Journal of Chromatography A, 1217 (2010) 6496–6502

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Separation of post-translational modifications in monoclonal antibodies by exploiting subtle conformational changes under mildly acidic conditions

Shiyi Wang, Roxana Ionescu, Norbert Peekhaus, Jin-yu Leung, Sha Ha, Josef Vlasak*

Merck Research Laboratories, West Point, PA, USA

ARTICLE INFO

Article history:
Received 12 May 2010
Received in revised form 11 August 2010
Accepted 16 August 2010
Available online 21 August 2010

Keywords:
Antibody
Low-pH conformation
Cation-exchange HPLC
Glycosylation
Methionine oxidation

ABSTRACT

Chromatographic separation plays a key role in the identification, quantification, and characterization of protein variants. Here we describe separation of species containing two post-translational modifications (glycosylation and methionine oxidation) in the Fc fragment of a monoclonal antibody. The method is based on cation-exchange chromatography under mildly acidic conditions that destabilize mainly the CH2 domain. Our data suggest that the separation is not mediated by the chemical modification itself, but rather by subtle structural changes induced by the chemical modification in the domain-decoupled conformation that monoclonal antibodies adopt around pH 4. Compared to other procedures already described in the literature, this method demonstrates an improved separation and allows purification of species in the native fold for additional functional characterization. This approach of separation under conditions where the protein assumes an alternative conformation could find a more general utility for the separation of chemical modifications in proteins.

1. Introduction

Monoclonal antibodies are assembled from immunoglobulin domains, four involving the heavy chain (for IgG) and two involving the light chain (Fig. 1). The second constant domain in human IgG heavy chain, referred to as the CH2 domain, contains the conserved, glycan-bearing Asn297 (EU numbering). Compared to other antibody domains, the CH2 domain has a relatively low thermal stability [1–3], which can be further decreased by lowering solvent pH [1–3]. At pH ≤ 3.8 conformational transition occurs in the CH2 domain, weakening the interaction between CH2 and CH3 domains and thus destabilizing the CH2 domain [4] (Fig. 1).

Post-translational modifications may impact the conformation of the CH2 domain and, consequently, its stability. The role of N-linked glycosylation in the conformation of the CH2 domain has received significant attention as the affinity to Fcγ receptors (FcγR) is affected by the glycosylation pattern [5,6]. The conformation of some glycoforms and of a fully deglycosylated antibody have been reported by X-ray crystallography [7], NMR spectroscopy [8], hydrogen/deuterium exchange mass spectrometry [9], and by susceptibility to protease digestion [10]. Changes in the conformation are also reflected in the thermal stability of the CH2 domain. Antibodies with shorter glycan chains on Asn297 show lower melting temperature (Tm) of their CH2 domain compared to antibodies with typical glycosylation; complete removal of the glycans further decreases the Tm [11].

Methionine oxidation is another chemical modification known to change the stability and conformation of the CH2 domain [10,12,13]. Two oxidation sensitive methionine residues – Met252 and Met428 (EU numbering) are located at the interface of the CH2 and CH3 domains (Fig. 1). Although some methionine residues prone to oxidation belong to the CH3 domain, it is mostly the CH2 domain stability that is impacted by the oxidation [12]. Interestingly, some regions impacted by methionine oxidation are also impacted by glycan removal [13].

While the aforementioned chemical modifications change the conformation of the CH2 domain, there are only few reports showing that these antibody variants can be separated by chromatography [14–16]. Cation-exchange chromatography (CEX) has been successfully used to separate numerous modifications in monoclonal antibodies [6,17,18] and the results reported in this paper demonstrate that chromatographic separation can be improved under conditions that weaken the interaction between the CH2 and CH3 domains. Compared to typical conditions used for CEX separation of antibodies, the mildly acidic conditions described here enhance conformational changes induced by these post-

* Corresponding author at: Merck Research Laboratories, WP78A-31, Sumneytown Pike, West Point, PA 19486, USA. Tel.: +1 215 652 2585.
E-mail address: josef_vlasak@merck.com (J. Vlasak).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.08.044

