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Probing Cysteine Reactivity in Proteins by Mass Spectrometric EC-Tagging

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The on-line electrochemical tagging (EC-tagging) of cysteine residues in proteins during mass spectrometry is studied to probe the cysteine environment. Benzoquinone probes electrogenerated at a microspray electrode react with the thiol functions of the proteins within a microchannel and the products are analyzed by mass spectrometry. The fundamentals of the technique are discussed, with a focus on the kinetic aspects. The EC-tagging efficiency of the cysteine residues in proteins is used to probe their environment. Experiments with unmodified proteins and their chemically reduced forms highlight the strong effect of the cysteine site reactivity on the tagging efficiencies. This study highlights relevant parameters for such on-line electrochemical derivatization/MS detection strategies.

Keywords: cysteine markers • electrochemistry • electrospray • mass spectrometry • protein structures probing

Introduction

In proteins, cysteine residues are important for metal coordination, catalysis, and protein structure by forming disulfide bonds. Moreover, crucial cysteine residues are involved in modulation of protein activity and signaling events via redox reactions, chelation of transition metals and *S*-nitrosation. Cysteine is also the binding site in human albumin for biological and clinical small molecules such as platinum(II) anticancer drugs.[1,2]

The reactivity of cysteine in proteins is complex for reasons such as steric hindrance, charge distribution and solvation. It varies from one protein to another and specific competition with glutathione makes the system even more complex in biological fluids. The antioxidant character of cysteinyl proteins depends highly on the protein structure that for instance prevent the formation of disulfide bonds in albumin or reduce the antioxidant capacity of hemoglobin in comparison to glutathione.[3,4]

As nucleophiles, thiols have a reaction rate that depends on the protonation state of the sulfhydryl group. The primary structure of the biomolecule influences the thiol reactivity since the pK_a of the thiol is strongly dependent on the charged residues in the vicinity of the cysteine. It has been shown that Michael-type addition of sulfhydryl-containing peptides onto unsaturated groups have higher or lower rates when positive charges and negative charges are respectively in the vicinity, resulting to a decrease or an increase of pK_a.[5]

In electrospray ionization (ESI) mass spectrometry (MS), the application of an electric field to generate the spray has led to the consideration of emitters as on-line electrochemical flow-cells. Then, the inherent electrochemical aspect of electrospray[6−8] has opened the way to the study of electrochemically induced reactions[9−12] like rearrangements of biological molecules.[13−15] Moreover, electro-active probes have been developed to chemically derivatize weakly ionisable compounds,[16−21] and to label specific amino acids.[22] Recently, we have developed a polymer micro-ESI emitter comprised of a microband electrode.[23] This micro-flow-cell was shown to be an efficient controlled-current electrochemical flow-cell compared to many commercially available ESI sources.[24] Additionally, the upstream position of the microband electrode within the micromachined flow channel is a major advantage in the electrogeneration of tags to bind to molecules flowing above the electrode toward the Taylor cone. The oxidation of hydroquinone derivatives on the microband anode was studied to tag cysteine residues in peptides via a selective 1,4-Michael addition. When controlling both the electrode mass transport and the kinetics of the addition reaction, the application of on-line counting of cysteines in peptides to the identification of proteins by peptide mass mapping was achieved.[25,26]

We present here a method to probe cysteinyl sites in proteins. An analytical kinetic model is developed to predict tagging extents at the end of the microchannel prior to the Taylor cone. We show that the MS measurements of the extent of the EC-tagging reaction depend mainly on the reactivity of cysteine residues in the proteins and not on the ionization properties of adducts. The EC-tagging of β-lactoglobulin A (one free cysteine residue) was studied and compared with the multi-tagging of its reduced form (i.e., five free cysteine residues after reduction of the two disulfide bonds). Creatine phosphokinase and reduced insulin were probed by EC-tagging to complete the study.

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Figure 1. Schematic representation of the EC-tagging of cysteinyl proteins (P) by benzoquinone (BQ, dark dot) tags electrogenerated at the microspray electrode from hydroquinone (HQ, clear dot).

Experimental Section

Maple Calculation. The kinetic model is based on a set of differential equations that can be solved analytically with commercial software (Maple 9.5, Waterloo Maple Inc.) on an iMac G5 (2GHz PowerPC, 512MB DDR400 SDRAM) (see Supporting Information).

Materials. Insulin from bovine pancreas, creatine phosphokinase (CK) from rabbit muscle and β-lactoglobulin A from bovine milk (>90%) were purchased from Sigma (St Louis, MO). Tri-*n*-butylphosphine (TBP, 97%) and methoxycarbonyl-1,4-hydroquinone (methyl 2,5-dihydroxybenzoate) (99%) were from Aldrich (Milwaukee, WI). 1,4-Hydroquinone (> 98%), 1,4-benzoquinone (≥ 98%), L-cysteine (> 99.5%), lithium trifluoromethanesulfonate (purum), *N*,*N*-dimethylformamide (DMF, ~99%), acetic acid (AcOH, 99.5%) were from Fluka (Büchs, Switzerland). Methanol (MeOH, > 99.8%) was bought from Riedel-de Haën (Seelze, Germany). Acetonitrile (HPLC grade) was from Sds (Peypin, France). Deionized water (18.2 MΩ·cm) was prepared using a Milli-Q system from Millipore (Bedford, MA).

