

## Infrared Spectroscopic Evidence for Calcium Ion Interaction with Carboxylate Groups of Casein<sup>1</sup>

### ABSTRACT

Examination of the Fourier-transform infrared difference spectra of lyophilized whole caseins with and without  $\text{Ca}^{2+}$  ions provides direct empirical evidence for the interaction between these divalent ions and the carboxylate groups of glutamate and aspartate residues. In the absence of  $\text{Ca}^{2+}$ , the O-C-O stretching vibrations of these carboxylates give two characteristic infrared absorption bands near 1400 and 1575  $\text{cm}^{-1}$ . When  $\text{Ca}^{2+}$  ions are present, this pair of bands was observed to shift about 10  $\text{cm}^{-1}$  to approximately 1410 and 1565  $\text{cm}^{-1}$ , respectively. In addition, the difference in the frequency between the two peaks decreased. Such changes in the position of the carboxylate bands typically indicate that significant interaction has occurred between the carboxylates and the metal ion. This observation suggests that carboxylate groups may play an important role along side that of the serine phosphates as sites for  $\text{Ca}^{2+}$  ion binding in caseins.

### INTRODUCTION

A distinguishing structural feature of most caseins, in contrast to other milk proteins, is the occurrence of repeating sequences of serine phosphates. Interactions between these negatively charged groups and  $\text{Ca}^{2+}$  ions have long been the focal point of research on the func-

tional properties of caseins. The colloidal skim milk system is, however, a complex montage involving competing multiple equilibria (6), and several lines of evidence have pointed toward the importance of negatively charged carboxylate groups of glutamate and aspartate residues in caseins as potential participants in colloidal interactions (7). From a consideration of the equilibria that exist among skim milk components, it would seem likely that  $\text{Ca}^{2+}$  binding to casein carboxylates should also occur. Nevertheless, direct evidence has been lacking.

We have studied lyophilized whole casein, in the presence and absence of  $\text{Ca}^{2+}$  ions, by computer-assisted Fourier-transform infrared (FTIR) difference spectroscopy to search for more convincing evidence of this interaction. Small, but distinct, shifts in the frequencies of the pair of bands associated with carboxylate O-C-O stretching vibrations near 1400 and 1575  $\text{cm}^{-1}$  (Figures 1 to 3) have revealed that  $\text{Ca}^{2+}$  ions do interact and bind to casein carboxylate groups. In addition, these spectra suggest that minor changes occur in the overall conformation of the proteins upon interaction with  $\text{Ca}^{2+}$  ions.

### MATERIALS AND METHODS

Lyophilized whole casein, from the whole milk of an individual Jersey cow, was isolated and treated as summarized below; reference (1) gives a more detailed description. Milk from the cow was treated immediately after collection with phenylmethylsulfonyl fluoride to inhibit proteolysis by serine proteases. The milk was defatted by centrifugation and diluted 1:1 with distilled water. Casein was precipitated at pH 4.6 by addition of 1 N HCl. The precipitate was homogenized and redissolved (at pH 7.0) with NaOH. The sodium caseinate was reprecipitated, washed, and then resuspended. The

Received September 21, 1988.

Accepted January 18, 1989.

<sup>1</sup>Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

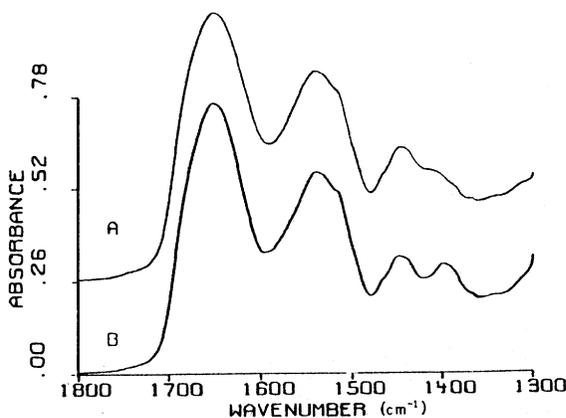


Figure 1. Original infrared spectra of  $\text{Ca}^{2+}$ -casein (A) and  $\text{K}^{+}$ -casein (B) as halocarbon mulls. (Data obtained with Nujol mulls were nearly identical.)

suspension was cooled to  $4^{\circ}\text{C}$ , centrifuged to remove residual fat, and then freeze-dried.

