

Nitration of Tyrosyl Residues in κ - and α_{s1} -Caseins

Abstract

Tyrosyl residues in κ - and α_{s1} -casein were nitrated with tetranitromethane and its effect on their properties determined.

Nitration of up to four of the eight tyrosines in κ -casein did not affect its α_{s1} -casein-stabilizing ability; additional nitration progressively decreased the stabilizing ability and this was totally absent after all eight tyrosyl residues were nitrated.

Nitrated α_{s1} -casein retained its calcium sensitivity but was unable to form a protective complex with κ -casein after nitration of four of its 12 tyrosyl residues. A complete tyrosyl nitration of κ - and α_{s1} -caseins and of a 1:10 κ/α_{s1} -complex was achieved at low molar ratios of tetranitromethane. This suggests that these proteins are devoid of major secondary or tertiary structures.

Interest in the mechanism of casein micelle formation, and more specifically of κ - and α_{s1} -casein interaction, has stimulated investigations of the role of various functional groups of these proteins in their interactions. Modification of the free amino groups of κ -casein by trifluoroacetylation (7), or carbamylation (3), resulted in the loss of its α_{s1} -casein-stabilizing ability. The loss of this protective action in the modified κ -caseins can probably be attributed to the resulting conformational or ionic changes, and should not be interpreted as evidence of direct involvement of the epsilon amino groups in the stabilization of α_{s1} -casein.

There has been an increasing awareness of the importance of tyrosine residues both in stabilizing the structure of proteins and in participating in their functions. The availability of tetranitromethane as a highly specific nitrating agent for tyrosyl residues (4) has led us to investigate the effects of this modification on the important properties of α_{s1} - and κ -caseins.

Materials and Methods

κ -Casein was isolated from acid-precipitated whole casein by the urea-sulfuric acid method (9); α_{s1} -casein was also prepared from the acid-precipitated casein by the urea-calcium chloride procedure (5).

Nitration of the α_{s1} - and κ -caseins was done at room temperature according to the procedure of Sokolovsky et al. (4) in 0.05 M tris

buffer at pH 8; nitration of the κ/α_{s1} -complex was done in 0.01 M imidazole buffer at pH 7.0. Aliquots of a 0.84 M solution of tetranitromethane (TNM) in 95% ethanol, to yield desired molar ratios of reagent, were added to 2.5% protein solutions. The pH of the reaction mixture was maintained at 8.0 with 0.1 N NaOH. After a reaction time of 2 hr, the reactants were separated on a 4.5- by 40-cm column of Sephadex G-25 equilibrated with tris buffer at pH 8.7, dialyzed, and lyophilized.

A κ/α_{s1} -casein complex (1:10) was prepared in pH 7.0, 0.01 M imidazole buffer containing 0.02 M CaCl_2 . Thirty minutes after addition of CaCl_2 , the complex solution was centrifuged to remove any precipitated or insoluble protein. The complex was then reacted with a 2.21 molar excess of tetranitromethane as aforementioned.

The degree of tyrosyl nitration in the modified proteins was measured by determining the amount of unmodified tyrosine remaining, using an automatic amino acid analyzer. 3-Nitrotyrosine, the only reaction product (4), could not be determined directly because it eluted together with phenylalanine with the gradient elution system used.

The ability of the nitrated κ -caseins (N- κ) to stabilize α_{s1} -casein, and of the nitrated α_{s1} -casein (N- α_{s1}) to be stabilized by κ -casein, was determined in the presence of 0.02 M CaCl_2 according to the procedure described by Zittle (8), using pH 7.0 imidazole buffer (0.01 M).