Methoxycarbonyl-1,4-benzoquinone was synthesized as already described.[27]

Reduction of Proteins. To reduce the disulfide bridges, 1 mg of purified protein was dissolved in 900 μL of H_2O. 100 μL of TBP at 10% in DMF ($4 \cdot 10^{-5}$ mol) was added to the protein solution according to the literature.[28] The mixture was stirred between 60 and 90 min. Then, the resulting mixture was lyophilized overnight to get rid of the excess reagent (TBP)[29] that can react with benzoquinone compounds.[30] Without lyophilization, the EC-tagging of the protein was indeed not obtained. The proteins were redissolved in degassed MeOH/H_2O/AcOH 50/49/1.

EC-Tagging. The proteins at 50 μM were sprayed with methoxycarbonyl-1,4-hydroquinone or 1,4-hydroquinone at 2.5 mM in degassed MeOH/H_2O/AcOH 50/49/1. The total acquisition time of the spectra was 1 min. A few minutes were needed to reach the steady state of the process. The EC-tagging was checked to be stable during 25 min.

The apparent tagging extents (a-TE%) and the MS tagging yield ($Yield_{MS}$) were calculated as 100 fold the sum of the peak intensity of the product(s) over the sum of the peak intensity of the product(s) and the starting protein.

Chemical Labeling and Ionization Experiments. The chemical formation of adducts was performed by adding benzoquinone BQ as a solid or in acetronitrile solution (from 250 to 2500 μM). The ratio BQ/protein or cysteine was varied from 0.05 to 0.7. The mixtures were analyzed by MS after 30−45 min of reaction. The reaction is rapid and is assumed to be total[27,31] to calculate the yields ($Yield_{MS}$) as 100 fold the sum of the peak intensity of the product(s) over the sum of the peak intensity of the product(s) and the starting protein.

Micro-Fabricated ESI Emitter. The polymer microspray fabrication has been already described in detail.[23] The surface of the electrode integrated at the bottom of the channel was 70 × 25 μm^2 (the length L was 70 μm, and the width l was 25 μm). The channel length from the electrode to the tip was L_{ch} = 2 cm and the cross-section was 30 × 35 μm^2 (the width d was 30 μm and the height $2h$ was 35 μm).

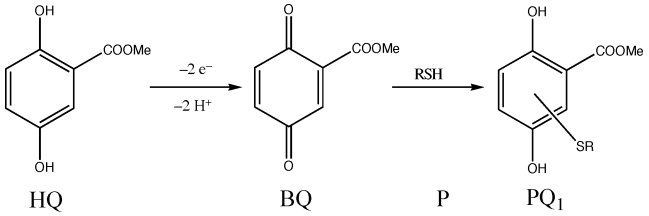
MS Setup. A LCQ DUO ion trap mass spectrometer (Finnigan, San José, CA) was used. The heated capillary was kept at 200 °C. In each experiment, the ion transmission parameters were optimized automatically in order to improve the detection of the analyte of interest (the unmodified biomolecule). The ESI interface was removed, and the microchip holder was mounted on the probe slide adapter of the mass spectrometer. The device was coupled to a syringe pump (kdScientific, Holliston, MA) to introduce the solution. The flow rate F_v was set to 250 nL·min^{-1} and the voltage applied was 3−4 kV. The distance from the outlet of the microchip to the entrance of the spectrometer varied between 1 and 2 cm in order to set the electrospray current and to optimize the signal and the trap injection time. The applied spray current I_{spray} was set at 120 nA.

Diffusion Coefficient Measurements. The electrochemical measurements were recorded on an Autolab PGSTAT 12 potentiostat from Metrohm (Herisau, Switzerland) using an undivided cell filled with a protected saturated calomel electrode as reference electrode, a glassy carbon electrode (3 mm diameter) as working electrode and a platinum wire as counter electrode. The electrochemical cell was filled with the spray solution (MeOH/H_2O/AcOH 50/49/1) at 0.1 M lithium trifluoromethanesulfonate and 2 mM hydroquinone. The working electrode was carefully polished with a suspension of 0.3 μm alumina (from Buehler, Lake Bluff, IL) before every experiment. Diffusion coefficients of 1,4-hydroquinone and methoxycarbonyl-1,4-hydroquinone were measured by chronoamperometry. The potential was set for 60 s at 0.8 V and at 1 V for 1,4-hydroquinone and methoxycarbonyl-1,4-hydroquinone respectively and the oxidation current was recorded (every 0.5 s). Diffusion coefficients were extracted from the slope of the straight line $I(t) = f(t^{-0.5})$ and were found to be 4×10^{-10} and 3.5×10^{-10} m^2·s^{-1} for 1,4-hydroquinone and methoxycarbonyl-1,4-hydroquinone. This difference can be attributed to the strong hydration of 1,4-hydroquinone in water compared to that of methoxycarbonyl-1,4-hydroquinone.

