

Kinetics of Change in Casein Aggregation as a Result of Solvent-Induced Structural Change

Sadakatsu Nishikawa,* Hua Huang,# Chiho Matsumoto, Mizuho Katayama, and Brian H. Robinson†

Department of Chemistry and Applied Chemistry, Faculty of Science and Engineering, Saga University, Saga 840-8502

†School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

(Received July 30, 2001)

The stopped-flow method has been applied to a kinetic study concerning structural changes in casein aggregates in an aqueous medium. The aggregation process has been followed in aqueous solutions of α-casein, the results of which have revealed the same rate parameters as those reported previously in a mixed protein system (sodium casein). It has been found that the additions of urea and 1-propanol to aqueous solutions of sodium casein lead to a breakdown of the casein aggregates, which is monitored by the decrease in the absorbance (turbidity) of the sample. From the concentration dependence of the apparent first-order rate constant and the amplitude of the reaction, it is proposed that the rate-determining step is the departure of a single molecule (monomer) or a small aggregate from a large aggregate. The apparent first-order rate constant is 37 s^{-1} in the presence of 1-propanol and 0.26 s^{-1} for urea. Furthermore, in a mixing experiment with 1-propanol, an additional process associated with structural changes in the aggregate has been observed at longer times (monitored by an increase of absorbance), the apparent first-order rate constant being about 0.03 s^{-1}. It is inferred that an intermediate is observed between the process of the breakdown of the aggregate which is quite stable in the presence of $CaCl_2$, and that of the formation of another stable aggregate, which may include 1-propanol.

It is well known that casein molecules form aggregates when calcium ions are present in the solution.[1,2] These are quite large aggregates consisting of various protein molecules. The stability of casein aggregates may be attributed mainly to their net negative charge.[3] In a previous study,[4] we showed that the formation of a stable casein aggregate in the presence of calcium ion can be observed by the stopped-flow method, and that the reaction proceeds cooperatively once the aggregation reaction has been initiated. On the other hand, the casein aggregates are also affected by the presence of additives, e.g. alcohols.[5–7] One of the reasons for the stability of the aggregate structure in aqueous solution could be related to the solvent environment, and to the structural characteristics of water. We recently studied the dynamical properties of aqueous solutions containing alcohols by means of ultrasonic absorption, and found supporting evidence for the view that alcohols with relatively large hydrophobic groups promote water structure.[8,9] On the other hand, urea is apparently able to disrupt the long-range order for water; that is, it may act as a structure breaker for water.[10] The effects of alcohols and urea on protein structures have been reported by a number of investigators.[11–15] However, the dynamic characteristics of a casein aggregate induced by solvent structural changes are not yet well established.

For these situations, it is desirable to know, first, how the α-casein (the main component of protein in casein) contributes to the aggregation process and, second, how the casein aggregates are influenced by the addition of water structure promoters and breakers. For this purpose, stopped-flow kinetic experiments have been performed for α-casein in aqueous solution; 1-propanol and urea have been chosen as additives.

Experimental

Chemicals. The casein sample (sodium casein) for additive-effect studies was the same as that described elsewhere.[4] α-Casein was purchased from Wako Chemical Co., Ltd. and used as received. 1-Propanol was distilled once at normal pressure. Purest-grade urea, calcium chloride, and calcium bromide were purchased from Wako Chemical Co., Ltd., and used without further purification. All sample solutions were prepared with the pH controlled at 7.2 using tris-HCl buffer (Tris(hydroxymethyl)aminomethane: 0.05 mol dm^{-3} and hydrochloric acid: 0.045 mol dm^{-3}). Water was deionized and filtered by a MilliQ SP-TOC filter System from Japan Millipore Ltd.

α-Casein did not readily dissolve into the buffer. It was dissolved slowly and the solution was filtered. The exact concentrations of α-casein in solutions were then determined using a UV-vis spectrophotometer at 280 nm.[16]

Measurements. The stopped-flow apparatus was similar to that previously described.[4] Changes to the apparatus were an offset electric circuit with a lower noise amplifier from Kairiku Denpa Co. Ltd., a 16-bit computer (NEC 9800) and a digital voltmeter (Advantest TR6845). The electric signal from the photomultiplier was sent to the wave memory through the offset circuit; also, the real voltages for the open and dark current from the photomultiplier and the voltage at infinite time of the reaction were received from the voltmeter into the computer. Most of the stopped-flow measurements were followed at 440 nm, since our apparatus had a relatively high sensitivity at this wavelength. The static absorbance (turbidity) was measured by means of a JOEL Ubest-30 spectrophotometer. The measurement temperature was 25 °C.

