

Preparation and Properties of Trifluoroacetylated κ -Casein

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Abstract

Blocking the free amino groups of κ -casein with trifluoroacetyl-groups resulted in markedly altered properties of the protein. Trifluoroacetylated κ -casein was not able to stabilize α_{s1} -casein in the presence of calcium ions, but this ability was fully restored after removal of the blocking groups. Trifluoroacetylated- κ -casein was susceptible to rennin action, but the liberated *para*- κ -casein was completely soluble and did not yield the typical *para*- κ -casein clot. This loss of characteristic properties is attributed to structural alterations produced by the change in ionic character or by steric effects of the blocking trifluoroacetyl-groups.

The stability of the casein micellar system can be attributed directly to the unique properties of κ -casein. Through complex formation with calcium-sensitive caseins, κ -casein imparts physical stability to casein micelles in the presence of calcium ions. Although much information regarding the chemistry of the caseins has accumulated, the physicochemical forces responsible for the interactions between κ -casein and the calcium-sensitive caseins have not yet been elucidated.

Various functional groups of κ -casein have been studied, to determine their participation in complex formations. κ -Casein modified by removal of phosphate groups (5) or sialic acid moieties (6) substantially retained the ability to form complexes with α_{s1} -casein. Similarly, studies with reduced and alkylated κ -caseins (3, 8) have indicated that neither free sulfhydryl groups nor intact disulfide bonds are essential for complex formation.

In the present investigation, we report the blocking of the free amino groups of κ -casein using S-ethyl-trifluorothioacetate, and its effect on the stabilizing properties, rennin sensitivity, and sedimentation behavior.

Materials and Methods

κ -Casein was isolated from the acid-precipitated casein of pooled milk by the urea-sulfuric acid method (11) and purified by precipitation

from ethanolic solution (4). Carboxamidomethyl- κ -casein (CAM- κ) was prepared according to the method previously reported (8).

Trifluoroacetylated κ -casein (TFA- κ) was prepared according to the method of Goldberger and Anfinsen (1), using S-ethyl-trifluorothioacetate (Eastman Organic Chemicals).¹ One gram of κ -casein was dissolved in 100 ml distilled water and the pH raised to 10.0 by adding N KOH. S-ethyl-trifluorothioacetate (2.5 ml) was added to the continuously stirred solution and pH maintained between 9.9 and 10.0 by adding KOH. The base uptake of the reaction ceased after approximately 45 min. The solution was adjusted to pH 4.5 with N HCl and the precipitate collected by centrifugation. The precipitate was dissolved at pH 7 and dialyzed exhaustively against 0.01 M imidazole buffer, pH 7, and finally against water, and lyophilized. Trifluoroacetylated-*para*- κ -casein was prepared under identical conditions, except that alkylation took place with a suspension of *para*- κ at pH 9.9-10.0.

Removal of the blocking trifluoroacetyl groups from the alkylated protein was done by dissolving 50 mg of TFA- κ -casein in 5 ml of molar piperidine at room temperature, and immediately placed in an ice bath. After two hours of incubation, the regenerated κ -casein was separated from the reagents by gel filtration on a 4.5- by 40-cm column of Sephadex G-25 with 0.01 M imidazole, pH 7, as solvent.

The ability of κ - and TFA- κ -caseins to solubilize α_{s1} -casein in the presence of calcium ions was measured according to Zittle's procedure (10).

The carboxamidomethyl derivatives of κ - and TFA- κ -casein were prepared according to the method previously reported (8).

Comparative sedimentation velocity experiments were done with a Beckman-Spinco Model E ultracentrifuge. One per cent solutions of reduced and alkylated κ - and TFA- κ -caseins in pH 7.0 phosphate buffer, ionic strength 0.1, were run simultaneously at 25 C and 52,640 rpm, one sample placed in a wedge window and the other in a standard cell having 12-mm Kel-F centerpieces.

¹ Mention of brand or firm name does not constitute an endorsement by the USDA over others of a similar nature not mentioned.

The electrophoretic patterns were obtained using 7% polyacrylamide 4.5 M urea with tris buffer, pH 9.2. All protein samples were reduced with mercaptoethanol before electrophoresis (7).