Results and Discussion

1. Mechanism and Kinetics. Figure 1 shows a schematic representation of a microchip electrospray emitter comprising basically a flow channel and a band electrode located at the bottom of the microchip. During electrospray from the microchip at a flow rate F_V of 250 nL·min^{-1}, benzoquinone tags (BQ)

Scheme 1. EC-Tagging Mechanism of a Cysteinyl Protein with Methoxycarbonyl-1,4- Hydroquinone



are generated at a microband electrode by oxidation of hydroquinone compounds (HQ) and react specifically with the thiol groups of the proteins (P) in the flow channel (Scheme 1). The products of the reaction at the end of the channel are analyzed continuously by ion trap mass spectrometry.

The addition of BQ tags on thiols has a very large equilibrium constant.[27,31] According to the number of cysteines n in P, adducts (PQ_i) are successively formed (i = 1...n) (Scheme 2). The mechanism for multi-cysteine-containing protein can be described by PQ_i are that representative of the tagged state but not of a defined molecule (i.e., for multi-cysteine-containing protein, PQ_1 represents several regio-isomers). It is assumed that every thiol group presents equal reactivity.

1.1. Analytical Kinetic Model. 1.1.1. Electrochemical Step. In the positive ionization mode, the reaction taking place at the high voltage microband electrode[32] as illustrated in Figure 1 is the oxidation of HQ considering its redox potential.[27] In the flow-cell microspray emitter, the convection-diffusion limiting current I_{lim} in a 2-D laminar Poisseuille flow can be classically calculated by[33−35]

$$I_{lim} = 0.925zFCl(LD)^{2/3}\left(\frac{F_V}{h^2 d}\right)^{1/3} \quad (1)$$

where z is the number of electrons per oxidized molecule, F is the Faraday constant, C and D are respectively the bulk concentration and the diffusion coefficient of the electroactive species, l and L the width and the length of the electrode, F_V the pressure-driven flow rate, $2h$ and d the height and the width of the channel.

The electrospray current I_{spray} is set by controlling the flow rate, the distance between the chip tip and the entrance of the MS, and the high voltage applied by the MS[8] (see Experimental Section). When the imposed electrospray current I_{spray} value is greater than the calculated HQ oxidation convection-diffusion limiting current I_{lim}, we can use Faraday's law to calculate the quantity of BQ tags produced at the electrode according to

$$N_{BQ} = \frac{I_{lim} t_e}{zF} \quad (2)$$

where $t_e = L/\bar{v}$ is the residence time of species on the top of the electrode and $\bar{v}$ is the mean flow velocity.

Conversely, when the imposed electrospray current I_{spray} value is lower than the calculated HQ oxidation current I_{lim}, the quantity of BQ tags produced at the electrode is

$$N_{BQ} = \frac{I_{spray} t_e}{zF} \quad (3)$$

Considering a fast transversal diffusion above the electrode, the initial concentration of BQ tags ($[BQ]_0$) can be assimilated to its mean concentration in the volume element over the electrode ($V_e = L \times d \times 2h$) after a time t_e (Table 1 and Figure 1). Post-electrode oxidation of HQ by the other species electrogenerated at the electrode such as oxygen is neglected.

Scheme 2

$$P + BQ \xrightarrow{k_1 = nk} PQ_1$$
$$PQ_1 + BQ \xrightarrow{k_2 = (n-1)k} PQ_2$$
$$\vdots$$
$$PQ_{n-1} + BQ \xrightarrow{k_n = k} PQ_n$$

Table 1. Parameters[a] for Calculation of Species Concentration in the Channel (N.B. I_{spray} = 120 nA)

general parameters	1,4-Hydroquinone	Methoxycarbonyl-1,4-hydroquinone
$F_V = 250$ nL·min^{-1}	$D = 4{\cdot}10^{-10}$ m^2·s^{-1}[b]	$D = 3.5{\cdot}10^{-10}$ m^2·s^{-1}[b]
$\bar{v} = 4.3$ mm·s^{-1}	$I_{lim} = 81$ nA[c]	$I_{lim} = 74$ nA[c]
$[HQ]_0 = 2.5$ mM	$[BQ]_0 = 101\ \mu$M[d]	$[BQ]_0 = 93\ \mu$M[d]

[a] The real cross-section geometry of the channel is in fact trapezoidal.[32] To bring this correction to the model, the width of the channel d was taken as $d_m = (d + l)/2$. The reaction time t in the full channel is then 4.7 s. [b] See Experimental Section. [c] According to eq 1. [d] According to eq 2.

1.1.2. Chemical Step. For a simplified analytical calculation of the extent of tagging in the microchip setup, complete mixing is assumed to be ideal in the finite volume V_e. The reaction occurs in this volume translating with the flow along the microchannel.