Present address: Chugai Pharmaceutical Co. Ltd., Japan

Results

First, results concerning a α-casein solution are presented. There was no optical-density change observed when a solution of α-casein was mixed with the solvent buffer. However, when the solution was mixed with $CaCl_2$ or $CaBr_2$ solutions, a change in optical density was observed. In the concentration range below 0.04 wt% α-casein, the trace after mixing could be analyzed by an equation for the first-order reaction, as follows:

$$Abs - Abs_\infty = |Abs_0 - Abs_\infty| \exp(-k_{app}t), \quad (1)$$

where k_{app} is the apparent first-order rate constant, Abs is the absorbance at time t, and Abs_0 and Abs_∞ are values at time zero and infinity. Before mixing with a salt solution, no aggregate is considered to exist, and then Abs_0 is approximated to be zero. Equation 1 is expressed by the voltages from the photomultiplier and the voltmeter by

$$\log\{(V_t - V_0)/(V_\infty - V_0)\} = \log\{(V_{00} - V_0)/(V_\infty - V_0)\} \exp(-k_{app}t), \quad (1')$$

where V_t is the real voltage at time t, V_{00} and V_∞ are those at time zero and at time infinity, and V_0 is the dark-current voltage. By applying semilogarithmic plots of Eq. 1′ and by the Guggenheim analytical procedure, the apparent rate constant (k_{app}) and the parameter associated with the reaction amplitude, $\log\{(V_{00} - V_0)/(V_\infty - V_0)\}$, can be determined. The amplitude corresponds to the difference between the absorbance at infinite time of the reaction and that at time zero, i.e. $\delta Abs = |Abs_\infty - Abs_0|$. When the two analytical procedures give similar values for k_{app} and δAbs, it is considered that a first-order reaction process is observed by the stopped-flow apparatus. The apparent first-order rate constants obtained are shown in Fig. 1; the result with $CaBr_2$ exhibited the same trend. The results in a sodium casein solution are also shown in the same figure for a comparison. The amplitude of the reaction showed a similar concentration dependence. A similar mixing experiment for an α-casein solution was carried out at 10 $mmol^{-1}$ $CaCl_2$; the data show the same dependence as that for sodium casein. These results support that the phenomena observed in the stopped-flow time range at 440 nm can be associated mainly with the aggregation reaction of α-casein induced by the presence of calcium ions in the solution.

In order to see the effect of the solvent characteristics on the aggregation reaction of casein, the following experiments were carried out. One solution contained urea or 1-propanol with $CaCl_2$ and the other was a solution of $CaCl_2$. Both were degassed by sound under reduced pressure. Sodium casein was slowly dissolved in the $CaCl_2$ solution in order to prevent the formation of bubbles at the desired concentrations before mixing experiments in the stopped-flow apparatus. When aqueous solutions of urea or 1-propanol were mixed rapidly with a $CaCl_2$ solution, no output voltage change was observed. In contrast, a clear signal change with time was found when casein was present in the solution with $CaCl_2$. Therefore, the observed signal change is thought to be associated with casein.

I. Solution Characteristics with Urea. The dependence

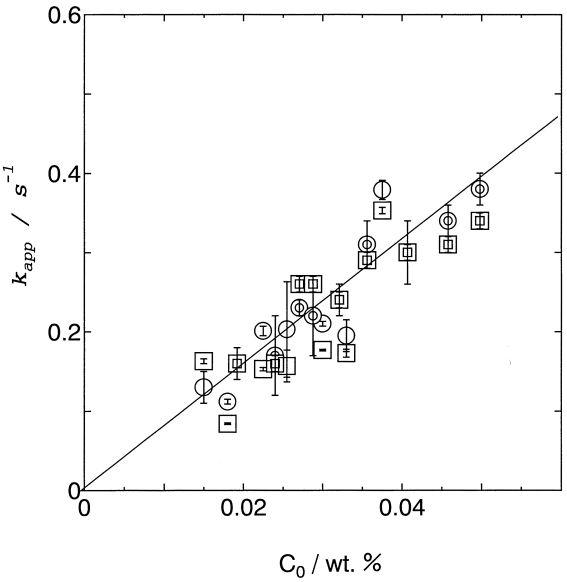


Fig. 1. α-Casein and sodium casein concentration dependence of the apparent first order rate constant, k_{app}, at 25 °C. ○: data for α-Casein solution with 15 mmol dm^{-3} $CaCl_2$ by Guggenheim method, □: by semilogarithmic method; ◎: data for sodium casein solution with 15 mmol dm^{-3} $CaCl_2$ by Guggenheim method, ▣: by semilogarithmic method.

of the absorbance on the casein concentration at various concentrations of urea was measured by a static spectrophotometer at 440 nm. In these solutions, the concentration of $CaCl_2$ was fixed at 10 mmol dm^{-3}, where casein aggregates are readily formed.[4] In Fig. 2, it can be seen that along with an increase in the urea concentration, the absorbance decreases. This would indicate that the casein aggregates, to some extent,