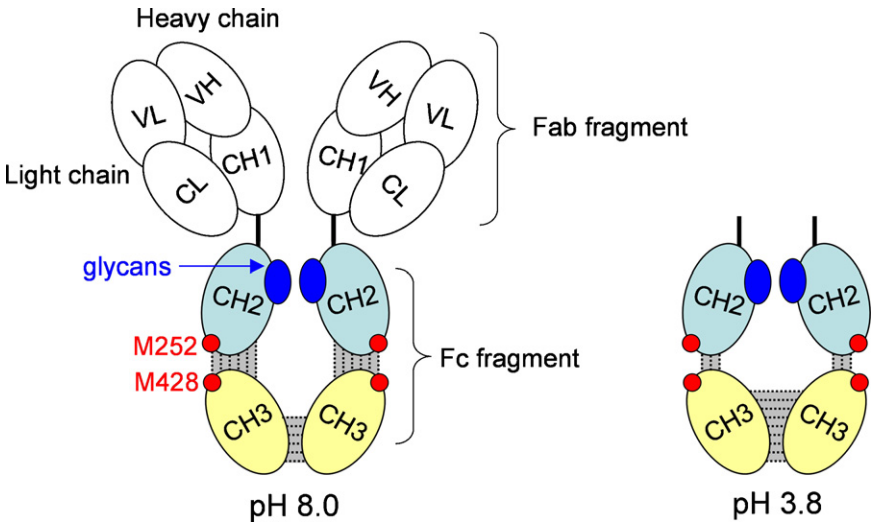

Fig. 1. Schematic drawing of an IgG antibody and the domain-decoupled conformation of the Fc fragment at pH 3.8 (adapted from [4]). Heavy chain domains: variable (VH), constant 1, 2, and 3 (CH1, CH2, CH3). Light chain domains: variable (VL), constant (CL). Two conserved methionine residues (252 and 428) at the interface of the CH2 and CH3 domains and the glycan chain on each heavy chain are indicated. The dotted lines on grey background indicate the relative degree of interaction between domains.

translational modifications, and provide the basis for the improved separation.

2. Materials and methods

2.1. Materials

Monoclonal antibody A and B were manufactured at Merck & Co., Inc using recombinant DNA technology. *Tert*-butyl hydroperoxide was purchased from Sigma (St. Louis, MO), PNGaseF from New England Biolabs (Ipswich, MA). Other chemicals including NaCl, sodium acetate, sodium phosphate, guanidine HCl, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), iodoacetamide, trypsin, trifluoroacetic acid (TFA), and acetonitrile were from Sigma (St. Louis, MO).

2.2. Cation-exchange HPLC (CEX)

CEX was performed on Agilent HPLC using a ProPac WCX-10 4 × 250 mm column (Dionex, Sunnyvale, CA) and a gradient elution (mobile phase A: 10 mM sodium acetate; mobile phase B: 10 mM sodium acetate, 1.0 M NaCl). The pH of the mobile phases was varied from 3.7 to 6.5. A constant flow rate of 1 mL/min was maintained. The NaCl gradient was increased 1% per minute, which is equivalent to 10 mM NaCl increase per minute. 1 M NaCl concentration of mobile phase B was maintained throughout the pH ranges, except for the experiments at pH 3.7, for which the NaCl concentration was 1.5 M due to the insufficient elution power of 1 M NaCl. The slope of the gradient was kept the same.

2.3. Oxidation of antibody B

Antibody B was oxidized with 5% *tert*-butyl hydroperoxide (tBHP) as described previously [19]. Antibody solution at 10 mg/mL formulated in 10 mM sodium phosphate pH 7.0 was mixed with tBHP to a final concentration of 5% and incubated at room temperature for 1 and 23 h. To quench the reaction the samples were thoroughly dialyzed into pre-cooled 10 mM sodium acetate pH 5.0. For circular dichroism measurements, the Fc fragment was prepared by papain digestion using immobilized papain following manufacturer's instructions (Fab Preparation kit, Pierce, Rockford, IL); the digestion was carried out at 37 °C overnight. The Fc fragment was purified using Protein A column included in the kit. The Fc fragment was then oxidized the same way as the whole IgG (using only 3.3 mg/mL of Fc fragment to maintain the same molar ratio).

2.4. Determination of methionine oxidation by peptide mapping

Antibody samples (100 μg) were denatured and reduced by adding 100 μL mixtures of 7.5 M Guanidine HCl, 2 mM EDTA, 100 mM Tris, pH 7.5 and 20 mM DTT. The mixture was incubated for 45 min at 40 °C followed by alkylation (13 μL of 10 mM Iodoacetamide, 45 min at 25 °C in the dark). The samples were then buffer-exchanged into trypsin digestion buffer (1 mM $CaCl_2$, 50 mM Tris, pH 7.6). Trypsin digestion was carried out at 37 °C for 5 h; antibody:trypsin ratio of 50:1. The reaction was stopped by adding 4 μL of 1% TFA (v/v) and put on Waters UPLC for analysis. The digest was analyzed by reversed-phase chromatography with UV detection; 35 μL of the digest was loaded onto an ACQuity UPLC BEH300 C18 1.7 μm, 2.1 × 100 mm column (Waters Corporation) kept at 40 °C; mobile phases A: 0.05% TFA (v/v) in water, B: 0.05% TFA (v/v) in acetonitrile. The peptides were eluted by a gradient of 2% B to 40% B in 50 min at a flow rate of 0.2 mL/min. The degree of oxidation was obtained by comparing the ratio of the oxidized and unoxidized peptides containing M252 and M428.

2.5. Determination of methionine oxidation by Protein A chromatography

Protein A chromatography was performed using POROS A/20, 4.6 × 100 mm Protein A column (Applied Biosystems, Foster City, CA). Mobile phase A: 20 mM Tris, 150 mM NaCl, pH 7.0; mobile phase B: 20 mM sodium acetate, 150 mM NaCl, pH 3.1; gradient: 5–60% B in 20 min at a flow rate of 4 mL/min.