Results and Discussion

The reaction of κ - and α_{s1} -caseins with tetranitromethane was carried out at various molar ratios to obtain caseins having different levels of tyrosine modification. Using molar ratios (TNM/Tyr) ranging from 0.22 to 4.42, nitration levels between 40 and 100% were obtained (Table 1). As can be seen from Table 1, complete nitration of α_{s1} - and κ -caseins was obtained with approximately a 2:1 ratio of tetranitromethane. This contrasts markedly with the nitration of most other proteins studied (4), in that less than complete nitration was obtained even though substantially higher ratios of tetranitromethane (50-100:1) were used. From nitration work with other proteins (4), it was found that the number of tyrosine residues nitrated in the absence of denaturants appeared to approximate the number of residues known to be "free" and exposed, as determined

TABLE 1. Nitration of κ - and α_{s1} -casein.

	Molar ratio TNM/ Tyr	3-NO ₂ - Tyr/ mole protein ^a	Tyro- sine nitrated (%)
κ -Casein (8 Tyr/mole)	0.22	3.2	40
	0.56	3.8	47
	1.11	4.7	59
	1.67	5.7	71
	2.21	7.4	93
	4.42	7.7	96
α_{s1} -Casein (12 Tyr/mole)	0.56	4.5	38
	1.11	7.5	63
	2.22	12.0	100

^a Determined by difference between original tyrosine content and remaining unmodified tyrosine.

by other reagents. Thus, in proteins devoid of major secondary or tertiary structures, all of the tyrosines should be reactive to tetranitromethane. The essentially complete tyrosine nitration obtained with both caseins at low molar ratios of reagent reflects the accessibility of the tyrosine residues and leads to the conclusion that neither α_s - nor κ -casein contains buried tyrosine residues. Thus, the data support the idea that little, if any, major secondary or tertiary structure is to be found in these caseins, confirming the optical rotatory dispersion studies (2) which ruled out the existence of any appreciable degree of alpha helix formation for these caseins in aqueous media. This, however, should not preclude the possible existence of some other specific structural form for these caseins in solution.

Although the nitrated caseins appeared to have normal solubility properties, they differed from the native proteins in several respects. The fully nitrated κ - and α_s -caseins were not able to penetrate the 8% acrylamide gel pores, as indicated by impacting of proteins in the gel slot during attempts at electrophoresis. The failure to penetrate the acrylamide gel even in the presence of 2-mercaptoethanol and 7 M urea, at both acid and alkaline pH's, indicates a high degree of aggregation, the nature of which is not understood but undoubtedly arises as a result of tyrosine nitration. Crosslinking has been reported in a number of proteins following reaction with tetranitromethane (1).

N- κ -Casein remained sensitive to the action

of rennin as was determined by the release of macropeptide into a 12% trichloroacetic acid supernatant. Flocculation of the para- κ -casein, however, decreased with increasing levels of nitration; the fully nitrated para- κ was totally soluble at neutral pH. The eight tyrosine residues of κ -casein are found in the para- κ portion of the molecule. The failure to produce a typical para- κ -casein clot can be attributed to altered aggregation properties arising from tyrosine nitration.

The stabilizing ability of the N- κ -caseins was measured by the solubilization of α_s -casein in the presence of 0.02 M CaCl₂. Results of this determination for N- κ -caseins having from three to eight nitrated tyrosines are shown in Figure 1. From the curves shown, it is evident that nitration of up to four tyrosine residues does not alter the protective ability of the modified κ -caseins when compared with that of the native protein. However, a very large decrease in stabilizing ability accompanies nitration of the fifth tyrosine residue, with further loss accompanying continued nitration; total loss of stabilizing ability accompanies full nitration (eight residues). The data indicate that a critical level is reached with the nitration of

