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The Effect of Reducing Agent TCEP on Translational Diffusion and Supramolecular Assembly in Aqueous Solutions of α -Casein

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Abstract

The translational diffusion coefficient is highly sensitive to the size change of diffusing species and is ideally suited for the study of molecular association. Here, we used translational diffusion measurements by pulsed-field gradient Nuclear Magnetic Resonance (PFG NMR) technique to investigate the role of disulfide bonds in the formation of a supramolecular gel-like structure in the concentrated solution of α -casein. To reduce disulfide bonds, we added a commonly used reducing reagent tris(2-carboxyethyl)phosphine (TCEP) to α -casein solution. We found that the disruption of a disulfide bond Cys36-Cys40 in α_{s2} -casein does not alter the translational diffusion or secondary structure of α -casein in dilute, 1 and 3 % (wt. %) solution. On the contrary, in concentrated, 15 % (wt. %) α -casein solution, in addition to the disruption of disulfide bonds, TCEP induced significant changes in gel properties. New long-lived intermolecular interactions formed, leading to the irreversible gel formation. While a few side reactions of TCEP (as well as other reducing agents, e.g., DTT) have been reported, this area is still understudied. Here, we provide new data on side reaction of the reducing agent TCEP in concentrated protein solution, suggesting that at high protein concentrations TCEP should be used with caution.

Graphical Abstract

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INTRODUCTION

Caseins represent approximately 80% of milk protein. The primary function of caseins in milk is nutritional as they serve as a source of amino acids as well as of calcium and phosphorus¹. In addition to nutritional value, caseins can act as molecular chaperones under stress conditions^{2–3}. Furthermore, biologically active peptides, including opioid peptides as well as immunostimulating, antithrombic, bactericidal, and anti-inflammatory peptides, have been isolated from casein proteins⁴, and several in vitro and in vivo studies have shown that caseins can be used for efficient pharmaceutical drug delivery⁵.

Such diverse functions of caseins are directly related to physicochemical characteristics of their molecules. Casein proteins include α_{s1} and α_{s2} -caseins (collectively called α -casein), β -casein, and κ -casein. In bovine milk, α -casein comprises about 65% of casein fraction, in which α_{s1} and α_{s2} -caseins are present in a ratio of 4:1 (w/w). Although unrelated in amino acid sequence, molecules of α_{s1} , α_{s2} , β , and κ -caseins are amphipathic, containing polar and hydrophobic domains, and all caseins do not possess a well-defined tertiary structure⁶. In fact, caseins were among the first proteins described as unfolded phosphopolypeptides, in which the intrinsic disorder is linked to their function^{7–9}. For example, the flexibility of casein molecules makes them susceptible to proteolysis and posttranslational modification, e.g., phosphorylation of α_{s1} , α_{s2} , and β -caseins or glycosylation of κ -casein¹⁰. Once phosphorylated, caseins bind large amounts of calcium ions or calcium salt such as calcium phosphate.

Due to the amphipathic nature of their molecules, all caseins display a strong tendency to associate, though the degree and type of association differ between caseins. The association

of caseins is controlled by a fine balance of hydrogen bonding, hydrophobic interactions, and electrostatic repulsion that depend on temperature, pH, and ionic strength¹¹. In milk, caseins form large (~108 Da, 50–500 nm) and stable colloidal particles – casein micelles that are held together by protein-protein interactions and calcium phosphate bridges^{12–13}. While the structure of casein micelles have been extensively investigated, the association and the nature of intermolecular interaction of casein molecules within the micelle still remain under debate as several models of casein micelle have been proposed¹⁴. The difficulty of elucidating the exact structure of a casein micelle stems from the heterogeneity of size and micelle composition, its dynamic nature and dependence on environmental conditions, and from the tendency of purified caseins to self-associate and aggregate into diverse structures ranging from dimers to amyloid fibrils^{15–18}. Accordingly, studying the association and the nature of intermolecular interaction of casein molecules remains an important task.

NMR diffusion measurements provide a sensitive and direct method for studying the association in protein solutions because the translational diffusion coefficient is inversely proportional to the size of diffusing species and is highly sensitive to obstacles present in solution¹⁹. In our recent study, we used pulsed-field gradient NMR spectroscopy to investigate the translational diffusion in aqueous solutions of α -casein in a broad range of protein concentrations²⁰. We have found that if the concentration of protein increases above ~5% (wt. %), the translational diffusion of α -casein molecules demonstrates typical characteristics of the diffusion within the three-dimensional gel-like structure^{19, 21–24}. This gel-like structure is labile, and the lifetime of α -casein molecules in the gel is about 3.5 s. Furthermore, gel formation is reversible, and when the gel is dissolved, the translational diffusion coefficient of α -casein is the same as in the freshly prepared dilute solution at the corresponding α -casein concentration. Based on these results, we have proposed that the formation of gel-like structure in concentrated α -casein solutions results from noncovalent interactions between α -casein molecules (either hydrophobic or hydrogen bonds).