To produce lyophilized samples with known amounts of  $\text{K}^{+}$  and  $\text{Ca}^{2+}$ , the sodium caseinate was dissolved in  $\text{H}_2\text{O}$  at 20 g/L and the pH adjusted to 6.8 with  $\text{NaOH}$ . To one-half of the solution was added a stock aqueous solution of  $\text{CaCl}_2$  to give a final  $\text{Ca}^{2+}$  concentration of 15 mM to the other half, a  $\text{KCl}$  solution, to yield  $[\text{K}^{+}] = 45 \text{ mM}$ . (This concentration of  $\text{K}^{+}$  results in a solution of comparable ionic strength to that of the sample with  $\text{Ca}^{2+}$ .) Finally, the two solutions were again freeze-dried.

A few milligrams of each of the two lyophilized, whole caseins were mulled with Nujol and with perhalocarbon oil; spectra were measured of thin films of each mull spread between  $\text{KBr}$  plates. All spectra were obtained on a Nicolet 170SX FTIR spectrometer equipped with a water-cooled Globar source, a germanium-potassium bromide beamsplitter, and a broad range, liquid nitrogen cooled mercury-cadmium-telluride (MCT) detector. Each spectrum consisted of 4000 single-sided interferograms, co-added, phase-corrected, zero-filled once, apodized with the Happ-Genzel function, and fast Fourier transformed. Nominal instrument resolution was  $2 \text{ cm}^{-1}$  so that there was one data point every  $1 \text{ cm}^{-1}$ . Second derivative spectra were calculated by a simple analytic

procedure that used every data point, as described previously (2, 11).

## RESULTS AND DISCUSSION

Figure 1 shows the original spectrum of whole, dried casein without  $\text{Ca}^{2+}$  ( $\text{K}^{+}$  is the counterion present in this case) [B] and with  $\text{Ca}^{2+}$  present [A]. Few differences are obvious, except for a band at  $1400 \text{ cm}^{-1}$  [B], which becomes a weak shoulder near  $1407 \text{ cm}^{-1}$  when  $\text{Ca}^{2+}$  is present [A]. Even when second derivative spectra are compared, as in Figure 2, only minor changes appear; the principal one again is a shift in the band at  $1401 \text{ cm}^{-1}$  [B] to higher value ( $1408 \text{ cm}^{-1}$ ) [A]. Other noticeable differences are the disappearance of a weak shoulder about  $1575 \text{ cm}^{-1}$  (Figure 2B) and a change in the relative intensities of the bands near  $1690$  and  $1654 \text{ cm}^{-1}$ .

Calculation of the difference between the two original spectra (Figure 1B minus Figure 1A) is illustrated in Figure 3. [The two spectra were scaled prior to subtraction so that the peak intensity of the band at  $1515 \text{ cm}^{-1}$  in the two second derivative spectra (Figure 2), associated with the ring stretching mode of tyrosine residues, had the same value.] In Figure 3, the peaks above zero absorbance difference represent features that have higher intensities in the spectrum of the sample containing  $\text{Ca}^{2+}$  than in

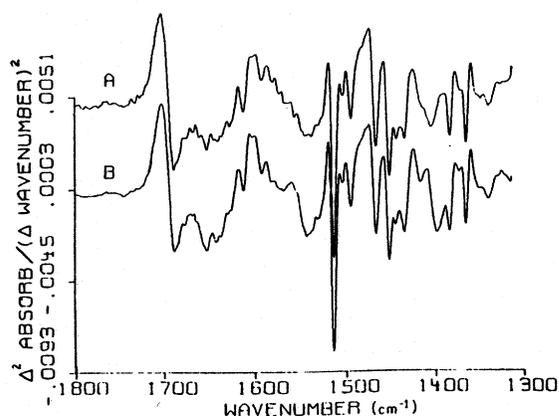


Figure 2. Unsmoothed second derivative spectra of  $\text{Ca}^{2+}$ -casein (A) and  $\text{K}^{+}$ -casein (B). (These data were calculated from the spectra shown in Figure 1.)

