenon, though the observed phenomena was not well interpreted.

The solution turbidity, τ, is related to the molecular weight of the aggregate, M_w, and the concentration, as[14]

$$\tau = QM_w[A_{s+n+1}], \quad (8)$$

where Q is a constant. Using Eqs.7 and 8, and comparing Eq. 2, the apparent rate constant, k_{app} observed by the stopped-flow method is found to be $(A_0-A_{s0})k_f$ and the reachtion amplitude, $\log\{(V_{100}-V_0)/(V_\infty-V_0)\}$, corresponds to $QM_w[A_{s+n+1}]$.

First, following the above relations, we consider the concentration dependence of the apparent rate constant, k_{app}. As can be seen in Fig. 3, plots of k_{app} vs. C_0 seems to indicate a linear increase with almost a zero intercept at 10 and 15 mmol dm^{-3} $CaCl_2$. These may mean that the concentration of small aggregates is much smaller than that of free casein molecules in a relatively dilute solution because $C_0=[A_0]+s[A_{s0}]$. From the slopes of these plots, we obtained rate constants of k_f=2.8 (wt% $s)^{-1}$ for a solution with 10 mmol dm^{-3} and k_f=7.6 (wt% $s)^{-1}$ for that with 15 mmol dm^{-3}. With increasing $CaCl_2$ concentration, the plots gave a slightly curved line and a positive intercept. Therefore, we reluctantly show the experimental results at 30 mmol dm^{-3} $CaCl_2$ without having determined the rate constant. Such an unexpected dependence might mean that an approximated analytical equation (Eq. 7) is not satisfied at higher concentrations of $CaCl_2$. Also, according to previous studies,[11,14] it is said that a von Smolucowski-type reaction proceeds for the formation of larger micelles; this process can be expressed as

$$[M_w] = [M_w(0)](1+k'C_0 t), \quad (9)$$

where $[M_w(0)]$ is the molecular weight at t=0, and k' the rate constant for the coagulation process. Then, the observed time dependence of the optical density change may deviate from single exponential decay at a relatively high concentration of $CaCl_2$. Actually, at concentrations of more than 0.05 wt% casein, the time courses of the stopped-flow could not also be fitted to the single exponential decay function. These results may indicate that more complex reactions start at around these conditions.

Small aggregates or submicelles are expected to be spongy protein particles with molecular weight, 250000.[12] It is said that they consist of 10 or 12 casein molecules. These estimations are quite reasonable if we take into account the constituting molecular structures of casein, namely α-, β- and κ-casein. The mean free casein concentration is roughly estimated from the analytical concentration. If we take the mean molecular weight of monomer casein to be 25000, the obtained rate constants, k_f, are converted to be 7×10^4 mol^{-1} dm^3 s^{-1} at 10 mmol dm^{-3} $CaCl_2$ and 1.9×10^5 mol^{-1} dm^3 s^{-1} at 15 mmol dm^{-3} $CaCl_2$. These rate constants are those for the first step for the aggregation in Eq. 3. The fact that the larger rate constant is obtained at higher concentrations of $CaCl_2$ may be considered as follows. The greater binding of Ca^{2+} ions to the casein molecule makes the repulsive interaction between the proteins decrease, thus facilitating the aggregation. This speculation is also supported by the $CaCl_2$ dependence of the rate parameters, which is considered later. The processes after the second one are considered to be very rapid since the stationary state approximation is well satisfied.

Next, the concentration dependence of the reaction amplitude is considered. As can be seen in Fig. 4, it increases monotonously with the concentration; such a dependence reflects an increase of the larger micelle concentration, $[A_{s+n+1}]$. Unfortunately, however, the quantitative analytical correlation between the absorbance measured by the static method (Fig. 1) and the amplitude of the reaction (Fig. 4) is not given at this stage because the observation cell of the stopped-flow apparatus consists of cylindrical quartz with a 2 mm diameter light pass length. The monotonous increase of the reaction amplitude is easily speculated from Eqs. 7 and 8. Qualitatively, both of the dependences observed by the static and dynamic methods are very similar eah other. This confirms that the observed time dependent phenomenon is associated with the formation of larger casein mielles induced by binding of the calcium cations.

The $CaCl_2$ concentration dependences on the apparent rate constant and the reaction amplitude seem to reflect the specificity of calcium ion binding to the casein. As can be seen in Figs. 7 and 8, they increase for lower concentrations of $CaCl_2$, but tend to reach their plateaus when the casein concentration is low. Such profiles of the rate parameters may indicate that the number of the binding sites in the casein molecule is

limited. With increasing the calcium concentration, the rate increases, which is seen in solutions with 0.03 wt% casein and in the dilute solution of $CaCl_2$ with 0.02 wt% casein. When most of the binding sites are occupied by calcium ions, the aggregation process may tend to reach a constant value.

In conclusion, it was found in the present study that there is a very rapid and simple process regarding the casein micelle formation in aqueous media during the early stage of the reaction process. The time dependence is described by a single exponential time constant, and can be well interpreted in terms of the aggregation reaction mechanism. In a concentrated solution of casein, further, more complex reactions are speculated to take place.

Further dynamical studies concerning the solutions of individual proteins in casein are desired for elucidating more details concerning molecular aggregation mechanism.

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