In the case of the simplest addition between BQ tags and a protein P containing one cysteine the rate law follows a first-order kinetics for each reactant:[36,37]

$$v = -\frac{d[BQ]}{dt} = -\frac{d[P]}{dt} = \frac{d[PQ_1]}{dt} = k[BQ][P] \quad (4)$$

v is the rate of the reaction, k is the rate constant and [BQ], [P], and $[PQ_1]$ represent respectively the concentration of the electrogenerated BQ tags, the concentration of a protein P containing one cysteine residue and the concentration of the single-cysteine-containing product PQ_1 at the time t. The integrated rate law gives

$$\frac{1}{[BQ]_0 - [P]_0} \ln\left[\frac{[P]_0([BQ]_0 - x)}{[BQ]_0([P]_0 - x)}\right] = kt \quad (5)$$

where $[BQ]_0$ and $[P]_0$ are the initial concentrations of the electrogenerated BQ tags (i.e., at the electrode) and of the single-cysteine-containing protein (Scheme 2), and $x = [PQ_1] = [BQ]_0 - [BQ] = [P]_0 - [P]$.

This calculation can be applied to the consecutive stages when the protein possesses several cysteine units (Scheme 2). The use of the Maple software is then convenient to solve the differential equation systems, as the analytical solutions are too cumbersome to derive manually (see the program for a five-step chemical reaction, i.e. five-cysteine-containing protein, in the Supporting Information).

1.2. Numerical Validation of the Model. Recently, we have developed a finite-element model treating the emitter as a flow chemical reactor to study the consecutive reactions in the microchannel and to optimize the conditions for cysteine counting in peptides.[38] The comparison of the concentrations [BQ], [P], and $[PQ_n]$ at the end of the channel from the analytical kinetic model shows a very good correlation with the simulated data, validating the present analytical kinetic model as illustrated in Figure 2 for methoxycarbonyl-1,4-hydroquinone.

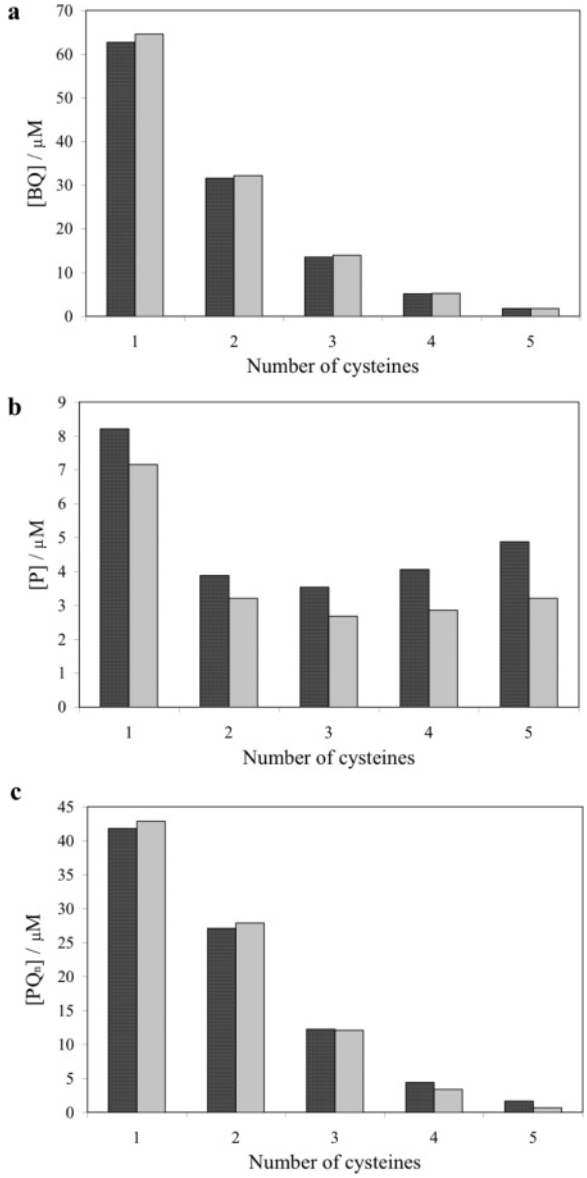


Figure 2. Analytical model validation. The concentration of benzoquinone BQ (**a**), protein P(**b**) and fully tagged product PQ_n (**c**) were calculated from single-cysteine-containing to five-cysteine-containing protein by numerical simulation for a 2D cross section of the channel geometry[38] (black bars) and by analytical kinetic model (grey bars for $[BQ]_0 = 107$ μM, $[P]_0 = 50$ μM, $k = 5000$ $M^{-1}\cdot s^{-1}$ and $t = 5$ s corresponding to a rectangular channel geometry with $l = d = 30$ μm).

1.3. Experimental Validation of the Model. Tagging of L-cysteine ($[P]_0 = 200$ μM) by methoxycarbonyl-1,4-hydroquinone ($[HQ]_0 = 20$ mM) has been previously investigated and the Michael addition rate constant has be determined to be very large in the spray medium MeOH/H_2O/AcOH 50/49/1.[27,37] The apparent EC-tagging extent, a-TE% was 99%. To take into account MS ionization phenomena induced by the tag,[10,39] a calibration curve was determined giving the MS response as a function of the true mixture composition (Figure 3). The effective e-TE% (i.e., in bulk) extracted from the curve is 76%.

The analytical kinetic model predicts a 67% TE% value for tagging with methoxycarbonyl-1,4-hydroquinone, which correlates rather well with experimental e-TE% of 76%. These data validate the use of the analytical kinetic model to predict the tagging efficiency.

