Proteomics approaches to study the redox state of Cysteine containing proteins

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Summary. - All the proteins synthesized in a cell undergo several post-translational modifications that are essential in their functional regulation. Among these, the change of the redox state of Cysteine residues is assuming a great interest: this modification in fact, represents a very dynamic and regulated balance. There are several reversible oxidative events that can occur and that are difficult to detect. In this work we describe a methodology useful to recognize and to select Cysteines containing proteins on the basis of their redox state. The strategy is based on the selective labeling of the interested proteins and allows their visualization by Western Blot, enrichment by affinity chromatography and finally the identification of the protein and of the modified Cysteine residues by mass spectrometry. This methodology can be used in proteomic studies to recognize redox-sensitive Cysteine containing proteins and nitric oxide targets.

Key words: redox state, Cysteine, nitrosylation, mass spectrometry.

Introduction

In the last twenty years there has been an increasing interest about the effects occurring in an oxygen rich environment: several works have focused their attention on the negative effects that high concentrations of oxygen produce generating the so called “oxidative stress”. This phenomenon takes place when the pro-oxidant - antioxidant balance is perturbed, leading to a potential damage [1]. This event is strictly related to the variations of oxygen concentration and occurs when the rate of generation of reactive compounds exceeds the detoxification capacity of the cell [2]. Although this has to be considered an important biological event, often involved in many pathologies, recently it has been highlighted that the modulation of oxygen concentration is an important physiological mechanism in the regulation of many biological pathways. The balance of oxygen, and also of oxidative and reducing species (the “redox state”), changes in dependence of the cellular compartments modifying protein activity and function. It is known that the most important redox buffer in the cell is constituted by GSH which contains one Cysteine residue that is responsible of the buffering activity [3]. This tripeptide contributes also to the compartmentalization of the redox state in the cell: the ratio GSH-GSSG is 3:1 in the ER, to favour the oxidative processes occurring during protein folding, and 300:1 in the cytosol, determining a reducing environment [4]. Chemically, the aminoacid most sensitive to a change...
in the redox state is the Cysteine in which the reactivity is due to the thiol group. In most intracellular proteins thiol groups are strongly buffered against oxidation by the highly reducing environment inside the cell; moreover their sensitivity depends on the chemical environment present in the protein [5] with the consequence that only accessible protein thiol groups, with high thiol-disulphide oxidation potentials, are likely to be redox sensitive.

In this context, it is important to highlight that only the reversible oxidation of Cysteine plays a regulatory role while the irreversible modifications are related to damage of the protein activity. The oxidation of Cysteine to produce a disulphide bond is the first post-translational modification that occurs during the assembly of the nascent protein, often involving the action of some specific enzymes. It was thought that this modification is permanent in the life of the protein, but it has been instead realized that the redox state of Cysteine residues is a dynamic process that changes during the life time of the proteins, modifying their structure and modulating their activity [6, 7]. Evidences of this come from the correlation between the oxidation of specific Cysteine residues present in some kinases and the activation of these proteins [8], as well as from the observation that the oxidation of Cysteine residues present on the protein surface leads to inhibition of some phosphatases activity [9].

There are also other reversible oxidative processes that occur by chemical mechanisms without requiring any enzymatic activity: among these, the formation of sulfenic acid (Cys-SOH) and of S-nitrosylated Cysteine residues (Cys-S-NO) are becoming relevant in the comprehension of the modulation of the protein function in response to redox changes [10].

It is therefore essential to identify proteins containing Cys residues sensitive to oxidation but only few studies have attempted to examine global changes in disulphide bonds formation following ROS exposure. [11, 12]. A special agent able to oxidize Cysteine residues at an intermediate oxidative grade has been recognized in a gas different from oxygen, but very important and reactive: the nitric oxide. It is increasingly becoming appreciated that nitrosylation of protein sulphydryl groups represents an important NO dependent post-translational modification that is involved in signal transduction cascades [13]. The reactivity of nitric oxide is due to its radicalic nature. Moreover, in presence of oxygen, nitric oxide is able to produce several reactive nitrogen species (RNS), causing the so called “nitrosative stress”. Although the importance that this modification is assuming, this is very difficult to study because the S-NO bond is very labile.

