Post-translational phosphorylation affects the IgE binding capacity of caseins

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Abstract IgE response specific to those molecular regions of casein that contain a major phosphorylation site was analyzed using native and modified caseins and derived peptides. This study included (i) the naturally occurring common variants A1 and A from β- and αs2-caseins, respectively, which were purified in the native form and then dephosphorylated, (ii) a purified rare variant D of αs2-casein which lacks one major phosphorylation site, and (iii) the native and dephosphorylated tryptic fragment f(1–25) from β-casein. Direct and indirect ELISA using sera from patients allergic to milk showed that the IgE response to caseins is affected by modifying or eliminating the major phosphorylation site.

Key words: Allergy; IgE; Casein; Phosphorylation site

1. Introduction

The functional properties of proteins are modulated by numerous post-translational modifications that act directly or influence the secondary–tertiary folding of the molecule. Such post-translational processes also influence the immunoreactivity of proteins and especially of allergens, which are mostly glycoproteins, where the deglycosylation lowers immunoreactivity [1,2]. Glycosylation is not the only post-translational event affecting protein allergenicity [3].

Much effort has been devoted to identifying the domains responsible for the allergenic potential of allergens. To localize mainly the linear epitopes, a logical approach consists in using peptides, either prepared by chemical synthesis or resulting from enzymatic hydrolysis. Recently, recombinant allergens subject to deletions or substitutions were obtained for various molecules, including calcium binding proteins, and proved helpful in identifying IgE binding domains (linear and/or conformational epitopes) for pollen [4–6] and peanut [7]. However, the IgE immunoreactivity of recombinant allergens may be lower than that of natural molecules, probably due to a deficiency in the post-translational events [8].

In cow's milk, whole casein accounts for 80% of the total protein content and presents an interesting post-translational feature, i.e. phosphorylation of some residues involved in Ca^{2+} binding. This occurs in three out of four of its constituents (i.e. β-, αs1- and αs2-casein). These caseins can exhibit different degrees of phosphorylation, the phosphorylated seryl residues being mostly grouped together in major phosphorylation sites.

In previous studies, we have demonstrated that whole casein acts as a potent allergen in cow's milk allergy and that each of the different casein fractions (β-, αs1-, αs2- and κ-casein) can induce a marked IgE response [9]. Co- and/or cross-sensitization to the different caseins was observed in most cow's milk allergic patients, suggesting that both distinct and common epitopes may occur on these proteins. These different caseins, encoded by four different genes, have very few amino acid sequence homologies [10,11]. A rare homologous sequence present in the three Ca^{2+} binding caseins is the highly conserved major site of phosphorylation, i.e. SerP-SerP-SerP-Glu-Glu corresponding to sequences (66–70) of αs1-casein, (17–21) of β-casein, (8–12) and (56–60) of αs2-casein [12].

Modification of such a well-characterized site in casein thus appeared to be an original way to characterize an important epitope. This strategy benefits from the natural occurrence in milk proteins of different genetic variants with substitution(s) and/or deletion(s) [13].

The influence of phosphorylation on the immunoreactivity of the Ca^{2+} binding caseins was studied using either IgG from immunized animals or IgE from sera of allergic patients as a marker. The reactivity of these Ig isotypes was studied on various forms of purified bovine caseins (native, enzymatically dephosphorylated, or constitutively deleted for the phosphorylation site), and on the native and dephosphorylated form of the β-casein fragment 1–25 which comprises the cluster sequence of phosphorylated serine residues [14].

2. Materials and methods

2.1. Sera

Human sera from patients with clinical symptoms of cow's milk allergy were obtained from hospital banks of sera. These sera were tested for specific anti-bovine β- and αs2-casein IgE using the original enzyme immunoassay (EIA) developed in our laboratory [9]. Fifty-three sera with a large range of specific IgE were thus selected for the study. The concentrations of specific IgE ranged from 0.1 to 330 IU/

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Abbreviations: Ig, immunoglobulin; ELISA, enzyme-linked immunosorbent assay; EIA, enzyme immunoassay; E/S, enzyme/substrate ratio; RP-HPLC, reversed phase high performance liquid chromatography; TFA, trifluoroacetic acid; HSA, human serum albumin; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography

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ml (i.e. 0.2–750 ng/ml). Eight control sera were obtained from non-allergic human volunteers.