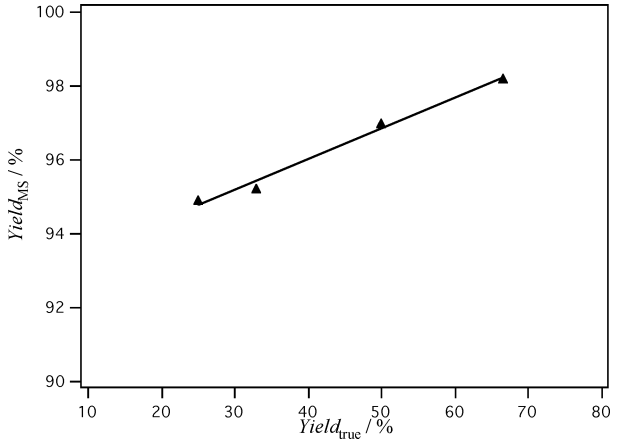


Figure 3. Calibration curves of the MS tagging yield ($Yield_{MS}$) as a function of the bulk yield ($Yield_{true}$) for the reaction of L-cysteine with methoxycarbonyl-1,4-benzoquinone. The mixtures were prepared by adding BQ equivalent (from 0.25 to 0.7) to 200 μM of L-cysteine. The fitting equation is $Y = 92.68 + 0.083514\ X$.

Table 2. Tagging Extent Obtained by Calculation and MS Experiments for a Single-cysteine-containing Biomolecule[a]

Hydroquinone compound	k_{cys} ($M^{-1}\cdot s^{-1}$)[b]	tagging extent (%) analytical TE%[c]	apparent a-TE%[d]	effective e-TE%[d]
Methoxycarbonyl-1,4-hydroquinone	5000	79	31	26
1,4-Hydroquinone	210	9	25	5

[a] The addition reaction time in the microchannel is 4.7 s. [b] Rate constant of Michael addition of L-cysteine in MeOH/H_2O/AcOH 50/49/1.[27,37] [c] Extents were calculated thanks to eq 5 with initial concentration of Table 1, a time residence $t = 4.7$ s and rate constants relative to L-cysteine in MeOH/H_2O/AcOH 50/49/1. [d] Experimental data were obtained with β-lactoglobulin A, which possesses one free cysteine (Figure 5).

2. EC-Tagging of Proteins. The analytical kinetic model for EC-tagging has been applied to the study of β-lactoglobulin A tagged by methoxycarbonyl-1,4-benzoquinone. This tag has been shown to be efficient because of its reactivity and the stability of its associated hydroquinone form, even in the presence of oxygen.[27] It is therefore an ideal probe to tag cysteine in proteins and the physical parameters relative to the tag are given in Table 1. The tagging extent TE% (Table 2) predicted by the kinetic model for a singly cysteinyl biomolecule is calculated using the rate constant measured for the tagging of L-cysteine, ($TE\% = 100\cdot[PQ_1]/([P] + [PQ_1]) = 100\cdot[PQ_1]/[P]_0$).

From one point of view, short residence times in the microchannel are required to avoid the reaction running to completion, so that simultaneous MS detection of both the untagged and tagged proteins can be obtained with a mass shift corresponding to the mass of the benzoquinone tags.

The apparent experimental a-TE% (i.e., the consumption of P) for the EC-tagging of β-lactoglobulin A (one free cysteine) by methoxycarbonyl-1,4-hydroquinone at a concentration ratio of 50 μM/2.5 mM was found to be 31%. The mass spectrum obtained is given in Figure 4. In comparison with the analytical model (Table 2), methoxycarbonyl-1,4-hydroquinone provides a much lower conversion than expected (31 ± 10% a-TE%

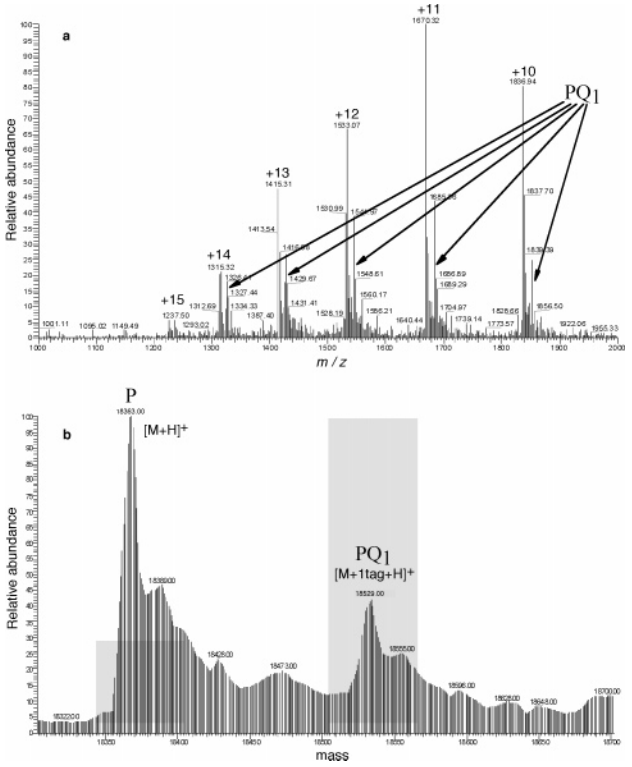


Figure 4. Microspray mass spectrum of β-lactoglobulin A (50 μM) infused with methoxycarbonyl-1,4-hydroquinone (2.5 mM) (**a**). Deconvolution mass spectrum (**b**). Grey bars give the predicted distribution of the species according to the analytical model. P and PQ_1 indicate respectively the untagged and tagged protein.