At the same time, α -casein contains α_{s1} and α_{s2} -caseins, of which α_{s2} -casein has two cysteine residues (Cys36 and Cys40) that can form intra or intermolecular disulfide bonds^{25–26}. There are several reducing agents that are typically used to break the disulfide bonds. Tris(2-carboxyethyl)phosphine (TCEP) is frequently chosen over dithiothreitol (DTT) because it is odorless and nonvolatile, and, unlike DTT, is resistant to air oxidation and remains active at acidic pH or at pH above 7.5^{27–28}. It is generally accepted that besides the disruption of disulfide bonds, the addition of TCEP to protein solution does not cause any changes. In this study, we used TCEP to break disulfide bonds in α_{s2} -casein. The goal of the present study is to investigate the effect of TCEP on the translational diffusion of α -casein in dilute and concentrated solutions and to reveal whether the disulfide bonds play any role in the formation of a supramolecular gel-like structure in the concentrated solution of α -casein. We expected that in the dilute solution, the disruption of a disulfide bond by TCEP might lead to a conformational change in α_{s2} -casein and, consequently, to a change of the hydrodynamic radius of its molecule, which, in turn, lead to a change of its translational diffusion coefficient. In the concentrated solution, we expected no change in the translational diffusion of α -casein upon the addition of TCEP. This was because previously observed formation of a gel-like structure in the concentrated solution of α -casein was reversible²⁰,

suggesting that disulfide bonds were not involved in its formation, and the disruption of a disulfide bond would change only local electrostatics with minimal effects on the intermolecular interactions between α -casein molecules. Unexpectedly, we discovered a side reaction of TCEP, leading to the formation of the irreversible gel, which can only result from the formation of stable long-lived intermolecular bonds. Overall, our results show that in the concentrated solution of α -casein, the addition of TCEP triggers additional chemical processes that alter the mechanism of α -casein self-association.

EXPERIMENTAL METHODS

Materials

Bovine α -casein (C6780, 70 % purity) was purchased from Sigma-Aldrich and used without further purification. As we have shown previously²⁰, impurities (if present) or α_{s1} - and α_{s2} -caseins are indistinguishable as far as their translational diffusion coefficient is concerned. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl, 98 % purity) and *N*-(1-Pyrenyl)maleimide were also purchased from Sigma-Aldrich (75259 and P28, respectively). Alexa Fluor 532 (A-10255) was purchased from ThermoFisher Scientific.

To confirm the presence of disulfide bonds and their disruption by TCEP, α -casein was covalently labeled with *N*-(1-Pyrenyl)maleimide or with Alexa Fluor 532 at 10:1 label: α -casein molar ratio for 1.5 hrs at room temperature. The excess label was removed using Amicon spin concentrators (MilliporeSigma, Burlington MA). The absorption of labeled protein was recorded with Evolution 60S UV-Vis spectrophotometer (ThermoFisher Scientific, Waltham, MA). The fluorescence of labeled protein was recorded with PTI QuantaMaster fluorimeter (Horiba Scientific, Piscataway, NJ).

For NMR measurements, samples were prepared at protein concentrations 1, 3, and 15 % (wt. %) by dissolving the lyophilized powder of α -casein in D₂O in order to minimize the signal from water protons. Before adding TCEP to protein solution, it was dissolved in D₂O, and the solution pH was measured using the pH-meter SevenEasy S20 (Mettler-Toledo AG, Switzerland) and adjusted accordingly to 6.85 by adding microliter quantities of NaOD.

For CD spectroscopy, samples were prepared using H₂O at protein concentrations of 7 % for far-UV and 0.15 % for near-UV experiments.

The molar ratio of α -casein to TCEP was maintained at 1:10 at each concentration of α -casein used in this study.

NMR spectroscopy

All NMR measurements were performed at 298 K on a 400 MHz Bruker Avance-III TM spectrometer equipped with gradient system that allowed a maximum gradient, g , of 28 T/m (e.g., 2800 G/cm). Temperature was calibrated using a set of test samples with known diffusion coefficients²⁹. Self-diffusion coefficients (hereinafter referred to simply as diffusion coefficients) were measured using the stimulated-echo pulse sequence (PGSTE)³⁰. ¹H experiments were performed using 48 different values of g , the gradient pulse duration δ of 2 and 5 ms, the time between the leading edges of gradient pulses Δ = 50 and 800 ms, the

time interval between the first and the second radiofrequency pulses τ ranging from 6 to 10 ms, and the recycle delay of 4400 ms. ^{31}P experiments at the resonance frequency of 162 MHz were performed using 25 different values of g , the gradient pulse duration $\delta = 1$ ms, the time between the leading edges of gradient pulses $\Delta = 25$ and 100 ms, the time interval between the first and the second radiofrequency pulses $\tau = 6$ ms, and the recycle delay of 3000 ms. ^{31}P spectra were referenced to 85 % orthophosphoric acid H_3PO_4 .

The analysis of NMR diffusion data was performed as described previously²⁰. Monoexponential diffusion attenuation of the spin echo amplitude, $A(g^2)$, was described by the following equation with a single diffusion coefficient:

$$\frac{A(g^2)}{A(0)} = \exp(-\gamma^2 \delta^2 g^2 t_d D), \quad (1)$$

where $A(0)$ is the spin echo amplitude at $g = 0$, γ is the gyromagnetic ratio for protons, and $t_d = \Delta - \delta/3$ is the diffusion time. In this case, the diffusion coefficient was determined from the linear slope of the attenuation, plotted using a semi-logarithmic scale, according to Eq. 1. Multi-exponential diffusion attenuation was described by the spectrum of diffusion coefficients:

$$\frac{A(g^2)}{A(0)} = \sum_i p_i \exp(-\gamma^2 \delta^2 g^2 t_d D_i). \quad (2)$$

Here p_i is the relaxation weighted fraction of the component with the diffusion coefficient D_i . All relevant details are discussed in the Results section.

