Journal of Chromatography B, 879 (2011) 1338–1344

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Review

Analysis of the oxido-redox status of plasma proteins. Technology advances for clinical applications☆

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ARTICLE INFO

Article history:
Received 16 August 2010
Accepted 24 November 2010
Available online 3 December 2010

Keywords:
Reactive oxygen species
Serum albumin
Sulphydryl groups
Focal segmental glomerulosclerosis
Glomerulonephritis

ABSTRACT

Reactive oxygen species (ROS) are potentially implicated in renal pathology. Direct evidence is available for animal models of glomerulonephritis but the demonstration of ROS implication in human diseases is only circumstantial and requires further experimental support. One problem limiting any evolution is the brief life of ROS (in terms of milliseconds) that makes it difficult their direct detection '*in vivo*'. An alternative is to look at the products of oxidation of proteins that remain in blood as a signature of ROS activity. Recent data have shown the presence of oxidation products of albumin (sulfonic ^{34}Cys albumin) in serum of patients with focal-glomerulosclerosis, that is a primary glomerular diseases causing nephrotic syndrome. Structural studies based on spectroscopy and calorimetry strengthened the relevance of oxidation of the unique free SH groups of ^{34}Cys for conformation of albumin, in analogy with what already reported for other proteins. In this review, we present new developments on technologies for the detection of the oxido-redox potential of proteins that are based on the concept that oxidation is inversely correlated with their free content of sulphydryl groups. We describe, in particular, two new iodoacetamide-substituted cyanines that have been developed for labelling sulphydryl groups and can be utilized as stable dyes prior mono- and bi-dimensional electrophoresis. Proteins with low binding with iodoacetamide-cyanines may be considered as surrogate biomarkers of ROS activity. Standardization of these techniques and their acquisition in more laboratories would enable clinicians to plan screening studies on ROS in human diseases.

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Abbreviations: C3NIASO3 and C5NIASO3, iodoacetamide-substituted cyanines.

☆ This paper is part of the special issue "Enhancement of Analysis by Analytical Derivatization", Jack Rosenfeld (Guest Editor).

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doi:10.1016/j.jchromb.2010.11.030

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1. General considerations

Oxidants (ROS) are toxic for many organs and whenever their production overcomes the intra and extracellular defence, an organ damage is generated [1,2]. ROS are produced in living organisms to fulfil fundamental functions such as immunologic defence and energy production. In fact, they are utilized by leukocytes to kill bacteria and are produced in mitochondria during the respiratory chain. It is clear that control mechanisms are active to limit potential damage even the rapid degradation (in the order of milliseconds) implies that the majority of oxidants are toxic when produced within the cell [3]. Long-living ROS metabolites such as chloramines could, instead, be active for minutes and reach target organs if not buffered by specific systems [4]. Due to the variable time of activity, different protective mechanisms are operative: specific enzymes are deputed to block ROS within cells whereas peptides and proteins function as buffering systems in plasma [5–8]. Evolution in the field of antioxidant systems are now showing that the protective system involves a rather complex machinery both within and outside the cell that results in modulation of various signalling pathway and structural proteins [9–13]. Overproduction of ROS may occur when the quota of metabolic ROS is altered or not buffered by specific enzymatic pathways or proteins (such as in presence of inherited defect of coenzyme Q synthesis) [14] or more frequently when leukocytes are activated by immunologic triggers such as complement, immune complexes or ANCA [15,16]. Technologies for ROS detection are essential to discover pathological situations that may evolve towards overt damage if left untreated. Strategies for ROS analysis rely on different basic assumption. One is devoted to evaluation of the amount of ROS produced by nucleated blood cells by means of specific fluorescent labels that are activated by oxidants. This approach gives a dynamic idea of what is the general oxidative status in a given condition [17,18]. The other is tailored to evaluation of the oxido-redox potential of proteins and gives a static estimate of what really happened in a period that is a function of half life of a given protein. The two strategies are not alternative and may be utilized in parallel to gain a more representative picture of a clinical condition in evolution.

In this review, we will outline recent methodological advances based on group-specific labelling of oxidised proteins that also utilize electrophoresis as a separation tool. The final intent is to develop technologies that allow detection of trace amount of ROS in plasma utilizing proteins as surrogate biomarkers of their activity.

2. Oxidants, their generation and functional implications

Instable metabolites of oxygen and nitrogen represent the two major families of oxidants. They are known as reactive oxygen species (ROS) and reactive nitrogen species (RNS) respectively and include a panel of products deriving from the same molecule, i.e. superoxide superoxide (O°).