instead of 79% by calculation). This decrease in the reactivity of the addition reaction compared to that of the simple amino acid can be explained by several factors including difference in ionizations, variation of the local pK_a in proteins and steric hindrance.[40]

2.1. Ionization Effects. The tag can induce a difference in MS ionization efficiency. However, the mass spectrum of an equimolar mixture of β-lactoglobulin A and its tagged analogue shows a signal enhancement for the tagged compound over that of the untagged protein (see Supporting Information Figure S1). This effect is similar to that observed in Figure 3 for L-cysteine.

Calibration curves of the MS response as a function of a known mixture of β-lactoglobulin A and its associate adduct were carried out (Figure 5) and an effective e-TE% of 26% was determined.

Therefore, the observed decrease of the tagging efficiency for β-lactoglobulin A cannot be attributed to a difference in ionization.

2.2. Variation of pK_a and Steric Hindrance. The thiol pK_a is influenced by the peptidic chain primary structure.[41] At a given pH, a decrease or an increase of the pK_a lead respectively to an increase or a decrease in the observed addition rate according to the Bronsted equation

$$k = (10^{pK_a - pH} + 1)^{-1} k_{true} \quad (6)$$

where k_{true} is the rate constant when the thiol is fully deprotonated.[5,42]

According to eq 5, the e-*TE*% of 26% yields an observed rate constant $k = k_P = 760\ M^{-1}\cdot s^{-1}$ for the free cysteine of

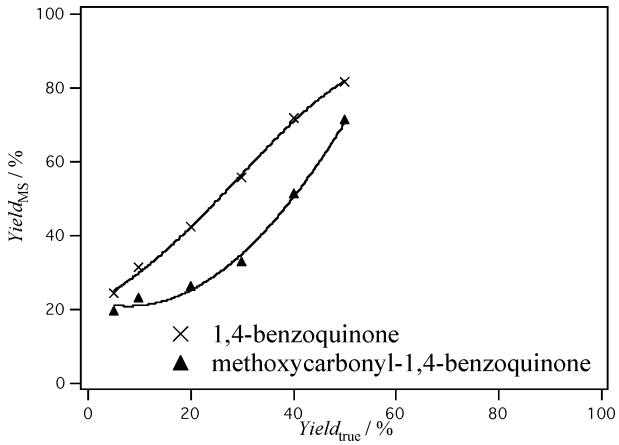


Figure 5. Calibration curves for the MS tagging yield ($Yield_{MS}$) as a function of the bulk yield ($Yield_{true}$) for the reaction of β-lactoglobulin A with methoxycarbonyl-1,4-benzoquinone and 1,4-benzoquinone. The mixtures were prepared by adding BQ equivalent (from 0.05 to 0.5) to 50 μM of β-lactoglobulin A. The fitting of the curves gives $Y = 613.57 + 592.61 \exp(-((X - 7.8623)/(142.26))^2)$ and $Y = 8.917 + 77.954 \exp(-((X - 61.596)/(45.194))^2)$ for methoxycarbonyl-1,4-benzoquinone and 1,4-benzoquinone, respectively.

β-lactoglobulin A. Having evaluated the addition rate constant, eq 6 can then be used to determine the local pK_{ap} compared to that of L-cysteine. Indeed, we can write assuming that k_{true} is the same for both the amino acid and the protein:

$$\frac{k_P}{k_{cys}} = \frac{10^{pK_{acys} - pH} + 1}{10^{pK_{ap} - pH} + 1} \quad (7)$$

where k_{cys} is the addition rate constant determined with L-cysteine (Table 2) and p$K_{a_{cys}}$ is the thiol pK_a of L-cysteine. This equation yields a local pK_{ap} of 8.8 when taking p$K_{a_{cys}} = 8$ for L-cysteine and pH = 3.3 (spray medium).

For comparison, we also tagged β-lactoglobulin A with 1,4-hydroquinone and in this case the e-TE% measured (Table 2) yields a rate constant value of $k_P = 105\ M^{-1}\cdot s^{-1}$. Considering eq 7 and the fixed thiol pK_{ap} value of β-lactoglobulin A in the medium, eq 8 must be satisfied

$$\left(\frac{k_P}{k_{cys}}\right)_{methoxycarbonyl-1,4-hydroquinone} = \left(\frac{k_P}{k_{cys}}\right)_{1,4-hydroquinone} \quad (8)$$

This ratio is however found three times larger for 1,4-hydroquinone than for methoxycarbonyl-1,4-hydroquinone showing the role of steric hindrance after consideration of pK_a effects.