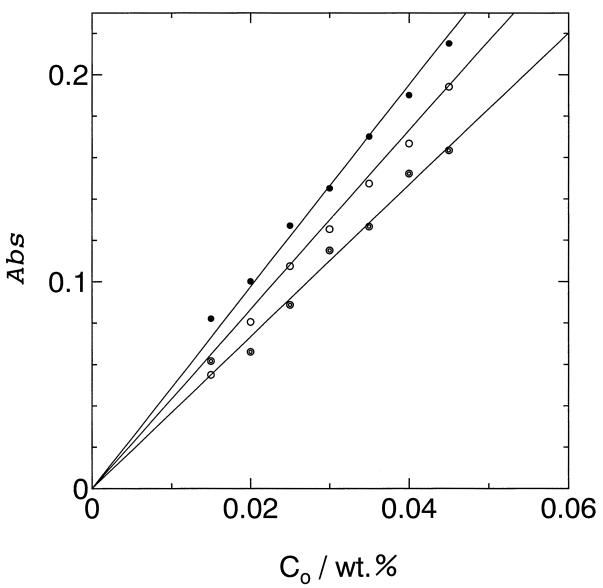


Fig. 2. Sodium casein concentration dependence of absorbance (440 nm) at various urea concentrations at 25 °C. ●: without urea, ○: 0.25 mol dm^{-3} urea, ◎: 0.50 mol dm^{-3} urea.

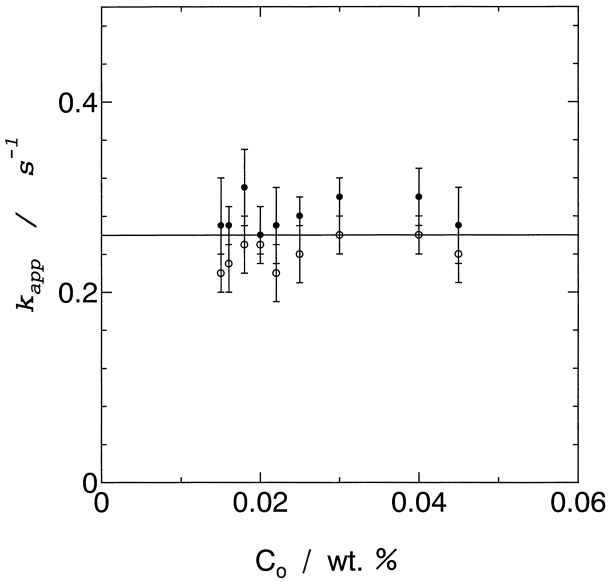


Fig. 3. Sodium casein concentration dependence of the k_{app} at 0.375 mol dm^{-3} urea at 25 °C. ○: calculated from semilogarithmic plots, ●: obtained from Guggenheim plots.

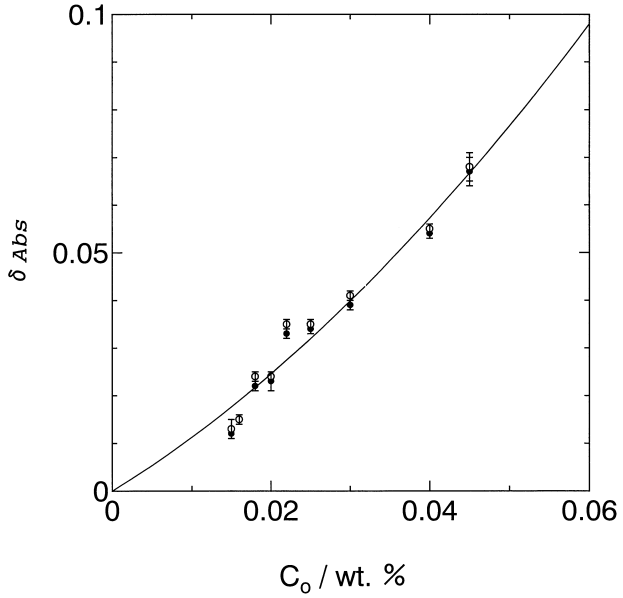


Fig. 4. Sodium casein concentration dependence of the amplitude of the reaction at 0.375 mol dm^{-3} urea at 25 °C. ○: calculated from semilogarithmic plots, ●: obtained from Guggenheim plots.

break down to smaller aggregates or monomers by addition of urea. It was previously shown that the absorbance increases through the formation of casein aggregates.[4] When a solution of urea is mixed rapidly with that of casein, a decrease in absorbance at 440 nm is observed, which is considered to correspond to a breakdown of the aggregate. The reaction trace was analyzed by Eq. 1′ and the apparent first-order rate constant (k_{app}) and the amplitude of reaction (δAbs) were determined. Figure 3 shows the casein concentration dependence of k_{app} for the breakdown of casein aggregates; the dependence of the amplitude of the reaction is shown in Fig. 4. The k_{app} values are independent of the casein concentration, whereas the amplitude of the reaction varies with the casein concentration. The urea concentration dependence of k_{app} and δAbs for the breakage process were also studied, and are shown in Figs. 5 and 6. The k_{app} value is independent of the urea concentration as well as the casein concentration. The δAbs value depends on both concentrations. The mean values (k_{app} and δAbs) obtained from the two analytical procedures mentioned above are indicated hereafter.