CD spectroscopy

CD measurements were performed using a Jasco-1500 spectropolarimeter at 298 K. Far-UV CD spectra were recorded for the 7 % α -casein sample in the absence or presence of 10 molar excess of TCEP in the range of 190–260 nm using a quartz cuvette with a path length of 0.01 mm. Near-UV spectra were recorded for the 0.15 % α -casein sample in the absence or presence of 10 molar excess of TCEP in the range of 250–330 nm using a quartz cuvette with a path length of 10 mm. The choice of protein concentrations was limited by allowable HT values and was made to have a representative dilute and concentrated protein solution. The spectra were recorded using 50 nm/min scanning rate, 0.1 nm spectral resolution, 4.0 s response, and 1.0 nm bandwidth. Reported spectra are averages of 3–5 scans and are expressed as mean residue molar ellipticity (MRE), $[\theta]$, calculated by using the relation:

$$[\theta] = \frac{M_0 \cdot \theta_\lambda}{100 \cdot C \cdot l}, \quad (3)$$

where M_0 is the mean residue molar mass, θ_λ is the measured ellipticity in degrees, and C is the protein concentration, and l is the path length.

RESULTS

TCEP disrupts disulfide bond in α_{s2} -casein

α -casein contains α_{s1} and α_{s2} -caseins at approximately 4:1 molar ratio. Only α_{s2} -casein has cysteine residues (Cys36 and Cys40), and these cysteine residues can form disulfide bonds^{25–26}. To verify the presence of disulfide bonds and confirm that TCEP disrupts them, we covalently labeled cysteine residues using thiol-reactive pyrene and Alexa-532 maleimides in the absence or presence of 10 molar excess TCEP and compared the intensity of absorbance and fluorescence spectra of labeled α -casein. Figure 1 summarizes these data. Both the absorbance and fluorescence spectra show markedly increased intensity when labeling was performed in the presence of TCEP, unambiguously demonstrating that TCEP disrupts disulfide bonds making cysteine residues available for labeling.

The effect of TCEP on the translational diffusion of α -casein in dilute solutions

The effect of TCEP on the translational diffusion of α -casein in dilute solutions was investigated at protein concentrations of 1 and 3 % (wt. %). The molar ratio of TCEP to α -casein was kept constant at 10:1, sufficient to reduce disulfide bonds in less than 10 minutes. Figure 2 shows ^1H NMR spectra collected for 0.1 M stock TCEP solution (top panel) and for the 3 % α -casein solution in the absence (middle panel) or presence (bottom panel) of TCEP. In the ^1H TCEP NMR spectrum, signals originating from residual water and two methylene protons are well separated, enabling the unambiguous measurement of the diffusion coefficient of TCEP. The diffusion attenuation of TCEP spin-echo signal intensities is shown in Figure 3A as a function of $k \cdot t_d = (\gamma \delta g)^2 \cdot t_d$. It has a monoexponential form over the three orders of magnitude. As expected based on its size, the diffusion coefficient of TCEP determined from the slope of diffusion attenuation is about an order of magnitude less than the diffusion coefficient of water molecules measured at the same temperature³¹ ($D_{\text{water}} = 2.70 \pm 0.10 \times 10^{-9} \text{ m}^2/\text{s}$) and equals $D = 4.20 \pm 0.16 \times 10^{-10} \text{ m}^2/\text{s}$ (Table 1).

When TCEP is added to the 3% α -casein solution, proton signals originating from protein (0.16 to 3.61 ppm) and TCEP (2.0 to 3.5 ppm) partially overlap. Therefore, to minimize the contribution from TCEP, the diffusion coefficient of α -casein was calculated using the integrated area of the peak between 0.16 and 2.0 ppm. Figure 3B shows two diffusion attenuations for the 3 % α -casein solution. Curve 1 is plotted using spin-echo signal intensities determined in the region from 0.16 to 2.0 ppm in the presence of 10 molar TCEP excess, and curve 2 corresponds to protein spin-echo signal intensities between 0.16 to 3.61 ppm recorded in pure α -casein solution. Both diffusion attenuations are monoexponential and coincide in the whole measurement range. The diffusion coefficient of α -casein, determined from the slope of diffusion attenuations, equals to $2.97 \pm 0.14 \times 10^{-11} \text{ m}^2/\text{s}$ (Table 1), which is in agreement with our previous data²⁰. Similarly, α -casein diffusion coefficients were identical in the 1 % α -casein solution in the presence or absence of 10 molar TCEP excess (data not shown). In the 1 % α -casein solution, the diffusion coefficient of α -casein was found to be equal to $8.56 \pm 0.40 \times 10^{-11} \text{ m}^2/\text{s}$. Collectively, these data show that the addition of TCEP to dilute solutions of α -casein (1 – 3 %) does not affect the translational diffusion of α -casein molecules, and that the disruption of disulfide bonds by

TCEP does not lead to conformational changes sufficient enough to change the translational diffusion of α -casein.

The effect of TCEP on the translational diffusion of α -casein in concentrated (15 %) solution

NMR diffusion measurements are particularly useful for studying concentrated protein solutions, where the application of optically-based methods is limited. Here, we took the advantage of our hardware capability to measure diffusion coefficients as low as $\sim 10^{-17}$ m²/s and applied the advanced methodology of diffusion data analysis developed previously for synthetic polymers^{23–24, 32} to investigate the effect of TCEP on the translational diffusion of α -casein in the concentrated (15 % or ~ 6 mM) α -casein solution where the translational diffusion is significantly slowed down. As with dilute solutions, we kept the molar ratio of TCEP to protein at 10:1. Previously, we observed a qualitatively and quantitatively different translational diffusion of α -casein in dilute and concentrated solutions²⁰. In dilute solutions, α -casein molecules remained compact, hydrodynamically comparable to rigid Brownian particles, whereas at the protein concentrations greater than 5 %, the molecules of α -casein reversibly self-assemble to form a labile three-dimensional gel-like network²⁰. At the protein concentration of 15 % used here, the gel-like network of α -casein molecules is readily formed.