2.6. Circular dichroism (CD) spectroscopy

CD measurements were performed on a Jasco-810 spectropolarimeter (Jasco, Inc., Easton, MD). The near-UV CD spectra for the Fc fragments (formulated in 10 mM sodium acetate, pH 3.7 or 4.9) were acquired at 20 °C with a protein concentration of 1 mg/mL using a 1 cm quartz cuvette. The scans were conducted between 260 and 310 nm; at 10 nm/min scan rate with a 4 s response time. All samples were measured once with accumulations of five mea-

surements. The mean residue ellipticity (MRE, deg cm^2 $dmol^{-1}$) was calculated. Spectra of blank buffer were measured and were subtracted prior to the MRE calculation.

2.7. Differential scanning calorimetry (DSC)

DSC measurements were conducted on a VP-DSC Capillary Cell Microcalorimeter (MicroCal, LLC, Northampton, MA) at protein concentration of 1 mg/mL, with a scan rate of 1 °C/min in the temperature range of 25–85 °C. The protein was dialysed in 10 mM sodium acetate buffer at pH 3.7 and 4.9 before the DSC measurement. Buffer/buffer scan was subtracted from buffer/protein scan. The thermograms were processed using the Origin 7.0 software and normalized to the molar concentration of the protein. The final excess heat capacity thermogram was obtained by interpolating a cubic baseline in the transition region.

3. Results

3.1. Separation of antibodies with different levels of glycan occupancy

This section describes a CEX HPLC-based method to separate antibodies that are fully glycosylated from those that are hemi-glycosylated (glycosylated only at one of the two heavy chains = half occupancy) and those that lack glycosylation altogether (aglycosylated = zero occupancy). The material represents an antibody (antibody A) in an early stage of development and it was known by alternative methods (reduced CE-SDS, mass spectrometry; data not shown) that it contains a non-negligible amount of partially glycosylated molecules. Although the procedure described below can be used for quantitative analysis of the individual species, the main objective is to allow purification of the fractions for additional structural and functional characterization. At pH 6.5 no separation of species with different glycan occupancy is observed for antibody A in the native form, but the presence of hemi-glycosylated species is apparent after treatment with PNGaseF (Fig. 2A). Enzymatic deglycosylation results in earlier elution of the main fraction (labeled as D/D297 in Fig. 2A) due to the conversion of Asn297 into Asp297. Importantly, a new basic peak is resolved (labeled as D/N in Fig. 2A). This peak represents the original hemi-glycosylated antibody which, after enzymatic deglycosylation, contains one Asp297 and one Asn297 (Asn297 without the glycan chain is not converted to Asp by PNGaseF). The fraction of species with zero glycan occupancy prior to PNGaseF digestion (labeled as N/N in Fig. 2A) constitutes a small fraction of the sample and it also becomes apparent after PNGaseF treatment.

When the chromatography is performed at pH 4.0 (Fig. 2B), several observations can be made. First, the antibody elutes at a significantly later time compared to at pH 6.5; due to the greater positive net charge at pH 4.0, and thus stronger interaction with the column (the same gradient was employed for both pH 6.5 and 4.0). Second, two basic peaks at approximately 58 and 68 min are well resolved from the main species eluting around 53 min. These peaks were collected and analyzed by SDS-PAGE and intact-protein mass spectrometry (not shown). The result indicated that the elution is in the order of the glycan occupancy: fully glycosylated (~53 min), followed by hemi-glycosylated (~58 min), and aglycosylated species (~68 min). Third, the enzymatically deglycosylated antibody elutes later than the native antibody (more basic), despite one (D/N) or two (D/D) additional Asp residues. Depending on the pKA, Asp297 could either carry a negative charge or be protonated and have the same net charge as Asn297 in the fully glycosylated antibody. Thus, without conformational changes, it would be expected that the deglycosylated antibody elutes either acidic or has unchanged elution compared to the native one. This is indeed the case when CEX is carried out at pH 6.5 (Fig. 2A), where acidic elution is seen for the deglycosylated antibody. In contrast, basic elution of the deglycosylated antibody is seen at pH 4.0 (Fig. 2B), which therefore is an indication of conformational changes in the protein; presumably in the CH2 domain, which shows destabilization at low pH [1–3]. Fig. 2C illustrates how the separation improves from pH 6.5 to pH 4.0.

