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An Optimized Strategy for ICAT Quantification of Membrane Proteins*

Claire Ramus‡, Anne Gonzalez de Peredo‡§, Cécile Dahout¶, Maighread Gallagher‡, and Jérôme Garin‡||

The work presented here focuses on the development of a method adapting isotope labeling of proteins with ICAT to the study of highly hydrophobic proteins. Conditions for the labeling of proteins were first established using two standard soluble proteins and iodoacetamidyl-3,6-dioxaoctanediamine biotin (PEO-iodoacetyl biotin). Results demonstrated the efficiency of the labeling in the presence of high concentrations of both SDS and urea. These conditions were then used to label a highly hydrophobic mitochondrial membrane protein, the adenine nucleotide translocator ANT-1, with PEO-iodoacetyl biotin and then with the cleavable ICAT reagent. The results presented here show that labeling of proteins with cleavable ICAT is possible and may even be improved in strong denaturing buffers containing both SDS at a concentration higher than 0.5% (w/v) and urea. These results open the possibility of applying the ICAT strategy to complex samples containing very hydrophobic proteins solubilized in urea-SDS buffers. The adaptability of the developed method is demonstrated here with preliminary results obtained during the study of membrane-enriched fractions prepared from murine embryonic stem cells. *Molecular & Cellular Proteomics* 5:68–78, 2006.

Membrane proteins are involved in a multitude of cellular processes such as signal transduction, cellular adhesion, ion transport, and drug resistance. Their identification provides clues to the understanding of cellular functions and mechanisms. Proteins bearing multiple transmembrane helices, which include many transporters and receptors, are very hydrophobic. Because such proteins are difficult to solubilize and are therefore often poorly represented as part of the total protein pool, their identification and quantification remain important challenges. Considering the biological and pharmacological importance of this protein class with over 50% of current drug targets being membrane proteins (1), it appears necessary to develop new methods that will

allow the study of changes in membrane protein expression.

Whereas two-dimensional electrophoresis (2DE)¹ can be used to identify and compare protein samples quantitatively (2, 3), this technique is not applicable for the study of highly hydrophobic proteins (4). In this context, despite significant improvements (5), 2DE is being gradually replaced by multi-dimensional protein identification technology (MudPIT) (6, 7). Such strategies appear better adapted to the analysis of hydrophobic proteins than 2DE as membrane suspensions can be used and the analysis focused on the surface-exposed portions of integral membrane proteins (8).

However, whereas different approaches are being developed for the quantitative profiling of proteins using mass spectrometry, very few studies report quantitative analysis of membrane proteins using chemical labeling such as ICAT. In a typical ICAT experiment, samples to be compared are individually labeled by the light or heavy form of the ICAT reagent, resulting in the detection of a pair of peaks for each peptide in common between the two samples when analyzed by nano-LC-MS (9). The peptides labeled by the light or the heavy ICAT isoforms co-elute on the liquid chromatography reverse phase column, and the ratios between the MS signal areas for the low and high mass components of these pairs of peaks provide an accurate measure of the relative abundance of the peptides and thus of the relative levels of protein expression in the original samples. In a classical ICAT analysis, all of the protein labeling and preparation steps are carried out in solution. This technique has been used for the quantitative analysis of a total yeast extract carried out by Han *et al.* (10). It is of note that during the course of the study by Han *et al.*, among the hundreds of proteins analyzed, quantitative data concerning true membrane proteins were scarce because of the low abundance of these proteins and to the fact that ICAT labeling requires the presence of both exposed soluble regions of sufficient length to generate one or more tryptic peptides and a cysteine residue present in the extramembrane material to be accessible to the reagent. Consequently, during such large scale quantitative proteome analyses, hy-

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¹ The abbreviations used are: 2DE, two-dimensional electrophoresis; ANT-1, adenine nucleotide translocator; PEO-iodoacetyl biotin, iodoacetamidyl-3,6-dioxaoctanediamine biotin; c-ICAT, cleavable ICAT; LAPAO, 3-laurylamido-*N,N'*-dimethylpropylaminooxide; ES, embryonic stem; ESd, differentiated ES; Ccam, carbamidomethylated cysteine; Cpeo, cysteine modified with PEO-iodoacetyl biotin.

drophobic proteins remain largely underrepresented.

One strategy for studying membrane proteomes in depth is to apply specific methods, such as biological fractionation, to enrich the hydrophobic protein core present in the fraction of interest (11–13). The present work was aimed at adapting the ICAT protocol to experimental conditions that would allow ICAT labeling of fully solubilized and denatured core membrane proteins. Therefore, we set up and tested new conditions that allow ICAT labeling in the presence of high concentrations of SDS. In this study, cysteine labeling with PEO-iodoacetyl biotin, a chemical reagent whose structure is close to that of the ICAT reagent, first allowed us to optimize conditions for the labeling of two cysteine-rich non-membrane proteins, ovalbumin and β -lactoglobulin, and ICAT labeling was then performed on the bovine mitochondrial ADP/ATP translocator, a highly hydrophobic 33-kDa protein containing six α -helical transmembrane domains. Finally our newly developed ICAT labeling technique was tested on an enriched membrane protein fraction prepared from murine embryonic stem cells.

EXPERIMENTAL PROCEDURES

Biological Material

Standard Proteins—Ovalbumin (chicken) and β -lactoglobulin (bovine) proteins were from Sigma.

ADP/ATP Translocator—The ADP/ATP translocator was isolated from bovine heart mitochondria as a carboxyatractyloside-carrier complex in the presence of 0.05% 3-laurylamido-*N,N'*-dimethylpropylaminoxide (LAPAO) according to the procedure described by Block *et al.* (14). The recovered ADP/ATP translocator-enriched fraction (obtained in 50 mM Na₂SO₄, 10 mM Tris, pH 7.3, 1 mM EDTA, 0.05% LAPAO) was precipitated in acetone at –20 °C overnight. After this, the precipitate was washed at least twice in water before being solubilized in buffer U8S4 (50 mM Tris-HCl, pH 8.3, 5 mM EDTA, 8 mM urea, 4% (w/v) SDS), and protein concentration was determined by a Bradford assay (15).