The reaction traces as a function of time for more concentrated solutions of casein and urea are found to be complex because they cannot be fitted to a single exponential function.

II. Solution Characteristics with 1-Propanol. The results are represented for casein aqueous solutions mixed with 1-propanol aqueous solutions. The concentration of $CaCl_2$ was fixed at 3.75 mmol dm^{-3} in this mixing experiment. Figure 7 indicates a representative stopped-flow trace. A rapid decrease in the absorbance was observed in a time range of less than 200 ms, which was followed by a gradual increase in the absorbance during a few tens of seconds. These two opposite time traces are readily distinguished. Figure 7-b indicates the trace for the shorter time course and Figure 7-c that for the longer one. Both of them are well expressed by a single exponential function of time. The faster process was analyzed by

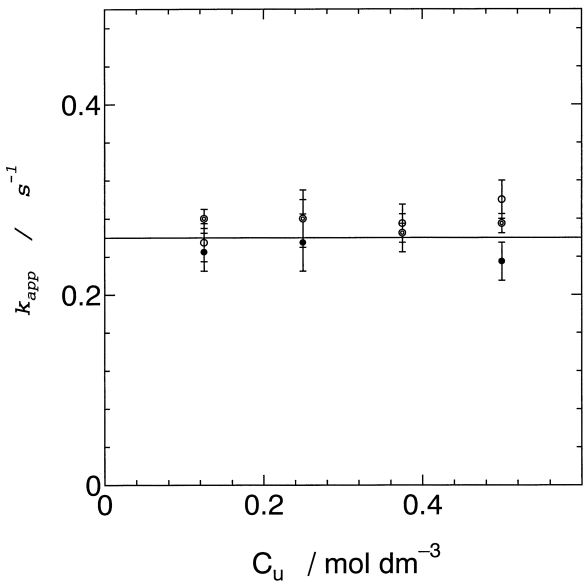


Fig. 5. Urea concentration dependence of the k_{app} at various casein concentrations. ●: 0.02 wt% casein, ○: 0.03 wt% casein, ◎: 0.04 wt% casein.

Eq. 1′ in the concentration range of casein from 0.012 to 0.032 wt%. The k_{app} values with 12.5 vol% of 1-propanol are shown in Fig. 8 as a function of the casein concentration. It is again clearly independent of the casein concentration, though the amplitude of the reaction is dependent on the casein concentration (Fig. 9). The dependence of the faster process on 1-propanol was also examined (Figs. 10 and 11). Along with an increase in the alcohol concentration, the apparent first-order rate constant increased slightly. There was also an increase in the amplitude of the reaction with the alcohol concentration.

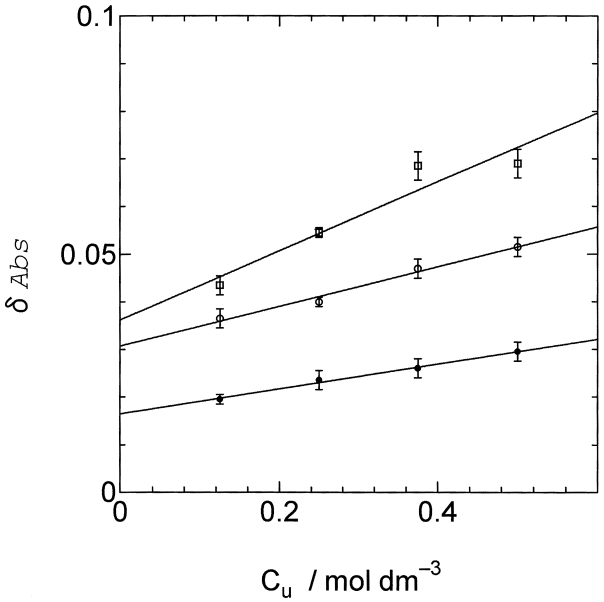


Fig. 6. Urea concentration dependence of amplitude of reaction at various casein concentrations. ●: 0.02 wt%, ○: 0.03 wt%, □: 0.04 wt%.

The apparent first-order rate constant for the slower process is independent of the casein concentration at about 0.03 s^{-1} (Fig. 12). However, the amplitude depends on the concentration (Fig. 13). This process may be associated with a further structural change in the casein aggregates.

Discussion

From the results of the α-casein solution, the aggregation process observed in the stopped-flow time range is mainly associated with α-casein induced by calcium ion, even in solutions of the mixed protein (sodium casein). Although the concentration range is restricted, the aggregation reaction proceeds cooperatively, as shown in a previous report.[4] Dalgeish et al. studied the aggregation dynamics of α-casein with calcium ions.[17] Their reaction was accompanied by precipitation with large aggregates, while the present kinetics was carried out under a homogeneous condition for restricted concentrations of α-casein and calcium ion.

