Electrochemical Modifications of Proteins. 1. Glycitolation

Ph. Cayot,*,† L. Roullier,‡ and G. Tainturier†

Département de biochimie-physicochimie, École nationale supérieure de biologie appliquée à la nutrition et à l'alimentation, and Laboratoire de synthèse et d'électrosynthèse organométalliques, Université de Bourgogne, B.P. 138, 21004 Dijon Cedex, France

When an electrochemical method is tentatively applied for N-alkylation of proteins, some Maillard reactions occur on the counter electrode. A low level of reductive N-alkylation of casein was obtained on a cathodic electrode, but the method unfortunately remains poorly efficacious in comparison with those using a hydride donor such as sodium cyanoborohydride. Electroassisted reductive N-alkylation is suitable only for basic proteins such as histones or lysozyme. For other proteins, an alternative consists of either methylating carboxylic residues to increase the value of their isoelectric pH or coating them with a cationic detergent.

Keywords: *Reductive alkylation; glycitolation; glycation; glycosylation; electrochemistry; amino group*

INTRODUCTION

Glycoproteins display essential biological activities. The lectins are responsible for cellular recognition; the antifreeze glycoproteins protect fishes, insects, and plants in cold areas of the globe from freezing (Feeney and Yeh, 1993); the O- and N-glycosylations [the term "glycosylation" is reserved for the natural fixation of an osidic residue on a protein in a cell during the biosynthesis (Figure 1)] confer to proteins allergenic or hormonal (thyroglobulines, hypophyser gonadotrophines) properties (Turner, 1992), enhance viscosity (submaxillary mucines) or foaming power (egg white glycoproteins) (Kinsella, 1981; Cheftel et al., 1985; Kinsella and Whitehead, 1988), and give stability to milk casein micelles owing to the electrostatic repulsion of sialic acid residues (Dalgleish, 1984). Payne and Young (1995) found that damage to meat due to frozen storage could be minimized by injecting intravenously antifreeze glycoproteins from Antarctic cod into lamb before slaughter. We could imagine, for example, that, owing to this technique, the damage on agricultural crops, stored fruits or vegetables, due to freezing would be prevented and that, as suggested by Mizuno et al. (1997), the quality of ice cream could be increased by reducing the dimensions of the ice crystals (Feeney and Yeh, 1993) without using monosaccharides or salt to lower the melting point (eutectic mix).

By synthesis of neoglycoproteins, these wonderful properties could be domesticated. Some searchers hope to create, and put into practice, lure substances analogous to lectins to obtain cellular antiadhesion drugs (Sharon and Lis, 1993). By glycation at a low level of modification using mono- or polysaccharide, several authors increased the emulsion stabilizing power (and sometimes the emulsion capacity) of casein, β-lactoglobulin, bovine serum albumin, plasma protein, 11S soy globulin, and lysozyme or carp myofibrillar proteins (Dickinson and Galazka, 1991; Dickinson and Euston, 1992; Dickinson, 1992; Kato et al., 1992; Matsumodi et al., 1995; Shu et al., 1996; Saeki, 1997; Nacka et al., 1998). [Glycation corresponds to an alkylation of lysyl residues of protein by Maillard reaction. This protein modification is obtained by heating the mixture of saccharides and proteins. The Maillard (1912) reactions occur during food cooking and also in the process of

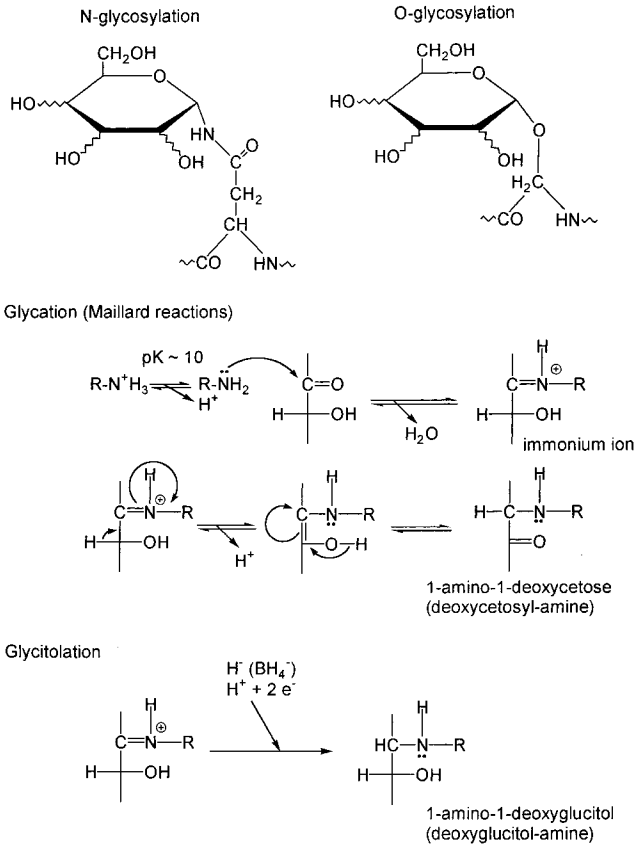

Figure 1. Differences between glycosylation, glycation, and glycitolation.

* Author to whom correspondence should be addressed [fax (33) 03.80.39.66.84; e-mail cayot@u-bourgogne.fr].

† Département de biochimie-physicochimie.

‡ Laboratoire de synthèse et d'électrosynthèse orgaométalliques.

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cellular aging (Cerami et al., 1987).] Using a similar glycation, Kato et al. (1993) increased the stability of bovine trypsin and maintained its activity at high temperature, but the glycation is a time-consuming technique of protein modification: generally, it needs a long heating of protein powder [see, for example, Kato et al. (1992)] or protein in suspension in a buffer at pH 6.5 [see, for example, Nacka et al. (1998)]. The modification level is always difficult to determine a priori. Moreover, the Maillard reactions are very complex ones and led to a lot of different products [see, for example, Belitz and Grosch (1987), Varouyan et al. (1994), and Friedman (1996)].