Stem Cell Protein Preparations

60 × 10⁶ totipotent murine embryonic stem cells (ES cells) or early differentiated murine embryonic stem cells (ESd cells) were resuspended in 10 mM Tris, pH 7.5, complemented with an anti-protease mixture (Complete, Roche Diagnostics). Lysis was performed using a cell disrupter system (Constant Systems, Northants, UK) in one-shot mode at 350 bars. The resulting lysate was submitted first to a short centrifugation at 1000 × *g* at 4 °C to eliminate nuclei and intact cells. The postnuclear supernatant was ultracentrifuged for 1 h at 100,000 × *g* at 4 °C (in an MLA-80 rotor). For the enrichment of membrane proteins in the samples, pellets were first resuspended in an alkaline buffer (0.1 M Na₂CO₃, pH 11) and centrifuged for 45 min at 100,000 × *g* at 4 °C. The Na₂CO₃ pellets were resuspended in 0.1 M Na₂CO₃, 0.5 M NaCl, pH 11 before centrifuging for 45 min at 100,000 × *g* at 4 °C. Pellets, containing the insoluble proteins, were further washed in water and finally resuspended in buffer U8S4 (see above) to perform ICAT labeling. After protein concentration determination, equal amounts of each sample (ES and ESd preparations) were modified by ICAT cleavable reagents (Applied Biosystems, Framingham, MA) as described under “Isotope Labeling of Cysteines.”

Isotope Labeling of Cysteines

Depending on the experiment, proteins were solubilized using one of the following buffers: buffer U8S4 (see above), buffer U8S0.5 (50 mM Tris-HCl, pH 8.3, 5 mM EDTA, 8 mM urea, 0.5% (w/v) SDS), buffer U8S0 (50 mM Tris-HCl, pH 8.3, 5 mM EDTA, 8 mM urea), buffer U6S0.05 (50 mM Tris-HCl, pH 8.3, 5 mM EDTA, 6 mM urea, 0.05% (w/v) SDS), or buffer U0S0 (50 mM Tris-HCl, pH 8.3, 5 mM EDTA). Protein disulfide bonds were reduced by 10 mM tributylphosphine for 30 min at 37 °C. Cysteine residues were then alkylated by either PEO-iodoacetyl biotin (Pierce) (45 min in the dark at 37 °C) or cleavable ICAT (c-ICAT, Applied Biosystems) (2 h in the dark at 37 °C). The alkylation reaction was quenched with DTT before loading labeled proteins on a 12% polyacrylamide gel and submitting them to electrophoresis (16). After migration, proteins were colored by Coomassie staining. In the case of complex mixtures, 100 μ g of proteins from the enriched membrane fraction of either ES or ESd cells were labeled with light and heavy c-ICAT, respectively, before mixing together. One-quarter of the resulting mixture was then loaded on a 12% SDS-PAGE gel and submitted to a short migration to fractionate the mixture into 10 bands, an adaptation of a method described by Ferro *et al.* (11).

ANT-1 Immunodetection

For Western blotting purposes, 20 μ g of membrane proteins from differentiated stem cells were separated by SDS-PAGE and transferred onto a nitrocellulose sheet according to the standard method (17). Immunodetection was carried out using a polyclonal antibody (diluted at 1:1000) raised against the bovine ANT-1 protein. The immune complexes were revealed by protein A-peroxidase (Pierce) at a dilution of 1:5000 and ECL reagent (Amersham Biosciences).

In-gel Digestion of the Proteins and Peptide Extraction

Each gel band was excised and prepared for mass spectrometry analysis. Gel bands were destained by repeated cycles of incubation in 25 mM NH₄HCO₃ for 15 min and then with 50% (v/v) acetonitrile in the same buffer (25 mM NH₄HCO₃) for 15 min (18). After drying by vacuum centrifugation, the bands were incubated with a reducing solution (25 mM NH₄HCO₃ containing 10 mM DTT) for 30 min at 37 °C. Alkylation was performed with an alkylating solution (25 mM NH₄HCO₃ containing 55 mM iodoacetamide) for a further 30 min at 37 °C. Bands were then washed several times with the destaining solutions and finally in pure water for 15 min before being dehydrated with 100% acetonitrile. In-gel digestion was performed using trypsin (sequencing grade, Promega, Madison, WI) at a 1:20 protease to protein ratio in 25 mM NH₄HCO₃ for 5 h at 37 °C. Peptides were extracted from the gel using passive diffusion in the following solutions: 50% CH₃CN, then 5% formic acid, and finally 100% CH₃CN. The extract was dried by vacuum centrifugation; peptides were then resolubilized in an aqueous trifluoroacetic acid solution for MALDI-TOF/MS analysis.

Concentration of Peptides Covalently Modified with c-ICAT

c-ICAT-labeled peptides were purified using the avidin column supplied in the c-ICAT kit (Applied Biosystems) according to the manufacturer's instructions. The bound peptides were eluted using 30% CH₃CN, 0.4% TFA. After drying, cleavage of the acid-labile moiety of the c-ICAT reagent was performed as recommended by the manufacturer (37 °C in 95% TFA for 2 h) before nano-LC-MS/MS analysis.

MALDI-TOF/MS Analysis

Peptide mixtures were analyzed with a MALDI-TOF/MS mass spectrometer (Autoflex, Bruker Daltonics) in reflector mode. 0.5 μ l of

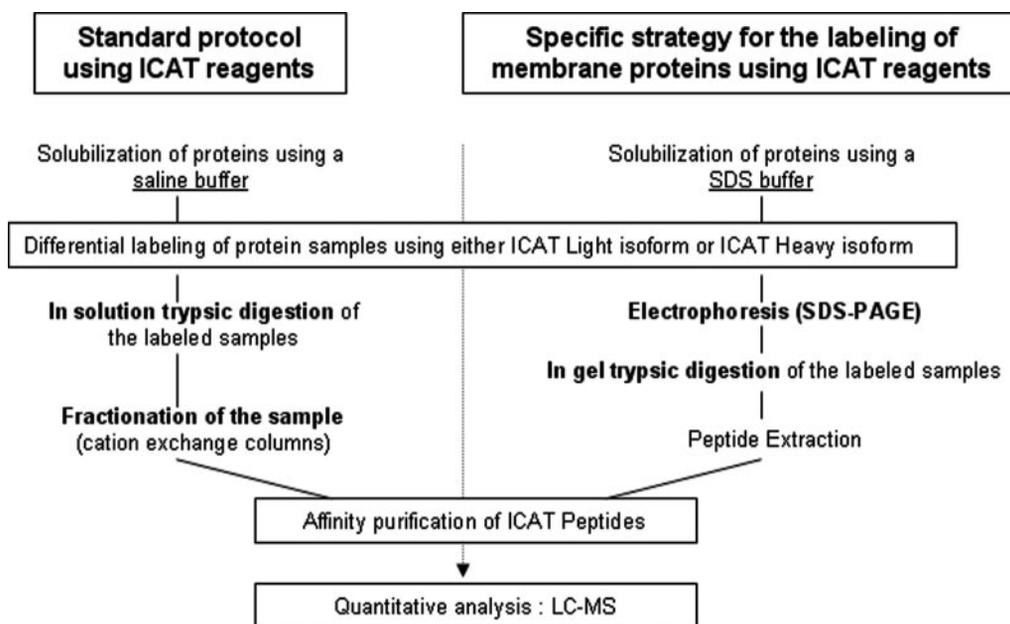


FIG. 1. **Schematic representation of ICAT labeling strategies.** Standard conditions for ICAT labeling of proteins are shown in the *left-hand panel*; our modified conditions, adapted to the study of membrane proteins, are shown on the *right-hand panel*. The major modifications to the protocol are as follows. 1) During the standard ICAT protocol, proteins are solubilized and labeled in a saline buffer; the strategy developed for quantifying differential expression of membrane proteins requires the use of SDS to correctly solubilize and label such proteins. 2) In the standard procedure, after labeling, proteins are digested with trypsin in solution before being cleaned and fractionated by cation exchange chromatography; because of the presence of SDS in the solubilization buffer of the modified protocol, labeled membrane protein mixtures must be cleaned and fractionated by SDS-PAGE migration before trypsin digestion can be performed in-gel. In both protocols ICAT-labeled peptides are isolated by affinity chromatography making use of the biotin tag before relative quantification of the peptide pairs by nano-LC-MS.