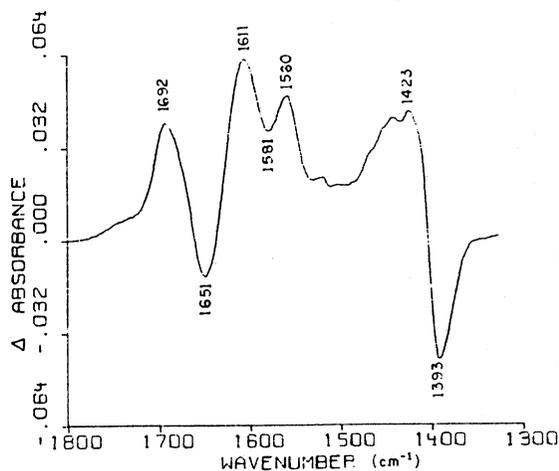


Figure 3. Unsmoothed difference spectrum calculated from the two original infrared spectra (shown in Figure 1), normalized at  $1515\text{ cm}^{-1}$ . [Here the ordinate represents the computed difference in the absorbance intensities at each data point when the spectrum of  $\text{K}^+$ -casein (Figure 1B) is subtracted from the spectrum of  $\text{Ca}^{2+}$ -casein (Figure 1A). Bands with negative intensity values are attributable to vibrations of the  $\text{K}^+$ -caseinate; positive bands are presumed to arise from the periodic motions of the  $\text{Ca}^{2+}$ -caseinate.]

the one with  $\text{K}^+$  ion only. Peaks below zero represent bands whose height and area is greater in the casein sample with just  $\text{K}^+$  ion. Where the two original spectra have no bands or each has a peak at the same frequency position, with identical shapes, intensities, and widths, the difference spectrum will have only a horizontal line at zero absorbance. Note, where a peak in one spectrum lies closer than about one full band-width in frequency from another peak in the second spectrum, their frequency positions, as measured by the maximum of the positive peak and the minimum of the negative peak which appear in the difference spectrum, will generally be shifted in opposing directions from the true values of the respective component bands in the original spectra.

Figure 3 (which has its y-axis expanded about six times relative to that in Figure 1) immediately reveals that the spectra are not identical. The presence of  $\text{Ca}^{2+}$  ions has induced a number of small, but significant, changes in the spectra. Between  $1775$  and  $1325\text{ cm}^{-1}$ , one may see four bands with positive intensity at  $1692$ ,  $1611$ ,  $1560$ , and  $1423\text{ cm}^{-1}$

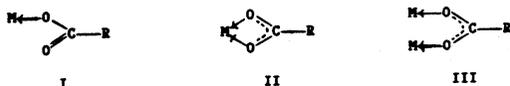
and three negative bands at  $1651$ ,  $1581$ , and  $1393\text{ cm}^{-1}$ . The first four represent bands that appear at new positions, or bands whose intensities increase, in the spectrum of the casein with  $\text{Ca}^{2+}$  compared with that for the casein with just  $\text{K}^+$ ; the three negative features represent peaks found in the spectrum of the latter ( $\text{K}^+$ ), which are either greater than the comparable band in the former ( $\text{Ca}^{2+}$ ) or which disappear when  $\text{Ca}^{2+}$  is present.

For each sample, two bands, one between  $1400$  and  $1425\text{ cm}^{-1}$  and the other between  $1560$  and  $1585\text{ cm}^{-1}$ , are associated with the O-C-O stretching vibrations of carboxylate groups (9, 12). In the former, both C-O bonds stretch in-phase with one another. This is commonly denoted as the symmetric mode. In the latter (antisymmetric mode), the two bonds move out-of-phase relative to one another. For simple alkali (Group Ia) metal acetates, bonding is largely ionic with relatively little covalent character. In such cases, the observed frequency of these two vibrations is about  $1415$  and  $1580\text{ cm}^{-1}$  (9). For the protein lysozyme, the reported value for the antisymmetric stretch appears about  $1565\text{ cm}^{-1}$  (12). For calcium acetate, where a greater measure of covalency is expected, the reported O-C-O stretching frequencies are  $1418$  and  $1538\text{ cm}^{-1}$  (5).