For these reasons, some authors preferred reductive N-alkylation of primary amino groups to Maillard reactions for modification of proteins. The glycitolated derivatives of whole casein, α_s- and β-casein, or β-lactoglobulin, prepared from monosaccharides with sodium cyanoborohydride as alkylating agent, generally have better rheological, emulsifying, or foaming properties than the starting protein (Colas et al., 1988; Courthaudon et al., 1989; Kinsella and Whitehead, 1988; Bertrand-Harb et al., 1990; Cayot et al., 1991a,b). (We have chosen the term "glycitolation" to indicate the fixation of saccharide residue on protein by reductive alkylation and to distinguish this reaction from natural glycosylation and "Maillard glycation". The reactions and the products are different in the three cases.) These N-alkylated proteins could be also protected against thermal denaturation (Waniska and Kinsella, 1984; Kitabatake et al., 1985).

Reductive alkylation needs a nucleophilic hydride as hydrogen donor, for example, sodium boro- or cyanoborohydride, dimethyl-, trimethyl-, or triethylamine, or pyridine borane (Means and Feeney, 1971; Borch et al., 1971; Geoghegan et al., 1981; Cabacungan et al., 1982). These reagents are thought to hydrogenate the Schiff base produced in the first stage by the nucleophilic addition of an amino group of the protein on the carbonyl group (aldehyde or ketone) of the saccharide (Figure 1). If a definite modification level is desired, the excess of reagent must be destroyed or eliminated. Moreover, because of the toxicity of the reagents, it must be ensured that their elimination reaches completion if studies on living species have to be carried out.

To replace hydride donors, Tainturier et al. (1992) proposed the use of an electrochemical method. The reduction of the imine produced by nucleophilic attack of an amino group on the reducing end of the saccharide should occur on a cathodic electrode. This technique should allow the scientist to choose a priori the modification level, only by switching off. Unfortunately, because of the instability of aliphatic imines, data on their electrochemical reduction are very scarce. The half-wave potential reduction of aromatic imines lies from −0.6 (Schiff base of hexylamine and pyridoxal-5′-phosphate at pH 6 and 25 °C; Blasquez et al., 1989) to −1.75 V relative to that of methylbenzylideneaniline (Schiff base of methylbenzaldehyde and aniline, at pH 7, 25 °C; Siegerman, 1975).

A limit of this technique also deals with the half-wave potential ($E_{1/2}$) of the saccharide reagent. For example, the values of galactose $E_{1/2}$ at 25 °C are −1.6 V at pH 7, −1.75 V at pH 7.5 (Siegerman, 1975), and −1.98 V at pH 8 (Overend et al., 1961). The glucose $E_{1/2}$ at 25 °C is −1.87 V at pH 8 (Overend et al., 1961). Moreover, other functional groups borne by proteins are potentially reducible. For example, carboxylic groups are reducible by a strongly negative electrode. It is known that aspartic and glutamic acids are reduced in two waves ($E_{1/2}$ = −2.1 and −2.6 V). Consequently, to hold the applied potential at a value lower than −2.0 V, an assumption must be made that carboxylic groups of proteins are not modified. Cystinyl residues are more prone than carboxylic groups to electrochemical reduction. The half-wave potential of homocystine is −1.79 V (Siegerman, 1975). We will present our results dealing with reduction of disulfide bonds in a forthcoming paper (2. Reduction of disulfide bond). In the present work, according to the above data, we will hold the potential higher than −2.0 V.

Table 1. Experiment Plan[a] of Glycitolation by the Electrochemical Method in the Presence of CTMA Bromide

parameter	level −1	level +1
[R−NH_2] in mol·L^{-1}	0.0117	0.023
corresponding to [protein] of g·L^{-1}	22.5	45
galactose/R−NH_2 molar ratio	5.43	54.3
CTMA/protein molar ratio	20	100
ethanol proportion in % (v/v)	33	66
working electrode potential (V)	−1.6	−1.8
[LiCl] in g·L^{-1}	4	8
temp (°C)	20	40

[a] The two levels of the experiment plan are denoted −1 and +1.

MATERIALS AND METHODS

The whole casein was prepared from bovine milk by isoelectric precipitation (pH 4.6), and β-casein was isolated according to the method of Cayot et al. (1992). The proteins were dialyzed and freeze-dried. Histones and lysozymes were supplied by Sigma-Aldrich (St. Quentin, France), glucose and galactose by Merck (Darmstadt, Germany).

The electrochemical device was a glassy, double-walled cell, thermostated by water. The cathode (working electrode) was either—for the first experiments with whole caseinate—a layer of mercury, the upper part of which was continuously renewed by magnetic stirring, or a glassy carbon beaker containing a magnetic bar. The anode was a platinum strip, and the reference electrode from Tacussel (Villeurbanne, France) contained a saturated aqueous solution of calomel [for other experimental details, see Tainturier et al. (1992)]. The constant potential generator was purchased from Tacussel. Reference and anodic electrodes were separated from the reaction mixture by porous glass.

Before electrochemical reduction, the dissolved dioxygen was eliminated by bubbling the solution with dinitrogen (if present, the dioxygen would be reduced at $E_{1/2}$= −0.1 V at pH 7; Bard and Faulkner, 1980). Protein and saccharide were dissolved in a Britton−Robinson buffer (1 mL of pure acetic acid, 1 mL of 85% phosphoric acid, 1 g of boric acid; the pH was adjusted with 1 M LiOH solution, and the final volume was completed to 50 mL with deionized water) containing lithium chloride (generally 4 g·L^{-1}) as a supporting electrolyte.