Results and Discussion

The reaction of S-ethyl-trifluoroacetate with κ - and *para*- κ -caseins proceeded rapidly, and in *para*- κ -casein, complete stabilization was obtained in 10 min. No free amino groups were detected in the TFA-proteins by reaction with dinitrofluorobenzene, indicating that the ϵ -amino groups of all nine lysine residues had been masked. The TFA- κ -casein had solubility properties comparable to native κ -casein; TFA-*para*- κ -casein, in contrast to native *para*- κ -casein, was soluble above pH 6. As could be expected, blocking the free amino groups markedly altered the electrophoretic behavior of the TFA-proteins. A comparison of the gel electrophoretic patterns in Fig. 1 demonstrated the increased anionic properties of the modified proteins. Trifluoroacetylated- κ had an apparent electrophoretic mobility approximately twice that of the κ -casein; whereas, TFA-

para- κ migrated toward the anode in contrast to the cathodic migration of underivatized *para*- κ . Although not very evident from the patterns in Fig. 1, the acylation procedure preserved the individual components of the respective proteins. The solubility of the TFA-*para*- κ at neutral pH's suggests use of this derivative in certain enzymatic studies (hydrolysis with trypsin, chymotrypsin, etc.) where a soluble protein is desired.

Trifluoroacetylation of the ϵ -amino groups of κ -casein results in a loss of its ability to stabilize α_{s1} -casein in the presence of calcium ions. This effect is demonstrated in the solubility curves in Fig. 2, wherein only an insignificant amount of stabilization is obtained with TFA- κ compared to native κ -casein. Loss of this protective ability of κ -casein can be directly attributed to incorporation of the TFA-groups per se, rather than to denaturation resulting from the reaction conditions. Removal of the blocking TFA-groups by incubation with molar piperidine restored the full stabilizing ability to κ -casein (Fig. 2) and regenerated the original κ -casein components (Fig. 1).

Failure of the TFA- κ to stabilize α_{s1} -casein is undoubtedly due to the inability of the protein to enter into the necessary complex with α_{s1} -casein. The lack of complex forma-

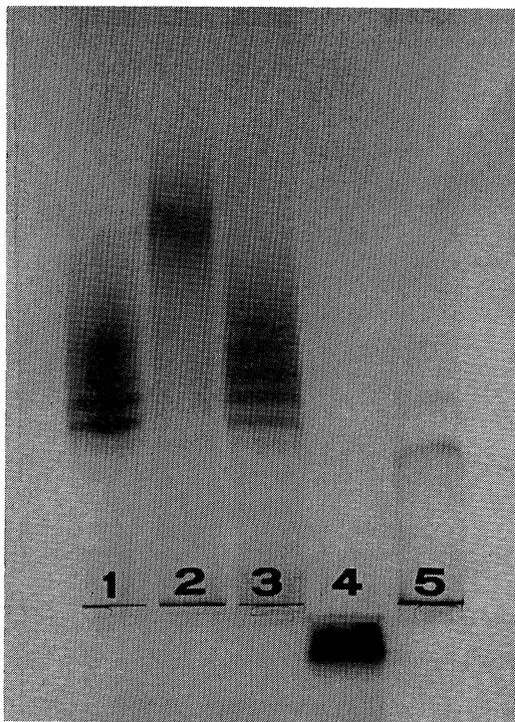


FIG. 1. Polyacrylamide gel electrophoresis of κ - and TFA- κ -caseins. 1. κ -Casein. 2. TFA- κ -casein. 3. κ -Casein obtained after removal of blocking TFA-groups. 4. *Para*- κ -casein. 5. TFA-*para*- κ -casein.

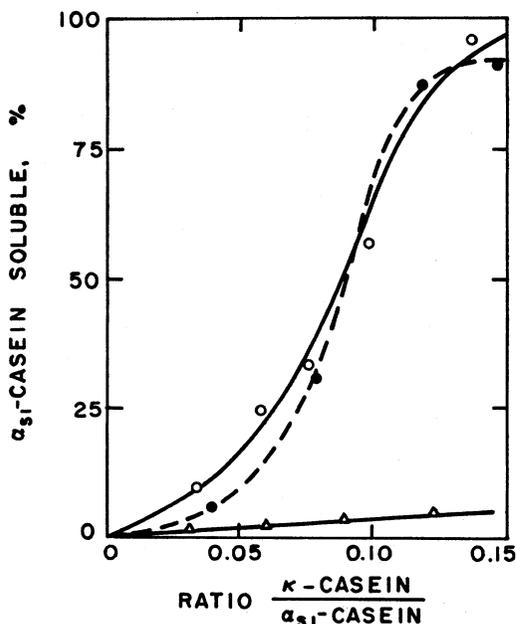


FIG. 2. Solubilization of α_{s1} -casein in the presence of 0.02 M CaCl_2 by κ -casein, (-O-); TFA- κ -casein, (- Δ -); and regenerated κ -casein (-.-.-) obtained after removal of TFA-groups.