sample was mixed with the same amount of matrix solution (α -sinapinic acid saturated in 50% CH_3CN , 0.1% TFA) and loaded on a target before analysis.

Nano-LC-MS and Nano-LC-MS/MS Analyses

The dried gel-extracted tryptic peptides were solubilized in 95% water (v/v) containing 2.5% acetonitrile and 2.5% trifluoroacetic acid for nano-LC-MS and nano-LC-MS/MS analysis (CapLC and Q-TOF Ultima, Waters, Milford, MA). The method consisted of a 50-min run at a flow rate of 200 nl/min using a two-solvent gradient: solvent A (2% acetonitrile, 97.9% water, 0.1% formic acid) and solvent B (80% acetonitrile, 19.9% water, 0.1% formic acid). The system includes a 300- μm \times 5-mm PepMap C_{18} precolumn (LC-Packings, Dionex, Sunnyvale, CA) to concentrate peptides before injection onto a 75- μm \times 150-mm C_{18} column (LC-Packings) directly coupled to the mass spectrometer.

Protein Identification and Quantification

Proteins were identified from MS/MS data with Mascot 2.0 software (Matrix Science, www.matrixscience.com). Database searches were performed on the Swiss-Prot_Trembl data bank specifying several variable amino acid modifications: acetylation, oxidized methionine, ICAT light/heavy, and carbamidomethylated cysteines. Identification of all ICAT peptides was manually confirmed by inspection of MS/MS spectra. Quantification was done manually after integration of peaks for both isoforms of each labeled peptide identified on reconstituted chromatograms. Reconstituted chromatograms were obtained after extraction of a specific mass (± 0.1 Da) from the nano-LC-MS data using MassLynx software (Waters).

RESULTS

General Strategy—Membrane proteins are often difficult to extract from their native environment without encountering difficulties because of insolubility and protein loss through precipitation. High concentrations of detergents, such as SDS, allow the solubilization of most proteins, even highly hydrophobic ones, as the detergent mimics the native lipid bilayer environment of the proteins. However, in such conditions, the labeling of cysteine residues present on hydrophobic proteins with ICAT probes requires adaptation of the standard protocol that is not compatible with the presence of high concentrations of SDS (19). Fig. 1 underlines the major differences between the ICAT standard protocol and the strategy that was set up during the course of this work to obtain quantitative expression data on membrane proteins. Usually, protein samples are first labeled with ICAT in a saline buffer, mixed, and then submitted to trypsin digestion. In our modified method, as proteins are first labeled with ICAT in the presence of high amounts of SDS, their migration on a 12% polyacrylamide gel is necessary before trypsin digestion can be performed. During electrophoresis, excess ICAT and other buffer components incompatible with the tryptic digestion are separated from the protein samples. Thus, the in-gel treatment of the proteins replaces the cation exchange column used for cleaning and fractionation of the samples after “in-solution” trypsin digestion in the classical protocol.

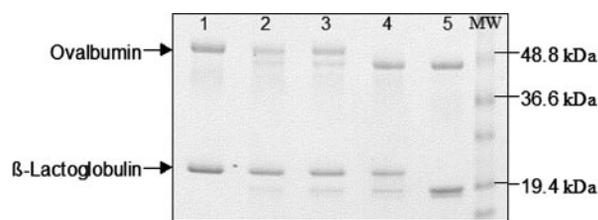


FIG. 2. Efficiency of PEO-iodoacetyl biotin labeling of ovalbumin and β -lactoglobulin in different buffer conditions. Ovalbumin (~45 kDa) and β -lactoglobulin (~20 kDa) were labeled with PEO-iodoacetyl biotin in different buffer conditions. Labeling efficiency was estimated by the induction of a band shift on 12% SDS-PAGE gels visualized by colloidal Coomassie Blue staining (10 μ g of total protein/lane). Lane 1, buffer U8S0.5; lane 2, buffer U6S0.05; lane 3, buffer U8S0; lane 4, buffer U0S0; lane 5, unmodified proteins solubilized in buffer U8S0.5.

As the yield of in-gel alkylation with iodoacetamide has been shown to be close to 100% (20), cysteine residues of the ICAT-labeled proteins were further in-gel alkylated with iodoacetamide to allow the identification of cysteine peptides that may have only partially reacted with ICAT. Similarly to the classical ICAT method, ICAT-labeled tryptic peptides were then concentrated by affinity chromatography on an avidin column prior to their analysis by nano-LC-MS and nano-LC-MS/MS.

Optimization of Labeling Conditions in SDS Buffers—To set up experimental conditions offering the ability to label proteins with ICAT in highly denaturing buffers, optimization of the labeling conditions was first performed with ovalbumin and β -lactoglobulin, two soluble proteins often used as models for the evaluation of alkylation as they contain six and seven cysteine residues, respectively. Cysteine labeling was carried out using an ICAT analog, PEO-iodoacetyl biotin. This reagent is composed of a biotin affinity tag, a linker, and a specific thiol-reactive group. It reacts with cysteine residues by the same alkylation reaction as the one implicated in ICAT labeling (19). Being far less expensive than ICAT reagents, PEO-iodoacetyl biotin allowed us to test several buffer conditions for labeling. The effects of both a chaotropic agent (urea) and a detergent (SDS) were tested on the labeling of ovalbumin and β -lactoglobulin. During the labeling reaction, temperature was not allowed to exceed 37 °C to avoid protein carbamylation that would occur on lysine residues at high temperatures in the presence of urea (21). After the labeling step, proteins were analyzed by SDS-PAGE (Fig. 2). When a protein is modified by PEO-iodoacetyl biotin, an increase in mass of 414 Da per cysteine is induced. Thus, complete alkylation of ovalbumin and β -lactoglobulin with this molecule should induce a mass shift of 2484 and 2898 Da, respectively. Taking advantage of this easily detectable evidence of modification, labeling was roughly estimated from the migration shift of the proteins on the SDS-PAGE gel at this stage of the work. Fig. 2 demonstrates the effect of different buffers during the labeling step. Protein labeling was first performed in the absence of both urea and SDS (lane 4, buffer U0S0): when

