Phosphorylation of β-Lactoglobulin under Mild Conditions

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β-Lactoglobulin was phosphorylated with different molar ratios of $POCl_3$/protein (20, 40, and 80) in the presence of either triethylamine or hexylamine (6 mol of base/mol of $POCl_3$) in aqueous conditions. Urea–PAGE, SDS–PAGE, and isofocusing patterns showed increasing negative charges and little cross-linking on β-lactoglobulin molecule. The circular dichroism spectra showed that phosphorylation has disordered the secondary structure of β-lactoglobulin. The phosphorylation-induced structural and electrostatic changes have displaced modified β-lactoglobulin solubility minima toward lower values.

Keywords: *Phosphorylation; β-lactoglobulin*

INTRODUCTION

β-Lactoglobulin is the major component of whey proteins and differs from caseins by the absence of post-translational modifications, especially phosphorylation (Swaisgood, 1982). The primary and secondary structures of this protein are known (Timasheff et al., 1966a,b; Townend et al., 1967; Braunitzer et al., 1972; McKenzie et al., 1972; Preaux et al., 1979; Casal et al., 1988). The solubility of this protein is peculiar and quite different from that of casein as its minimal solubility in the isoelectric range is still higher than 60% (Kinsella, 1983). This well-defined protein can serve as an ideal substrate for studying protein modifications, especially phosphorylation.

Phosphorylation of proteins has been used to modify several proteins (Willimitzer and Wagner, 1975; Sung et al., 1983; Matheis and Whitaker 1984a,b, 1987; Chobert et al., 1987, 1989a; Huang and Kinsella, 1986a,b, 1987). Although β-lactoglobulin was previously phosphorylated (Woo et al., 1982; Woo and Richardson, 1983), the conditions used in those studies were rather drastic and involved the use of very high molar ratios (2000–4000) of phosphorus oxychloride ($POCl_3$). The aim of this work was to optimize the phosphorylation of β-lactoglobulin using low molar ratios of $POCl_3$ (20–80) and modifying reaction conditions.

MATERIALS AND METHODS

Materials. β-Lactoglobulin (a mixture of variants A and B) was purchased from Laiteries Triballat, Noyal/Vilaine, France, and purified according to the procedure of Mailliart and Ribadeau Dumas (1988). All other chemicals were of reagent grade.

Phosphorylation. The general procedure of Sitohy et al. (1994) was followed under the following conditions. β-Lactoglobulin was dissolved in water at a concentration of 1.25% and placed in an ice bath under continuous stirring. Amounts of $POCl_3$ equivalent to 20, 40, and 80 molar ratios (MR) (moles of $POCl_3$/mole of protein) were dissolved in carbon tetrachloride at a concentration of 20% and added dropwise regularly to the protein solution during 30 min. Equivalent amounts of the organic base triethylamine or hexylamine were added simultaneously to the reaction mixture. The amount of base required for the reaction was calculated as 6 mol of base/mol of $POCl_3$ unless otherwise stated. The reaction medium was then left for 10 min for complete separation of the organic from the aqueous layer, which was recovered and submitted to extensive dialysis against distilled water for 4 days with several changes. The purified phosphorylated proteins were lyophilized and kept at −20 °C until analysis. Covalently bound phosphorus was determined in the modified proteins according to the procedure of Bartlett (1959) as modified by Sitohy et al. (1994).

Electrophoresis. Urea–PAGE was carried out according to the procedure described by Chobert et al. (1989b). SDS–PAGE was performed according to the method of Laemmli (1970) on 15% acrylamide running gel and 3.2% acrylamide stacking gel. Electrofocusing was performed in the pH range 3–7 on ready-to-use isoelectric focusing gels (FMC, Rockland, MD). A marker kit of p*I* in the range 3.5–7.35 was used (Pharmacia LKB, Uppsala, Sweden).

Reversed-Phase HPLC. Fifty microliters of protein samples (1 μg/μL) was injected into a Nucleosil C_{18} column (SFCC, France) equilibrated in solvent A (0.15 M NaCl/HCl, pH 2.5), and the elution was obtained by using a linear gradient from solvent A to solvent B (60% acetonitrile and 40% solvent A) in 32 min. Both the column and the solvents were maintained at 40 °C. The flow rate was 1 mL/min, and the absorbance was read at 214 nm.