Proton spectra of the 15% α -casein solution in the absence (spectrum 1) or presence (spectrum 2) of TCEP are shown in Figure 4A. In contrast to the 3 % α -casein solution, a strong overlap of protein and TCEP signals is observed, not permitting the separation of contributions from α -casein and TCEP to the diffusion attenuation. Accordingly, the diffusion attenuation recorded for the 15% α -casein solution determined using the integrated area of the peak between 0.16 and 2.0 ppm contains two contributions, from α -casein and TCEP (Figure 4B). As expected, it has a non-monoexponential form due to the difference in diffusion coefficients of α -casein and TCEP. Surprisingly, the detailed analysis revealed that instead of two components (due to α -casein and TCEP) the observed diffusion attenuation has a three-exponential form, described by the following equation:

$$\frac{A(g^2)}{A(0)} = p_1 \exp(-\gamma^2 g^2 \delta^2 t_d D_1) + p_2 \exp(-\gamma^2 g^2 \delta^2 t_d D_2) + p_3 \exp(-\gamma^2 g^2 \delta^2 t_d D_3), \quad (4)$$

where D_1 is the diffusion coefficient of slowly diffusing molecules, D_2 is the intermediate diffusion coefficient, D_3 is the diffusion coefficient of fast diffusing molecules, and p_1 , p_2 , and p_3 are relative populations of respective diffusion attenuation components. The solid line represents the best data fit curve calculated using the following values of diffusion coefficients and populations: $D_1 = 3.40 \pm 0.17 \times 10^{-15}$ m²/s and $p_1 = 0.014$, $D_2 = 3.00 \pm 0.14 \times 10^{-13}$ m²/s and $p_2 = 0.001$, $D_3 = 3.80 \pm 0.15 \times 10^{-10}$ m²/s and $p_3 = 0.985$ (Table 1). The insert in Figure 4B shows the diffusion attenuation after subtracting the component with diffusion coefficient D_3 .

In order to establish the correspondence of three diffusion attenuation components to molecular species present in solution, we analyzed the dependence of the shape of proton

spectra on the magnitude of pulsed-field gradient g . Figure 5 shows that when g increases, the proton signal between 2.0 and 3.5 ppm rapidly decreases. However, the proton signal between 0.16 and ~2.6 ppm remains visible even at the highest value of g . This signal is similar to the signal from α -casein protons shown in Figure 2, except that it is significantly broadened. Accordingly, the components of the diffusion attenuation characterized by smallest and largest diffusion coefficients D_1 and D_3 correspond to the molecules of α -casein and TCEP, respectively. We note that both diffusion coefficients are smaller than those observed in the 3 % α -casein solution, as expected due to concentration (viscosity) effects. We also note that the diffusion coefficient of α -casein is smaller than in the pure 15 % solution of α -casein ($4.10 \pm 0.21 \times 10^{-15} \text{ m}^2/\text{s}$ at $t_d = 100 \text{ ms}$) previously observed²⁰.

At this point, it remains unclear which species in solution are characterized by the diffusion coefficient D_2 . Indeed, the value of D_2 is greater than the diffusion coefficient of α -casein ($3.40 \pm 0.17 \times 10^{-15} \text{ m}^2/\text{s}$) in the solution with protein concentration 15 %, but smaller than in the 3 % α -casein solution ($2.97 \pm 0.14 \times 10^{-11} \text{ m}^2/\text{s}$). Thus, one can assume that some α -casein molecules do not join gel-like structures, and that the coefficient D_2 corresponds to these α -casein molecules. Alternatively, one cannot exclude that D_2 corresponds to TCEP molecules, the translational diffusion of which slowed down due to the interaction with protein molecules. To resolve this conundrum unambiguously, we used ^{31}P -diffusion measurements.

Figure 6 shows a ^{31}P spectrum acquired in the 15 % aqueous solution of α -casein mixed with TCEP at 1:10 molar ratio (spectrum 1). The spectrum displays three well separated resonances, indicating the possibility of measuring the TCEP and α -casein diffusion coefficients independently. For identifying resonances, we acquired ^{31}P spectra for aqueous solutions of α -casein (spectrum 2) and TCEP (spectrum 3) alone. The ^{31}P spectrum of α -casein (spectrum 2) displays a single resonance centered at about 2–3 ppm, which is due to the high content of phosphorylated serine residues located in specific phosphorylation site motifs^{13, 33–34}. Resonances in the ^{31}P spectrum of TCEP (spectrum 3) at about 15–18 ppm and 58–60 ppm belong to TCEP and its P-oxide TCEPO, respectively^{35–36}. Figure 7 shows the diffusion attenuation of spin-echo amplitude for the peak between 15 and 18 ppm in the 15 % aqueous solution of α -casein acquired at different diffusion times. The diffusion attenuation is monoexponential over the three orders of magnitude and no dependence on diffusion time is observed, indicating that the diffusion of TCEP molecules in the 15 % α -casein solution is unrestricted. A similar result was obtained for the peak between 58 and 62 ppm (data not shown). The diffusion coefficient of TCEP calculated from the slope of the diffusion attenuation is equal to $3.80 \pm 0.15 \times 10^{-10} \text{ m}^2/\text{s}$. As expected, due to concentration effects, the diffusion coefficients of TCEP ($3.80 \pm 0.17 \times 10^{-10} \text{ m}^2/\text{s}$) and water ($2.30 \pm 0.09 \times 10^{-9} \text{ m}^2/\text{s}$) are similarly reduced as compared to the dilute solution of α -casein ($4.20 \pm 0.16 \times 10^{-10} \text{ m}^2/\text{s}$ and $2.70 \pm 0.11 \times 10^{-9} \text{ m}^2/\text{s}$, respectively). Thus, the TCEP species in the 15 % solution of α -casein is characterized by a single diffusion coefficient, and the interactions of TCEP with α -casein do not affect its translational diffusion, likely due to fast exchange with TCEP molecules of bulk solution. Accordingly, the diffusion coefficient D_2 can only correspond to α -casein molecules. Due to hardware restriction on the maximum value of pulsed-field gradient g , ^{31}P -diffusion measurements of extremely small diffusion coefficients such as the diffusion coefficients of α -casein in concentrated solutions are not