In this work we describe a methodology to discriminate the redox state of Cysteine residues present in the proteins. The method is based on a differential alkylation of redox sensitive cysteines by using different alkylating agents: one of these is linked to a biotin tag that allows the visualization of the modified proteins including the characterization of the peptides and also the selection and the purification of the labeled peptides/proteins. Moreover, the strategy was adapted to the study of S-nitrosylated proteins: the protocol that has been set up is similar to the method described by Jaffrey [14], but it contains some modifications about the reagents used to block the free Cysteine residues. The protocol developed in this work allows the identification and the characterization of nitric oxide targets and of the S-nitrosylated residues. This approach can be applied to reach many information useful for the comprehension of the physiological role of nitric oxide.

**Results**

**Analysis of reduced and oxidated Cysteine residues**

The study of the redox state of a Cysteine residue is complicated by the possibility of scrambling of disulphide bridges that can occur without any enzymatic involvement because of the reversibility of the process. In this context the recognition of the original redox state requires the freezing of the redox state by an irreversible reaction that is usually constituted by an alkylation.

To set up a methodology useful to recognize the redox state of the Cysteine residues present in the proteins we have used Ovalbumin as a model protein as it contains several Cysteine residues, present in different redox states (four of the six Cysteine residues are in the reduced state and the other two are involved in a disulphide bond [15]). NEM was used to alkylate the reduced Cysteines while IAM alklylation was performed on gel after DTT reduction. After tryptic digestion the peptides mixture analyzed by MALDI-TOF MS showed a 62% of sequence coverage and it was possible to recognize Cys513 and Cys5120 alkylated by IAM while the other residues were labeled by NEM. These data are in agreement with the observations published by Thompson and Fisher [15].

The alkylating agents have shown a good efficiency and selectivity, so they were used also in the labeled form linked to a biotin tag: as it is shown in Fig. 1A, both the molecules were able to alkylate free Cysteine residues with the same efficiency and specificity. Moreover the tagged alkylating agents offer an important advantage because the labeled molecules
incubation: Ponceau stained membrane (up) and anti biotin of Ovalbumin after biotinylated NEM (on the left) or NEM (on the right); tryptic digestion of alkylated Ovalbumin by biotinylated strategy. For this reason the purification of intact proteins that have been alkylated. To optimize this strategy all the Cysteine residues present in Ovalbumin were reduced by DTT and then alkylated by biotinylated NEM: the protein was then hydrolyzed by trypsin and the tryptic peptides mixture was analyzed by MALDI-TOF MS (Fig. 2A). The MS analysis shows several peaks compatible with the theoretical tryptic peptides of Ovalbumin and many of these correspond to the peptides containing Cysteine residues that are alkylated by biotinylated NEM: the comparison between the MALDI spectrum of the unbound peptides (Fig. 2B) and the spectrum of the eluting step (Fig. 2C) shows the efficiency of the affinity chromatography in the selective enrichment of the labeled peptides. The presence of several peaks in which the modified peptides show also an increase of 18 Da can be explained by the frequent observation of the hydrolyzed form of the NEM adduct [16].

This method is very useful to enrich modified peptides and to characterize them, but the most important improvement derives from the possibility to use the affinity chromatography in a proteomic strategy. For this reason the purification of intact modified proteins has been optimized. Ribonuclease has been used with this aim after reduction of all the four disulphide bridges by DTT and alkylation by biotinylated NEM: the MALDI spectrum of the tryptic mixture obtained after in solution digestion demonstrates that all the Cysteine residues are alkylated (data not shown). The same reaction was performed using biotinylated iodoacetamide and the modified protein was loaded on SDS-PAGE: the low molecular weight of Ribonuclease allows to see an increase of the apparent molecular weight in the alkylated form in comparison with the unmodified form (Fig. 3). This difference is in accordance with the incorporation of eight molecules of alkylation agent, that induces an increase of about 4 kDa. To

Fig. 1. - A: MALDI analysis of peptide (360-369) after tryptic digestion of alkylated Ovalbumin by biotinylated IAM (on the left) or NEM (on the right); B Western Blot anti biotin of Ovalbumin after biotinylated NEM incubation: Ponceau stained membrane (up) and Western Blot (down).