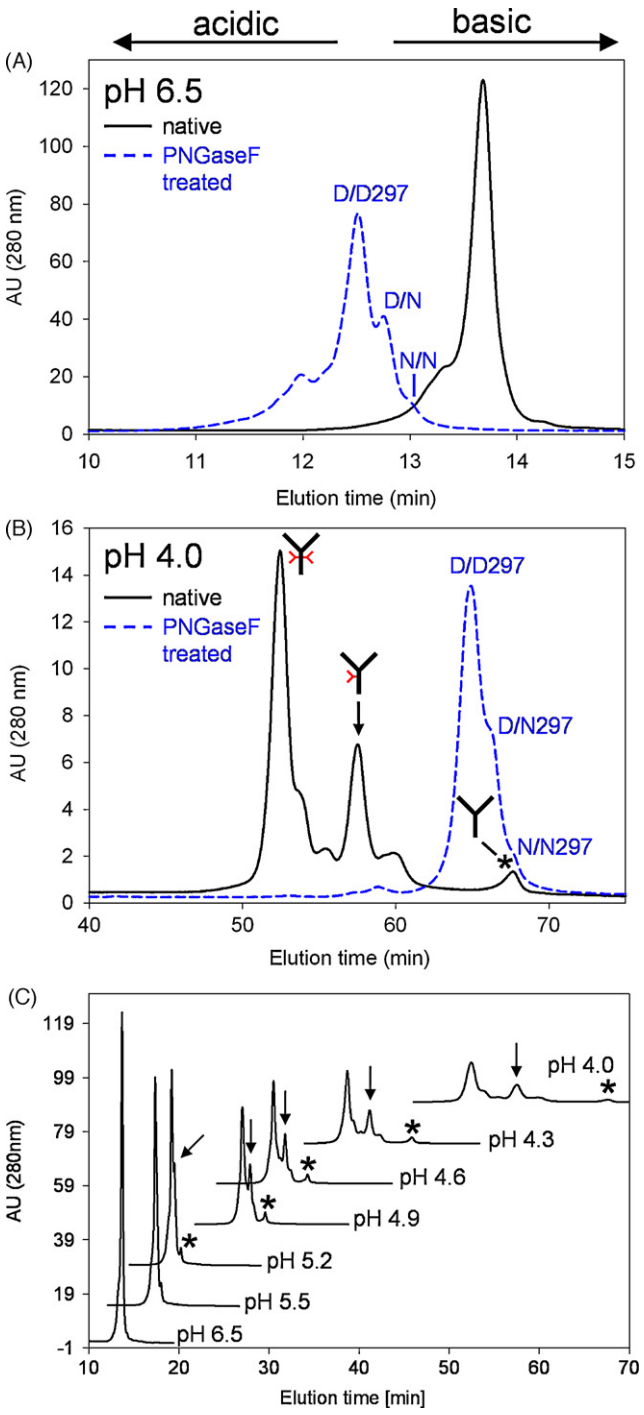

Fig. 2. CEX separation of the native and PNGaseF-treated (deglycosylated) antibody A at pH 6.5 (A), pH 4.0 (B), and at pH ranging from 6.5 to 4.0 (C). The same gradient was used for all conditions. D/D297 indicates that both heavy chains contain Asp at position 297, D/N – one heavy chain with Asp and one with Asn, N/N – both heavy chains with Asn at position 297. The arrows in (B and C) indicate the hemi-glycosylated variant, the asterisks (*) indicate the fully aglycosylated (N/N297) variant.

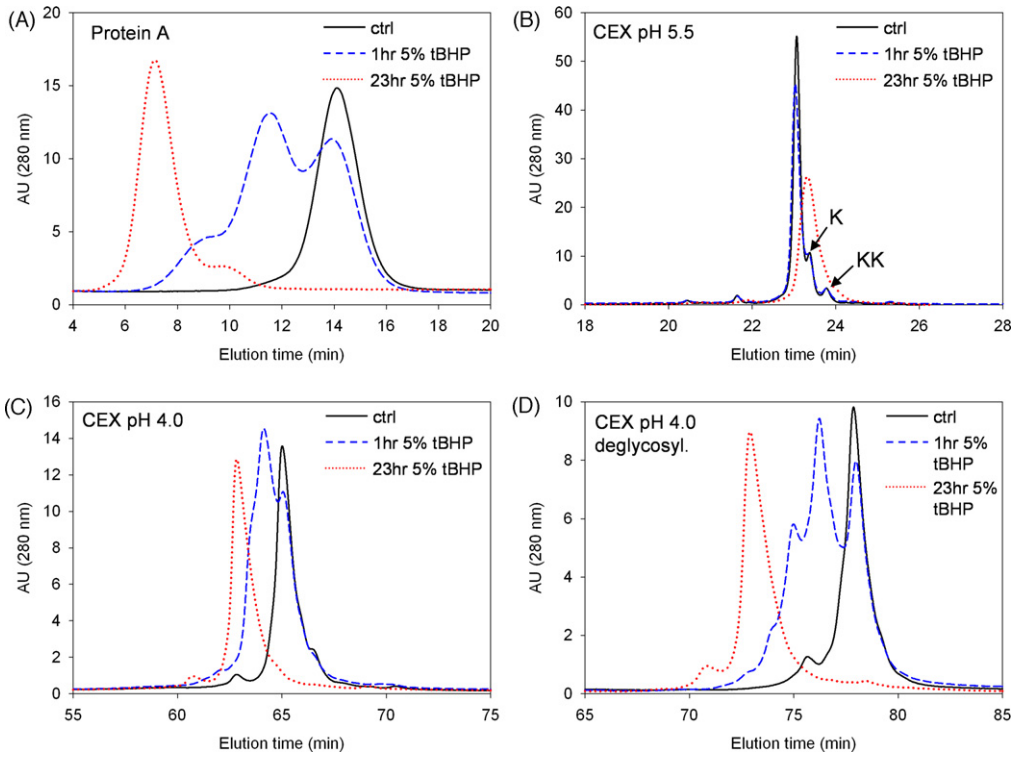

Fig. 3. CEX separation of partially (1 h 5% tBHP, dashed lines) and fully (23 h 5% tBHP, dotted lines) oxidized antibody B. K and KK in (B) indicated Lys variants; they are not resolved at pH 4.0 or by Protein A chromatography.