Esterification of whole casein was performed according to the method of Bello (1956) with a few adaptations: 10 g of freeze-dried casein was dispersed in 1 L of methanol, acidified with 8 mL of concentrated hydrochloric acid (nearly 12 M). The reacting mixture was stirred for 24 h at room temperature. The dialysis was realized against a dilute acidic aqueous solution to prevent hydrolysis of carboxymethyl groups. The esterified casein was finally lyophilized. The value of the isoelectric point of esterified casein was checked by isoelectrofocalization (50 mA, 1500 V, 30 W, 90 min) in Bio-Lyte 3/10 ampholines (Bio-Rad, Richmond, CA) using an LKB apparatus (Uppsala, Sweden). We laid 15 μL of protein solution (10 g·L^{-1}) on PAGplate 1804-101 gel (LKB). After focalization, the gel was stained with Coomassie blue. (No measure of esterification

degree has been done because we were interested only in isolelectric point evolution.)

Glycitolation in cetyltrimethylammonium (CTMA) bromide medium (hexadecyltrimethylammonium bromide) was performed according to an experimental plan at two levels and seven parameters (Table 1).

The first experiment corresponds to the line + + + − + − −; the others are deduced from the first one by circular permutation. The last line (8) is − − − − − − −. For example, conditions of experiment 2 were

$$[R{-}NH_2] = 0.0117\ M; \quad \frac{\text{galactose}}{R{-}NH_2}\ \text{molar ratio} = 54.3; \quad \frac{CTMA}{\text{protein}}\ \text{molar ratio} = 100$$

$$\text{EtOH proportion} = 66\%; \quad \text{potential} = -1.6\ V; \quad [LiCl] = 8\ g{\cdot}L^{-1}; \quad \text{temp} = 20\ °C$$

The ratio of N-alkylated primary amino groups of proteins was evaluated according to the method of Cayot and Tainturier (1997): the trinitrobenzenesulfonic acid (TNBS) method, defined by these authors in the conclusion of their paper, and/or total hydrolysis followed by amino acid analysis as described in this publication. The principle of the TNBS method rests on the specificity of primary amino groups reaction on TNBS: no reaction is possible with alkylated amino groups. The reaction produces the Mesenheimer complex, which absorbs at 420 nm [Figure 2; see for details Cayot and Tainturier (1997)]. The difference of optical density between the sample and the initial material before glycation or glycitolation indicates the modification level of protein amino groups. The amino acid analysis on the Beckman HPLC Gold 126AA (Fullerton, CA) allows the separation and the quantification of lysine and alkyl-lysine.

By comparison of the results of different measurements (number of primary amino groups, quantity of alkylated lysine in protein hydrolysate, quantity of homoarginine in guanidinated protein hydrolysate, quantity of trinitrophenylated lysine), it is possible to differentiate products stemming from the Maillard reactions and from reductive N-alkylation (Cayot and Tainturier, 1997). This differentiation is based mostly on the instability of N-alkylated lysyl moieties obtained by the Maillard reaction during acid hydrolysis (hydrolysis of 100 mol of Maillard alkylated lysyl groups at 110 °C, in 6 M HCl during 20 h gives nearly 50 mol of lysine, 20 mol of furosine, 10 mol of pyridosine, but a very few moles of alkyl-lysine; Finot et al., 1981; Figure 2). On the contrary, products stemming from reductive N-alkylation are stable during hydrolysis (Schwartz and Gray, 1977). The determination of homoarginine in a hydrolysate of the guanidinated protein or the quantification of primary amino groups of the protein by using the TNBS method gives the quantity of nonmodified lysyl residues regardless of the reaction of primary amino group with guanyldimethylpyrazole (Figure 2).

The procedures of protein modification by the Maillard reactions (heating method) or reductive alkylation by the cyanoborohydride use (chemical method) are described as follows:

(1) The dispersions of lysozyme (56 $g{\cdot}L^{-1}$) or histones (33 $g{\cdot}L^{-1}$) and galactose (72 $g{\cdot}L^{-1}$) were prepared in a Britton−Robinson buffer at pH 9 and divided in two groups. In the first group, the dispersions were heated at 40 °C during 24 h and in the second group, at 80 °C during 24 h. Other dispersions were made with Britton−Robinson buffer at pH 9: 19.6 $g{\cdot}L^{-1}$ for lysozyme, 38,7 $g{\cdot}L^{-1}$ for histones. These dispersions were mixed with sodium cyanoborohydride and stirred during 24 h at 20 °C [nearly the same conditions as in Cayot et al. (1991a,b)] to allow comparison with the conditions of electrochemical modification.

(2) A dispersion of caseinate (45 $g{\cdot}L^{-1}$) and galactose (22.5 or 225 $g{\cdot}L^{-1}$) containing CTMA bromide (71 $g{\cdot}L^{-1}$) and lithium chloride (4.8 $g{\cdot}L^{-1}$) was prepared in a mixture of ethanol and Britton−Robinson buffer (1/3; pH 6 and 9). Two types of treatment were applied to these caseinate dispersions. In one group, the dispersions were heated at 78 °C during 24 h. In another group, the dispersions were treated with sodium cyanoborohydride (15 $g{\cdot}L^{-1}$) at room temperature during 8 h.

Afterward, the products were dialyzed. The modification level of the final protein was evaluated by using different methods to compare the product to that of electroassisted N-alkylation as previously described.