compared with unmodified proteins (lane 5), no shift in mass is noticed for ovalbumin, whereas multiple bands appear for β -lactoglobulin, suggesting incomplete labeling. Although labeling efficiency was actually improved by the use of buffer U8S0 (lane 3) and was even better in buffer U6S0.05 (the classical ICAT conditions, lane 2), a slight band corresponding to unlabeled ovalbumin was reproducibly detected. Therefore we tried labeling conditions where a high percentage of SDS was included in the solubilization buffer, conditions that would be well adapted for solubilization of hydrophobic proteins. When 8 M urea and 0.5% SDS were used (lane 1, buffer U8S0.5), a complete mass shift was observed for both proteins, suggesting that complete labeling was achieved. The same result was obtained in the presence of 8 M urea and 4% SDS (not shown). To assess the labeling of ovalbumin and β -lactoglobulin by PEO-iodoacetyl biotin more accurately, protein gel bands (observed on Fig. 2) were excised and submitted to in-gel alkylation with iodoacetamide and tryptic digestion, and the resulting peptides were analyzed by MALDI-TOF/MS. This experiment confirmed the partial labeling by PEO-iodoacetyl biotin in the absence of SDS (buffer U8S0). In contrast, in the presence of SDS and urea (buffer U8S0.5), no cysteine peptide labeled with iodoacetamide could be detected (Fig. 3). The efficiency of the labeling and the identity of each PEO-iodoacetyl biotin-modified peptide were confirmed by nano-LC-MS and nano-LC-MS/MS (data not shown). Taken together, these results lead to the conclusion that, far from being detrimental, the presence of a high concentration of SDS, when associated with 8 M urea, enhances the labeling of these two soluble proteins, whereas the previous publications dealing with ICAT labeling insist on the importance of not exceeding 0.1% (manufacturer's protocol) or even 0.05% SDS (19).

Labeling of ANT-1, a Highly Hydrophobic Protein, with PEO-Iodoacetyl Biotin—We next tested these modified labeling conditions on a highly hydrophobic protein, the adenine nucleotide translocator ANT-1. This protein, containing four cysteines and six transmembrane domains (22), one of which contains a cysteine residue, can be considered as a model for multitransmembrane domain proteins. Because of its numerous transmembrane domains, ANT-1 is a highly hydrophobic protein and is poorly soluble in buffers that are classically used for ICAT labeling (6 M urea, 0.05% SDS). Therefore, ANT-1 was solubilized in the presence of 8 M urea using a range of SDS concentrations (1–4% (w/v)). The solubilized protein was then labeled with PEO-iodoacetyl biotin and loaded onto an SDS-PAGE gel.

When ANT-1 was labeled in the presence of 8 M urea and 4% SDS, the four ANT-1 cysteine residue-containing peptides were detected by MALDI-TOF/MS analysis as peptides bearing the PEO adduct, and for all these peptides, no mass fragments corresponding to peptides alkylated by iodoacetamide were detected in the same spectrum (Fig. 4, a and b). Partial solubilization of ANT-1 resulting in a loss of material