Most likely, the difference bands with negative intensity at  $1581$  and  $1393\text{ cm}^{-1}$  (Figure 3) result from the pair of carboxylate bands near  $1575$  and  $1400\text{ cm}^{-1}$  that are only partially resolved in the second derivative spectrum of the  $\text{K}^+$ -casein (Figure 2B). The positive difference bands at  $1560$  and  $1423\text{ cm}^{-1}$  (Figure 3) probably correspond to analogous features near  $1565$  and  $1410\text{ cm}^{-1}$  in the spectrum of  $\text{Ca}^{2+}$ -casein (Figure 2). Had the  $\text{Ca}^{2+}$  ions interacted only weakly, or not at all, with the carboxylate groups of the glutamate and aspartate residues of the proteins, one would expect to see little, if any, shift in frequency of these bands. The size of the observed frequency changes implies instead that appreciable interaction exists.

More important as an indicator of interaction than the absolute change in the frequencies of these two bands, however, is the variation in the frequency difference between them (3, 9). In simple ionic acetates, this value ranges from about  $165$  to  $155\text{ cm}^{-1}$  (5, 9). Increases in this value indicate monodentate covalent bonding

between one of the carboxylate oxygens and the metal atom bound thereto (I). A decrease by contrast typically implies that the both carboxylate oxygens have chelated the metal in bidentate fashion (II) or that the carboxylate oxygens are bridging two metal ions (III).<sup>2</sup>



For calcium acetate, the reported frequency difference is only about  $120\text{ cm}^{-1}$  (5), 35 to  $45\text{ cm}^{-1}$  less than the value observed for alkali acetates. These data imply that the carboxylate group has chelated the  $\text{Ca}^{2+}$ , probably in bidentate fashion (II).

From the difference spectrum in Figure 3, one may estimate frequency differences of not less than  $175\text{ cm}^{-1}$  and not more than  $150\text{ cm}^{-1}$ , respectively, for the casein with  $\text{K}^+$  as compared with the casein with  $\text{Ca}^{2+}$ . The observed decrease in the carboxylate frequency difference for the two casein samples is thus greater than  $25\text{ cm}^{-1}$ . This change is only slightly smaller than that for calcium acetate and clearly suggests that appreciable interaction occurs between the carboxylate groups of the caseins and the  $\text{Ca}^{2+}$  ions. Furthermore, this binding is either bidentate (II) or bridging (III) (3, 9).

All previous reports of  $\text{Ca}^{2+}$  interactions with casein carboxylates have come from studies of the individual caseins in solution and have relied on indirect evidence. Investigations by equilibrium dialysis of the purified caseins (4, 10) showed that  $\bar{\nu}$  (moles of  $\text{Ca}^{2+}$ /mole of caseinate) varies greatly with ionic strength. Near physiological conditions,  $\bar{\nu}$  approximates the number of phosphate residues, but much higher values are possible depending on envi-

ronmental conditions. Early results from  $\text{Ca}^{2+}$ -induced solubility profiles of  $\alpha_{\text{S}1}$ -casein (13) implicated  $\text{Ca}^{2+}$  binding sites exceeding by almost twofold the number of phosphate groups present. These observations were recently extended and quantitated by Farrell et al. (8) who correlated the salting-out parameter ( $k_1$ ) with  $\text{Ca}^{2+}$  binding and suggested that additional  $\text{Ca}^{2+}$  binding to carboxylates plays an important role in salting-in. Analysis of the solubility profiles for dephosphorylated  $\alpha_{\text{S}1}$ -casein dramatically points out the role of  $\text{Ca}^{2+}$  in the interaction with  $\alpha_{\text{S}1}$ -casein. Here  $k_1$  (7) was greater than that found for the native protein, indicating stronger  $\text{Ca}^{2+}$ -protein interactions in the dephosphorylated form. Given the association of the  $k_1$  with  $\text{Ca}^{2+}$  binding, an apparent  $K_{\text{diss}}$  for the interactions can be calculated to range from 4.5 to 7.4 mM, which is well within the range of free  $\text{Ca}^{2+}$  ion concentrations in milk serum (6). This being the case, one could argue that  $\text{Ca}^{2+}$ -casein interactions with non-phosphate groups should be observable. As outlined, the technique of FTIR difference spectroscopy has now provided the first direct, empirical evidence for  $\text{Ca}^{2+}$  interactions with caseins, at least for the solid state.