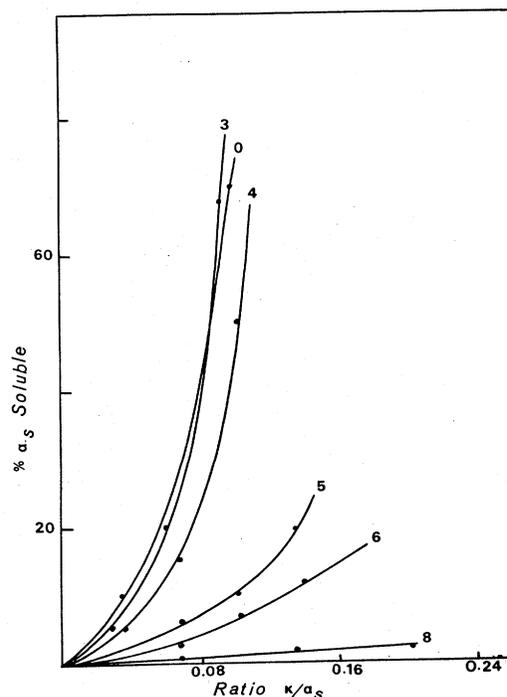


FIG. 1. Stabilization of α_s -casein by nitrated κ -casein. Numerals indicate number of tyrosine residues nitrated (κ -casein has total of 8 Tyr).

four residues. This level of nitration may be restricted to four specific tyrosyl residues not directly involved in formation of the critical κ/α_s complex. Nitration beyond four residues may involve those necessary for the formation of the complex or may simply reflect a level of tyrosine nitration at which sufficient change in ionic character is produced to prevent the normal protein interactions from occurring. Further studies such as peptide mapping will be necessary to determine whether the initial nitration occurs on specific tyrosine residues or whether it occurs in a random fashion among the various tyrosine residues.

The calcium sensitivity of the N- α_s casein was found to have decreased slightly with increased levels of nitration, but essentially complete precipitation of the protein was obtained at 0.02 M CaCl₂.

The retention of the calcium sensitivity by the N- α_s casein led us to question whether this modified protein was still able to enter into a protective complex with κ -casein and thus become insensitive to calcium ions. Consequently, the solubility of N- α_s -casein at three levels of nitration was determined in the presence of κ -casein (ratio 10:1, α_s/κ) and a calcium concentration of 0.02 M. The data obtained are presented in Table 2, together with data for unmodified α_s -casein. For all three levels of nitration, the amount of N- α_s -casein solubilized by κ -casein was no greater than the amount of native α_s -casein dissolved by 0.02 M calcium in the absence of κ -casein. It is thus apparent that tyrosine nitration changes molecular properties of α_s -casein such as tyrosine nitration alters κ -casein, and that the changes are sufficient to prevent formation of a calcium-insensitive α_s/κ -casein complex.

The mechanism leading to the formation of the α_s/κ complex has been the subject of much speculation. Application of tetranitromethane, a highly specific reagent capable of differentiating between "free" and "buried" tyrosine residues, should be helpful in elucidating the

forces involved in formation of this complex. Although α_s/κ -complexes are presumed to exist in the absence of calcium ions, it was desirable to form the initial complex in the presence of calcium, thus permitting its participation in the formation of the complex. A 10:1 (α_s/κ) complex was formed in the presence of 0.02 M calcium chloride and reacted with tetranitromethane at a ratio of 2.2:1 for 2 hr. No destabilization of the complex resulted from the nitration reaction. Amino acid analysis of the hydrolyzed complex revealed that all of the tyrosine residues were nitrated. This finding was unexpected, since a recent model of the casein micelle (6) is based on a core of α_s -casein forming a focal point around which the protective κ -casein molecules are oriented. One would anticipate that such a structure would not allow a reagent such as tetranitromethane full access to the tyrosine residues. The above findings, therefore, suggest that the structure of the α_s/κ -complex is one in which major portions of the molecules are accessible to the approach of reagents such as tetranitromethane or that all tyrosine residues are located in the external hydrophilic region of the complex.

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TABLE 2. Stabilization^a of nitrated α_s -caseins.

Tyrosine nitrated	Soluble
(%)	(%)
0	90
38	13
63	18
100	18

^a 10:1 α_s/κ ratio.

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