RESULTS

Electroassisted Modification of Sodium Caseinate with Galactose. At pH 6 and 40 °C, with an applied potential varying from −1.6 to −1.9 V during 24 h, no modification has been characterized by using the TNBS method and amino acid analysis of the hydrolysate (no alkylated lysine, no furosine, no lysinoalanine). After 24 h, at pH 8 and 40 °C, for −1.6 and −1.7 V, the molar modification level (TNBS method) is very close to 12% on average, but the results appear very irregular. Evaluation of the protein content (Kjeldahl method) of the product after dialysis and freeze-drying led to a value of modification level not far from 30%. The difference of the value of the modification level obtained by using the TNBS method and those by using the Kjeldahl method could be due to the presence of some high molecular products mixed with proteins after dialysis and present during the mineralization (Kjeldahl method). An incomplete dialysis or more certainly the presence of caramelization products (high polymers of saccharide could be produced by the applied tension) in the sample could distort the protein quantity evaluated before mineralization. The solution was actually brown on the opposite of the initial protein dispersion (milky). In conclusion, some reactions other than reductive alkylation should occur. At pH 8, 40 °C, and −1.8 or −1.9 V, the modification level evaluated by using the TNBS method is higher (∼14% after 24 h), but furosine was detected in the hydrolysate of the electromodified protein by amino acid analysis. The presence of furosine in an acid hydrolysate indicates the occurrence of Maillard reactions. Moreover, the evaluation was not very repeatable. At pH 9, 40 °C, and −1.62 V, the modification level obtained in two separate TNBS method experiments were, respectively, 24 and 32% after 24 h. These results are similar as those of Tainturier et al. (1992), but as the analysis of the acid hydrolysate of electromodified casein contains furosine, a question arises whether reductive alkylation and Maillard reaction simultaneously occurred.

New experiments (using a glassy carbon beaker) were performed to understand how modifications take place. According to Cayot and Tainturier (1997), the electromodification of casein was studied (Table 2). The modification levels, calculated on the one hand by the evaluation of the quantity of *N*-alkyl-lysine or lysine and, on the other hand, by quantification of homoarginine or by the TNBS method, are very different. The TNBS method and quantification of homoarginine indicate the number of unmodified lysyl residues. The *N*-alkyl-lysyl moiety coming from reductive alkylation [in this case, ϵ-*N*-[1-(1-deoxy-D-galactitol)]-L-lysyl residue) (Figure 1)] is stable toward acid hydrolysis, and its quantification gave the same modification level as the previous method. However, the *N*-alkyl-lysyl moiety obtained by the Maillard reactions [ϵ-*N*-[1-(1-deoxy-D-tagatosyl)]-L-lysyl residue)] was dealkylated in lysine during acid hydrolysis in ∼50% yield (Figure 2). The difference between the two values of the levels of

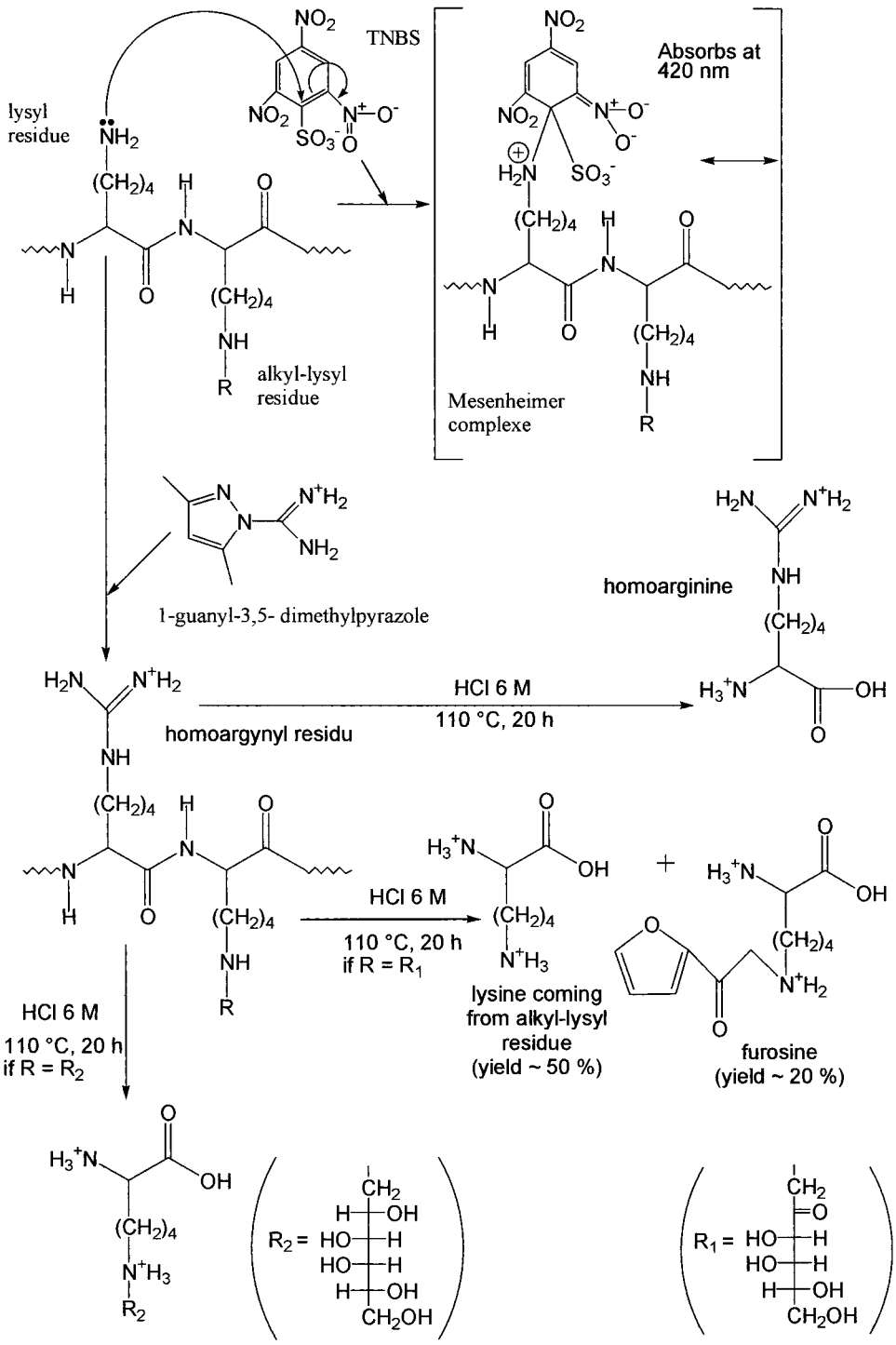

Figure 2. Quantification of lysyl residues of a protein: TNBS method by direct assay on protein and amino acid analysis of acid hydrolysates of protein. R_1 is the 1-deoxy-D-tagatosyl residue (coming from condensation of galactose by Maillard reactions), and R_2 is the 1-deoxy-D-galactitol residue (coming from condensation of galactose by reductive alkylation).

modification indicates that the reactions in the electrochemical cell are actually the Maillard reactions (Cayot and Tainturier, 1997).