possible. However, the analysis of the shape of α -casein diffusion attenuation is still possible using ^1H diffusion data after subtracting the TCEP component from the diffusion attenuation acquired for the 15% α -casein solution (Figure 4B, insert), leaving only the contribution of α -casein molecules. The solid line represents the best fit line of the experimental curve with two exponents using $D_1 = 3.40 \pm 0.17 \times 10^{-15} \text{ m}^2/\text{s}$, $D_2 = 3.00 \pm 0.14 \times 10^{-13} \text{ m}^2/\text{s}$,

$p'_1 = 0.93$, $p'_2 = 0.07$. Populations $p'_1 = \frac{p_1}{p_1 + p_2}$ and $p'_2 = \frac{p_2}{p_1 + p_2}$ are renormalized to the

content of α -casein molecules only, using p_1 and p_2 values determined using Eq. 4.

Together, diffusion data for the 15% concentrated solution of α -casein presented here and in our previous work²⁰ show that based on their translational mobility α -casein molecules are divided into two species. In the presence of TCEP, about 7% of α -casein molecules are highly mobile, and their diffusion coefficient is approximately equal to $3.10 \pm 0.14 \times 10^{-13} \text{ m}^2/\text{s}$ (Table 1). In contrast, the translational diffusion of major α -casein fraction (about 93%) is significantly, about two orders of magnitude, slower. Such a difference in diffusion coefficients suggests that slowly diffusing α -casein species form some type of a supramolecular structure. To gain a further insight into supramolecular structure formation in the concentrated solution of α -casein, we investigated the dependence of the shape of the diffusion attenuation on the diffusion time t_d .

Figure 8A shows that α -casein diffusion attenuation depends on t_d . Further, α -casein diffusion coefficients D_1 and D_2 and the population p'_1 (note, $p'_2 = 1 - p'_1$), calculated from diffusion attenuations (Table 2) shown in Figure 8A, are plotted vs. t_d in Figure 8B. While the diffusion coefficient D_2 (fast diffusing α -casein species) and the population p'_1 (hence, p'_2 as well) do not change with increasing diffusion time, the value of the diffusion coefficient D_1 (slow diffusing α -casein species) is inversely proportional to the diffusion time $D_1 \sim t_d^{-1}$, representative of a fully restricted diffusion^{19, 21–24}. Accordingly, the mean square displacement of slowly diffusing α -casein species, given by the equation $\langle r^2 \rangle = 6D_1 \cdot t_d$, also remains constant, e.g., $\langle r^2 \rangle \sim t_d^0$. The root-mean-square displacement $\sqrt{\langle r^2 \rangle} \approx 45 \pm 5 \text{ nm}$ provides the size-estimate of restrictions. Thus, in the 15 % solution of α -casein, in the presence of 10 molar TCEP excess, approximately 90 % of α -casein molecules form a supramolecular structure leading to their fully restricted diffusion.

Figure 8B shows that the population p'_1 of α -casein molecules undergoing the restricted diffusion does not depend on the diffusion time, showing that there is no molecular exchange between slow and fast diffusing α -casein species within the diffusion time range from 10 to 400 ms and suggesting that α -casein interchain interactions are long-lived. We attempted to prepare the 3% α -casein solution from the 15 % concentrated solution by adding water and periodically shaking the sample using Vortex. After three weeks, we performed diffusion measurements using the dissolved fraction of the sample. The intensity of protein signal in the proton spectrum was smaller than expected for the 3 % α -casein solution, indicating that the protein concentration was also smaller than expected. The diffusion attenuation shown in Figure 9 showed a markedly increased slope as compared to

the freshly made 3 % α -casein solution, consistent with the fact that the concentration of protein in the solution prepared by dilution of the 15 % α -casein solution is smaller than 3 %. The diffusion coefficient determined from the slope of the diffusion attenuation for the dissolved sample is equal to $9.20 \pm 0.41 \times 10^{-11} \text{ m}^2/\text{s}$, e.g., about three times greater than the diffusion coefficient of $2.97 \pm 0.14 \times 10^{-11} \text{ m}^2/\text{s}$ estimated for α -casein molecules in the 3 % α -casein solution. Because the addition of TCEP does not affect the diffusion properties of α -casein in dilute solution, using the concentration dependence for the diffusion coefficient of α -casein acquired in our previous work²⁰, we estimate that the concentration of α -casein in the dissolved sample is about 0.8 %. This concentration corresponds to approximately 25 % of all α -casein molecules dissolved and 75 % of α -casein molecules remaining in the gel-like structure. Given that the ratio of α_{s1} to α_{s2} -casein is approximately 4:1⁶, we conclude that both fractions of α -casein join the gel network.

Altogether, these data show that the addition of TCEP unexpectedly induces the formation of long-lived intermolecular bonds stabilizing the supramolecular structural gel network of α -casein.