2.2. Purification of different variants from αs2- and β-casein

Variant A1 from β-casein and variants A and D from αs2-casein were prepared from fresh milk samples of cows that were homozygous for these variants. These cows were identified and selected among genetically typed individuals listed in the records of the herd book of the Holstein (for β-casein variant A1 and αs2-casein variant A) and Tarine breeds (for αs2-casein variant D). Whole casein containing αs2-casein variant D was obtained by precipitation at pH 4.6 and selectively fractionated by addition of 40% propanol-1 at 4°C. After 2 h of slight agitation, the mixture was centrifuged at 17 000 rpm for 30 min at 4°C. The supernatant was discarded and the precipitate was solubilized in 6 M urea, dialyzed against water adjusted to pH 7.0 with NaOH, and lyophilized. Purification was achieved using a MonoQ anion exchange system [9]. αs2-Casein variant A and β-casein variant A1 were purified by selective precipitations followed by anion exchange chromatography as previously described [9].

2.3. Dephosphorylation of β- and αs2-casein

Native β-casein variant A1 was dissolved (10 mg/ml) in 50 mM sodium acetate buffer pH 5.8 (1% w/v) before adding potato acid phosphatase at a 1/500 (w/w) enzyme/substrate (E/S) ratio [15]. αs2-Casein variant A (15 mg/ml) was reacted in 50 mM sodium citrate buffer pH 7.5 with the same enzyme using a 1/30 (w/w) E/S ratio. The reaction was allowed to proceed for 20 h at 30°C for β-casein and for 30 h at 37°C for αs2-casein in a dialysis bath to dilute inorganic phosphate, which could inhibit the enzyme. In both cases, the pH of the solution was adjusted to 10 to inactivate the enzyme before dialysis against water (adjusted to pH 7.4 with 0.1 M NaOH) and lyophilization. Dephosphorylated forms were isolated by anion exchange chromatography [9].

2.4. Purity of the native and dephosphorylated caseins

Purity of the bovine caseins was assessed by reverse phase high performance liquid chromatography (RP-HPLC) using a C4-Vydac 250×4.6 mm column (Shandon). Elution was performed with a 40 min linear gradient from 25 to 45% of buffer B (acetonitrile–0.04% trifluoroacetic acid (TFA)) in buffer A (H_2O–0.1% TFA) at a flow rate of 1 ml/min. Detection was at 220 nm. The purity and immunoreactivity of the different native and dephosphorylated caseins were investigated by enzyme-linked immunosorbent assay (ELISA) as previously described [9,16]. Specific animal antisera were raised against αs1-, αs2-, κ-caseins and β-lactoglobulin in rabbit and against β-casein in guinea pig. Antisera were obtained by immunizing animals with each purified native protein. Since caseins are poorly immunogenic, they were first cross-linked using glutaraldehyde. Each animal was then first injected with 50 μg of each casein polymer using complete Freund's adjuvant, then boosted every 2 weeks in the presence of incomplete Freund's adjuvant.

2.5. Preparation and purification of the native and dephosphorylated fragment f(1–25) from β-casein

A three-step procedure consisting of solid phase extraction (anion exchange cartridges, QMA), RP-HPLC (C18) and anion exchange chromatography (QMA) was used to isolate casein phosphopeptide f(1–25)4P from a tryptic hydrolysate of bovine casein as described in [17]. An immobilized alkaline phosphatase (F7m column, Mobitec) was used for the preparation of the dephosphorylated fragment [17]. Purity of the isolated peptides was checked by analytical RP-HPLC (C18) and amino acid analysis [17], as well as by determination of the N-terminal group by the dansyl chloride method according to [18].