Checking the reactional cell after the period of applied tension revealed the accumulation of a paste around the porous glass of the counter electrode (anode). After analysis (Kjeldahl method and amino acid analysis), this paste appeared to contain caseinate. This observation indicates a migration of the protein toward the anode and not in the direction of the working electrode. This result is not unexpected: at pH >6 caseins are negatively charged; therefore, it is not completely surprising that they migrate toward the anode despite the mechanical agitation. At the anodic area, the electric potential was not controlled and the current field should be very high. This high current field should create an overheating around the electrode. There, because of the local high temperature, caseins underwent alkylation by the Maillard reactions.

Usually, electrochemists are not concerned with the negative charge of the species they want to reduce. These species are small molecules or small ions, either cations or anions. In our case, it seems that our macroions (proteins) could not reach the cathode due

Table 2. Modification Level (ML) of Whole Caseinate by the Electochemical Method in the Presence of Galactose[a]

conditions				% (mol/mol) of lysino-alanine	ML (% mol/mol) calcd from quantity evaluation of			
pH	*U* (V)	*T* (°C)	time (h)		AlkLys	Lys	Hrg	or method of TNBS
8	−1.9	20	24	0	0	1.2	nd[b]	nd
	−1.9	40	4	0.5	1	3	8	7.5
	−1.8	40	7	0	2.1	1.6	nd	nd
9.1	−1.8	40	5	0.8	2.2	5	nd	11
10.5	−1.9	40	5	1.6	3	9	16	nd

[a] The protein concentration was 45 g·L^{-1}, that is to say, [R−NH$_2$] ≈ 2.3 × 10^{-3} M, and galactose concentration was 22.5 g·L^{-1} (0.125 M). The conditions are described in this table. The ML values were calculated by quantification of alkyllysine (AlkLys) in electromodified protein hydrolysate, by comparison of lysine quantity (Lys) in control and electromodified protein hydrolysate, or by comparison of homoarginine quantity in the hydrolysates of guanidinated control and guanidinated electromodified proteins. With TNBS method, the ML was directly obtained by the difference between optical density of trinitrophenylated control and electromodified protein. [b] nd, not determined.

Table 3. Modification Level (ML) of Histones by Electrochemical Glycitolation[a]

		ML (% mol/mol)		
pH	time (h)	Hrg	AlkLys	Lys
8	24	11.3 ± 2.7	10.5 ± 1.8	7.0 ± 2.7
	24	nd[b]	11.7 ± 0.1	11.3 ± 4.7
9	8	nd	6.5 ± 0.4	7.7 ± 1.0
10	8	nd	2	6.8

[a] The alkylation of this basic protein has been done at different pH values. All other entry parameters were constant: $U = -1.75$ V; temp = 40 °C; [histones] = 16.5 g·L^{-1}; [galactose] = 26 g·L^{-1}. See the signification of ML in Table 2. For pH 10, we have only done one experiment. [b] nd, not determined.

to high electrostatic repulsions. The mechanical agitation should not be sufficient to overcome the electrostatic barrier.

Histones and Lysozyme Electromodification in the Presence of Saccharide. To verify the precedent assumption, basic proteins with a high isoelectric point (p*I*) were used. At 40 °C, a potential of −1.75 V was applied to a solution of histones (16.5 g·L^{-1}) and galactose (26 g·L^{-1}; moles of saccharide/mole of R−NH$_2$ ≈ 9.6) in a Britton−Robinson buffer containing LiCl (4 g·L^{-1}). The isoelectric potential of histones, checked by isoelectrofocalization, was p*I* ≈ 9.5. At pH 6 and during 8 h, no modification of the lysyl residues was obtained (no change between control and modified proteins in amino acid chromatogram of hydrolysates and in OD by TNBS method). For pH ≥8, a modification of lysyl residues of histones was observed (Table 3), and no furosine or lysinoalanine has been identified in the acid hydrolysate of electromodified histones. The similarity of modification levels (except at pH 10), whatever the calculation mode is, and the absence of furosine in the hydrolysate demonstrate that, in this case, no Maillard reaction occurred and that the modifications effectively arose from reductive N-alkylation for pH < p*I*. In addition, in contrast to our observations in the case of caseinates, no accumulation of paste of proteins on the anode was observed. At pH 10, the modification levels (ML$_{AlkLys}$ and ML$_{Lys}$) were low and not identical because the histones could be charged a little negatively.

The modification level after 24 h was low, <12% of alkylated amino groups. Compared to reductive N-alkylation obtained by the cyanoborohydride method (modification level not far from 80−88% in 24 h), the electroassisted reductive N-alkylation was not very efficient (Table 4). These results in Table 4 also indicate that the optimal pH value for the modification with electrochemical method is around 8 or 9. At pH 10, the modification level was lower than at pH 9 and, at pH 6, no modification was obtained. This last result is interpretable on the basis of p*K* values of ammonium/amino groups. At pH 6, all of the amino groups are protonated in non-nucleophilic ammonium moieties, unable to give a Schiff base. At pH 8, the pH value is not very far from the individual p*K* value of the protein's amino group and is also somewhat inferior to the p*I* of histones (~9). At pH 9, the imine formation should be enhanced by the increase of concentration of reactive amino groups. At pH 10, the charge of the histones is very close to zero or even slightly negative. The migration of protein to the working electrode should be difficult, and the reduction of imine would be sluggish.