3.2. Separation of monoclonal antibody species containing oxidized methionine residues

A similar approach using CEX and low-pH separation was tested for monoclonal antibodies containing oxidized methionine residues. It was reported previously that this chemical modification of Met252 (in the CH2 domain) and Met428 (in the CH3 domain) impacts the conformation and stability of the CH2 domain [12]. Partially and fully oxidized antibody (antibody B) was prepared as described previously in literature [19] by incubation with 5% *tert*-butyl hydroperoxide (tBHP) for 1 and 23 h, respectively. The degree of methionine oxidation was determined by peptide mapping (Table 1) and also assessed by Protein A chromatography [20] (Fig. 3A). After 23 h incubation the antibody is almost fully oxidized (both M252 and M428 are oxidized in both heavy chains) and, compared to the control, it elutes earlier from Protein A column as one peak (Fig. 3A, dotted trace). After 1 h incubation partial oxidation is observed, leading to formation of various antibody variants with different levels of oxidation, which generate multiple peaks by Protein A chromatography (Fig. 3A, dashed trace).

When analyzed by CEX under standard run conditions (pH 5.5 for antibody B) only minor separation of the fully oxidized molecules is apparent – oxidized molecules elute as slightly more basic species (Fig. 3B). Partially oxidized antibody shows comparable profile to the native one, presumably because the level of oxidation is insufficient to allow separation. Similar basic elution has been reported for other antibodies [21]. However, when CEX is run at pH 4.0, significantly greater separation is obtained and the oxidized antibody elutes acidic compared to the native antibody (Fig. 3C). For the partially oxidized sample a three-peak profile, similar to the Protein A separation is obtained. As anticipated, because glycan removal induces additional destabilization and conformational changes into the CH2 domain [11], improved separation of the oxidized species is observed after treatment with PNGaseF (Fig. 3D).

Similarly to the example of the hemi-glycosylated antibody, the separation improves gradually with decreasing pH (Fig. 4). For the glycosylated antibody, no separation between the native and fully oxidized sample is observed in the pH range 4.6–4.9. Below pH 4.6 the oxidized antibody elutes gradually more acidic and above pH 4.9 the oxidized antibody elutes slightly basic. For the deglycosylated antibody (treated with PNGaseF), similar switch in elution is observed, as the oxidized antibody elutes more acidic below pH 4.3 and more basic above pH 4.6. The separation is improved for deglycosylated antibody compared to the glycosylated sample and significant separation is observed throughout the entire pH range. We hypothesize that the structure of the CH2 domain after deglycosylation facilitates conformational changes post-oxidation, and these conformational changes enable separation at non-acidic pH.

Table 1
Percent of methionine oxidation in antibody B determined by peptide mapping.

	Met252	Met428
Control	2%	1%
1 h 5% tBHP	38%	17%
23 h 5% tBHP	100%	86%

3.3. Conformational changes detected by circular dichroism spectroscopy and differential scanning calorimetry

To gain an insight into structural changes that may explain the improved CEX separation of post-translational modifications under mildly acidic conditions, protein conformation was evaluated by circular dichroism spectroscopy (CD) and differential scanning calorimetry (DSC). Antibody B samples (oxidized, native, and deglycosylated with PNGaseF) were analyzed under two buffer conditions: pH 3.7 (corresponding to the optimal CEX separation) and pH 4.9 (corresponding to a poor CEX separation).

Variations in the tertiary structure were monitored by near-UV CD spectroscopy. To evaluate structural changes pertinent to CH2