An apparent first-order rate process was observed when sodium casein solutions containing aggregates were mixed with urea or 1-propanol. This is likely to be a reverse process when compared with that for the formation of aggregates, because the absorbance decreases after mixing. Looking at the concentration dependence of the apparent first-order rate constant and the amplitude of the reaction, the phenomena observed with urea may be the same as that observed in a shorter time range for additions of 1-propanol. The observed apparent first-order rate constant is independent of the casein concentration; also, the amplitude of the reaction increases with the casein concentration. However, the magnitudes of the rate constant and the amplitudes of the reaction for the additions of urea and 1-propanol are considerably different, although the concentration of

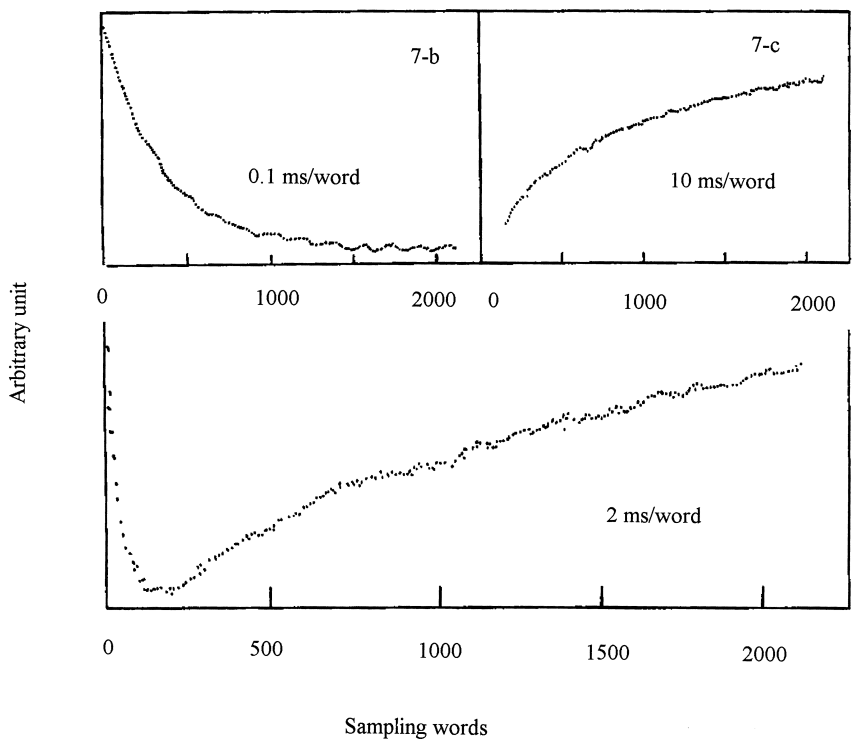


Fig. 7. Representative stopped-flow time trace at 0.025 wt% casein with 20 vol% 1-propanol at 25 °C at a wave length of 440 nm. The sampling time is 2 ms per word. Figure 7-b has been taken at the sampling time 0.1 ms per word and Fig. 7-c has been recorded at 10 ms per word. The negative increment with time corresponds to the decrease of the absorbance. The voltage is changed depending upon the magnitude of the amplitude of the reaction.

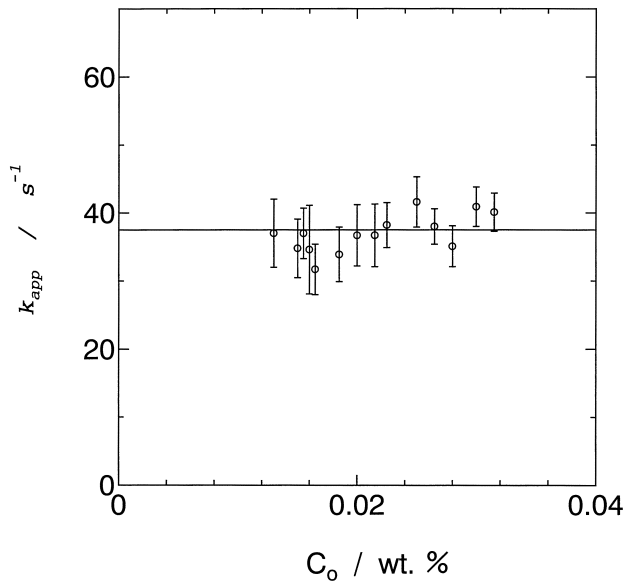


Fig. 8. Casein concentration dependence of k_{app} at 12.5 vol% 1-propanol at 25 °C.