Table 4. Modification Level (ML) of Lysozyme and Histones in the Presence of Galactose by Heating (Maillard Reactions) or by Adding Sodium Cyanoborohydride (Reductive Alkylation)[a]

modified proteins in the presence of galactose	ML in % mol/mol				furo-sine
	Lys	AlkLys	Hrg	TNBS	
lysozyme 40 °C	2.4 ± 2.0	4.8	0	0	0
lysozyme 80 °C	40 ± 8	18.7	62.5	(89.7[b])	+
lysozyme + NaCNBH$_4$	82 ± 1	76	82.6	82.4	0
histones 40 °C	4 ± 4	4.3	1.7	0	0
histones 80 °C	46 ± 12	28.3	70.3	(95.4[b])	+
histones + NaCNBH$_4$	81 ± 1	77	88	80.3	0

[a] See the signification of ML in Table 2. [b] Brown products were not eliminated by dialysis. The weighting operation of freeze-dried samples did not correspond to the weighting of the only modified proteins. Consequently, the ML$_{TNBS}$ was overvalued. The presence of furosine is denoted by +, its absence by 0.

The electroassisted reductive alkylation of egg lysozyme (p*I* = 9.5) and galactose (26.8 g·L^{-1}; moles of monosaccharide/moles of R−NH$_2$ = 6.2) at a reductive potential of −1.65 V was once again obtained. In this experiment no furosine was detected in the hydrolysate, and the values of modification level evaluated by different types of quantification were quite similar (ML$_{AlkLys}$ = 9.5 ± 2.0%, ML$_{Lys}$ = 12.7 ± 3.6%) but appeared very low in comparison to values reported in Table 4 dealing with chemical methods.

Modification of Methylated Caseinate. In nature, it is well-known that the basic proteins (p*I* > 7) are scarce. According to previous results, it seems that the occurrence of electroassisted N-alkylation needs two prerequisites for the pH values. First, the pH should be >8 to maintain a high proportion of nucleophilic unprotonated amino groups. Second, it should be lower to the p*I* of the protein to allow migration and subsequent reduction of imino groups at the cathode. To tentatively reduce the imino groups, even if the p*I* is <8, two possibilities have been investigated. The first one is to increase for a while the value of p*I*, that is to say, to increase the ratio of positive charges. Esterification of carboxylic groups of protein should reduce the number of negative charges. Figure 3 shows the modification level of esterified whole casein (45 g·L^{-1}) submitted to a potential of −1.75 V in the presence of galactose (225 g·L^{-1}; moles of saccharides/moles of R−NH$_2$ = 54.3) at pH 8 and 40 °C. The values found for modification level after 22 h are 15.8 ± 4.9% by quantification of N-alkylated lysyl groups and 14.0 ± 1.1% by lysine quantification in hydrolysate. Generally,

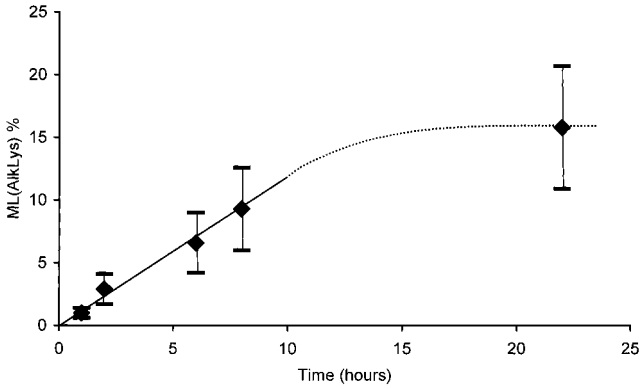

Figure 3. Modification level (ML) of esterified casein electromodified in the presence of galactose ($U = -1.75$, pH 8, 40 °C) as a function of applied current time. ML was calculated by quantification of alkylated lysine in hydrolysates of modified protein.

no significant difference between the values of modification level was observed (for example, after 2 h, $ML_{AlkLys} = 2.9\%$ and $ML_{Lys} = 3\%$; after 8 h, $ML_{AlkLys} = 9.3\%$ and $ML_{Lys} = 9.5\%$), and furosine was never detected in the hydrolysate, except in the product of 22 h of reaction. Because, in this last experiment, the final pH reached the value 8.8, this result could be interpreted by hydrolysis of some carboxymethyl groups, enhancing the number of negative charges and decreasing the p*I* value. In this hypothesis, some of caseins could reach the hot surrounding of the counter electrode to give thermoinduced Maillard reactions.

This hypothesis is reinforced by insight into the values of modification level during the experiment. After 22 h, the final value was as low as for electroassisted modification of basic proteins. We must specify nevertheless that, during the first period of the reaction, the modification level was nearly the same as those obtained by chemical method (10% after 10 h). After 10 h of reaction, we observed a decreasing of the modification rate (i.e., the increasing of modification level became sluggish). As the isoelectrofocalization showed that the p*I* of esterified casein is above 9.5 and as the pH value increased during the experiment, the decrease of the modification rate (Figure 3, after 8 h) could not be due to the simple change, without chemical modification, in the charge of protein. We assumed this phenomenon was originating from the hydrolysis of carboxymethyl groups in basic medium, which reduced the migration speed to the cathode. This assumption is confirmed by the following observations: when the initial pH value was 8.5 instead of 8.0, and the final value was 9.5 instead of 8.8, the overall modification level was only 4% after 23 h. When pH was maintained at the value 8.5, the modification level reached 8.2% after 7 h. This value is nearly the same as for the experiment described in Figure 3.