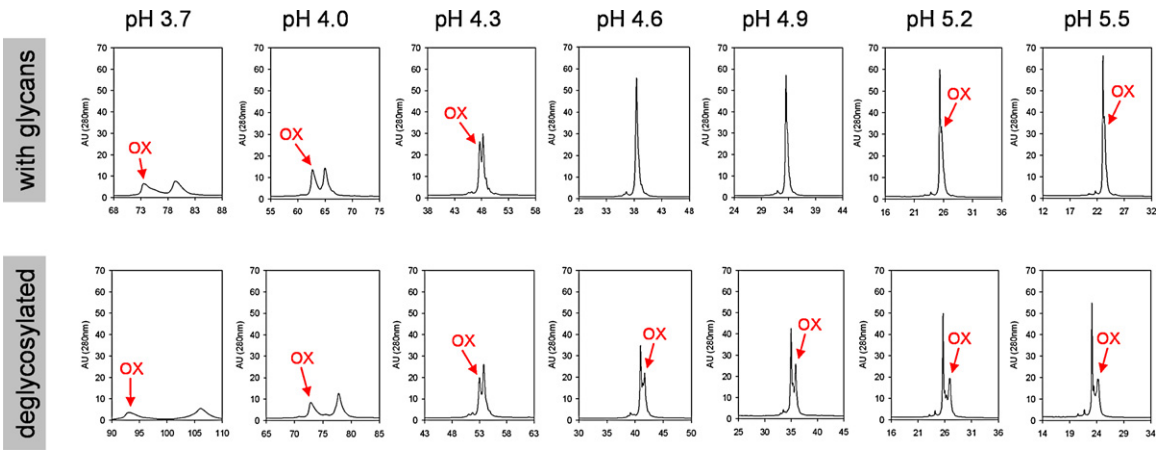

Fig. 4. CEX separation of antibody B at different pH. Native and fully oxidized sample was mixed 1:1 and run by CEX. Peak assignment was done by comparison with elution of individual samples (not shown); fully oxidized species are indicated with an arrow. The resolved oxidized antibody is indicated by an arrow. The same gradient was used for all conditions. Similar recovery was observed under all conditions.

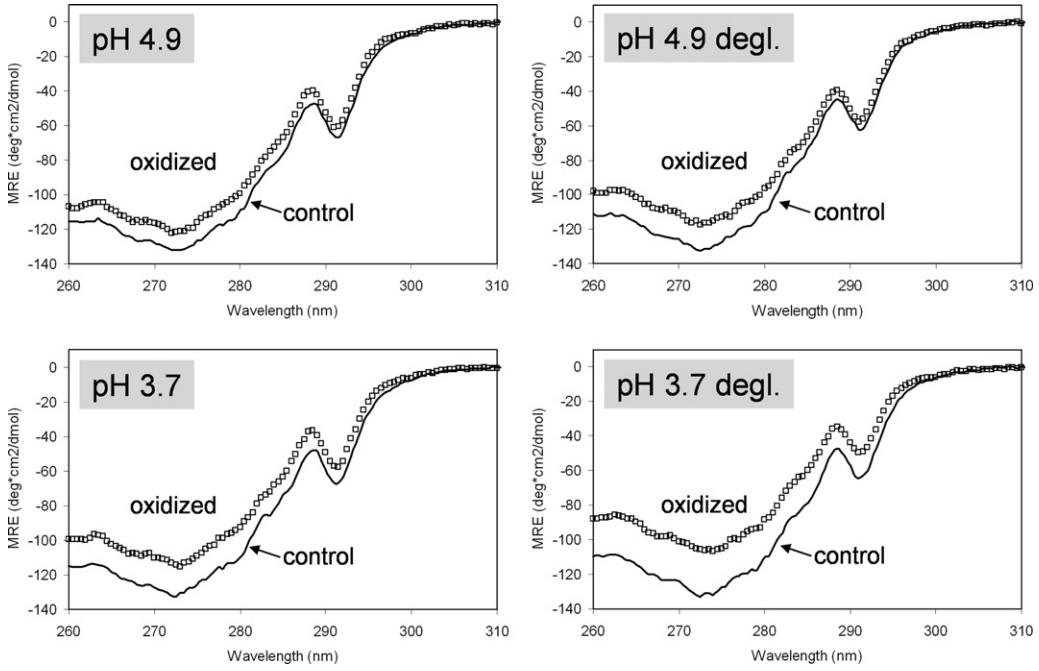

Fig. 5. Near-UV CD spectrum of the Fc fragment from antibody B, oxidized (square symbols) or control (solid lines), at pH 4.9 and 3.7, with and without PNGaseF treatment. In the left panel the control is unoxidized native antibody. In the right panel the control is unoxidized deglycosylated antibody.

and CH3 domains only, CD measurements were performed on oxidized and native Fc fragments, as opposed to the whole antibody molecules. Clear differences are observed between the oxidized and native Fc fragment, similar to those previously reported in literature [12]. In agreement with the CEX results, the spectral differences between oxidized and native are more pronounced at pH 3.7 compared to at pH 4.9 (Fig. 5, left panel) and this difference is further enhanced by deglycosylation after PNGaseF treatment (Fig. 5, right panel). Interestingly, no structural changes are detected between non-oxidized native and deglycosylated antibody under both pH conditions tested (Fig. 5).

The thermal stability of the native and oxidized antibody measured by DSC is shown in Fig. 6. Consistent with previous reports [12], the CH2 domain of the oxidized antibody unfolds at a lower temperature than the CH2 domain of the native antibody, regardless of the conditions. The difference is slightly greater at pH 3.7 compared to pH 4.9 and it is further increased after deglycosylation.

Altogether the biophysical measurements support the hypothesis that the conformational changes induced by oxidation are amplified at low pH via destabilization of the CH2 domain. These oxidation-induced conformational changes can be further augmented by the removal of Asn297 glycans. As shown in Figs. 5 and 6, we have observed the most changes in the CD spectra and the melting temperatures for antibodies with both oxidation and deglycosylation. The combined effect of deglycosylation and oxidation on the degree of conformational changes in the CH2 domain has also been reported from limited-proteolysis experiments [10]. In those experiments, oxidation by itself did not increase the sensitivity to proteases but it increased cleavage of a deglycosylated antibody.