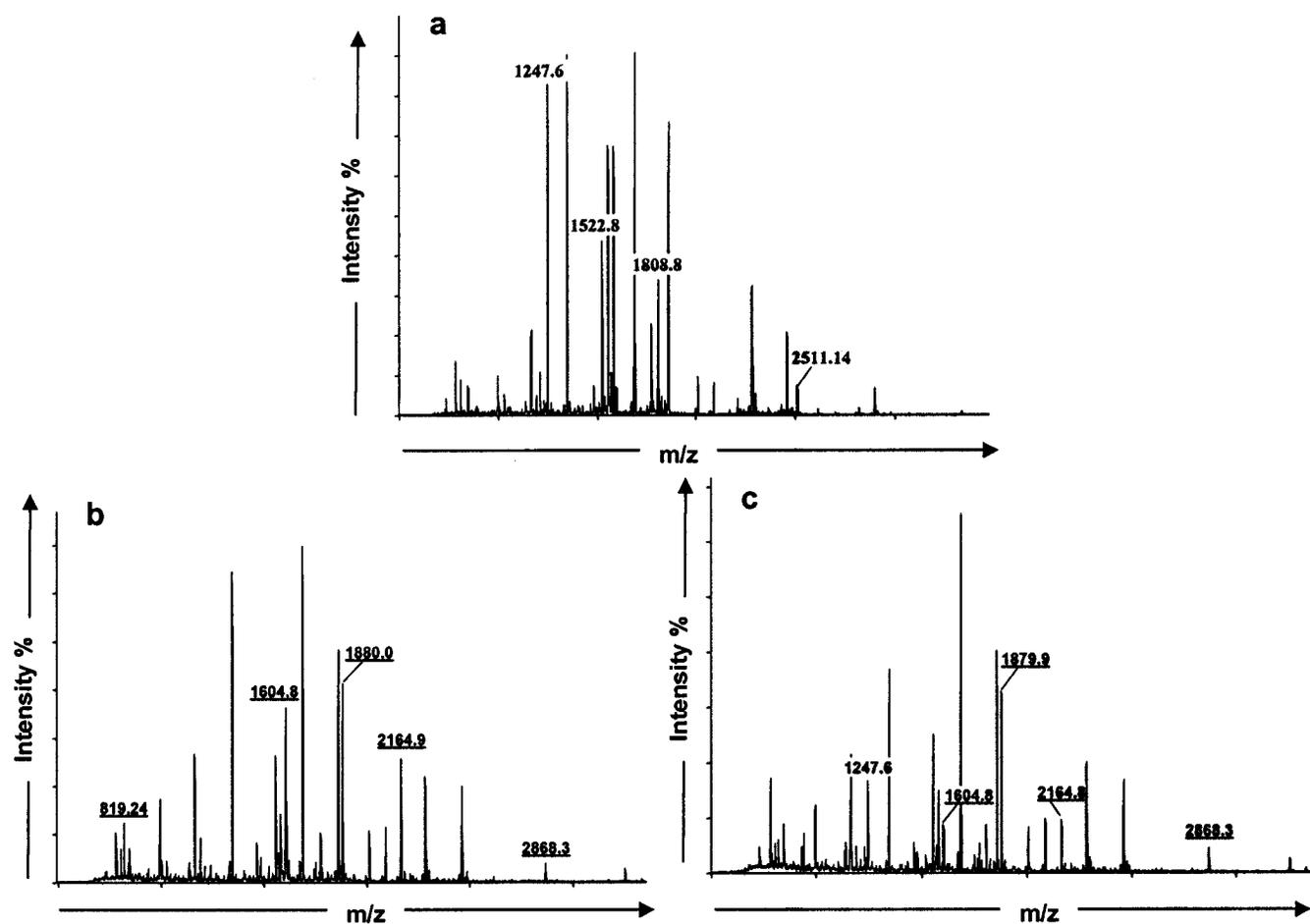


FIG. 3. **Ovalbumin labeling by PEO-iodoacetyl biotin: estimation of reaction efficiency by MALDI-TOF/MS.** After solubilization and labeling, ovalbumin was subjected to migration on a 12% SDS-PAGE gel. Bands were visualized by Coomassie Blue staining, excised, destained, reduced, alkylated by iodoacetamide, and submitted to in-gel trypsin digestion (as described under "Experimental Procedures"). The peptides obtained were analyzed by MALDI-TOF/MS to estimate the efficiency of PEO-iodoacetyl biotinylation. Peptide masses for cysteine peptides are shown. *Underlined* masses correspond to PEO-iodoacetyl biotin-labeled peptides, whereas masses of corresponding peptides containing a carbamidomethylcysteine modification are not underlined. *a*, unlabeled ovalbumin; *b*, ovalbumin labeled in buffer U8S0.5; *c*, ovalbumin labeled in buffer U8S0.

was observed when solubilization was performed in buffers with less than 4% SDS (not shown). In-gel alkylation, tryptic digestion, and MALDI-TOF/MS and nano-LC-MS analysis demonstrated that this loss of material was associated with a less efficient labeling reaction (data not shown). These observations indicate that, as expected, high SDS concentrations are necessary to obtain a good recovery and labeling of membrane proteins like ANT-1. Therefore, for all further experiments, the solubilization and labeling of ANT-1 were carried out in the presence of 8 M urea and 4% SDS. Analysis of the ANT-1 tryptic peptides by nano-LC-MS/MS confirmed the identity of all the peptides observed by MALDI-TOF/MS. Fig. 5a shows the fragmentation spectrum of peptide T22-PEO (EFTGLGN \underline{C} ITK). A characteristic signature of the presence of a PEO-labeled cysteine is illustrated by the presence of the following m/z ions: 227, 270, 314, 375, and 449 specific for the fragmentation of the reagent itself. Subsequently analysis

by nano-LC-MS was carried out to allow more accurate quantification of the labeling yield for each cysteine.

The entire quantification process is illustrated in Fig. 4, *c* and *d*, for peptide T22 (EFTGLGN \underline{C} ITK). After generation of the reconstituted ion chromatograms for both forms of each peptide, carbamidomethylated or labeled with PEO-iodoacetyl biotin (see Table I), the elution peaks corresponding to each form were integrated, and the ratio of the calculated areas was used to determine relative abundance. We are aware that quantifying two distinct forms of the same peptide, each one being modified by a different alkylating reagent (PEO-iodoacetyl-biotin and iodoacetamide, respectively), may induce a bias in the quantification. However, we found that it was the most convenient way to evaluate labeling efficiency. Based on this approximation, the abundance of three of the cysteine-containing peptides of ANT-1 modified by PEO-iodoacetyl biotin relative to the abundance of the