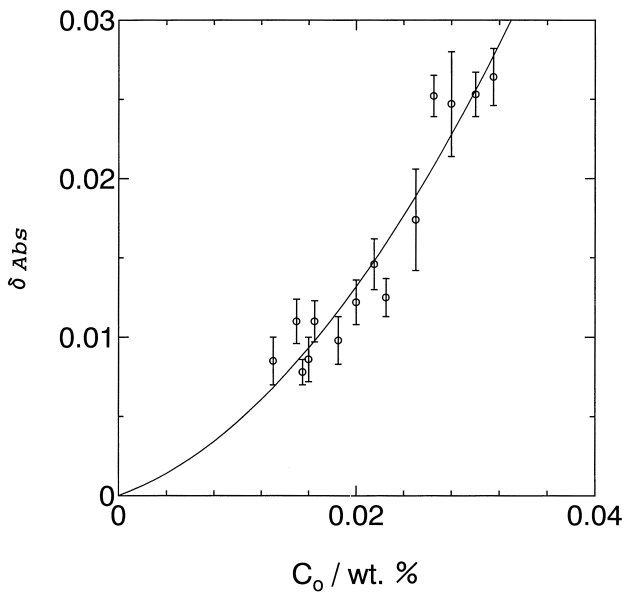


Fig. 9. Casein concentration dependence of the amplitude of the reaction at 12.5 vol% 1-propanol at 25 °C.

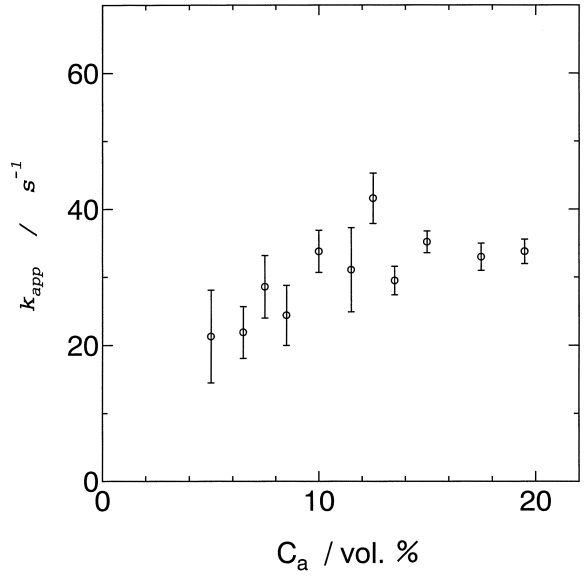


Fig. 10. Dependence of the apparent first order rate constant on 1-propanol concentration at 0.025 wt% casein at 25 °C.

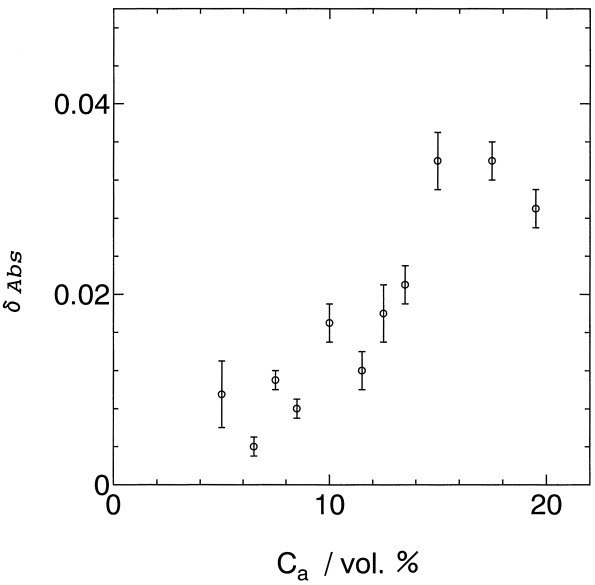


Fig. 11. 1-Propanol concentration dependence of the amplitude of reaction at 0.025 wt% casein at 25 °C.

urea is smaller than that of 1-propanol, as can be seen in Figs. 3, 4, 8 and 9.

Herskovitz et al.[14] have reported that proteins are denaturated by urea and alcohols, and that the denaturation process by alcohols is relatively rapid compared with urea, although the detailed time dependence has not been analyzed. The present study quantitatively supports their results. They have stressed that the hydrophobic ability of alcohols plays an important role in denaturation.

In a previous report,[4] we showed that the rate-determining step for the formation of the casein aggregates is the first step of the process expressed as

$$A_s + A \xrightarrow{k_f} A_{s+1} \rightarrow A_{s+2} \rightarrow \ldots A_{s+n+1}, \quad \begin{matrix} \nearrow & \nearrow \\ A & A \end{matrix} \qquad (2)$$

where A_s is a small aggregate of casein, A_{s+n+1} is a stable aggregate, and A is a monomer. Stable casein aggregates are formed quite rapidly once the first step of the above reaction has proceeded. If the observed process in the present experiment is the reverse one, the following reaction scheme may be presented:

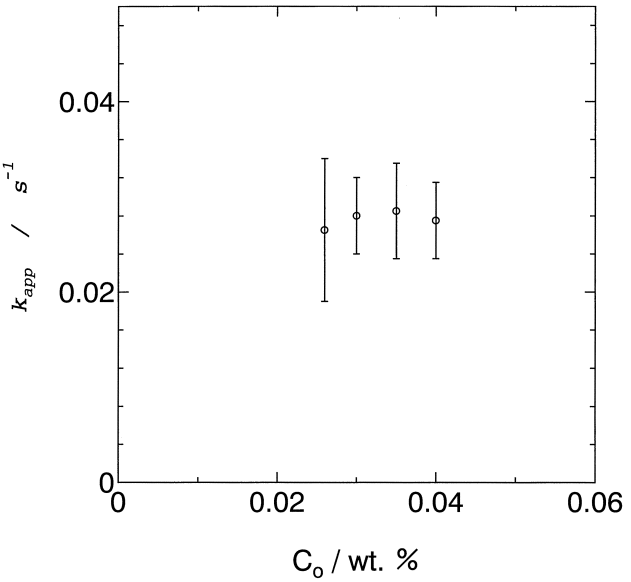


Fig. 12. Sodium casein concentration dependence of k_{app} for the formation process of the aggregate at 12.5 vol% 1-propanol at 25 °C.

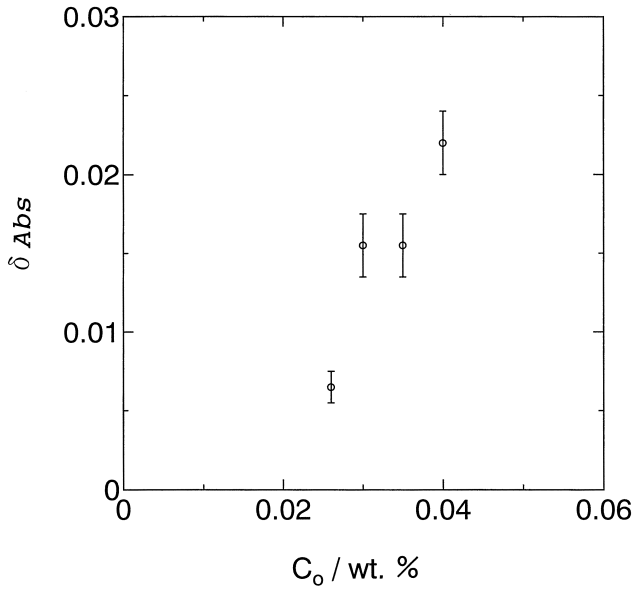


Fig. 13. Sodium casein concentration dependence of the amplitude of reaction for the formation process of the aggregate at 12.5 vol% 1-propanol at 25 °C.

$$\mathrm{A}_{s+n+1} \xrightarrow{k_b} \mathrm{A}_{s+n} \rightarrow \mathrm{A}_{s+n-1} \rightarrow \ldots \mathrm{A}_{s'}. \qquad \begin{matrix} \searrow & \searrow & \searrow \\ \mathrm{A} & \mathrm{A} & \mathrm{A} \end{matrix} \tag{3}$$

Again, we assume that the stable aggregates transform to smaller ones, $A_{s'}$, with the monomer in a stepwise manner, and that the rates of the steps beyond the first loss of the monomer are not the rate-determining. The small aggregate is not necessarily the same as that without additives. Thus, the rate equation gives

$$[\mathrm{A}_{s+n+1}] = ([\mathrm{A}_{s+n+1}]_0 - [\mathrm{A}_{s+n+1}]_\infty) \exp(-k_b t) + [\mathrm{A}_{s+n+1}]_\infty, \tag{4}$$

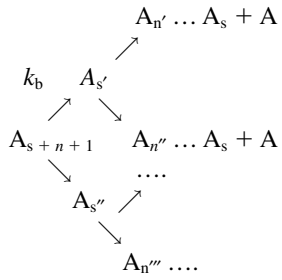
where $[\mathrm{A}_{s+n+1}]_0$ and $[\mathrm{A}_{s+n+1}]_\infty$ are the stable aggregate concentration at time zero and at time infinity, respectively. Before mixing, the casein aggregates may be regarded as being stable, and most of casein molecules participate in the formation of aggregates. It is seen that the time dependence of the concentration of casein aggregates may be expressed by a first-order reaction, and that the amplitude of the reaction increases with the casein concentration. This consideration suggests that casein aggregates disrupt cooperatively to smaller aggregates and monomers, when the first monomer departs from a stable aggregate. Hansen et al.[18] have analyzed neutron-scattering data, assuming that a casein aggregate consists of spherical submicelles. Thus, a small aggregate in the reaction under consideration may correspond to submicelles.

Urea is a water structure breaker, which denaturates many proteins.[10] On the other hand, 1-propanol is a promoter of water structure, and hydrogen-bond network may be further formed,[8] but it also denaturates proteins. The environments of proteins also influence their physical properties, especially the dynamic properties. Because solvent structural changes are much too fast to be observed in the stopped-flow time range,[8,9] the observed first-order rate process reflects structural changes in the casein aggregates. That is, the casein aggregates change into other forms only a following structural reorganization of the solvent.