Circular Dichroism Spectroscopy. CD spectra of phosphorylated β-lactoglobulin solutions were measured on a Jobin Yvon Mark VI dichrograph, and data were recorded on-line using a personal computer. The quartz cells used had a path length of 1 mm in the far-UV spectra (185–250 nm). All spectra were taken at 20 °C using β-lactoglobulin concentration of 10 mM, and the results were expressed as molar ellipticity [Θ]. Phosphorylated β-lactoglobulin concentrations were determined spectrophotometrically using for the calculation molecular absorption coefficient $E_{280} = 17\,600\ M^{-1}\ cm^{-1}$. The method of Provencher and Glockner (1981) was used to estimate α-helix and β-sheet contents.

Protein Solubility. Protein solubility was determined in the native and phosphorylated β-lactoglobulin in the pH range 2–8 according to the procedure of Chobert et al. (1991).

RESULTS AND DISCUSSION

Extent of Phosphorylation. β-Lactoglobulin was phosphorylated in aqueous media with $POCl_3$ (MR = 20, 40, and 80) yielding 7, 8, and 9 and 2, 5, and 7 mol of P/mol of protein when prepared in the presence of triethylamine or hexylamine, respectively. It is clear that the extent of phosphorylation was directly proportional to the molar ratio of $POCl_3$. It can also be observed that with a low molar ratio of $POCl_3$ (MR =

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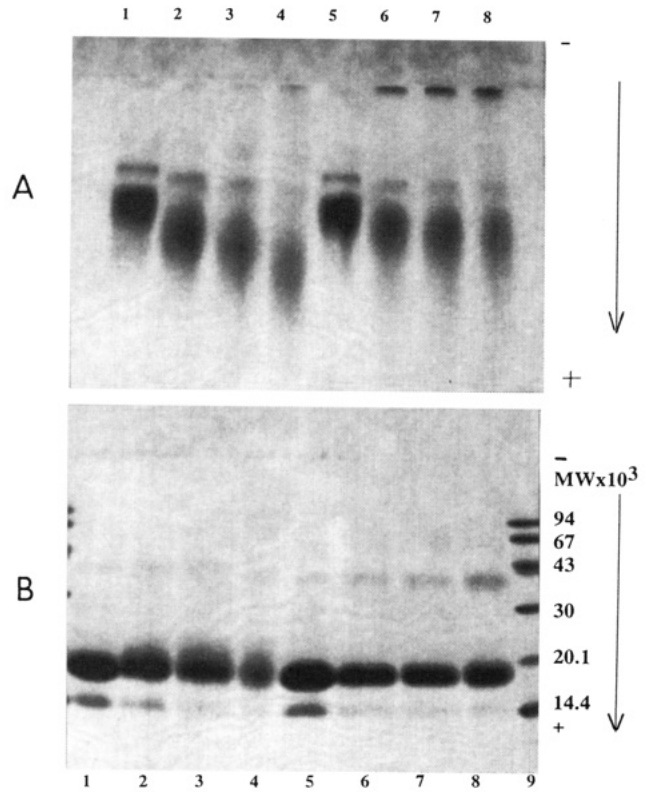

Figure 1. Electrophoretic patterns of phosphorylated β-lactoglobulin: (A) urea–PAGE; (B) SDS–PAGE. Samples 1 and 5 are native β-lactoglobulin. Samples 2, 3, and 4 are phosphorylated in the presence of TEA; the molar ratios $POCl_3$/protein were 20, 40, and 80, respectively. Samples 6, 7, and 8 are phosphorylated in the presence of hexylamine with the same molar ratios $POCl_3$/protein as samples 2, 3, and 4, respectively. Sample 9 is a molecular weight marker kit.