In non-phagocytic cells, O° is generated during oxidative phosphorylation in mitochondria where up to 1% of the electron flow escapes ATP synthesis and generates O° from O_2. The fate of O° is to undergo a rapid conversion to H_2O_2 by superoxide dismutase that may, in turn, be converted into water and H^+ by means of catalase and glutathione peroxide. In alternative, H_2O_2 reacts with Fe^{2+}, via the Fenton reaction that leads to the highly reactive molecule hydroxyl radical (OH°).

In inflammatory cells, H_2O_2 follows a different outcome: (a) in neutrophils, it is converted by myeloperoxidase into hypochlorous acid (HOCl) which reacts with thiols, tyrosil and methionyl groups of proteins; (b) in eosinophils, H_2O_2 interacts with bromoperoxidase to produce hypobromous acid which brominates proteins.

RNS include a series of metabolites (nitric oxide, nitrite, peroxynitrite) with functional implication mainly in vascular stability. Nitric oxide (NO°) the principle molecule derives from NADPH-dependent oxidation of arginine that is catalized by three isoforms of nitric oxide synthase (i.e. neuronal-nNOS, endothelial-eNOS and inducible-iNOS).

3. Chemistry of oxidized protein groups

Several groups of proteins are modified upon oxidation/nitrosylation (for a review see [19]). Among any other, modifications of protein thiols, tyrosine, methionine, histidine and tryptophan have been more frequently indicated by studies on kinetics of reactions. The former two (thiols, tyrosine) are mostly reactive with hypochlorous acid and chloramines, i.e. the oxidants with longer life, and are for this reason the reactive groups to confer antioxidant functions to proteins outside the cell.

3.1. SH

Thiol groups of cysteines are probably the most reactive groups of proteins [6,20,21]. They may undergo both reversible and stable changes depending to the entropy of the reaction. Upon mild oxidation SHs are converted into sulfenic and sulfinic acid (from SH to SOH and SO_2H) that represent intermediate steps and, due to reversibility, they can be a part of a regulatory process of protein function. Sulfonic acid (SO_3H) is, instead, the end product of the reaction that is actually irreversible and leads to protein degradation [12,22]. Thiol groups react also with NO° producing S-nitrosothiols. Oxidative modification of cysteines may have profound effects on protein conformation that varies for different proteins and play functional relevant changes (for changes of albumin see below). Enzymes and structural proteins are particularly susceptible to oxidative changes and contribute to cell dysfunction. Albumin is the most studied model of oxidative changes due to its particular structure containing 35 thiols linked in 17 disulphides bridges with an unique thiol free (^{34}Cys) in a catalytic pocket (see dedicated section) [23].

3.2. Tyrosine

Oxidation of tyrosine results from myeloperoxidase and peroxidase-catalysed reaction to give chloro and dichloro-tyrosine as well as RNS-catalised reaction to form NO_2-Tyrosine. All of these are considered pathways that takes place during inflammation in vascular bed.

3.3. Methionone

Mild oxidation of methionine leads to generation on methionine sulfoxide that can be further converted in methionine sulfone ($MetO_2$). Methionine oxidation has been mainly associated with regulation of a few enzyme (α1-protease inhibitor, riboneclease) or regulation of cytoskeleton (actin).

3.4. Histidine and tryptophan

Histidine and tryptophan oxidation is mainly linked to metal-catalysed oxidation in the case a protein possesses histidine or a tryptophan close to a metal-binding site. Examples of His and Try oxidation are myoglobin, other cardiac proteins and lens proteins.

4. Derivatization procedures for assessing the oxido-redox status of proteins

There is a vast literature addressing the laboratory approach to oxidised proteins. As a general role, and due to the wide range of changes induced by oxidation, mass spectrometry must be considered the basic approach for a real evaluation of different derivates. Dedicated mass spectrometry approaches have been developed and specific experimental conditions must be chosen in each case. The most simple is to determine the molecular mass of an oxidized protein in comparison to the native state since formation of oxidised derivates induces a slight increase of size. We utilized LC–MS for analysis of the molecular mass of oxidised albumin and could show a +54 KDa increment due to the presence of three more oxygens in the sulfonic group [24,25]. Mass spectrometry offers the possibility of a global evaluation of oxidation changes with the unique drawback that more than one technique is required. The description of mass spectrometry analysis of oxidised proteins is behind the scope of our review and readers are remanded to dedicated reviews [20].