In conclusion, the electroassisted N-alkylation of esterified caseins is hampered by hydrolysis of carboxymethyl groups, limiting the migration of proteins to the working electrode. Another significant disadvantage lies in the necessity of total hydrolysis to recover a product similar to a chemically N-alkylated protein. For these reasons, by analogy with sodium dodecyl sulfate (SDS) effect in electrophoresis of proteins, we have chosen to increase the positive charge of migrating species by coating the acid proteins with cetyltrimethylammonium ions (CTMA). [CTMA (C16) was chosen and not dodecyltrimethylammonium (C12) because of the price.] By this technique, we hoped to drive the protein toward the cathode regardless of its p*I* value.

Table 5. Modification Level of Whole Casein Modified by Addition of Sodium Cyanoborohydride or by Heating in the Presence of CTMA Bromide[a]

whole casein modified by	pH during the modification	ML_{Lys}	ML_{AlkLys}	furosine
reductive alkylation	6	47.4 ± 1.4	44 ± 2.0	0
	9	64.4 ± 1.0	64.6 ± 2.5	0
thermal glycation at 76 °C	6	51.2 ± 1.2	19.0 ± 4.3	++
	9[b]	45.9 ± 3.0	15.2 ± 3.5	++

[a] The reductive alkylation ($NaCNBH_4$) or the glycation by heating was done during 8 h at two different pH values. [b] The ML_{Hrg} is 81% for the glycated casein, obtained by heating at 76 °C and pH 9. See signification of ML in Table 2. The presence of furosine is denoted by +, its absence by 0.

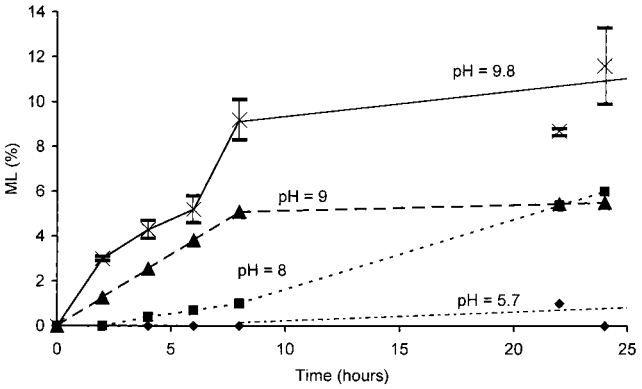

Figure 4. Modification level (ML) of lysyl residues of whole casein in the presence of CTMA bromide and galactose at −1.75 V and 40 °C, at different pH values.

The coating effect of CTMA ions on proteins was a concern in this case, making the formation of imines impossible. Thus, before any electrochemical experiments were performed, proteins were modifed in the presence of CTMA bromide. The reductive alkylation by the chemical method (sodium cyanoborohydride) was successfully realized (Table 5). According to the results, it seemed possible to obtain such modification by an electrochemical method with CTMA.

Reductive Alkylation of Casein in the Presence of CTMA Bromide. β-Casein (54.5 g·L^{-1}; i.e., 2.3×10^{-3} M protein; 0.025 M lysyl residues) was alkylated by galactose (225 g·L^{-1}; i.e., 1.25 M) at pH 9, 40 °C, and −1.75 V in a Britton−Robinson buffer containing CTMA bromide (72.8 g·L^{-1} of CTMA; 0.2 M, nearly 100 times molar excess relative to casein). [The critical micellar concentration of CTMA is 0.85×10^{-3} M between 25 and 35 °C (6.2×10^{-3} M at 25 °C for SDS; James and Lord, 1992); 0.1 M CTMA is perfectly soluble at 20 °C, according to Aldrich.] The modification level of the product was 9.3 ± 0.8%, and a little furosine was detected in the hydrolysate.

With whole casein, galactose, and CTMA, no modification was obtained at pH 6 (Figure 4). At pH 8, 9, or 9.8 in a Britton−Robinson buffer and 40 °C, the whole casein/CTMA complex (45 g·L^{-1} of casein; 72.8 g·L^{-1} of CTMA) was alkylated at −1.75 V (Figure 4) by galactose (225 g·L^{-1}; 1.25 M). The similarity of modification level values found by different methods and the absence of furosine (or the presence of only a few traces) in the hydrolysate of the product indicate the occurrence of reductive alkylation and no (or only a few) Maillard reactions.

Table 6. Comparison of Modification Level (ML) of Amino Groups and Ratio of Furosine Peak Area on Glycine Peak Area for Hydrolysate of Modified Whole Casein by Four Methods: Electrochemical Technique in Aqueous or Ethanolic/Aqueous Medium, Chemical Reductive Alkylation, or Heating[a]

	treatment of casein/CTMA mix	ML (%)	$A_{furosine}/A_{glycine}$
pH 6	no EtOH; −1.8 V; 40 °C; CTMA	nd	nd
	V_{EtOH}/V_{tot} = 1/3; −1.8 V; 40 °C; CTMA	1.7	0
	$CNBH_4$	29.9	0
	76 °C	11.5	0.354
pH 9	no EtOH; −1.8 V; 40 °C; CTMA	5.6	0.003
	V_{EtOH}/V_{tot} = 1/3; −1.8 V; 40 °C; CTMA	8.9	0.117
	$CNBH_4$	63.5	0
	76 °C	12.7	0.263

[a] nd, not determined. EtOH, ethanol; V, volume; V_{tot}, total volume.

It seems that the increase of pH to a value very close to the p*K* of the protein amino groups favors reductive alkylation, promoting the imine formation. When CTMA ions were added, the pH value should not determine the global charge of migrating species, but the modification degree obtained after 24 h is unfortunately low. Even so, this method seems a little less efficacious than the modification of esterified protein. Another inconvenience is the difficulty of isolating the modified protein. For this purpose, addition of an organic solvent of CTMA immiscible with water gave no result. Another method to separate the complex protein/CTMA would be adding sodium dodecyl sulfate, supposing that the affinity of CTMA for this detergent was greater than its affinity for protein. A very viscous dispersion was obtained. All other attempts of isolation of the product have also failed.