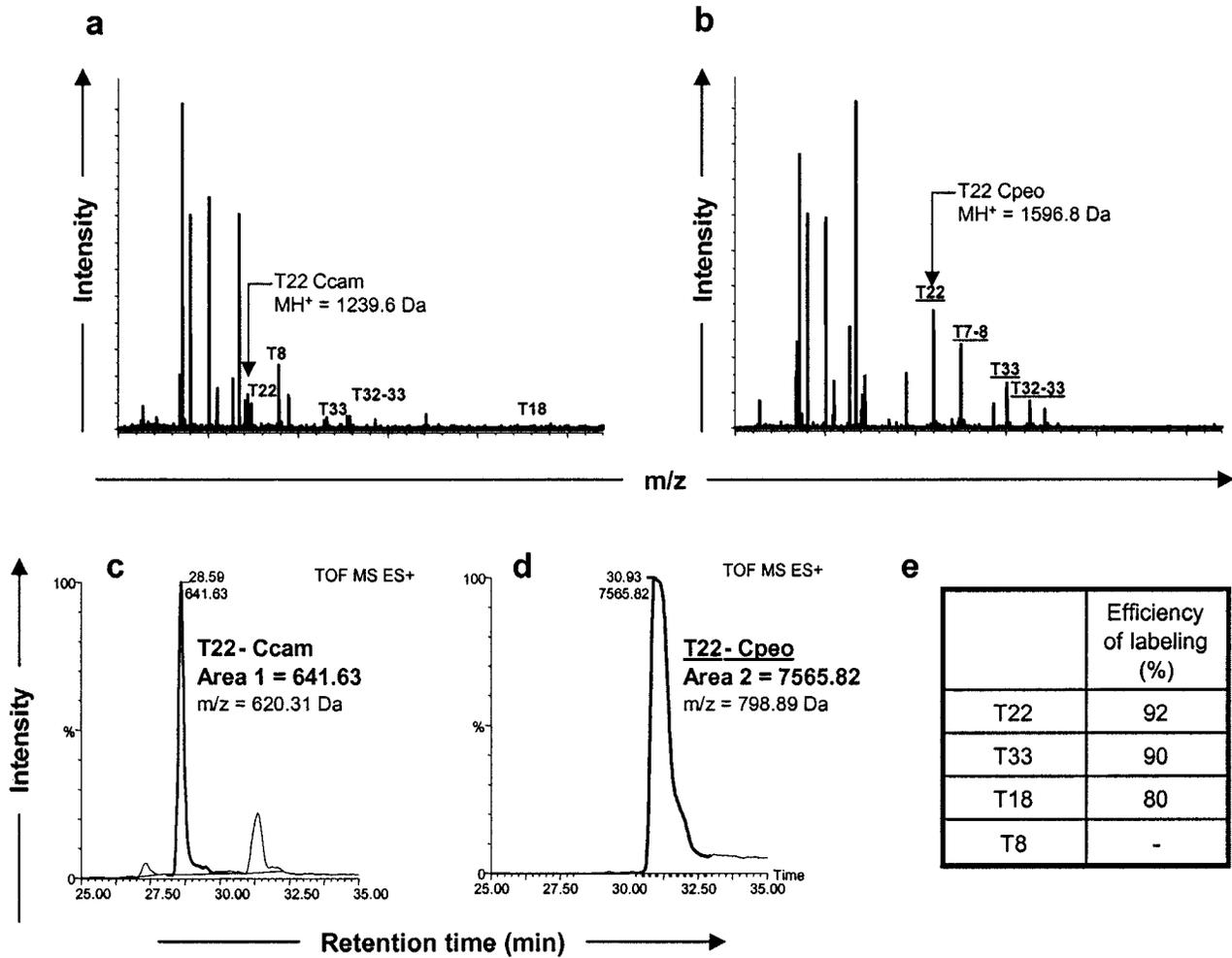


FIG. 4. MALDI-TOF/MS and nano-LC-MS analyses of ANT-1 protein after cysteine modification: evaluation of labeling efficiency using nano-LC-MS analysis. ANT-1 protein was solubilized in buffer U8S4, labeled with PEO-iodoacetyl biotin, and subjected to migration in its labeled and unlabeled forms on a 12% SDS-PAGE gel. Bands were visualized by Coomassie Blue staining, excised, destained, reduced, alkylated by iodoacetamide, and submitted to in-gel trypsin digestion (as described under “Experimental Procedures”). The peptides obtained were analyzed by MALDI-TOF/MS and nano-LC-MS to determine the efficiency of PEO-iodoacetyl biotin labeling. During reverse phase nano-LC-MS, PEO-modified peptides elute with a 2-min delay compared with the iodoacetamide-alkylated forms. The relative abundance of each form of T22 peptide was evaluated by comparing the ion chromatogram of each sister peptide and calculating the ratio of the areas measured by peak integration. Extracted ion chromatograms were obtained using a 0.1-Da mass tolerance window. *a*, mass fingerprint of ANT-1 alkylated by iodoacetamide; peptides containing carbamidomethylated cysteines (*Ccam*) are indicated. *b*, mass fingerprint of ANT-1 labeled with PEO-iodoacetyl biotin; peptides containing cysteines modified with PEO-iodoacetyl biotin (*Cpeo*) are indicated and underlined. *c*, nano-LC-MS analysis: reconstructed ion chromatogram of T22 peptide (*Ccam*); $m/z = 620.31$ Da. *d*, nano-LC-MS analysis: reconstructed ion chromatogram of T22 peptide (*Cpeo*); $m/z = 798.89$ Da. *e*, percent ratios of PEO-iodoacetyl biotin labeling versus carbamidomethylation for each ANT-1 tryptic peptide.

same peptides modified by iodoacetamide was calculated (Fig. 4e). The analysis of these peptides shows that the efficiency of labeling with PEO-iodoacetyl biotin was at least 80% when the reaction was carried out in the presence of 4% SDS and 8 M urea. Labeling of cysteine 56, present in peptide T8, could not be accurately quantified as it appeared in multiple peptide forms, resulting from miscleavage and post-translational modification (trimethylation on residue Lys-51).

Labeling of ANT-1 Protein with c-ICAT—The cysteine modification protocol was then applied to ANT-1 using c-ICAT reagents. Two experiments were conducted using ANT-1 ei-

ther modified by the light (^{12}C) or the heavy (^{13}C) isoform of c-ICAT. The labeling reaction was carried out in the optimized conditions described above for PEO-iodoacetyl biotin labeling (8 M urea, 4% SDS).

As a proof of principle experiment, mixtures of ANT-1 labeled by the two forms of c-ICAT were prepared at either 1:5 or 5:1 ($^{12}\text{C}/^{13}\text{C}$) ratios. ANT-1 mixtures were then submitted to SDS-PAGE, digested with trypsin, and analyzed by nano-LC-MS and nano-LC-MS/MS. Analysis of the ANT-1 tryptic peptides by nano-LC-MS/MS confirmed the identity of all the elution peaks detected by nano-LC-MS. For example, Fig. 5b

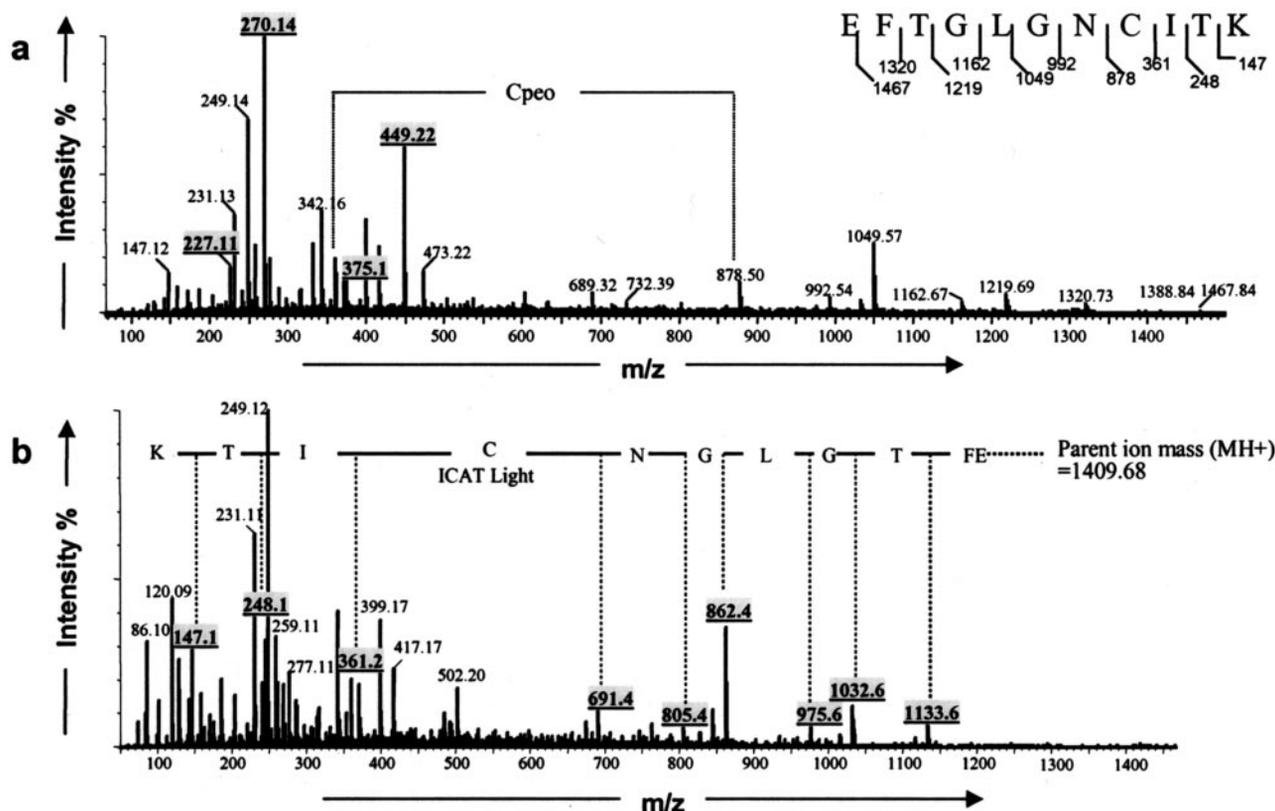


FIG. 5. Sequencing of the labeled ANT-1 T22 tryptic peptide by tandem mass spectrometry. T22 peptide (EFTGLGNCITK, $z = 2$) labeled with either PEO-iodoacetyl biotin or the c-ICAT light reagent was submitted to fragmentation by MS/MS. a, MS/MS spectrum of PEO-iodoacetyl biotin-labeled T22 tryptic peptide. b, MS/MS spectrum of c-ICAT light-labeled T22 tryptic peptide.