2.6. Phosphate determination

Phosphate groups were released from caseins by acid and heat treatments [19]. Phosphate groups were determined as inorganic phosphates by a colorimetric method in a microtiter plate assay [20] based on the formation of a complex between phosphomolybdate and malachite green using previously described reagents [21,22]. The phosphorylation of caseins was compared with a standard curve obtained using known amounts of phosphoserine.

2.7. Specific IgE ELISA

Specific IgE ELISA was performed as previously described [9,16]. Briefly, 96-well microplates (Nunc) were coated with 200 μl of purified protein or peptide (5 μg/ml) in 0.025 M ethylenediaminetetraacetic acid (EDTA) carbonate buffer pH 9.3. The plates were incubated for 18 h at 4°C and further saturated for 18 h at +4°C with 300 μl of EIA buffer (0.1 M potassium phosphate buffer pH 7.4 containing 0.1% human serum albumin (HSA), 0.4 M NaCl, 0.001 M EDTA and 0.01% sodium azide). Just before use, the plates were washed in an automatic plate washer (Titertek) with 0.01 M potassium phosphate buffer pH 7.4 containing 0.05% Tween 20.

Sera from allergic patients (50 μl) were dispensed per well at an appropriate dilution. After reaction for 24 h at 20°C followed by extensive washing, 50 μl/well of a solution of an anti-human IgE mouse monoclonal antibody (mAb) (i.e. clone BS17) labelled with acetylcholinesterase (AChE) was added. After a further 18-h incubation at 20°C, the plates were washed before revealing bound AChE by addition of 200 μl/well of Ellman reagent [23]. The enzymatic reaction was monitored at 414 nm using a Titertek plate reader.

Eight sera from non-allergic patients were used as negative controls. Non-specific binding was also evaluated by dispensing positive sera into HSA-coated wells.

Specific IgE were quantified as already described [16]. No interference with specific IgG was observed. The detection limit of specific IgE, calculated as the concentration corresponding to the average non-specific binding plus 3 standard deviations, was approximately 0.1 IU/ml (i.e. 0.23 ng/ml).

2.8. Indirect IgE ELISA

According to the amount of specific IgE measured in individual patients, sera were selected for complementary competitive ELISA. Immune or control sera (50 μl) were first incubated for 6 h by mixing at 20°C with 50 μl of increasing concentrations of inhibitors (ranging from 0.001 to 4 nmol/ml for caseins and 0.01 to 10 μg/ml for peptides) in EIA buffer. HSA was used as a control to evaluate the non-specific binding at high inhibitor concentrations. Aliquots (50 μl) of the mixture were then added to the antigen-coated wells. The different steps of the assay were then performed as described above.

3. Results

3.1. Purification and characterization of the dephosphorylated β- and αs2-caseins

Enzymatic dephosphorylation of purified αs2-casein variant A and β-casein variant A1 was checked by fast protein liquid chromatography (FPLC). In both cases, numerous peaks were observed. Late eluting minor peaks corresponding to native caseins (namely αs2- and β-casein) were detected. Inversely, the major peak corresponding to the most dephosphorylated form was poorly retained by anion exchange chromatography. In each case, the major peak was collected and characterized.

Absence of contamination of the dephosphorylated caseins by native forms was checked by RP-HPLC. Chromatographic patterns of both the native and the isolated dephosphorylated forms of the β- and αs2-caseins are shown in Fig. 1A,B. Each of the dephosphorylated caseins was eluted at a different retention time than its native counterpart. Due to the elimination of the phosphate groups, the dephosphorylated fractions were more hydrophobic and thus had higher retention times than the native forms. However, dephosphorylated αs2-casein showed a minor peak which could correspond to the native form. This possible contamination was estimated to be less than 5%.