We will discuss here recent developments on the laboratory approach to oxidised proteins that utilize derivatization and electrophoresis. They are alternatively based on 'in gel' or 'before gels' derivatization and are always designed to determine the extent of free thiol groups of proteins. The basic assumption is that thiols are almost always involved in oxidation and any decrease in titrable free SH in a given protein is an indirect marker of oxidative stress. This is particularly true for albumin (see below) that is the major antioxidant outside the cell. Derivatization with PEO-Maleimide and Iodoacetamide cyanines represent two available tools for evaluating free SH in proteins.

4.1. PEO-Maleimide

Maleimide is the classical dye for SH groups. It reacts with SH of proteins at pH 6.5–7.5 to form a stable thioether bond (Fig. 1). In PEO-Maleimide the biotin probe is linked to maleimide with an arm consisting of polyethylene oxide. This dye has been used for 'in gel' derivatization and enables selective staining of single proteins since they are labelled prior separation. The system based on biotin/avidin reaction allows various possibilities (e.g. fluorescence, chemiluminescence, visible).

Peo-Maleimide has been successfully utilized for staining the free ^{34}Cys of serum albumin in which case, total serum proteins are fractioned, after labelling, by gradient electrophoresis in one dimension [25]. As shown in Fig. 2, PEO-Maleimide is comparably sensitive for labelling SH of serum albumin as cyanines of new derivatization and can be utilized in tandem with one dimension electrophoresis as a good alternative of the later dyes.

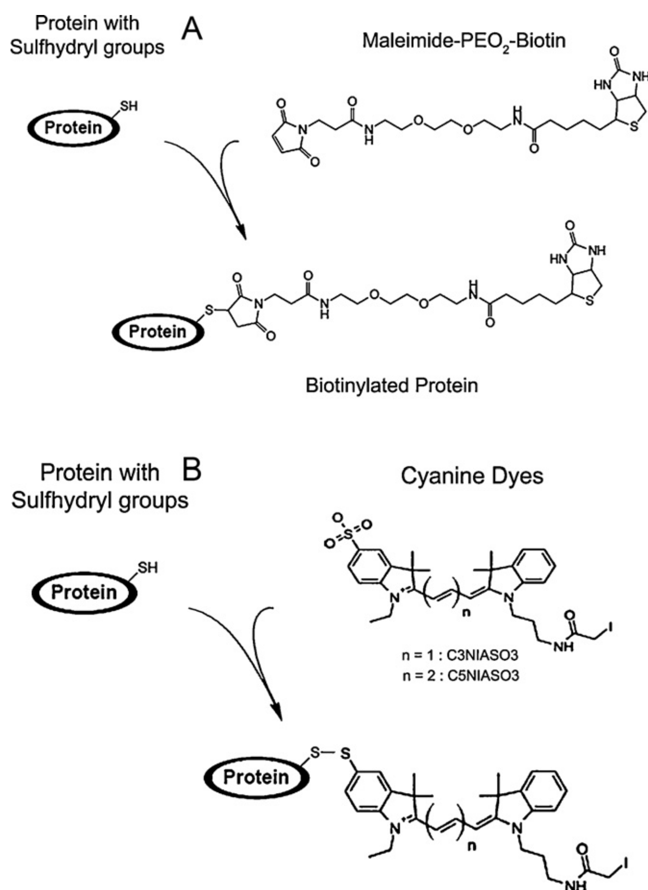

Fig. 1. (A and B) Schematic reaction between thiolic groups of proteins and maleimide-PEO2-biotine or the two new iodo-acetamide cyanine dyes C3NIASO3 and C5NIASO3. A detailed description of the reactions is provided in Section 4.

4.2. Iodoacetamide cyanines

In recent years an effort has been done to develop technologies that enable the derivatization of specific groups in proteins before their separation with two-dimensional gels. A new technique, called two dimensional differential display gel electrophoresis (DIGE), is the result of this evolution. It is a multiplexing system that allows the simultaneous comparison of site-selective labelled proteins in different mixtures [26] separated in a single run. It has been developed in mid nineties from the synthesis of matched sets of fluorescent N-hydroxysuccinimidyl ester cyanines (NHS) with different excitation-emission wave lengths [27,28]. For this reason, NHS can be utilized to label separate mixtures of proteins that are run in parallel and are analysed simultaneously owing to differentiate fluorescent spectra [29]. The clear advantage is that different samples are subject to the same procedure that limits in some way experimental variability and produces separate 2D images of the same gel. Cyanines were first devised to label primary amino groups of proteins (i.e. lysine ε and *N* terminal α amino groups) that are of widespread occurrence but it was then realized that *N* groups are common sites for protease digestion that should limit the proteomic characterization of labelled proteins.