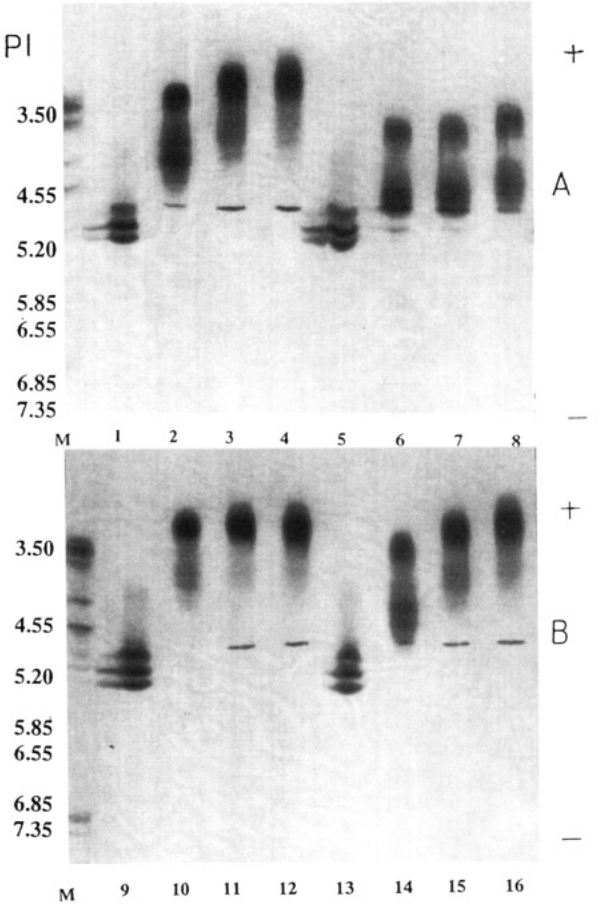

Figure 2. Isofocusing electrophoretic patterns of phosphorylated β-lactoglobulin: (A) samples 1–8, same as in Figure 1; (B) samples 9 and 13, native β-lactoglobulin; samples 10, 11, and 12, phosphorylated with $POCl_3$ (MR = 80) and TEA added during the reaction at ratios of 5, 5.5, and 6 mol of TEA/mol of $POCl_3$, respectively; samples 14, 15, and 16, phosphorylated with $POCl_3$ (MR = 80) and TEA added at the reaction start at ratios of 2.5, 5.0, and 5.5 mol of base/mol of $POCl_3$, respectively. Sample M, p*I* marker kit.

20) and in the presence of triethylamine (6 mol of TEA/mol of $POCl_3$) the extent of phosphorylation was quite significant (7 mol of P/mol of protein) and higher than that achieved by the conventional procedure using 50-fold higher molar ratios of $POCl_3$ in the presence of inorganic base (Woo et al., 1982). However, it was lower when hexylamine (6 mol/mol of $POCl_3$) was used. The relatively high extent of phosphorylation of β-lactoglobulin achieved by using low molar ratios of $POCl_3$ was because of the tertiary amine triethylamine, which deprotonates the protein amino groups without reacting with $POCl_3$ as the inorganic bases do. Moreover, using the reagents at quite low concentrations has not lead to subsequent dephosphorylation of the modified protein as is seen under the conventional conditions.

β-Lactoglobulin was also phosphorylated with constant molar ratio of $POCl_3$ and variable ratios of TEA, which was added either at the reaction start or during the reaction. When TEA was added at the reaction start at ratios of 2.5, 5.0, and 5.5 mol of base/mol of $POCl_3$, the resultant yields of phosphorylation were 7, 8, and 10 mol of P/mol of protein, respectively, showing a direct proportionality between the base concentration and the extent of phosphorylation. The same trend was noticed when TEA was added parallelly during the reaction at ratios of 5.0, 5.5, and 6 mol/mol of $POCl_3$ which gave rise to phosphorylation yields of 7, 8, and 9 mol of P/mol of protein, respectively. It is evident from these results that the concentration of the added base is essential for the reaction and that the maximal phosphorylation was only obtained when the concentration of the base in the reaction medium was about 5.5–6.0 mol/mol of $POCl_3$. This amount is sufficient to neutralize the protons released during the reaction. It can be also observed that the phosphorylation yield was slightly higher when the base was added at the reaction start.

Electrophoresis. The urea–PAGE electrophoregram of phosphorylated β-lactoglobulin is presented in Figure 1. It shows a gradual displacement of the bands corresponding to the modified samples toward the anode depending on the extent of phosphorylation. The most important migration is due to higher phosphorylation yields (Figure 1A). These results show the increase of the negative charges on the protein molecule resulting from the covalently bound phosphate groups. The SDS–PAGE electrophoretic patterns of the same samples (Figure 1B) show only a few cross-links, confirming the previous conclusion that most of the covalently attached phosphate groups did not react further. The use of an aqueous medium and a low molar ratio of $POCl_3$ may account for the absence of intensive cross-linking. This result is different from those obtained by Woo et al. (1982) that showed considerable dimerization and polymerization of the phosphorylated β-lactoglobulin.