Phosphate determination revealed 1.6 nmol of phosphate for 0.32 nmol of native β-casein while no phosphate group was detected after dephosphorylation. On the other hand, a significant concentration of phosphate (two phosphate groups per molecule) was found in the purified dephosphorylated αs2-casein. This could result either from a contamination by the native form (see above) or from a resistance of some

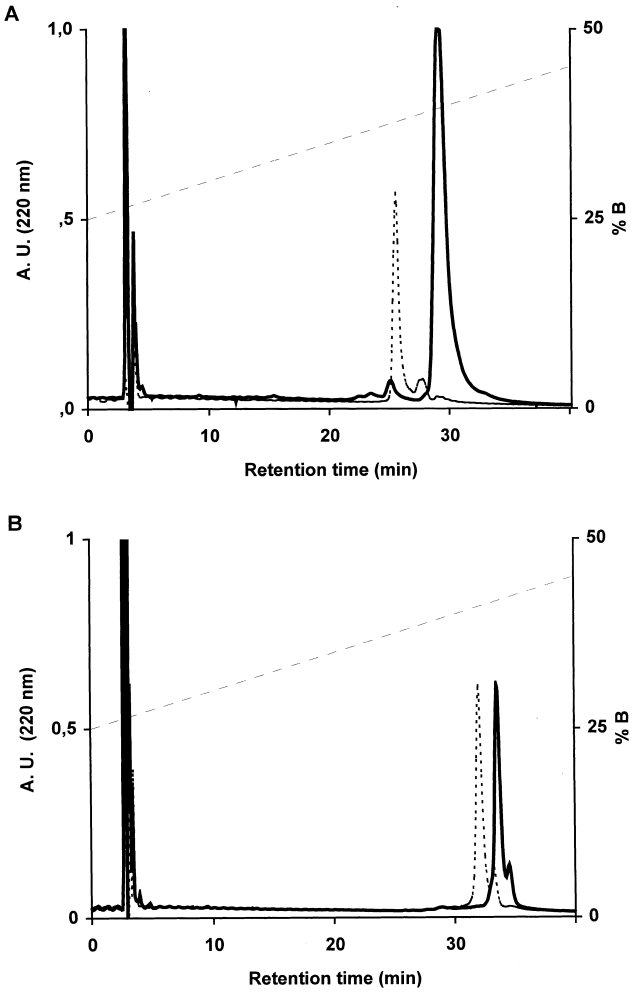

Fig. 1. RP-HPLC chromatograms of the different purified caseins: (A) native and dephosphorylated αs2-casein (in bold); (B) native and dephosphorylated β-casein (in bold). Detection at 220 nm is expressed in absorbance units (A.U.). Concentration of buffer B is expressed in % (%B).

groups to enzymatic degradation. However, these two residual phosphate groups represent less than 20% of the initial phosphate content of the native molecule, thus demonstrating the efficient action of the phosphatase.

3.2. Purification and characterization of the deleted αs2-casein variant D

After selective precipitation, FPLC revealed marked heterogeneity of the deleted αs2-casein (data not shown). The major peak was collected and analyzed by RP-HPLC, demonstrating the absence of contamination by the other caseins (i.e. κ-, αs1- and β-caseins). These results were confirmed by immunoanalytical methods, using specific antisera raised against each of these different caseins.

3.3. Purification and characterization of native and dephosphorylated fragment f(1–25) from β-casein

The three-step procedure separates phosphopeptides and non-phosphorylated peptides [17]. The yield of β-casein f(1–25)4P from 240 mg of lyophilized soluble fraction of a tryptic hydrolysate of bovine casein was 3.3 mg. After enzymatic dephosphorylation of 18.1 mg of β-casein f(1–25)4P collected from several purification runs, the yield of the dephosphorylated fragment β-casein f(1–25)0P was 11.7 mg. The peptides were clearly identified by determination of amino acid composition, phosphorus content and N-terminal end group.

3.4. IgG binding capacity of the different caseins

Competitive ELISA was performed using specific polyclonal anti-β- and anti-αs2-casein antibodies raised against each of the purified caseins. No difference was observed between the native and dephosphorylated forms of β-casein in the inhibition of the binding of specific guinea pig anti-β-casein polyclonal antibodies to immobilized native β-casein.