tion is due either to structural changes induced by the new ionic character of the κ -casein molecule or to loss of an amino group function essential to the interaction of these proteins. Stereochemical hindrance arising from the incorporated TFA-groups should also be considered as a possible cause for loss of stabilizing ability.

Digestion of TFA- κ with rennin did not result in a visible clot formation normally accompanying *para*- κ -release; however, examination of the digest mixture by gel electrophoresis demonstrated the presence of TFA-*para*- κ resulting from the cleavage of the rennin-sensitive site in κ -casein. Failure of the liberated *para*- κ to clot can be attributed to a change in aggregation properties resulting from derivatization of the amino groups.

Simultaneous sedimentation analysis of reduced and alkylated κ -casein and TFA- κ -casein (Fig. 3) indicated that significant changes in the sedimentation behavior result from trifluoroacetylation of the free amino groups. Trifluoroacetylated- κ -casein exhibited a hyper-sharp boundary that sedimented at a rate approximately half that of the native κ -casein. These changes in sedimentation behavior might arise from conformational changes in the protein or from a lower degree of aggregation attributable to an increased net negative charge of the derivatized protein. Of the two possibilities, the latter appears more likely, since an absence of major secondary or tertiary structure in κ -casein has been indicated by optical rotation studies (2) and by studies of buried tyrosine residues (9). Further physicochemical studies are required before this behavior can be adequately explained.

Loss of characteristic properties of native κ -casein resulting from alkylation of the free amino groups should not be interpreted to mean that the amino groups per se are essential to the normal properties of κ -casein. Rather, the foregoing results suggest that any significant alteration in structure or charge distribution may result in a loss of these properties. Further, in spite of the inability to demonstrate significant secondary or tertiary structures in κ -casein, it is evident that the complex normally formed between α_{s1} - and κ -casein requires a certain degree of structural order and freedom to permit the necessary interactions leading to complex formation. Studies with additional derivatives, specific for other functional groups, will be required before the mechanism of complex formation can be elucidated.

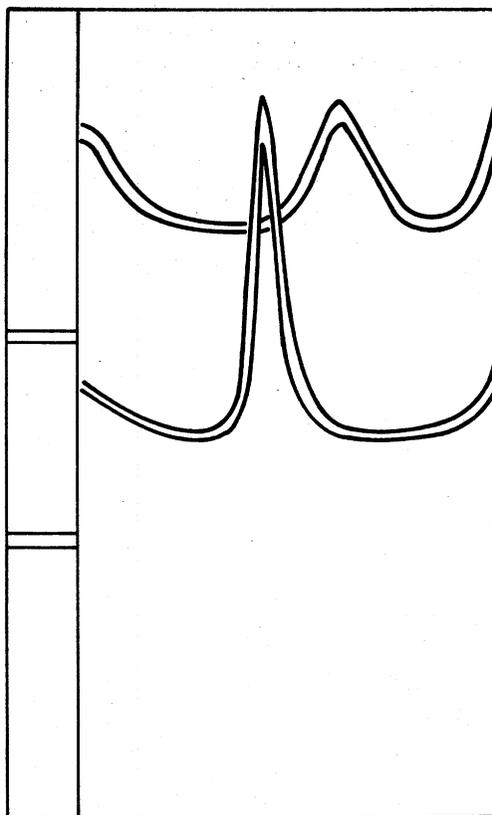


FIG. 3. Comparative sedimentation velocity patterns of κ -(upper) and TFA- κ -caseins (lower).

Acknowledgments

The author thanks Dr. M. E. Noelken for performing the sedimentation velocity experiments and Mrs. M. V. Wondolowski for her technical assistance.

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