TABLE I
Theoretical masses of each cysteine peptide of ANT-1 under different labeling conditions

Alkylation by iodoacetamide or PEO-iodoacetyl biotin induces an increment of mass of 57 and 414 Da per cysteine, respectively.

| Tryptic cysteine peptide | Mass of the unlabeled form (Ccam) | Mass of the labeled form (Cpeo) |
|--------------------------|-----------------------------------|---------------------------------|
| T8 | 931.5 | 1288.68 |
| T7-8 | 1392.75 | 1749.92 |
| T22 | 1239.6 | 1596.78 |
| T33 | 1644.71 | 2001.89 |
| T18 | 2796.35 | 3153.52 |

illustrates the fragmentation spectrum of the c-ICAT light-labeled ANT-1 T22 peptide. Quantification of each form of the labeled cysteine-containing peptides (heavy and light) was performed as described above. The mean difference between the observed and expected ratios for the four pairs of ANT-1 cysteine-labeled peptides was evaluated, demonstrating an average error of less than 20% (Table II). These results are in agreement with ICAT reaction yields described previously in the literature (23).

Application of Our Labeling Strategy to a Complex Protein Mixture—To fully test our new ICAT labeling procedure, we next applied it to a differential proteomic study carried out on

TABLE II
Comparison of observed and expected ratios of c-ICAT-labeled ANT-1 peptides

ANT-1 protein was labeled with both c-ICAT light and heavy isoforms in buffer U8S4, and the two labeled proteins were then mixed at different ratios before SDS-PAGE gel loading. Nano-LC-MS was used to quantify each form of the cysteine peptides (ICAT light or heavy). Observed and expected ratios are shown.

| Mixture | Peptide identified | Observed ratio ($^{12}\text{C}/^{13}\text{C}$) | Mean \pm S.D. | Expected ratio ($^{12}\text{C}/^{13}\text{C}$) |
|---------|--------------------|--|-----------------|--|
| 1:5 | T22 | 0.16 | 0.23 \pm 0.06 | 0.2 |
| | T18 | 0.25 | | |
| | T33 | 0.28 | | |
| 5:1 | T22 | 4.79 | 4.91 \pm 0.22 | 5 |
| | T18 | 4.78 | | |
| | T33 | 5.17 | | |

mouse embryonic stem cells. A pellet enriched in membrane proteins was prepared by differential centrifugation from totipotent (ES) and early differentiated (ESd) mouse stem cells (see “Experimental Procedures”).

First of all, the efficiency of solubilization and labeling of such fractions, using either classical ICAT labeling conditions (buffer U6S0.05) or our optimized conditions (buffer U8S4), were tested. To this aim, we took advantage of the presence

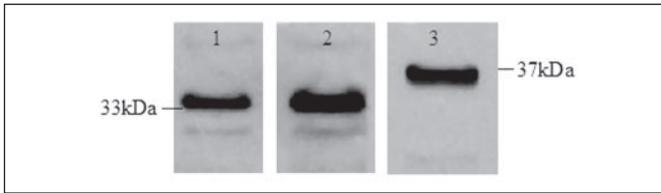


FIG. 6. **Immunodetection of the ANT-1 protein in membrane-enriched embryonic stem cell protein fractions.** A membrane-enriched fraction was prepared from differentiated embryonic stem cells by differential centrifugation as described under “Experimental Procedures.” The membrane pellet was solubilized in buffer U6S0.05 (*lane 1*) or buffer U8S4 (*lanes 2 and 3*). Proteins were left unlabeled (*lanes 1 and 2*) or were labeled with ICAT light reagent (*lane 3*). After migration, gels were transferred to PVDF membrane and immunoblotted for ANT-1. The molecular mass of unlabeled ANT-1 is 33 kDa; the molecular mass of ICAT-labeled ANT-1 protein is 37 kDa.

of ANT-1 protein in these murine protein fractions, and we analyzed one sample (membrane proteins from ESd cells), solubilized in each of these buffers, by SDS-PAGE and Western blot using an anti-ANT-1 antibody. Fig. 6 shows the result obtained for the Western blot: when using buffer U6S0.05 for the solubilization of proteins, a lower quantity of ANT-1 protein was observed (*lane 1*) compared with the signal obtained when solubilization was performed in buffer U8S4 (*lane 2*). This demonstrates that buffer U6S0.05 was not optimal for the solubilization of this hydrophobic protein, and we anticipate that this finding can be extrapolated to many other hydrophobic proteins present in this mixture. Furthermore the efficiency of ICAT labeling of ANT-1 in the protein mixture was tested (*lane 3*). Full labeling of ANT-1 was achieved as illustrated in *lane 3* by the band at 37 kDa on the polyacrylamide gel corresponding to the labeled protein and the total absence of any residual unlabeled ANT-1 (33 kDa).

Quantification of the ANT-1 labeling yield in both conditions (buffer U8S4 or U6S0.05) was performed by nano-LC-MS on c-ICAT light-labeled ANT-1. For that purpose, the SDS-PAGE region corresponding to the labeled and unlabeled forms of ANT-1 (30–40 kDa) was excised, washed, reduced, and in-gel alkylated with iodoacetamide. After in-gel digestion with trypsin, peptides were extracted and affinity-purified before analysis by nano-LC-MS and nano-LC-MS/MS. For each of the two buffer conditions tested (U8S4 or U6S0.05), the reconstituted chromatograms of the four cysteine peptides either alkylated by ICAT or by iodoacetamide were obtained; the areas under the corresponding peaks were calculated and compared to give the relative quantity of each of the forms of the cysteine peptides present in the samples. Results demonstrate the presence of a higher quantity of ANT-1 peptides when samples were treated in the presence of buffer U8S4, confirming the previous observation made through the Western blot experiment. Whereas the four cysteine-containing peptides could be analyzed when ANT-1 was solubilized in buffer U8S4, only the T22 tryptic peptide could be detected, and thus quantified, when the protein

was labeled in buffer U6S0.05. For this peptide, it turned out that the ICAT labeling efficiency was similar in the two buffer conditions ($95 \pm 5\%$).