Fig. 2. - MALDI-TOF analysis of peptides mixture of Ovalbumin after alkylation by biotinylated NEM, tryptic digestion and affinity chromatography: in panel A the total input is shown while in panels B and C a zoom of m/z range 1548.0-1666.0 of respectively the unbound and the eluted peptides is shown. Cys* indicates residues that are alkylated by biotinylated NEM.
This protocol could be used in a proteomic approach to study the redox state of Cysteine containing proteins: this could be important to analyze the cellular response to different redox changes as well as to study the function of strategic Cysteine residues located in functional regions of the protein. For this reason we have focused our attention on the possibility to adapt this methodology on a complex mixture coming from HeLa cells: in particular the membrane proteins are very interesting because the presence of reduced Cysteine residues in an oxidant environment (the extracellular one), or of residues that can be sensitive to redox state changes, suggests a specific functional role for these residues. The method was therefore tested on membrane proteins to verify the efficiency of the alkylation by biotinylated NEM, in the free or in the biotinylated form, in a complex protein mixture. In particular, the proteins were alkylated by NEM first, and then by biotinylated NEM and also by only one of the two reagents as a control. The two reagents are equivalent and completely label the free cysteines (data not shown). The tagged proteins can be purified by affinity chromatography and separated by SDS-PAGE to allow their identification by peptide mass fingerprint. This method, described for the selection of reduced Cysteine containing proteins, can be alternatively modified to select proteins that contain oxidized Cysteine residues: this is interesting for the Cysteine residues that are in the oxidized state although the environment is a reducing one, or when they change their redox state after an oxidative event, such as oxidative stress or other related stimuli. Moreover, the optimization of a method useful to analyze oxidized Cysteine containing proteins becomes very important when considering the difficulty to see a disulphide bond by mass spectrometry. This investigation requires the labeling of the oxidized Cysteine residues by the biotinylated alkylation reagent, and this is possible only after blocking the reduced Cysteine residues so that they can not undergo scrambling reactions and can not react with the labeled alkylation reagent. All these conditions can be respected performing first of all the alkylation of free Cysteine residues by NEM, then the oxidized Cysteine residues, in particular the residues involved in disulphide bonds, are reduced by DTT and finally alkylated by biotinylated NEM. We have tested this modified strategy using cytoplasmatic proteins from HeLa cells. The Western Blot analysis indicates again that the method is specific and that the blocking is complete because the signal is present only after DTT treatment. These experiments were performed in basal conditions, but this strategy is useful also to study redox state changes: the Western Blot analysis of Cysteine containing proteins after hydrogen peroxide
treatment shows an increase of the intensity of the signal after the oxidizing treatment (data not shown). In conclusion, the method described here produces proteins that can be selected on the basis of the redox state of the Cysteine residues.

**Analysis of nitrosylated cysteine residues**

One of the most interesting reversible redox changes that can occur on a Cysteine residue is represented by the nitrosylation: this post-translational modification consists in a partial oxidation of the thiol group. It is very difficult to detect, in particular by mass spectrometry [17], because the S-NO bond is very labile. Also in this case a modification of the method described for the detection and the selection of reduced Cysteine residues is useful for the detection and identification of S-nitrosylated proteins. In particular the methodology set up for the analysis of S-nitrosylated proteins modifies the protocol described for the analysis of oxidized Cysteine residues containing proteins by substitution of the DTT reduction with a specific treatment for the NO moiety removal. It is known from literature that Sodium Ascorbate is able to reduce nitrosylated thiols [18]. Before using it in a complex peptides mixture, we tested the possibility that this treatment could interfere with the presence of disulphide bridges. We demonstrated that there is no interference by using a synthetic peptide (whose sequence is CDGRCGVR): the disulphide bond present in this peptide is broken in presence of DTT, but it remains stable after Sodium Ascorbate treatment (data not shown). It is therefore possible to block the free Cysteine residues, as described before, by NEM alkylation, then to specifically remove the NO moiety by Sodium Ascorbate producing now free Cysteine residues that can be alkylated by biotinylated NEM (Fig. 4). To test the method we chose as a model protein Ovalbumin because of its similarity to BSA that is known to be nitrosylated in vitro [19], and because it contains only 6 Cysteine residues instead of 35.

The results obtained from the application of the methodology on Ovalbumin are described on the Western Blot in Fig. 5 where the signal relative to the reduced Cysteine (lane 2) and to the S-nitrosylated residues (lane 4) are shown. The difference in the intensity is due to the consideration that not all the free Cysteine residues are susceptible to nitrosylation [13], and this explains also the increase in the molecular weight of the protein in lane 2 in comparison with the nitrosylated one. We have to remember indeed, that in this case the intensity of the signal in Western Blot is proportional to the number of residues that are labelled in each molecule, while the Ponceau stained membrane indicates that the amount of the protein loaded in each lane is equivalent. Moreover, the absence of a signal in Western Blot in lane 3 indicates again the efficiency of the blocking step. We can then conclude that the method works efficiently and offers the possibility to select and purify the proteins after a specific labelling. Moreover the tag remains on the interested Cysteine residue until the end of the purification, allowing the identification of the modified residue by mass spectrometry.