The same phenomenon was also observed in inhibition studies with αs2-casein, using native αs2-casein-coated plates. Dephosphorylated as well as deleted forms of the protein appeared as efficient as the native variant A counterpart in inhibiting the binding of specific rabbit anti-αs2-casein polyclonal antibodies. In the case of β- and αs2-caseins, all the inhibition curves obtained with the different forms of the proteins were identical.

3.5. IgE binding capacity of native and dephosphorylated β-casein and fragments

Using direct ELISA in microplates coated with either native or dephosphorylated bovine β-casein, 53 sera from patients allergic to β-casein were analyzed for specific IgE against both forms of the protein. In all sera, the IgE response to dephosphorylated β-casein was lower than that to native β-casein. In particular, four sera showed no IgE response to dephosphorylated β-casein while 15 others exhibited a twofold lower IgE response. In 15 sera, the IgE response to dephosphorylated β-casein was 25–40% lower than to the native molecule. For the last 19 sera, the intensity of the IgE response to dephosphorylated β-casein was 80–100% of the response to native β-casein.

Competitive ELISA was performed on six sera selected

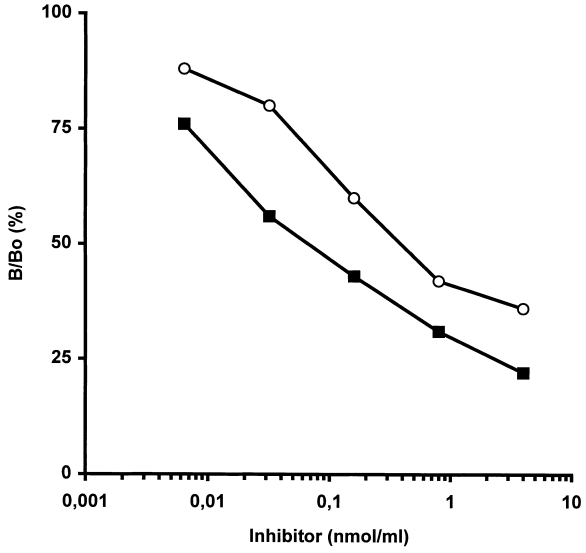

Fig. 2. Indirect ELISA. Inhibition of the binding of specific anti-β-casein (β-CN) IgE to immobilized native β-CN by phosphorylated (■) and dephosphorylated β-CN (○). B and Bo represent the bound enzyme activity measured in the presence or absence of inhibitor, respectively. The results are expressed in terms of B/Bo as a function of the logarithm of the dose.

from those showing a high IgE response to native β-casein. The dephosphorylated form always significantly inhibited IgE binding to native β-casein. For two sera, similar inhibition curves were obtained for native and dephosphorylated β-casein. However, for the four other sera, dephosphorylation induced a loss of recognition. Concentrations of dephosphorylated β-casein up to five-fold higher than those of the native form were needed to obtain the same inhibition (i.e. 50%) of the binding, as shown in Fig. 2 for one representative serum.

Moreover, the same observations were made on the isolated amino-terminal part of the protein using four sera which exhibited strong IgE binding to immobilized fragment f(1–25) of β-casein. IgE binding inhibition by dephosphorylated fragment f(1–25) was greatly limited when compared to the phosphorylated peptide. This is shown in Fig. 3 for one serum for which competition was totally absent using the dephosphorylated fragment.

3.6. IgE binding capacity of the different forms from αs2-casein

Direct ELISA was used to test 29 sera positive to variant A of αs2-casein for their IgE response to immobilized variant D of αs2-casein. Absence of IgE response to deleted αs2-casein was only observed for one serum. Comparison of IgE levels obtained with both variants revealed a great heterogeneity of the response. For most sera, the recognition of these two forms was not equivalent. However, as many sera exhibited a higher response to the variant A as to the deleted variant D. This was particularly evident when the IgE response to deleted αs2-casein was expressed as a percentage of the IgE response to the non-deleted form, showing a Gaussian distribution with a median close to 100%.