This result clearly shows that the decrease of the tagging rate of β-lactoglobulin A by methoxycarbonyl-1,4-hydroquinone from the predicted value of 79% to the experimental value of 26% does not only result from the thiol increased pK_{ap} in the protein but also from other factors. The steric hindrance acting on the EC-tag can be considered to have a relevant influence on the decrease of the kinetics. Indeed, free access of the probe to the cysteine is notably hindered by the protein chain even in denaturing solvents as the 3D structure is conserved by the presence of disulfide bridges even at low pH.[43,44]

In conclusion, the low tagging extent e-TE% observed with β-lactoglobulin A is basically associated to kinetic limiting factors linked with the cysteine environment (i.e., the pK_a value

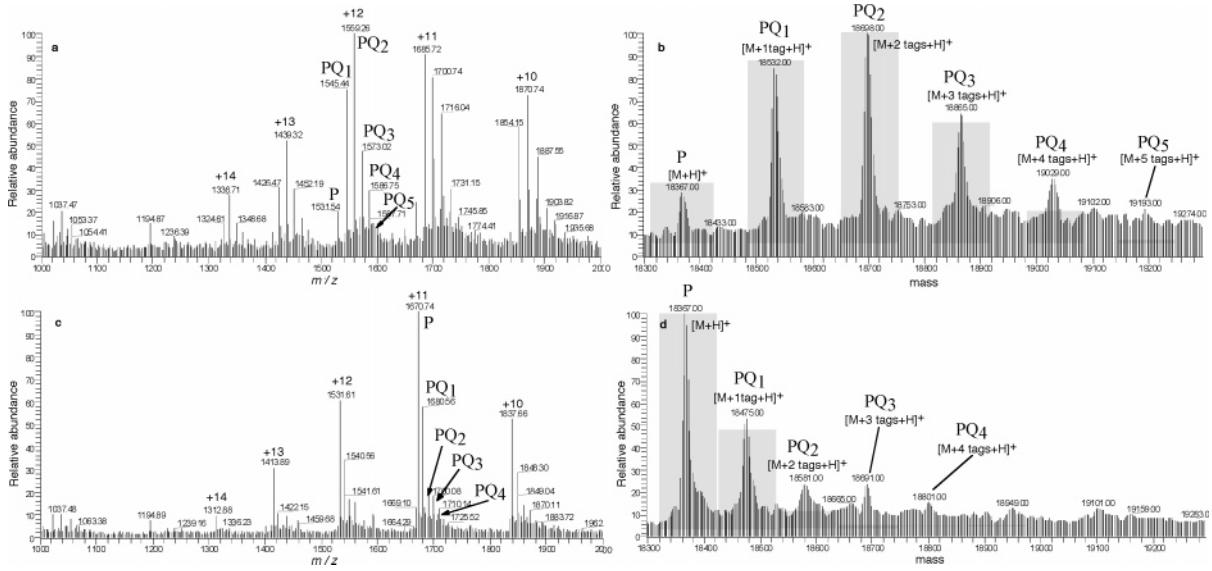


Figure 6. Microspray mass spectra of reduced β-lactoglobulin A (50 μM) infused with methoxycarbonyl-1,4-hydroquinone (2.5 mM) (**a**) and 1,4-hydroquinone (2.5 mM) (**c**). Deconvolution mass spectra of reduced β-lactoglobulin A (50 μM) infused with methoxycarbonyl-1,4-hydroquinone (2.5 mM) (**b**) and 1,4-hydroquinone (2.5 mM) (**d**). Grey bars give the predicted distributions of the species according to the analytical model. P and $PQ_{1...5}$ indicate respectively the untagged and successively tagged proteins.

and the steric hindrance). The EC-tagging is shown to probe the general thiol reactivity by the mass spectrometric measurement of the extent of the tagging reaction.

2.3. Multi-EC-Tagging of Reduced β-lactoglobulin A. To explore the effect of steric hindrance, we studied the tagging of β-lactoglobulin A, but after chemically reducing it. Indeed, the cleavage of the two disulfide bonds of the protein will alter completely the tertiary structure, due to almost complete unfolding of the protein chain. Reduced β-lactoglobulin A was EC-tagged using methoxycarbonyl-1,4-hydroquinone and 1,4-hydroquinone as shown in Figure 6a,b and c,d, respectively. As predicted by the analytical kinetic model, methoxycarbonyl-1,4-hydroquinone provides clearly a very efficient cysteine labeling when compared to 1,4-hydroquinone. Indeed, the tagging of the five cysteines of reduced β-lactoglobulin A can be observed as shown in Figure 6b. By comparing mass spectra of reduced and nonreduced forms of the protein, the method gives access to the number of disulfide bonds. Besides, comparison with the kinetic model that fits perfectly (Figure 6b and 6d) shows clearly the role of the steric hindrance for the nonreduced protein.