Two new iodoacetamide-substituted cyanines, C3NIASO3 and C5NIASO3 (Fig. 1), have been then synthesized starting from hemicyanine and utilized for labelling SH residues in plasma proteins (Figs. 3 and 4). The interest in iodoacetamide cyanynes was also supported by their potential implication in evaluating the oxido-redox status of proteins. The basic chemistry for the synthesis of iodoacetamide-cyanines and best experimental conditions for

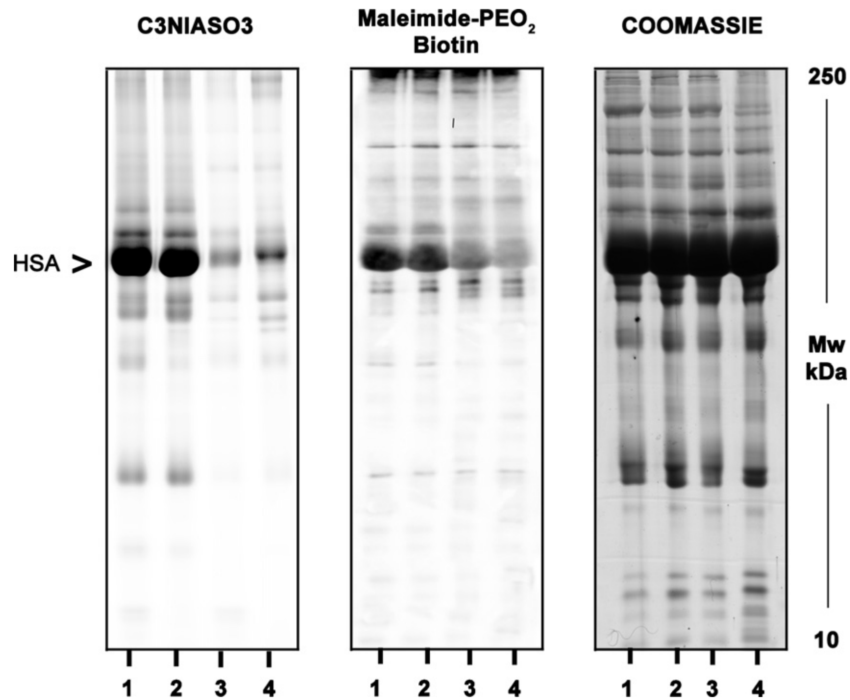

Fig. 2. Analysis by C3NIASO3 and maleimide-PEO2-biotin of plasma proteins from two normal patients (samples 1 and 2) and from two patients with focal segemental glomerulosclerosis (samples 3 and 4). In all cases, serum samples normalized for protein content (50 μg) were pre-labelled with C3NIASO3 (the more efficient of the two iodoacetamide cyanines) or with maleimide-PEO2-biotin and were then separated with SDS-PAGE. Coomassie Blue silver colloidal showed comparable amount of albumin. The binding of C3NIASO3 with albumin and other proteins was remarkably reduced in the case of FSGS patients.

their utilization have been described in the paper by Bruschi and coll. [30]. Specificity was also tested utilizing unlabelled iodoacetamide as inhibitor of the binding of C3NIASO3 and C5NIASO3 (not shown) and utilizing equimolar mixtures of standard proteins containing variable amounts of free SH and disulphides (Phosphorilase b, BSA, Ovalbumin, Carbonic anhydrase and Soybean trypsin inhibitor) (Fig. 3). Results indicated a good sensitivity/specificity and absence of staining with proteins lacking a SH group (see band