4. Discussion

Protein conformation plays a critical role in ion-exchange-based chromatography techniques because large regions of the protein

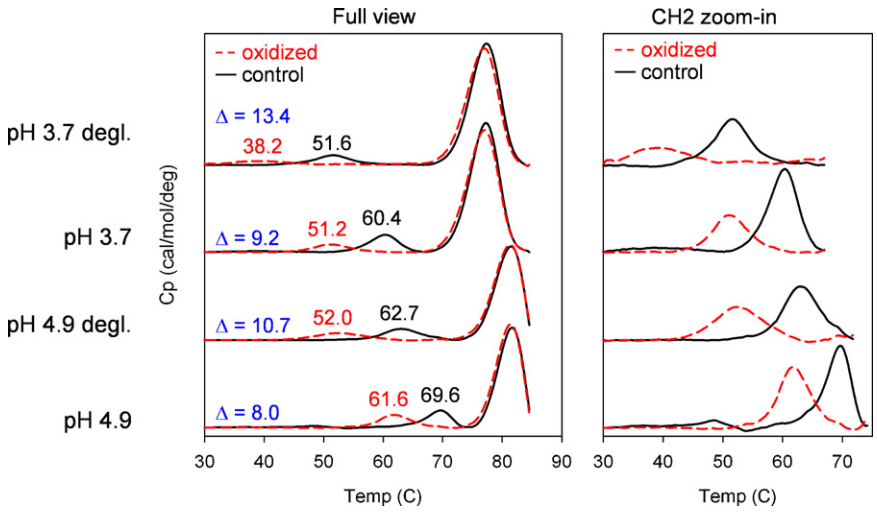

Fig. 6. Temperature-induced unfolding (determined by differential scanning calorimetry) of antibody B, oxidized or native, at pH 4.9 and 3.7, with and without PNGaseF treatment. The large-amplitude transition represents the unfolding of the Fab fragment and the CH3 domain. The small-amplitude transition (right panel) at lower temperatures represents the unfolding of the CH2 domain. The assignment of unfolding events to protein domains was described elsewhere [3].

surface are expected to be involved in the separation process [22]. Therefore, species with post-translational modifications can be resolved from the native protein based not only on the presence of a different chemical entity, but also based on structural differences induced by the new entity, which can be further amplified by solvent conditions.

It is now well established in the literature that glycan removal and methionine oxidation change the conformation of the monoclonal antibody Fc fragment [11,12]. Despite the structural changes, poor (if any) separation was achieved by CEX at pH 5 or higher for IgG1 fractions containing different levels of glycan occupancy (Fig. 2C) or Met oxidation (Fig. 4). Significantly better separation of these species was obtained when the charge distribution at the protein surface was changed by lowering the pH to approximately 4. It was previously reported in the literature that antibodies undergo limited conformational changes between pH 3.5 and 4.0 [23], and this conformation is distinct from the alternative immunoglobulin fold present in monoclonal antibodies below pH 3 [24]. This conformational change is supported by calorimetric studies from which it was inferred that the CH2/CH3 interactions, mediated by about 17 residues on a surface of 400 Å, are significantly weakened at pH 3.8 compared to pH values in the neutral range [4], see also Fig. 1. Our data support the conformational change at low pH. Fig. 4 shows that the relative elution of the oxidized and non-oxidized antibody changes in the pH region between 4.3 and 5.2. Compared to non-oxidized antibody, oxidized antibody elutes more acidic at pH below pH 4.3 but basic at pH 5.2 and above. This behavior seems to reflect the conformational switch occurring in this pH range – the low-pH conformation is impacted by methionine conformation differently than the conformation at pH 5.2 or higher. Similar switch is also observed for oxidized molecules that have been fully deglycosylated (Fig. 4, bottom panel).

Our results suggest that the above post-translational modifications in monoclonal antibodies are better resolved when the IgG1 adopts the domain-decoupled conformation [4] around pH 4. The presence of domain-decoupled conformation is strictly pH dependent and cannot be populated by destabilizing the CH2 and CH3 domains by other perturbations. This conclusion was drawn from the observation that the post-translational modifications described in this paper could not be resolved by CEX at neutral pH in the presence of urea (data not shown).

The separation of antibodies with and without glycosylation presented in this paper provides advantages compared to methods already presented in the literature. A CEX method for the characterization of glycosylation of monoclonal antibodies with elution at pH 6.0 was described [15]. The method resolved two populations with different glycosylation patterns, but it did not succeed in separating species with different levels of glycan occupancy (they all eluted in one peak). An alternative approach for the separation of monoclonal antibodies with different types of glycosylation was made using Protein A or Protein G chromatography [25]. Although the method is based on the same principles of differential impact of glycan composition on CH2/CH3 interface at low pH (since elution from Protein A is achieved at low pH), the peaks resolved by Protein A and Protein G chromatography are much broader than those reported in this paper and no distinct peaks are observed for species with different levels of glycan occupancy (even at 30% fraction of species with zero occupancy). Obtaining well-separated peaks is desirable when the method is intended for peak purification and subsequent biochemical and functional characterization of the post-translational modification. For the example of the hemi-glycosylated variants of antibody A, we could readily obtain fractions with >90% purity and evaluate their functional properties.