TCEP-induced structural changes in α -casein detected by CD spectroscopy

To examine structural changes in α -casein induced by the addition of TCEP, we used far-UV and near-UV CD spectroscopy (Figure 10). Far-UV CD spectroscopy provides information about the content of α -helical and β -sheet secondary structural elements and of disordered conformation. The far-UV spectra acquired for the 2 % and 7 % α -casein solutions in the absence of TCEP shows a large negative peak at $\sim 206 \text{ nm}$ and a shoulder at about 222 nm (Figures 10 A–B), suggestive of a combination of α -helical and disordered conformations, and, possibly, of the β -sheet conformation, in agreement with literature data³⁷. The addition of TCEP has no effect on the far-UV CD spectrum of α -casein in dilute (2 %) solution, indicative of no change in its secondary structure (Figure 10A). However, a significant change in the far-UV CD spectrum of α -casein is observed in concentrated (7 %) solution. The spectrum displays a large negative peak around $\sim 220\text{--}222 \text{ nm}$, a negative peak at $206\text{--}208 \text{ nm}$, and a positive peak at $\sim 192 \text{ nm}$. The amplitude of the negative peak at $\sim 220\text{--}222 \text{ nm}$ increases significantly, becoming greater than the amplitude of the peak at $206\text{--}208 \text{ nm}$. These changes indicate the increase of secondary structure content in the presence of TCEP.

Near-UV α -casein spectra acquired for the 0.15 % solution in the absence or presence of 10 molar TCEP excess show a broad minimum with fine structure between $\sim 258\text{--}283 \text{ nm}$ (Figure 10C). The near-UV CD spectrum arises from aromatic residues (phenylalanine, tyrosine, and tryptophan) located in different asymmetric environments in a structured protein as well as from the contribution of disulphide bonds. Peaks around 283 nm and 292 nm were previously associated in α_{s1} -casein with tyrosine and tryptophan contributions, respectively³⁸. A negligible change in the near-UV spectrum of α -casein at the concentration of 0.15 %, induced by the addition of TCEP, is observed. This change reflects the change in the aromatic residues (primarily tyrosines) environment that could be caused by tertiary structural and disulfide bond perturbations (in agreement with far-UV CD data). Based on our diffusion data, we do not expect the contribution from self-assembly of α -casein molecules at 0.15 % concentration. In addition, based on literature data, the change in

the near-UV spectrum due to aggregation of α -casein is much more pronounced, and a general increase in molar ellipticity and concurrent red shift are observed³⁹.

DISCUSSION

In this work, we investigated the translational diffusion of α -casein in the presence of reducing agent TCEP using NMR diffusion measurements. Our data show that the addition of TCEP does not affect the translational dynamics and physico-chemical properties of α -casein in dilute solutions. This result is consistent with the fact that α -casein does not have a well-defined three-dimensional structure and indicates that the disruption of a disulfide bond that can be formed by Cys36 and Cys40 in α_{s2} -casein does not change the hydrodynamic radius of the molecule. This result is also consistent with the general expectation that the addition of TCEP with the purpose of disulfide bond reduction does not cause any side reactions altering the protein. Our CD spectroscopic data further support this notion by showing no changes in the secondary structure of α -casein upon the disruption of Cys36-Cys40 disulfide bond in α_{s2} -casein by TCEP.

A different picture emerged when we added TCEP to the concentrated, 15 % (~ 6 mM) α -casein solution. Previously²⁰, we observed that in the absence of TCEP, at the concentration of α -casein 15 %, ~90 % of α -casein molecules formed a labile gel-like network stabilized by noncovalent intermolecular interactions with the lifetime of α -casein molecule within the network of ~3.5 s. TCEP altered the properties of α -casein gel-like network. While the distance between junction sites (45 ± 5 nm) was close to the size of restrictions estimated in the absence of TCEP (~50 nm) suggesting the formation of a similar structure, the gel lability was significantly reduced as judged by the lack of molecular exchange, during the diffusion time up to 400 ms, between α -casein molecules joined to the gel network (~93 %) and those that remained free in solution (~7 %). Furthermore, the attempt to dissolve this strong α -casein gel led to only a partial release of α -casein molecules back into solution. Based on our estimates, only ~ 25 % of α -casein molecules left gel after three weeks. These results suggest the formation of much stronger intermolecular interactions between α -casein molecules in the presence of TCEP, in addition to noncovalent intermolecular interactions stabilizing α -casein gel network in its absence. We note that gel structure may become less labile in the presence of a few strong and long-lived intermolecular interactions, in the presence of an equilibrium of many weak forming and re-forming short-lived intermolecular interactions, or the combination of both. Regardless of the nature of these bonds, the molecules of TCEP did not participate in long-lived intermolecular interactions, because upon the addition of TCEP to α -casein solution, no dependence of TCEP diffusion decay on diffusion time, representative of the fast exchange on the NMR time scale between TCEP bound to the protein and free in solution, and no signal broadening in the NMR spectrum of TCEP was observed.

At this point, we can only speculate about the nature of such interactions. The ionic character of TCEP in solution due to the presence of a basic group (ternary phosphorus) and three acidic carboxylic groups makes it behave in a fashion similar to strong electrolytes³⁶. It is known that changes in ion levels have pronounced effects on the conformation of disordered proteins and may lead to their enhanced aggregation⁴⁰. As follows from the

composition and distribution of charged residues, α -casein can be classified as a weak polyampholyte, and its charge-driven association is sensitive to changes in the environment due to the shielding of electrostatic interactions or counterion coordination⁴¹. While such effects remain small in dilute solution, where the distances between protein molecules are large, they would be prominent at high protein concentrations where the interactions between protein molecules become significant. Our CD data reveal an increase of secondary structure content in α -casein, which could account both for the intra-molecular and inter-molecular structure formation. It is possible that the change in electrostatic environment around α -casein due to the addition of TCEP leads to the formation of salt bridges or other inter-molecular bonds that stabilize gel structure. These bonds may be both strong and long-lived or weak and short-lived as mentioned above.