Indirect ELISA using immobilized variant A was used to test the immunoreactivity of the different forms of αs2-casein. IgE binding could be partially or completely inhibited by each of the three different forms of αs2-caseins. However, these experiments show a range in the efficiency of inhibition obtained with these three forms. The non-deleted variant A always appears a better competitor than deleted variant D. As far as totally dephosphorylated αs2-casein is concerned, huge amounts are required to inhibit the IgE binding. This was particularly striking with four sera where 6–30-fold more αs2-casein variant D and 50-fold more dephosphorylated αs2-casein than variant A was necessary to reach the same binding inhibition (IC_{50}). Typical inhibitions are shown in Fig. 4.

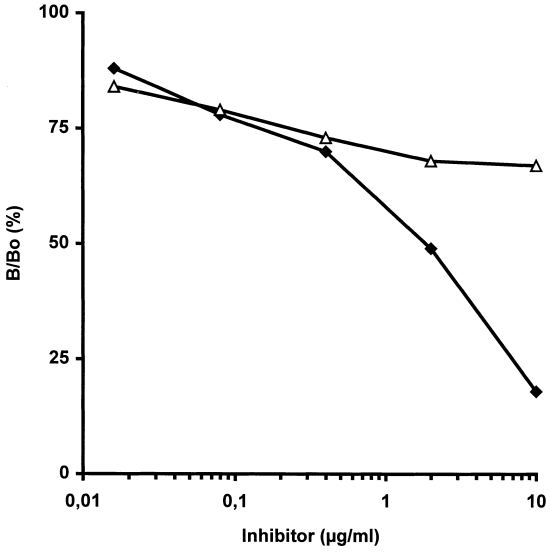

Fig. 3. Indirect ELISA. Inhibition of the binding of specific anti-β-casein (β-CN) IgE to immobilized fragment f(1–25) from native β-CN by phosphorylated (■) and dephosphorylated (△) fragments f(1–25) from β-CN. B and Bo represent the bound enzyme activity measured in the presence or absence of inhibitor, respectively. The results are expressed in terms of B/Bo as a function of the logarithm of the dose.

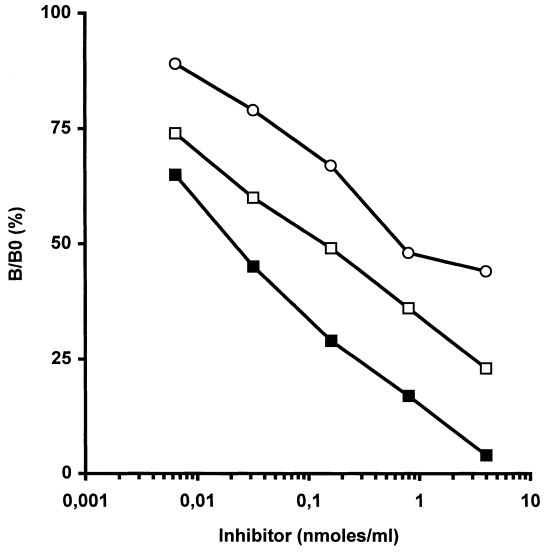

Fig. 4. Indirect ELISA. Inhibition of the binding of specific anti-αs2-casein (αs2-CN) IgE to immobilized native αs2-CN variant A by phosphorylated αs2-casein variant A (■), deleted αs2-casein variant D (□) and dephosphorylated αs2-casein variant A (○). B and Bo represent the bound enzyme activity measured in the presence or absence of inhibitor, respectively. The results are expressed in terms of B/Bo as a function of the logarithm of the dose.

4. Discussion

Small et al. [24] found that 80% of the phosphorylation sites were in β-turn predicted regions. Major phosphorylation sites in bovine caseins are hydrophilic and correspond to a loop structure which could include only β-turn as regular secondary structure [25]. The occurrence of β-turn in a phosphopeptide from casein was also described by Huq et al. [26], while Wahlgren et al. showed that phosphopeptide from β-casein is highly flexible [27]. These differing characteristics of primary and secondary structures were in accordance with predictions of immunogenic and/or antigenic domains [28–30]. Furthermore, the immunoreactivity of caseinophosphopeptides could result from their liberation during intestinal proteolysis and their resistance to further degradation by digestive enzymes [31].