The isofocusing patterns of the phosphorylated β-lactoglobulin samples presented in Figure 2 show a decrease of the isoelectric points (as compared with the native protein). This decrease is proportional to the extent of phosphorylation. This is also due to the increase of the negative charges of the modified protein molecule due to the covalently bound phosphate groups. Among the samples phosphorylated in the presence of TEA, the highly phosphorylated aliquots showed more

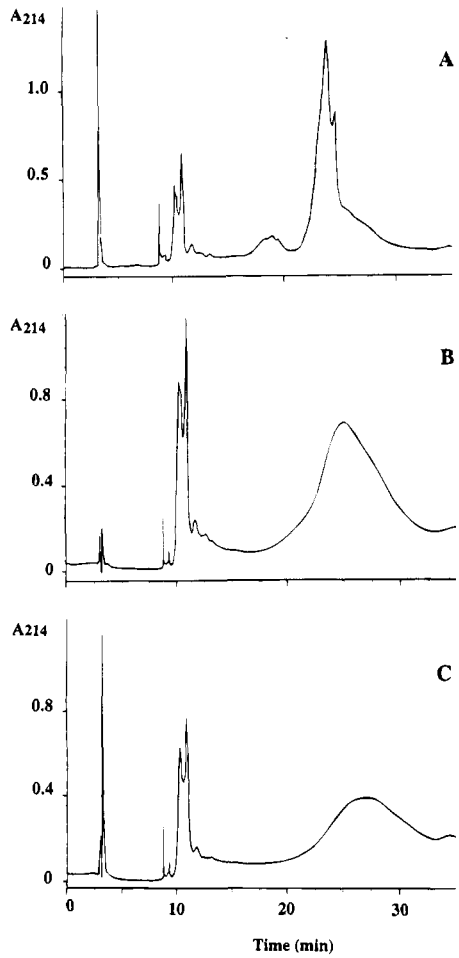

Figure 3. RP-HPLC profiles of phosphorylated β-lactoglobulin: (A) native β-lactoglobulin (β-LG); (B) β-LG (7 mol of P/mol of protein); (C) β-LG (9 mol of P/mol of protein).

homogeneous pattern (p*I* about 3), while the lower phosphorylated samples displayed a pattern with two distinctive areas of bands corresponding to p*I* 3.2 and 4.4 compared to p*I* of about 5.2 for the native β-lactoglobulin. This may show that the phosphorylation goes through an intermediate stage. This phenomenon is better seen in the less substituted samples phosphorylated in the presence of hexylamine. In conclusion, to obtain more homogeneous "phosphoproteins", the extent of phosphorylation should not be inferior to 9 mol of P/mol of protein.

This phenomenon was further examined in samples phosphorylated in the presence of different molar ratios of TEA while keeping a molar ratio of $POCl_3$ equal to 80 and when the base was added either regularly during the reaction time or once at the reaction start (Figure 2). It can still be observed that samples displaying lower phosphorylation separate into two distinctive p*I* areas. However, the phosphorylated proteins prepared by adding TEA regularly during the reaction were more homogeneous than those prepared when TEA was added at the reaction start. This may show that the reaction procedure may also affect the homogeneity of the phosphorylated products and not only the extent of modification.

Reversed-Phase HPLC Profiles. The RP-HPLC profiles of the phosphorylated β-lactoglobulin samples are presented in Figure 3. It can be observed that the peak corresponding to the native β-lactoglobulin was broader in the phosphorylated samples, which seems to be related to the extent of phosphorylation.

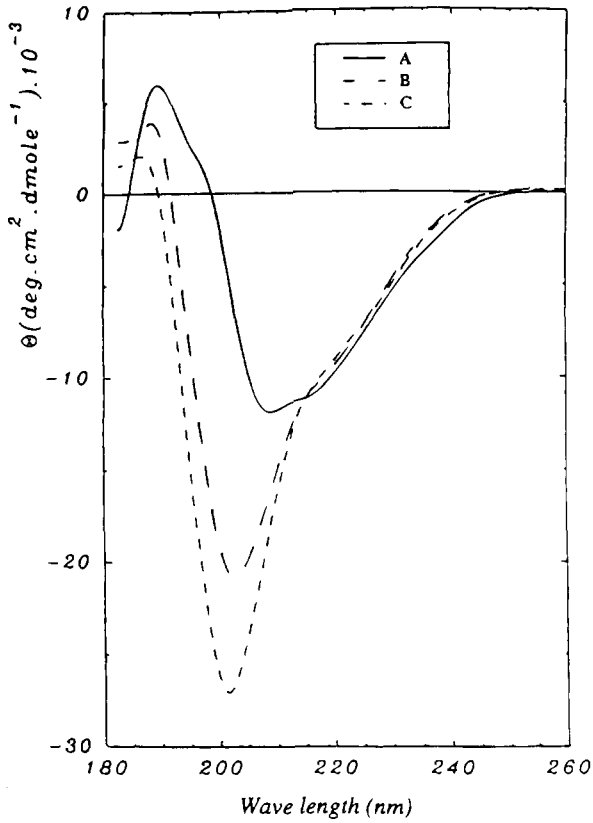

Figure 4. Far-UV circular dichroic spectra of phosphorylated β-lactoglobulin: (A) native β-lactoglobulin (β-LG); (B) β-LG (7 mol of P/mol of protein); (C) β-LG (9 mol of P/mol of protein).