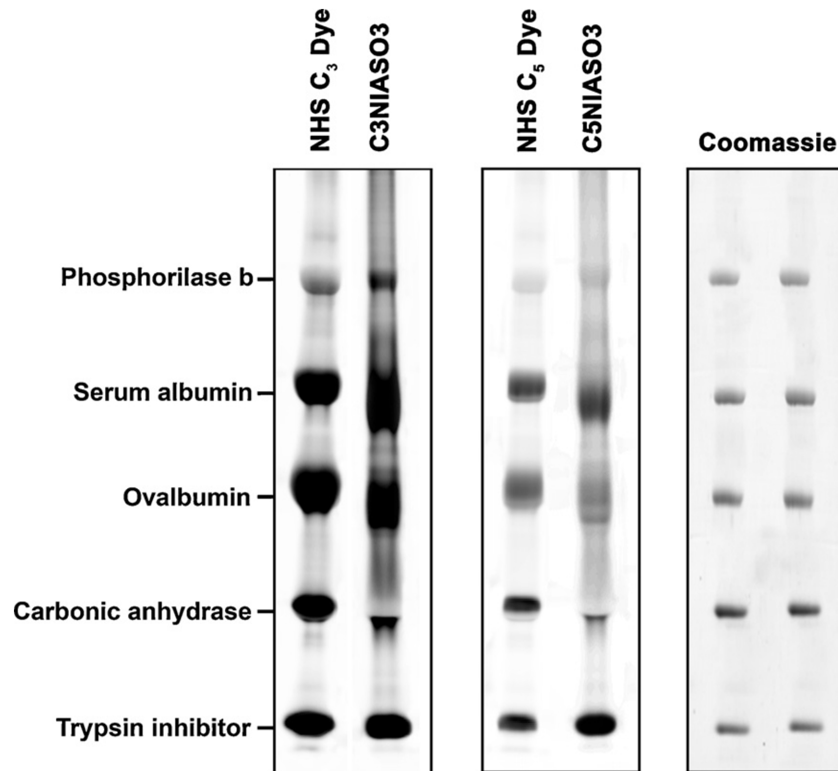

Fig. 3. Comparison of the sensitivity of different dyes available for differential display electrophoresis (DIGE) C3NIASO3, C5NIASO3 and Cyanines-NHS. All these dyes were utilized for labelling the same mixture of standard proteins tat were characterized by different content of SH groups: Phosphorilase b 97 kDa (209.37 ng), BSA 67 kDa (259.37 ng), Ovalbumin 45 kDa (459.37 ng), Carbonic anhydrase 30 kDa (259.37 ng) and Soybean trypsin inhibitor 20.1 kDa (250 ng).

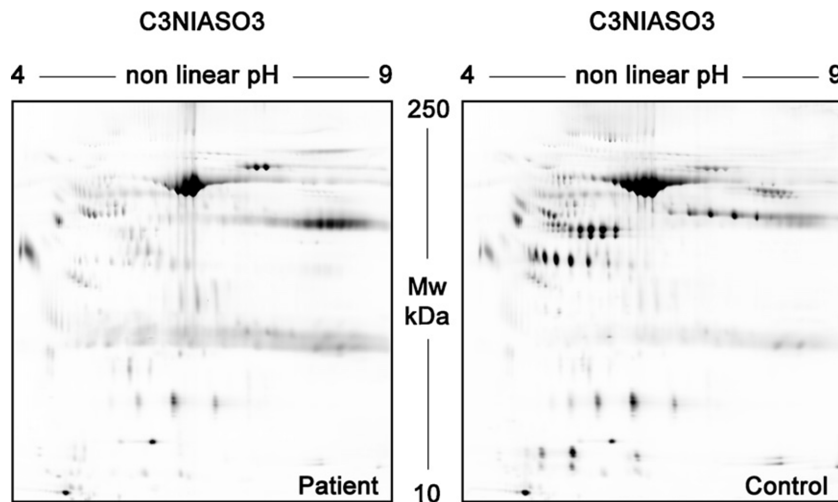

Fig. 4. One normal serum and one from a patient with focal segmental glomerulosclerosis (50 μg of proteins both) were pre-labelled with the same cyanine (C3NIASO3) and were then analysed by two dimensional electrophoresis. After electrophoresis gels were analysed labelling for the specific fluorescence band (Ex 532/Em 555) of the dye.

with 30 kDa, carbonic anhydrase in Fig. 3). Overall, results supported the utilization of both C3NIASO3 and C5NIASO3 for *in vivo* studies (see below).