In addition, it is expected that disulfide bonds do not form in the presence of TCEP²⁷. Our control experiments, where α -casein was labeled with cysteines-specific dyes pyrene or Alexa 532, showed that TCEP efficiently disrupted disulfide bonds. However, it has been reported that even in the presence of excess TCEP some disulfide bonds can re-form. For example, in bovine insulin, ~13% of the disulfide bonds were observed in the presence of excess TCEP⁴². Likewise, the addition of reducing agents DTT and TCEP to lysozyme and bovine serum albumin (BSA) led to the formation of scrambled disulfide bonds, both inter- and intramolecular⁴³. Protein-protein interactions are promoted at high protein concentrations^{44–45}. Of the two α -casein fractions, α_{s2} -casein contains cysteine residues (Cys 36 and Cys 40)¹³. Accordingly, one can assume that at high protein concentrations, e.g., at crowded conditions, new intermolecular disulfide bonds could form between α_{s2} -casein molecules upon the disruption of intramolecular disulfide bonds by TCEP. The molecule of α_{s2} -casein has two –SH groups that can form only one intramolecular, but two intermolecular disulfide bonds. Thus, in the presence of dynamic equilibrium of breaking and re-forming disulfide bonds, the probability of α_{s2} -casein molecule to remain within the gel network is higher than to remain “free”. Apparently, the molecules of α_{s1} -casein become incorporated into the gel structure via multiple non-covalent interaction with both α_{s1} -casein and α_{s2} -casein molecules. However, in the presence of TCEP, they remain trapped within the gel structure for a longer time via contacts with disulfide-bonded α_{s2} -casein molecules.

Collectively, our results show that the addition of TCEP to α -casein solution led to the formation of the hierarchy of intermolecular interactions of different strength, which include non-covalent interactions that form in the absence of TCEP and new interactions induced by TCEP. These results have implications for using TCEP in protein solutions. TCEP is widely used to reduce disulfide bonds, to maintain free sulfhydryl groups, and for subsequent structural analysis of proteins^{27–28, 46–47}. It is often a preferred reducing agent due to its strong reducing power, high stability, wide pH range, and the possibility of cysteine labeling in its presence. However, there are a few reports of side reactions, including the conversion of a cysteine residue to alanine by heating in the presence of TCEP⁴⁸, the cleavage of peptide bond of peptides and proteins at azido homoalanine⁴⁹, and a slow cleavage of cysteine-containing proteins under mild conditions⁵⁰. Here, we report that the addition of TCEP definitely alters the solution structure at high protein concentration of α -casein, e.g., at crowded conditions.

CONCLUSION

In summary, we show that the addition of reducing agent TCEP in excess disrupts the disulfide bond between cysteine residues Cys36 and Cys40 in α_{s2} -casein, but does not affect the translational diffusion and does not alter the secondary structure of α -casein in the dilute solution. At the same time, in the concentrated solution of α -casein, the addition of TCEP triggers additional chemical processes that alter the mechanism of α -casein self-association leading to the formation of non-labile and insoluble gel. This can be explained by the formation of new, stabilizing gel structure intermolecular interactions, which form in addition to noncovalent intermolecular interactions existing in the absence of TCEP. Based on our results, we propose that intermolecular disulfide bonds can be one type of such interactions. The probability of intermolecular disulfide bond formation in dilute solution is low. Accordingly, our results show that the addition of TCEP to the concentrated solution of α -casein can induce additional side processes at the intermolecular level. Similar changes could occur in the concentrated solutions of other proteins, and, hence, TCEP should be used with caution.

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ABBREVIATIONS:

NMR	Nuclear Magnetic Resonance
PFG	pulsed-field gradient
IDP	intrinsically disordered protein
CD	circular dichroism
RMS	root-mean square
TCEP	tris(2-carboxyethyl)phosphine
DTT	dithiothreitol
UV	ultraviolet
MRE	mean residue molar ellipticity

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Figure 1. Absorption (A,C) and fluorescence (B,D) spectra of α -casein labeled with *N*-(1-Pyrenyl)maleimide (A-B) or Alexa Fluor 532 (C-D). Spectra of the protein labeled in the absence of TCEP are shown in blue; spectra of the protein labeled in the presence of 10 molar excess TCEP are shown in red. Thin black and dashed lines in (B) show control spectra collected for unlabeled α -casein and water for reference.

Figure 2. ^1H NMR spectra of TCEP and α -casein.

^1H NMR spectra are shown for 0.1 M aqueous TCEP solution (top), and for the 3 % aqueous solution of α -casein in the absence (middle) and presence (bottom) of TCEP at the molar ratio of 1:10 (protein:TCEP), respectively. Spectral regions used to calculate the diffusion coefficient are shaded.

Figure 3. Diffusion attenuations of TCEP and α -casein in dilute solutions.

(A) The diffusion attenuation for 0.1 M aqueous solution of TCEP is plotted using the integrated peak area between 2.5 and 3.5 ppm (see Figure 1). Solid line represents a monoexponential diffusion attenuation, described by Eq. 1, fitted to experimental data. (B) Diffusion attenuations for the 3 % aqueous solution of α -casein in the absence (black squares) and presence (open circles) of TCEP, added at the molar ratio of 1:10 (protein:TCEP), are plotted using the integrated peak area from 0.16 to 3.61 ppm and 0.16 to 2.0 ppm, respectively. The diffusion attenuations are identical; hence, the diffusion coefficients of α -casein are the same in the absence or presence of TCEP.