We used genetically deleted variants and chemically modified forms of caseins to evaluate the influence of post-translational modifications, such as phosphorylation, on the immunoreactivity of the caseins. Potato acid phosphatase was used to dephosphorylate β- and αs2-casein as previously described for β- and αs1-casein [15,32,33]. Total dephosphorylation of αs2-casein was more difficult. Our preliminary studies showed that yeast alkaline phosphatase was inefficient and SDS–

PAGE demonstrated the presence of degradation products (data not shown). We therefore decided to dephosphorylate αs2-casein with acid phosphatase under drastic conditions: pH 7.5 to avoid precipitation of the casein [34] and an unusual E/S ratio to compensate for the reduced enzyme activity at this pH [32].

As expected, dephosphorylated αs2- and β-casein eluted more rapidly in FPLC anion exchange chromatography than the native forms [35]. Dephosphorylated forms can also be distinguished from native caseins by RP-HPLC analysis, where the loss of hydrophilic groups leads to stronger interactions [15,36]. This was also verified for variant D of αs2-casein which lacks sequence 51–59, which includes a major phosphorylation site [37,38]. However, chromatographic behavior during anion exchange chromatography was more complex. The enriched fraction of αs2-casein variant D gave a heterogeneous chromatographic pattern independently of contamination with other caseins, perhaps due to the presence of different isoforms of this variant.

Using specific animal antisera raised against the isolated whole native molecule of each of the pure casein fractions, we observed no significant modification of the immunoreactivity of β- and αs2-casein, either after dephosphorylation or after deletion of a major phosphorylation site. This demonstrated that no drastic structural change occurs after removal of phosphate groups and that most of the polyclonal antibodies specifically raised against pure αs2- and β-casein were not directed against the major phosphorylation site and were not affected by the phosphorylation of serine residues. Moreover, the immunoreactivity of casein seems unaffected by any conformational change induced by reducing the phosphorylation.

This finding raised the question of the possible involvement of such a highly conserved sequence as a common cross-reactive epitope for IgE from patients allergic to caseins.

Interestingly, the same four human sera used in the two sets of experiments involving β- and αs2-casein gave similar results. Dephosphorylation or deletion led to decreased recognition of each casein by the IgE of these four sera. Comparison of the results obtained in the direct vs. competitive inhibition ELISA suggests that dephosphorylation very likely lowers the affinity of IgE for caseins rather than the specific IgE titer itself. Without underestimating the possible conformational change induced by these modifications, it is very likely that at least part of the anti-casein IgE is directed against domains comprising a major phosphorylation site. This is supported by the fact that peptide f(1–25) from β-casein was less well recognized by IgE after dephosphorylation. High immunoreactivity of a major phosphorylation site then appears to be a potential explanation for the cross-reactivity between caseins (as previous studies have suggested) [9]. This agrees with the work of Otani et al. [39] demonstrating that polyclonal anti-αs1-casein antibodies cross-react with β-casein and that the antigenicity of αs1-casein was lost after dephosphorylation. Furthermore, cross-reactivity between αs2- and αs1-caseins was also reported with an anti-αs2-casein monoclonal antibody [40]. In the same study, binding of this monoclonal anti-αs2-casein antibody to αs2-casein partially depended on its degree of phosphorylation.

Although the involvement of a major phosphorylation site as an allergenic epitope could lead to IgE cross-reactivity between caseins, the possible extension of this cross-reactivity to other proteins containing this domain remains to be investigated. On the other hand, the potential allergenicity of caseinophosphopeptides should be taken into account when considering their biological interest and possible use in functional foods [41].

In conclusion, the major phosphorylation site appears to be an important allergenic epitope in caseins, and its alteration will affect the allergenicity of the caseins. In the case of milk allergy, milk protein variants, especially deleted isoforms which naturally exist in cow's and goat's milk, would be valuable in epitope mapping and investigation of the impact of structural post-translational modifications on allergy.

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