The multi-EC-tagging of proteins by methoxycarbonyl-1,4-hydroquinone was further tested with creatine phosphokinase (CK) and reduced insulin. By reduction, insulin splits into two polypeptidic chains. The B-chain contains mainly basic amino acids of the protein, conferring to the polypeptide a higher ionization in the spray medium (MeOH/H_2O/AcOH 50/49/1) compared to that of the A-chain which is not detected.[45] The B-chain of insulin is efficiently EC-tagged by methoxycarbonyl-1,4-hydroquinone since the untagged species is no longer observed. The fully tagged product appears greatly enhanced relative to the 1,4-hydroquinone reaction (see Supporting Information Figure S2). CK, which contains four free cysteine residues,[46] is EC-tagged on every residues. As for the previous case, MS gives peaks ranging from the untagged protein to the quadruply tagged protein, thus providing the on-line counting of the cysteine units (Figure 7). The experiments also provide information on the CK structure in the spray medium as under native conditions, only one cysteine unit is accessible for labeling.[47] These results therefore show that CK unfolds efficiently in the spray medium (MeOH/H_2O/AcOH 50/49/1) to lead to the quadruple tagging reaction.

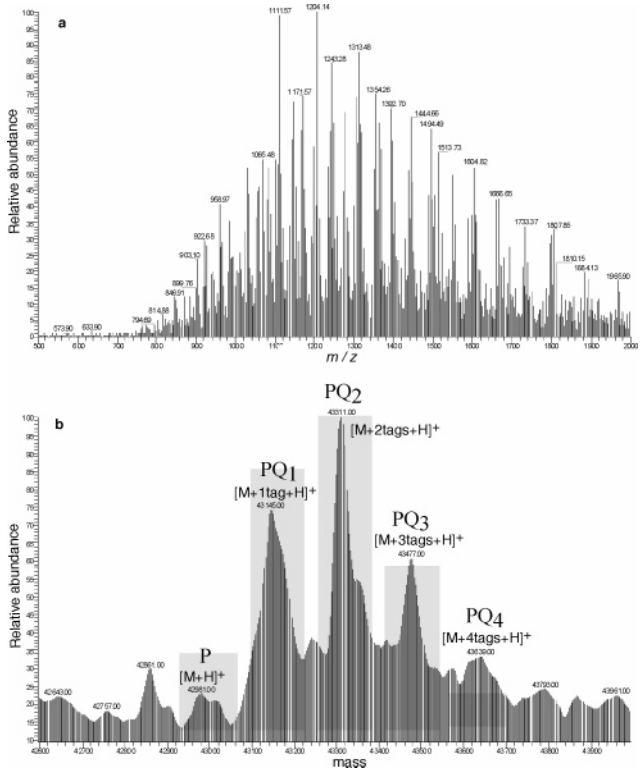


Figure 7. Microspray mass spectrum of creatine phosphokinase (50 μM) infused with methoxycarbonyl-1,4-hydroquinone (2.5 mM) (**a**). Deconvolution mass spectrum (**b**). Grey bars give the predicted distribution of the species according to the analytical model. P and $PQ_{1...4}$ indicate respectively the untagged and successively tagged proteins.

This last study demonstrates that methoxycarbonyl-1,4-hydroquinone is suitable for the multi-EC-tagging at the protein level. For multi-cysteine-containing unfolded protein, the analytical model fits perfectly well with the apparent tagging (Figures 6 and 7). Whatever the individual cysteine reactivity, the increase of the rates (i.e., nk) especially and compensation phenomena between higher and lower reactive thiols reduce the divergence.

Conclusions

The EC-tagging technique, by nonquantitative labeling, was shown to allow quick detection of cysteine-containing proteins by detection of the specific mass shift(s) corresponding to the marker. An analytical kinetic model taking into account the electrochemical and the chemical steps of the labeling reaction was developed to predict the tagging extent before MS analysis. Validated by comparison with numerically simulated data, the model was shown to fit perfectly well to the EC-tagging experiments on unfolded multi-cysteinyl-containing protein.

In addition, experiments with unmodified proteins and their chemically reduced forms show the strong effect of the cysteine site reactivity on the EC-tagging efficiencies. Chemically reducing the proteins prior to tagging leads both to a successive multi-tagging of the cysteine units liberated by the reduction of disulfide bonds and to an increase in the reaction kinetics. Methoxycarbonyl-1,4-hydroquinone was found to be efficient to tag up to five cysteines in biomacromolecules.

When decoupled from ionization factors eventually induced by the tag, the EC-tagging by methoxycarbonyl-1,4-hydroquinone reveals itself as an useful tool to probe the thiol reactivity of a single-thiol-containing protein. A similar study may be carried out for singly cysteinyl peptides that are by far the most common cysteinyl peptides resulting from proteolytic digestion of the proteome.[48] For peptides or small molecules presenting negligible steric constraint, the EC-tagging based on mass spectrometric detection could thus directly provide the thiol pK_a values that are commonly determined by potentiometric and electrophoretic means.

EC-microreactors directly coupled to ESI−MS are attractive tools that can be extensively used for probing the surface accessibilities of specific amino acids. The development of specifically targeted tags will help providing information about the protein microenvironment.

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Note Added after ASAP Publication: This manuscript was originally published on the Web 02/21/2006 with an error in Scheme 1. "RS" should have been "RSH". The version published on the Web 03/01/2006 and in print is correct.

Supporting Information Available: The program for a five-step chemical reaction; the mass spectrum of an equimolar mixture of β-lactoglobulin A and its tagged analogue shows a signal enhancement for the tagged compound over that of the untagged protein (Figure S1); and the fully tagged insulin product appears greatly enhanced relative to the 1,4-hydroquinone reaction (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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