Figure 4.

(**A**) ^1H NMR spectra are shown for the 15 % aqueous solution of α -casein in the absence (curve 1) and in the presence of 10 molar TCEP excess (curve 2). (**B**) The diffusion attenuation collected at $t_d = 100$ ms for the 15 % aqueous solution of α -casein in the presence of 10 molar TCEP excess is plotted using the integrated peak area between 0.16 and 3.61 ppm. The solid line represents the best fit curve of Eq. 4 to experimental data. Three exponents are needed to fit the data. The insert shows the diffusion attenuation of α -casein after the component with the largest diffusion coefficient $D_3 = 3.80 \pm 0.15 \times 10^{-10} \text{ m}^2/\text{s}$ was subtracted. The solid line in insert represents the best fit line using two exponents characterized by $D_1 = 3.40 \pm 0.17 \times 10^{-15} \text{ m}^2/\text{s}$ and $D_2 = 3.00 \pm 0.14 \times 10^{-13} \text{ m}^2/\text{s}$.

Figure 5. The dependence of ^1H NMR α -casein spectrum on the magnitude of pulsed-field gradient g .

^1H NMR spectra are shown for the 15 % α -casein solution with TCEP at different values of pulsed-field gradient amplitude g as indicated by different colors. Only the signal from α -casein molecules is observed at large values of g .

Figure 6. ^{31}P NMR spectra of α -casein and TCEP.

^{31}P NMR spectra of α -casein were collected for 15 % concentrated solution in the presence (top panel) or absence (middle panel) of TCEP. The ^{31}P NMR spectrum of TCEP (bottom panel) is shown for comparison. The spectra are not shown to scale.

Figure 7. Diffusion attenuations of ^{31}P TCEP spin-echo signal.

The diffusion attenuations were collected for TCEP added to the 15 % α -casein solution at the 10:1 molar ratio at different diffusion times: $t_d = 25$ ms (diamonds), 50 ms (circles), and 100 ms (stars). No dependence on t_d was observed.

Figure 8. The dependence on diffusion time t_d .

(A) Diffusion attenuations of α -casein, acquired for the 15 % solution in the presence of TCEP (10:1 molar ratio) at diffusion times $t_d = 50, 100, 200$, and 400 ms, are shown after subtracting TCEP contribution. Solid lines indicate the slope of the component characterized by diffusion coefficient D_1 (slowly diffusing α -casein species). (B) α -casein diffusion coefficients D_1 (open squares), D_2 (open triangles), and the population p_1 (solid squares) are plotted as functions of diffusion time, showing that only D_1 depends on t_d . Solid line has the slope of t_d^{-1} , demonstrating that experimental D_1 values are inversely proportional to t_d .

Figure 9. The reversibility test.

Diffusion attenuations are shown for the solution of α -casein, containing the fraction of α -casein molecules that left gel after dissolving for three weeks, (curve 1) and for the freshly prepared 3 % α -casein solution (curve 2). The diffusion coefficient measured for the dissolved fraction of α -casein is larger than in the 3 % α -casein solution, indicating that the protein concentration of the dissolved α -casein fraction is less than 3 %.

Figure 10. CD spectra of α -casein.

(A-B) Far-UV spectra were recorded for α -casein solution in the absence (blue) or presence (red) of 10 molar excess of TCEP. Panel A shows far-UV spectra for 2 % α -casein solution. Panel B shows spectra for 7 % α -casein solution. While no changes are observed in dilute (2 %) solution, a significant change is observed in concentrated (7 %) solution. (C) Near-UV spectra were recorded for the 0.15 % α -casein solution in the absence (blue) or presence (red) of 10 molar excess of TCEP. No changes are observed in near-UV CD spectra, whereas far-UV spectra significantly change upon the addition of TCEP.

Table 1.

Diffusion coefficients of different species in 1 %, 3 %, and 15 % solutions of α -casein.

Molecular species	Self-diffusion coefficient, m ² /s		
	1 % α -casein	3 % α -casein	15 % α -casein
Water	-	$2.70 \pm 0.11 \times 10^{-9}$	$2.30 \pm 0.09 \times 10^{-9}$
TCEP	-	$4.20 \pm 0.16 \times 10^{-10}$	$3.80 \pm 0.15 \times 10^{-10}$
α -casein species	$8.56 \pm 0.40 \times 10^{-11}$	$2.97 \pm 0.14 \times 10^{-11}$	$3.00 \pm 0.14 \times 10^{-13}$ $3.40 \pm 0.17 \times 10^{-15}$

Table 2.

Diffusion coefficients and populations of α -casein species measured at different diffusion times in the 15 % solution of α -casein.

Diffusion time, ms	Self-diffusion coefficient, m ² /s		Population	
	$D_1 \cdot 10^{-15}$	$D_2 \cdot 10^{-13}$	p'_1	p'_2
50	6.84 ± 0.48	3.10 ± 0.16	0.072 ± 0.002	0.0928 ± 0.002
100	3.42 ± 0.17	3.01 ± 0.15	0.069 ± 0.001	0.0931 ± 0.001
200	1.71 ± 0.10	3.05 ± 0.18	0.071 ± 0.002	0.0929 ± 0.002
400	0.86 ± 0.09	3.09 ± 0.26	0.070 ± 0.003	0.0930 ± 0.003