5. Group specific protein oxidation

Direct determination of ROS/RSN is difficult due to a very short life of practically all metabolites (in the order of milliseconds) and also in the case of more stable compounds, such as chloramines, there are not available technologies allowing direct evaluation in plasma. One chance is the use of surrogate markers such as the product of reaction of ROS/RNS with proteins. Owing to their stability, oxidation products of plasma proteins can retain the fingerprint of the initial modification and be utilized as marker of the oxidative stress. In fact, proteins can scavenge up to 75% of reactive radicals in plasma where glutathione and antioxidant enzymes are less concentrated and probably inadequate to blunt a severe stress [31]. Technologies for detecting oxidized proteins are in progress and a few have already been utilized for human studies. One is based on the assumption that the residual anti-oxidant potential of proteins is inversely correlated with their oxido-redox potential and can be determined by evaluating the amount of chloramine that is buffered in a solution. The inverse of residual anti-oxidant function gives an estimate of the amount of oxidation products (AOPP) present in serum at any given time. The second technique is based on the knowledge that oxidants react with SH groups of proteins. The amount of free SH of proteins, in particular of albumin, is therefore indirectly correlated with oxidation and gives a numerical estimation of this process.

5.1. AOOP

Starting in the early 90, advanced oxidation products or AOPPs have been utilized in several papers as surrogate markers of oxidation and elevated levels have been documented in several diseases with demographic impact such diabetes mellitus and IgA glomerulonephritis. When present, AOPPs predict the progression of renal lesions, atherosclerotic cardiovascular events and death [32–37]. In spite of these important pathologic implications we still lack a clear structural characterization of AOPPs. Capeillere-Blandin et al. [33] identified albumin as the main AOPP product in plasma confirming previous data indicating albumin as the major target of oxidant stress in uremia [34,35,38]. The technique for AOPPs is based on the determination of the power of plasma to buffer chloramine T that can be evaluated by spectroscopy and gives therefore an indirect estimate of the extent of the buffering SH groups of albumin. Recent studies reported AOOP levels in plasma of a wide cohort of patients with renal IgA nephropathy [39] and demonstrated a linear correlation with the clinical outcome. In an evolution of this study, AOPPs levels were correlated with the amount of SH groups of albumin in the same study cohort showing an inverse correlation between the two parameters confirming the assumption above (Fig. 5).

5.2. Plasma albumin

The indication of albumin as the most important plasma component undergoing oxidation seems the logical consequence of the fact that it is the most abundant protein in plasma [20,21]. Moreover, it retains a myeloperoxidase activity and is, for this reason, vulnerable to oxidation by hypochlorous acid (HOCl). Albumin oxidised '*in vitro*' presented extensive sulfonation (SO_3^-) of the free sulphydryl group of 13 Cys (^{34}Cys, ^{75}Cys, ^{101}Cys 1, ^{124}Cys, ^{265}Cys, ^{278}Cys, ^{279}Cys, ^{289}Cys, ^{360}Cys, ^{361}Cys, ^{392}Cys, ^{448}Cys, ^{461}Cys, ^{514}Cys, ^{567}Cys); in parallel, the S residue of several methionone (^{123}Met, ^{298}Met, ^{329}Met, ^{446}Met, ^{548}Met) was transformed into methionine sulfoxide ($C_5H_9NO_2S$). The search for nitro, chlorine and bromine derivatives was negative in all cases [20,21]. This finding suggest a conformational derangement with rupture of disulphide bonds

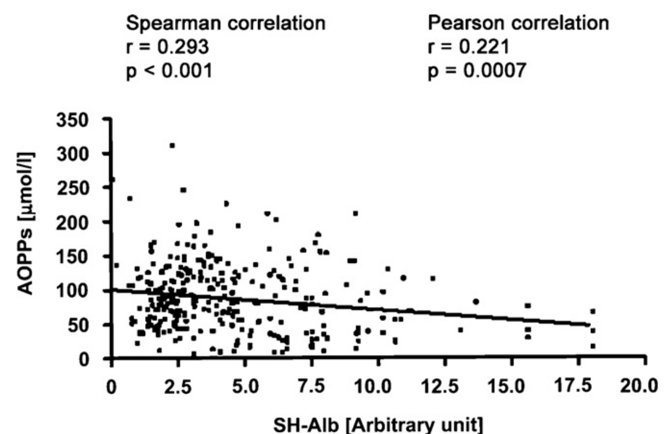

Fig. 5. Inverse correlation between the amount of AOPPs and the content of free sulphydryl groups (SH) of albumin in 200 patients affected by IgA mesangial glomerulonephritis at different stages of renal impairment.

that was confirmed by differential calorimetry (see below). Studies in a recent past have addressed the presence of oxidized products of albumin in two groups of patients affected by renal disease, i.e. nephrotic syndrome and IgA nephropathy [24,25,39] and in patients undergoing hemodialysis [35,38]. The analysis has been done utilizing a panel of techniques all giving separate picture of oxidation (LC–ESI-MS/MS, specific labelling with PEO-Maleimide, iodoacetamide cyanines, electrophoretic titration curves and differential scanning calorimetry (DSC).