CD Spectrum. The data in Figure 4 represent the CD spectra of native β-lactoglobulin and of two phosphorylated β-lactoglobulin samples (7 and 9 mol of P/mol of protein). It is evident that phosphorylation has changed the secondary structure of β-lactoglobulin. This may be generally characterized by an increase of the unordered at the expense of the ordered forms, especially that of α-helix which decreased from about 10% in the native protein to about 7 and 6% after phosphorylation. The β-sheet (about 47% in the native protein) was slightly changed to 49 and 45% depending on phosphorylation degree. Consequently, the level of the unordered form increased slightly from 43% for the native to 44 and 49% for the low and high phosphorylated protein, respectively. These results agree with those of Woo et al. (1982).

Protein Solubility. The data in Figure 5 show the solubility curves of the phosphorylated β-lactoglobulin samples as compared to the native protein. It can be noticed that the isoelectric points of the modified β-lactoglobulins were displaced toward the acidic values, according to the extent of phosphorylation. This agrees well with the obtained isoelectrofocusing patterns (Figure 2). This shift is obviously due to the increase of the negative charges on protein molecules. A similar change was previously observed after the phosphorylation of other proteins (Sung et al., 1983 ; Chobert et al., 1989). It can also be seen that the minimum solubility of β-lactoglobulin was reduced by phosphorylation from about 70% to about 40−20% according to the extent of phosphorylation. The exceptionally high solubility of the native β-lactoglobulin at the isoelectric range was explained by Kinsella (1983) by the fact that the attractive forces were unable to prevail. Hence, it may be suggested that the introduction of phosphate groups might have changed the fine equilibrium of electrostatic charges. Morr et al. (1973) stated that most of whey

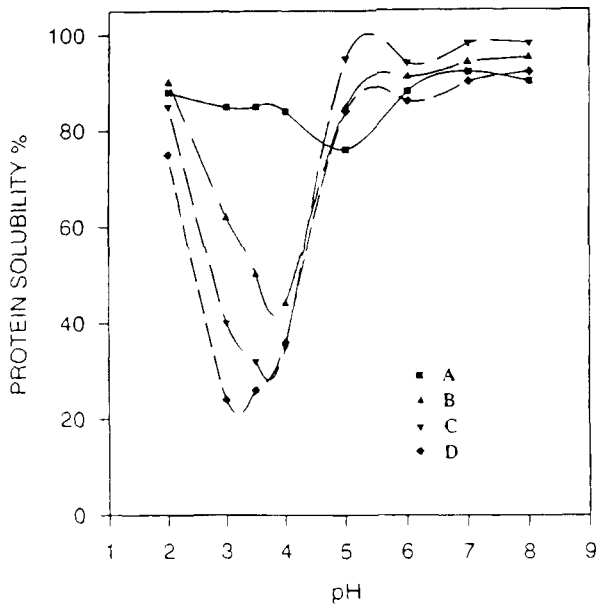

Figure 5. pH–solubility curves for phosphorylated β-lactoglobulin: (A) native β-LG; (B) β-LG (7 mol of P/mol of protein); (C) β-LG (8 mol of P/mol of protein); (D) β-LG (9 mol of P/mol of protein).

protein concentrates are highly soluble at the isoelectric region except for their metaphosphate complex, which exhibits a drastic reduction in solubility at pH values lower than 6. This reduction in solubility was attributed to the presence of metaphosphate ions which complexed and precipitated the positively charged protein molecules at the isoelectric point. Alternatively, as the solubility behavior can also be driven by the protein conformation changes (Frank, 1993), the changes in the secondary structure of the phosphorylated β-lactoglobulin (Figure 4) may partially account for the reduction of solubility at the isoelectric region. It is clear that the reduction of the isoelectric point solubility of β-lactoglobulin by phosphorylation might find some applications.

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