The results presented in this study demonstrate that two post-translational modifications, glycan occupancy and methionine oxidation, can be better resolved in monoclonal antibodies by cation-exchange chromatography under mildly acidic conditions than at pH 5 or higher. Although we have not evaluated this approach across a wide spectrum of monoclonal antibodies, we have successfully used it in another, unrelated antibody to isolate hemi-glycosylated species. Together with the fact that these two post-translational modifications are located in the conserved region of the molecule, it is likely that this approach could be applicable to a broad spectrum of antibodies. In addition, future experiments may demonstrate the validity of this approach for other post-translational modifications in monoclonal antibodies or in other proteins.

Acknowledgements

We wish to thank to Yang Wang and Lorenzo Chen for critical review of the manuscript.

References

[1] S.P. Martsev, Z.I. Kravchuk, A.P. Vlasov, G.V. Lyakhnovich, FEBS Lett. 361 (1995) 173.
[2] T.J. Kamerzell, J.D. Ramsey, C.R. Middaugh, J. Phys. Chem. B 112 (2008) 3240.
[3] R.M. Ionescu, J. Vlasak, C. Price, M. Kirchmeier, J. Pharm. Sci. 97 (2008) 1414.
[4] V.M. Tischenko, V.M. Abramov, V.P. Zav'yalov, Biochemistry 37 (1998) 5576.
[5] R. Jefferis, J. Lund, Immunol. Lett. 82 (2002) 57.
[6] R.J. Harris, Dev. Biol. (Basel) 122 (2005) 117.
[7] M.J. Feige, S. Nath, S.R. Catharino, D. Weinfurtner, S. Steinbacher, J. Buchner, J. Mol. Biol. 391 (2009) 599.
[8] Y. Yamaguchi, M. Nishimura, M. Nagano, H. Yagi, H. Sasakawa, K. Uchida, K. Shitara, K. Kato, Biochim. Biophys. Acta 1760 (2006) 693.
[9] D. Houde, J. Arndt, W. Domeier, S. Berkowitz, J.R. Engen, Anal. Chem. 81 (2009) 2644.
[10] H. Liu, G. Gaza-Bulseco, T. Xiang, C. Chumsae, Mol. Immunol. 45 (2008) 701.
[11] Y. Mimura, P. Sondermann, R. Ghirlando, J. Lund, S.P. Young, M. Goodall, R. Jefferis, J. Biol. Chem. 276 (2001) 45539.
[12] D. Liu, D. Ren, H. Huang, J. Dankberg, R. Rosenfeld, M.J. Cocco, L. Li, D.N. Brems, R.L. Remmele Jr., Biochemistry 47 (2008) 5088.
[13] D. Houde, Y. Peng, S.A. Berkowitz, J.R. Engen, Mol. Cell Proteomics 9 (8) (2010) 1716.
[14] J.F. Shen, M.Y. Kwong, R.G. Keck, R.J. Harris, Techniques in Protein Chemistry VII, Academic Press, Inc., 1996, p. 275.
[15] G. Gaza-Bulseco, A. Bulseco, C. Chumsae, H. Liu, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 862 (2008) 155.
[16] J. Valliere-Douglass, A. Wallace, A. Balland, J. Chromatogr. A 1214 (2008) 81.
[17] R.J. Harris, B. Kabakoff, F.D. Macchi, F.J. Shen, M. Kwong, J.D. Andya, S.J. Shire, N. Bjork, K. Totpal, A.B. Chen, J. Chromatogr. B 752 (2001) 233.
[18] J. Vlasak, R. Ionescu, Curr. Pharm. Biotechnol. 9 (2008) 468.
[19] A. Bertolotti-Ciarlet, W. Wang, R. Lownes, P. Pristatsky, Y. Fang, T. McKelvey, Y. Li, Y. Li, J. Drummond, T. Prueksaritanont, J. Vlasak, Mol. Immunol. 46 (2009) 1878.
[20] H. Pan, K. Chen, L. Chu, F. Kinderman, I. Apostol, G. Huang, Protein Sci. 18 (2009) 424.
[21] C. Chumsae, G. Gaza-Bulseco, J. Sun, H. Liu, J. Chromatogr. B Anal. Technol. Biomed Life Sci. 850 (2007) 285.
[22] F.E. Regnier, Science 238 (1987) 319.
[23] D. Ejima, K. Tsumoto, H. Fukada, R. Yumioka, K. Nagase, T. Arakawa, J.S. Philo, Proteins 66 (2007) 954.
[24] J. Buchner, M. Renner, H. Lilie, H.J. Hinz, R. Jaenicke, T. Kiefhabel, R. Rudolph, Biochemistry 30 (1991) 6922.
[25] G. Gaza-Bulseco, K. Hickman, S. Sinicropi-Yao, K. Hurkmans, C. Chumsae, H. Liu, J. Chromatogr. A 1216 (2009) 2382.