The unique finding in normal plasma albumin was the constant presence of a methionine sulfoxide ($C_5H_9NO_2S$) residues at ^{329}Met and ^{548}Met that are to be considered as physiologic signals of aging of the protein whose half-life is 19 days. Besides sulfonation of the two Met residues above, plasma albumin in patients treated with hemodialysis presented only two reversible sulfenic (SO_2^-) derivates of ^{169}Cys and ^{265}Cys, one methionine sulfoxide residue at ^{123}Met in two cases and in one case ^{401}Tyr presented bromine (Br). It is important to stress that ^{34}Cys, that is the unique free SH group of albumin out of other 34 linked in 14 disulphide bridges in conditions of normal enthalpy, is not modified in albumin of patients undergoing hemodialysis and that the structural characteristics of the molecule such as electrical charge and conformation appears unmodified. Therefore, there are striking differences with oxidised albumin (*in vitro* model and patients with FSGS) that presented sulfonation (SO_3-) of the free ^{34}Cys in all cases (see below).

Musante and co-workers [25] described the presence of oxidation products of albumin in serum of patients with focal-glomerulosclerosis (sulfonic-^{34}Cys albumin) suggesting that ROS had been produced and buffered by protein SH groups. The same authors confirmed loss of free ^{34}Cys in albumin that suggests oxidation [40]. Finally, the analysis by FACS with specific fluorochromes that are activated by oxidants [17] showed an active oxidative-burst in circulating neutrophils [18].

The same authors attempted a detailed analysis of oxidised albumin for which they utilized electrophoretic titration, spectroscopic and calorimetric analysis. The first approach clearly showed the formation of an acidic component in the range of pH between 4.6 and 6 due to the addition of a sulfonic acid residue in place of a free SH group [41].

Fluorescent spectra indicated an increased hydration of the aromatic Trp that is exposed due to molecular perturbation (Fig. 6). The analysis of thermodynamic parameters [30] measured by differential scanning calorimetry confirmed the structural data above and showed important modifications of the swelling and the unfolding of oxidized albumin. Structural alterations are so profound that they cannot readily be justified by the presence of only few hydrogen bonds as the simulation of molecular dynamic as suggested by Kawakami et al. [21]. These data strengthen therefore the relevance of oxidation of the unique free SH groups of ^{34}Cys for conformation of albumin, in analogy with what already reported after sulfonation of free SH in other proteins. It can be suggested that in analogy with Ccd25b protein [42] sulfonation of ^{34}Cys facilitates the formation of hydrogen bonds with adjacent amino acids and deeply modify the enthalpy of the molecule.