The mixture of CTMA bromide and protein in the aqueous buffer was heterogeneous and could be at the origin of the low modification level. The lowest phase was quite limpid (like a true solution); the supernatant was turbid and sticky. The CTMA bromide is soluble in water, like sodium dodecyl sulfate, but the equimolar mixture of these two electrolytes is an opalescent suspension. The aspect of a mixture of protein and CTMA bromide in the reactional buffer is identical and could be explainable on the basis of electrostatic attractions between proteins and CTMA as it could be supposed between sodium dodecyl sulfate and CTMA. Because of this heterogeneous character, the temperature of the reaction mixture is of great importance. At 20 °C and pH 9 (the same conditions as before except the value of temperature), no modification was obtained. At 20 °C, the high viscosity of the medium should lower the mobility of the species, hampering formation of the imino group (low mobility of galactose) and migration of the species toward the cathode. Moreover, at this temperature, CTMA is poorly soluble in water.

As CTMA is soluble in ethanol (but insoluble in acetone) and as ethanol is completely miscible with water, a small quantity of ethanol was introduced into the reaction mixture to facilitate the alkylation. We obtained a turbid but quasi-homogeneous mixture. Unfortunately, the electroreductive alkylation in such conditions did not give better results than in a 100% aqueous buffer. The found modification level of whole casein (32.1 g·L^{-1}) after 8 h, at −1.80 V, pH 9, and 40 °C, with galactose (160.7 g·L^{-1}) and CTMA (52 g·L^{-1}) in 25 mL of aqueous buffer and 10 mL of ethanol, was 5.8 ± 0.6%; a similar value (5.6 ± 0.9%) was found for whole casein in aqueous buffer (25 mL) and deionized water (10 mL) in the same conditions.

To improve the reaction, we tentatively increased the ratio of ethanol (33−66 v/v % instead of 28% previously) in an ethanol Britton−Robinson aqueous medium mixture, but the analysis of the product showed a resulting enhancement of the Maillard glycation (see below).

The results obtained (data not shown) owing to the experimental planning described in Table 1 gave the polynomial function eq 1, which shows that every entry parameter affects the modification level of the protein (exit parameter), except in the study domain the cathodic potential. The polynomial function, obtained by multiplying the experimental matrix and the column vector of one exit parameter and which models the effects of entry parameters on the result, is the following:

$$\mathrm{ML} = 5.54 - 0.86X_{[\mathrm{protein}]} + 0.88X_{[[\mathrm{galactose}]/[\mathrm{protein}]} - 1.06 \times [\mathrm{CTMA}]/[\mathrm{protein}] + 1.28X_{\mathrm{VetOH/Vtot}} + 1.02X_{[\mathrm{LiCl}]} + 1.88X_{t°\mathrm{C}} \quad (1)$$

This equation shows that it is difficult to improve the alkylation level (no predominant factor). Moreover, it does not represent the exact reality (some real MLs are different from calculated MLs). The use of these screening experiments also concerns the evaluation of furosine quantity in hydrolysate (i.e., the extent of Maillard reactions; see experimental planning in Table 1). We noticed that the presence of ethanol at 33%, as at 66% (v/v), induces a glycation by Maillard reactions (data not shown). The ratio of galactose to protein, the potential, and the temperature have a great influence on the exit parameter (ratio of the furosine area to the glycine area in the aminogram of hydrolysate) as seen on Pareto representation (not shown) and according to the polynomial function

$$\frac{A_{\mathrm{furosine}}}{A_{\mathrm{glycine}}} = 0.076 + 0.035X_{[\mathrm{galactose}]/[\mathrm{protein}]} + 0.023X_U + 0.017X_{t°\mathrm{C}} \quad (2)$$

The modelization is quite satisfactory (calculated ratios were similar to real ratios) to analyze the events. The first observation concerns the quantity of furosine in the hydrolysate of modified protein. Traces of furosine or the absence of furosine was previously observed without ethanol, but, in the presence of a great quantity of ethanol, the Maillard reactions are favored before N-reductive alkylation (Table 6). This observation is interpreted by considering that ethanol probably destroyed the positive coat around the protein. This speculation is consistent with the observed enhancement of the Maillard reactions when the concentration of galactose is increased. A similar effect is obtained by increasing the cathodic potential, which should correspond to an increase of the temperature around the counter electrode, and occurrence of subsequent thermoinduced Maillard reactions. Therefore, the addition of ethanol does not increase the reductive alkylation but the Maillard reactions. In conclusion, alkylation by an electrochemical method seems to be very limited and difficult to improve.

Conclusion. Owing to the necessity of complete elimination of cyanoborohydride and its degradation products for further experiments [see, for example, Courthaudon et al. (1989), Bertrand-Harb et al. (1990),

Cayot et al. (1991a,b), Chobert et al. (1991), Lee et al. (1979), and Nacka et al. (1998)], the field of electrochemical methods to glycitolate proteins appeared to be promising. Nevertheless, it seems impossible to have reductive alkylation with acid protein: as the reaction occurs at pH ≥8 (not far from the p*K* value of amino groups to allow the imine formation), most of the protein is charged negatively. At this pH, the protein cannot migrate to the working electrode (cathode), where the reduction of the imine occurs (product of the reaction between protein and reductive carbohydrates). With basic proteins or proteins coated with cationic detergent, the reductive alkylation is possible, but its results appear to be very disappointing compared to those of chemical N-alkylation. The simultaneous reduction of carbohydrates and the degradation of the medium could be responsible for the low modification level.

Another use of electrochemical methods has been investigated: it seems that they could cut disulfide bonds of proteins (Cayot, 1993). This study will be described in a forthcoming paper.

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