The main difference of the casein aggregate disruption in urea and 1-propanol solutions is the magnitude of the first-order rate constants. In the solution with 1-propanol, it is 37 s^{-1}, while it is around 0.26 s^{-1} for the urea solution. Although the effect of additives on the water structure is very different, both additives disrupt the casein aggregates to smaller constituents of casein.

Urea as a denaturant for peptide is considered to bind to the exposed amide linkage.[19,20] Therefore, aggregates are disrupted from the surface of the aggregates when urea is added to the solution. This may reflect the slower rate of disruption of the aggregates.

On the other hand, it is said that the effect of alcohols as denaturants for polypeptides is due to a hydrophobic interaction between alcohol and peptides.[19] Alcohols with a relatively large hydrophobic group may then bind more easily to casein molecules and the stable aggregates would change into smaller ones. Another factor is that because 1-propanol has a relatively high hydrophobicity it may have an ability to penetrate into casein aggregates and thus disrupting them. This factor may make the disruption rate of the casein aggregates faster in 1-propanol than in urea. As can be seen in Fig. 10, the rate constant tends to increase with the 1-propanol concentration. This may reflect the effect of the penetration of alcohol into the casein aggregates. Then, a more plausible disruption mechanism of the larger aggregate through the penetration of 1-propanol may be a division into several small aggregates if the penetration process is faster than that of the disruption, as follows:



(5)

This reaction model may also give the same rate equation as Eq. 4 when the first step is the rate-determining step. Therefore, a stable aggregate disrupts easily compared with the case of urea, which reflects the greater rate constant.

Another different phenomenon observed in aqueous solutions with 1-propanol as compared with urea is the observation of a further slow process with an apparent rate constant of about 0.03 s^{-1} (Fig. 12). This is associated with an increase in the absorbance, and may correspond to the formation of a different aggregate of casein, possibly mixed aggregates of casein with 1-propanol. It is hard at this stage to interpret this process quantitatively because the observed concentration range is restricted and the amplitude of the reaction is small. The stable casein aggregates may be broken in a short time, and then another aggregate with a different structure may be created in a subsequent process. This phenomenon may also relate to the contrary effect of 1-propanol and urea on the water structure. Because such different phenomena are not observable by static experiments, the observation of "intermediates" of the casein aggregate shows the advantage of transient dynamic studies.

Further studies concerning the solvent structural effect on the aggregation reaction should be examined for a more precise understanding of the detailed reaction mechanism.

This work is partly supported by a Grant-in-Aid for Scientific Research (B) (No 11695054) from the Ministry of Education, Science, Sports and Culture.

References

1 D. F. Waugh, C. W. Slattery, and L. K. Creamer, *Biochemistry*, **10**, 817 (1971).
2 T. Wade, T. K. Beatie, W. N. Rowlands, and M. A. Augustin, *J. Dairy Res.*, **63**, 387 (1996), and references therein.
3 M. L. Green and G. Crutchfield, *J. Dairy Res.*, **38**, 151 (1971).
4 S. Nishikawa, M. Tatsusue, T. Inoue, and T. Yasunaga, *Bull. Chem. Soc. Jpn.*, **64**, 3515 (1991).
5 D. S. Horne and T. G. Parker, *J. Dairy Res.*, **50**, 425 (1983) and references therein.
6 M. Ahmed and E. Dickinson, *Colloids Surf.*, **47**, 353 (1990).
7 N. A. Bringe and J. E. Kinsella, *J. Dairy Res.*, **53**, 359 (1986).
8 S. Nishikawa, M. Mashima, and T. Yasunaga, *Bull. Chem. Soc., Jpn.*, **48**, 661 (1975).
9 S. Nishikawa and M. Ueda, *J. Phys. Chem.*, **97**, 3539 (1983).
10 H. F. Frank and F. Frank, *J. Chem. Phys.*, **48**, 4746 (1968).
11 M. E. Goldberg, R. Rudolph, and R. Jaenicke, *Biochemistry*, **30**, 2790 (1991).
12 H. B. Bull and K. Breese, *Biochemistry*, **17**, 2121 (1978).
13 S. Nilsson, L. Piculell, and M. Malmstm, *J. Phys. Chem.*, **94**, 5149 (1990).
14 T. T. Herskovitz, B. Gadegbeku, and H. Jaillet, *J. Biol. Chem.*, **245**, 588 (1970).
15 K. Murakami, *Bull. Chem. Soc. Jpn.*, **61**, 3043 (1988).
16 C. A. Zittle, *J. Dairy Sci.*, **44**, 2101 (1961).
17 D. G. Dalgleish, E. Paterson, and D. S. Horne, *Biophys. Chem.*, **13**, 307(1981).
18 S. Hansen, R. Bauer, S. B. Lomholt, K. B. Quist, T. S. Pedersen, and K. Mortemsen, *Eur. Biophys. J.*, **24**,143 (1996).
19 L. Peller, *J. Phys. Chem.*, **63**, 1199 (1959).
20 E. E. Schrier, R. T. Ingwall, and H. A. Scheraga, *J. Phys. Chem.*, **69**, 298 (1956).