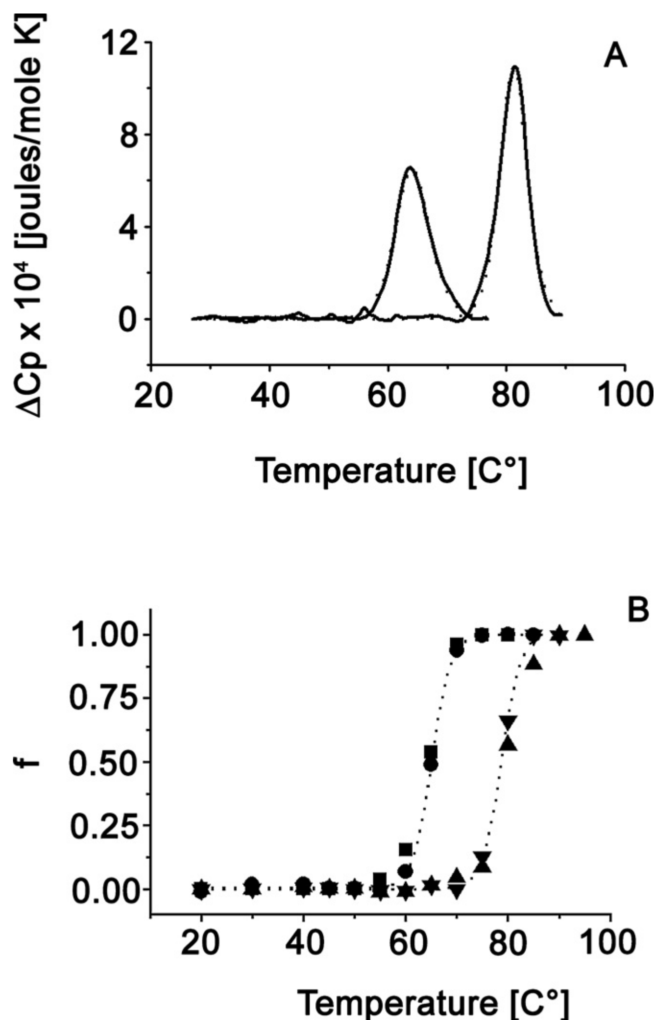

Fig. 6. Differential scan calorimetry (DSC) analysis (A) and fluorescent/UV spectra (B) of serum albumin purified from a normal donor and from a patient with glomerulosclerosis. The same two samples were analysed by different thermodynamic techniques in order to obtain detailed information on the unfolding status of the proteins. In the former case (A) normal albumin is indicated with a dot line, pathological albumin with a continuous line. For fluorescent spectra (B) normal albumin is indicates with (●), pathological albumin with (▲); for UV spectra normal albumin is (■) and the pathological with (▼). The overall result from the two techniques below gives an estimate of '*f*' that is the denatured fraction with normal albumin that presents a Tm lesser than pathological albumin.

6. Oxidants in chronic renal diseases

In spite an implication of oxidants in several diseases appears intuitive, a clear evidence for a role of ROS is limited to experimental animal models and a few genetic conditions characterized by mutations in relevant mithochondrial genes. In the former case, studies have provided evidence for a role of oxidants in leukocyte dependent glomerulonephrites (for a review see Shah [43]) that occurs when activated cells infiltrate glomeruli. The presence of infiltrates in glomeruli is necessary for determining an immediate contact of ROS with cells due to the rapid degradation of ROS (in the order of milliseconds) [3].

In animals, the infusion of stable oxidants such as adriamycin [44,45] and puromycin [46] produces renal lesions and nephrotic syndrome that mimic minimal change disease and/or focal glomerulosclerosis.

In humans, mutations of genes coding for enzymes deputed to Coenzyme Q synthesis [14,47] are associated with the same clinical and pathological conditions suggesting that a defect in the synthesis of molecules deputed to contrast ROS is to some extent equivalent to an increase in ROS production in terms of biological equilibrium. In spite of the evidence from animal models and the evidence in children deficient in Coenzyme Q, no clear proof that oxidants are implicated in human renal pathologies are available. Studies in humans are particularly difficult mostly because of the short life ROS and the unique chance we were left in the near past was the possibility to find out the products of their interaction with membrane especially of red-blood cells [48]. Now the development of technologies for analysis of the oxido-redox potential of

circulating proteins offers an opportunity to extend our approach to various pathologies and reinforce the awaited hope to developed surrogate biomarkers of clinical outcomes.

Current applications are limited to the determination of advanced oxidation products, or AOPPs, in chronic conditions which present or develop renal lesions such diabetes mellitus and as IgA glomerulonephritis (see dedicated section). Available results have shown that when present, AOPPs [32–35] predict the progression of renal lesions, atherosclerotic cardiovascular events [32,36,37] and death.

7. Perspectives

The availability of techniques for determining the oxido-redox status of plasma proteins offers an opportunity to gain information on the implication of these metabolites in human diseases. There are new possibilities to address at least three specific points: (a) one is the direct evaluation of ROS generation by neutrophils in blood by means of fluorescent dyes, (b) the other two allow the detection of the signature of ROS in proteins by means of a functional essay (AOPPs) or by determining the extent of SH consumption in albumin during the buffering of ROS. All represent clear advances that can determine some evolution in the field.

We need that these techniques are standardised in more laboratories and validated using internal standards shared by different groups. Clinicians should plan the formation of bio-banks of cells and serum of patients affected by clinical conditions in which ROS are potentially involved. Confirmation of preliminary results would open to new strategies for a proper follow-up of patients and validation of new therapeutic tools that are emerging in renal diseases.

Acknowledgements

This work was supported by the Italian Ministry of Health and by the Renal Child Foundation. Authors also acknowledge Fondazione Mara Wilma e Bianca Querci for financial support to the project ‘Ruolo dello stress reticolare nello progressione del danno renale e tumorale’ and Fondazione La Nuova Speranza for supporting the project ‘Progetto integrato per la definizione dei meccanismi implicati nella glomerulo sclerosi focale. Dalla predisposizione genetica alla regolazione della produzione di fattori cellulari e circolanti’.

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