![Fig. 4. - Strategy to label the nitrosylated Cysteine residues in proteins containing reduced Cysteine residues and disulphide bonds.](image1)

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![Fig. 5. - Analysis of Ovalbumin after nitrosylation and Biotin Switc protocol by using NEM and biotinylated NEM to alkylate free Cysteine residues: Ponceau stained nitrocellulose (A) and Western Blot (B) are shown.](image2)
Discussion

There are many post-translational modifications that play important roles in regulating the function of several proteins. The ability to exert a regulating role is mainly due to the reversible character of these modifications. Many of these reactions occur without an enzymatic involvement and often the newborn bonds are very labile and can be lost during the experimental conditions used to detect them. Changes in the redox state of Cysteine residues constitute one important example of this dynamic regulation, as it is demonstrated by the increasing interest in this field during the last years and by the discovery of an important role of the redox regulation in many physiological and pathological processes.

Nevertheless the high reactivity of the Cysteine residues represents a serious problem for the detection of these reversible modifications and the only possibility to study them is to use an irreversible reaction to label the interested residues and to freeze the reactive residues as it was in the physiological condition studied. For these reasons we set up a methodology useful to recognize free Cysteine residues but also the oxidized ones if they are involved in disulphide bonds formation or in S-nitrosylation. In particular, the methodology described here allows a proteomic analysis useful to monitor the redox state of cysteine containing proteins in basal conditions or in presence of several stimuli. The most important advantage offered by this methodology is represented by the presence of the biotin tag on the alkylating agent that gives the possibility to select and to purify labelled peptides and proteins.

A specific enrichment of tagged proteins from a complex protein mixture has been set up and this is the base for an alternative proteomic strategy, that can be 2DE independent, using the affinity chromatography as a separation step: by using this method it is possible to have the enrichment of the interested proteins, that often are less represented and whose presence is therefore masked by the more abundant proteins. The enrichment step can be performed not only on proteins, that can be then separated and identified, but also on peptides; in this case the eluting fractions can be analyzed by MS and MS-MS, after chromatographic separation, in order to recognize the sequence of the peptides and, most important, the modified Cysteine residues. The method described here is similar to the method published by Snyder [20] but there are some differences in the reagents used to block and to label the Cysteine residues. This protocol takes advantages of affinity chromatography step because the high affinity between biotin and streptavidin is used for the binding step, but an efficient reduction of disulphide bonds by DTT is sufficient to elute in a specific way the bound proteins. Moreover, after the purification, all the Cysteine residues are in the reduced state. The method here optimized and described allows to identify the specific residue that is S-nitrosylated and this could be very important for the comprehension of the mechanisms by which the S-nitrosylation regulates protein function and metabolic pathways.

The relevance that reversible oxidations of Cysteine residues can have in physiological conditions has been underestimated for many years, and only recently redox state changes are becoming object of an increasing interest in several biological fields. Moreover it has becoming evident that these modifications are not only a representation of oxidative stress events, but they also constitute a regulatory mechanism that can be identified such as “redox switch” [21]. The oxidation of Cysteine residues has been compared with the phosphorylation: it seems that these two post-translational modifications have several common characteristics and, first of all, an important role in signaling mechanisms [9]. One important example of protein activity modulation mediated by redox switch is constituted by the transcriptional factor OxyR: with the increasing of the oxidation degree, the cooperative effect of the DNA binding capacity of OxyR decreases [10]. If the redox balance is known to be involved in a lot of transcriptional events, not many information have been obtained about proteins involved in signaling pathways. For example it has been described that the NMDA receptor function is redox modulated [22, 23] but what is evident is that it is very important to distinguish among different oxidation levels, in particular between the formation of S-nitrosylated Cysteine residues and disulphide bonds. The consequences of these modifications on the protein structure, and finally on its function and activity, are different. In this context nitric oxide is assuming an important role, in particular in neurophysiological studies: many evidences suggest in fact that this gas can act as a neurotransmitter, but the mechanisms by which it operates have to be unraveled and the methods presented in this work can be very useful to clarify this issue.

REFERENCES