In addition to the spectral changes discussed for the carboxylate groups themselves, there are significant differences observed in the amide I region ( $1700$  to  $1600\text{ cm}^{-1}$ ). These differences imply that small but real changes have occurred in the average relative proportion of the different types of secondary structure present in one or more of the four component proteins [ $\alpha_{\text{S}1}$ -,  $\alpha_{\text{S}2}$ -,  $\beta$ -, and  $\kappa$ -] in the whole casein. Bands at  $1654$  and  $1645\text{ cm}^{-1}$  have been shown by previous work (2, 11) to be associated with  $\alpha$ -helices and disordered or aperiodic structure, respectively. Bands near  $1690\text{ cm}^{-1}$  are usually due to turns, although one cannot absolutely rule out possible contributions by the high frequency component of  $\beta$ -strands. Finally, bands below  $1635\text{ cm}^{-1}$  are typically due to the low frequency components of  $\beta$ -chains.

The observed difference bands in the amide I region consist of two positive intensity bands at  $1692$  and  $1611\text{ cm}^{-1}$  and one negative band at  $1651\text{ cm}^{-1}$ . Their positions and relative intensities suggest that the  $\text{K}^+$ -casein has somewhat more helix, while the  $\text{Ca}^{2+}$ -casein has a few more turns,  $\beta$ -structure, or both. In an

<sup>2</sup>Note  $\text{Ca}^{2+}$  typically exhibits approximately octahedral coordination. For simplicity structures I-III schematically depict only one or two of the six coordination sites on the metal ion (M). In a protein, for example, the remaining sites might be occupied by oxygens from other carboxylate groups, phosphate groups, hydroxyls, amides, or even bound water molecules.

earlier study, we used Raman spectra to estimate the average relative conformations of these caseins (1). We reported that the casein submicelles ( $K^+$ ) had approximately 18% helix, 27%  $\beta$ -structure, 39% turns, and 16% unordered (unspecified). The micelles ( $Ca^{2+}$ ), by contrast, exhibited about 14% helix, 27%  $\beta$ -structure, 41% turns, and 18% unordered. (Because these Raman estimates could be in error by as much as  $\pm 3\%$ , we refrained from comment on the small differences in the calculated values for the two casein samples.) Now infrared difference spectroscopy corroborates the validity of the general trends only suggested by the previous study.

The small decrease in percentage  $\alpha$ -helix and increase in amount of turns observed in the infrared difference spectra (Figure 3) upon addition of  $Ca^{2+}$  ion seems to indicate that some of the amino acid residues which interact with the  $Ca^{2+}$  ions are part of helical strands in the submicelles which partially uncoil upon specific interaction with  $Ca^{2+}$  ions. This hypothesis, however, must await further testing by other experimental data or by computer modeling of casein structure.

#### CONCLUSIONS

The FTIR difference spectroscopy has been shown to be capable of disclosing the interaction of  $Ca^{2+}$  ions with the carboxylate side chains of lyophilized caseins. In addition, this technique reveals that this interaction results in small, but clearly detectable, changes in the average overall secondary structure of these milk proteins. In the future, FTIR spectroscopy may find potential application as a rapid, non-destructive monitor of similar interactions in real food samples. Such methodology has the potential for providing food scientists and engineers a means for better understanding and ultimately predicting the relationships between

protein structure and function in dairy and other food processing.

#### ACKNOWLEDGMENTS

The authors thank Thomas M. Muller and Kevin N. Newman for their technical assistance in obtaining the infrared spectra.

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