We finally performed a comparative study of membrane proteins from ES and ESd cells by c-ICAT labeling. In accordance with the labeling procedure described previously, membrane-enriched pellets from either ES or ESd cells were solubilized in buffer U8S4 and labeled with light and heavy c-ICAT, respectively. After labeling, protein samples were mixed at a 1:1 ratio and loaded onto a polyacrylamide gel. The migration on the gel was shortened to fractionate the mixture into ten 2-mm gel bands (11). In-gel digestion with trypsin was performed on each gel band. The tryptic peptides were then extracted from the gel, and c-ICAT-labeled peptides were concentrated by avidin chromatography as described under “Experimental Procedures.” Each eluted fraction was then analyzed by nano-LC-MS and nano-LC-MS/MS. Several ICAT-labeled proteins were identified. For example, in a gel band that corresponds to high molecular weight proteins, the ICAT-labeled peptides allowed the identification and quantification of several true transmembrane proteins such as a receptor tyrosine kinase-like protein (Q8BKG3), a sodium/potassium-dependent ATPase (Q6PIE5), and the CD29 antigen (P09055). The quantification step of the overall data generated during this experiment is in progress.

DISCUSSION

The study of membrane proteins is a key area of proteomics as these proteins may include for example receptors involved in important signal transduction pathways or cell surface proteins representing biomarkers or easily accessible therapeutic targets. Because membrane proteins are difficult to solubilize in the buffers classically used for the first dimension of 2DE (containing little or no ionic detergents), their identification through this method has proved to be a challenge. On the other hand, with the development of systematic analysis of proteins in complex mixtures by nano-LC-MS/MS, the preliminary isolation of an individual protein in a given 2DE gel spot is no longer a prerequisite for its identification and quantification. This allows the use of strategies based on partial fractionation of the protein mixture by SDS-PAGE followed by nano-LC-MS/MS analysis of several one-dimensional gel bands. This approach is particularly well suited to membrane proteins as they are efficiently solubilized in buffers containing the anionic detergent SDS. Although this approach has been used successfully for the characterization of several complex samples, including membrane samples (24), it presents a major drawback: the quantitative information, which was easy to obtain in the two-dimensional gel strategy by comparing spot signal intensities between two samples, is almost impossible to extrapolate from the comparison of one-dimensional gel migration profiles of two samples. In the latter case, differential studies, which are the core of most current proteomic projects, can be conducted using either nano-LC-MS/MS- or

nano-LC-MS-based strategies. Recently relative quantitation of proteins was described using nano-LC-MS/MS (25, 26). In such approaches, the number of peptides per protein is considered as an indicator for protein abundance. Other approaches rely on isotopic labeling of proteins, for example with reagents like c-ICAT, followed by nano-LC-MS analysis of pairs of peptides (light *versus* heavy peptide). This allows accurate quantitative analysis of proteins identified by nano-LC-MS/MS. However, such an approach has rarely been used up to now to study fractions enriched in membrane proteins probably due to the fact that ICAT labeling of proteins had been described, by the manufacturer and in previous studies (19), to be poorly compatible with SDS.

The reason for the incompatibility of ICAT alkylation of cysteine residues with SDS concentrations higher than 0.05% (19) or 0.1% (manufacturer's protocol) is unclear. SDS is an anionic detergent with a long, flexible alkyl tail, which is able to contract hydrophobic interactions with all combinations of amino acids and will bind to the protein at a high mass ratio (1.4 g of SDS/g of protein) basically independent of the amino acid composition and the protein sequence (27). These hydrophobic interactions with the detergent usually lead to unfolding and denaturation of proteins. Moreover, the very drastic action of SDS is also due to its anionic head, which breaks ionic interactions between proteins and maintains an important electrostatic repulsion between SDS-protein complexes, preventing aggregation and promoting solubilization of the sample (28). One reason why alkylation of cysteine residues might be difficult in an SDS-protein complex could be that cysteine residues are less accessible in such a complex (19). Indeed, in a protein-detergent micelle, detergent head groups are known to form a barrier to the diffusion of water-soluble compounds (29). However, alkylation of proteins with small compounds like iodoacetamide can be performed in buffers containing SDS as is the case for example in classical 2DE electrophoresis protocols where proteins are reduced and alkylated directly after focalization on the IPG strips during the equilibration step in the presence of 2% SDS and 6 M urea before the second dimension (30).

In this report, we show that ICAT labeling is efficient in the presence of up to 4% SDS provided that high concentrations of urea are included in the reaction buffer. Labeling conditions were optimized using an ICAT analog and two standard soluble proteins. The extent of protein modification could be evaluated by SDS-PAGE and was also monitored by MALDI-TOF/MS and nano-LC-MS at the level of the cysteine-containing peptides themselves. In agreement with previously published results using ICAT (19), our results confirm that urea improves the efficiency of the labeling reaction compared with a labeling reaction performed in native conditions. Being a strong chaotrope, urea disrupts hydrogen bonds and electrostatic interactions inside the proteins and leads to denaturation and unfolding of the polypeptide chain, thus improving the accessibility of cysteine residues to alkylating reagents.

However, we found that in buffers containing little or no SDS (8 M urea or 6 M urea + 0.05% SDS), residual unlabeled proteins were still visible. When the amount of SDS in these urea-containing buffers was increased, the labeling reaction, far from being compromised, was actually improved, and the proteins were almost totally modified in the presence of 8 M urea and 4% SDS. These conditions were then used to label a highly hydrophobic protein, the adenine nucleotide translocator ANT-1, with PEO-iodoacetyl biotin, a low cost ICAT analog. Alkylation of the different cysteine residues of the protein was monitored by MALDI-TOF/MS fingerprinting, and the yield of the modification for each cysteine-containing peptide was estimated by nano-LC-MS. The labeling reaction in our modified conditions for all four ANT-1 cysteine residues was found to be very efficient ($\geq 80\%$). A very interesting result concerns cysteine 128, which is part of one of the transmembrane domains of ANT-1 probably packed in a "shell" of SDS molecules: the good yield of labeling (80%) of this cysteine demonstrates that even a residue that is present in a very hydrophobic region of a protein can be efficiently labeled under our experimental conditions. All these results were confirmed by performing the labeling of ANT-1, in the same urea/SDS buffer, using the c-ICAT reagent. As expected, alkylation with this reagent was also very efficient. Indeed the isotope coded linker chain of c-ICAT, located between the protein reactive group and the biotin moiety, is more hydrophobic than the linker chain of the original ICAT molecule or that of PEO-iodoacetyl biotin, which contains several oxygen atoms. Thus, one could expect that the c-ICAT compound may penetrate inside the SDS micelle more easily than the latter two reagents.

The results presented here show that labeling of proteins with c-ICAT is possible, and even improved, in strong denaturing buffers containing urea and SDS at a concentration higher than 0.5%. In previous studies dealing with the optimization of the ICAT labeling procedure (19), the effect of increasing concentrations of urea or SDS was tested independently. The presence of 0.5% SDS alone was found to be detrimental compared with labeling in native conditions. We can speculate that, for some proteins, SDS alone may not be able to disrupt the structure, coating the outside of the protein and hampering the nucleophilic attack of cysteine residues on the iodoacetyl moiety of the ICAT reagent compared with native conditions. To fully denature proteins in the presence of SDS alone, samples may be heated or urea may be added. In our conditions, the presence of both urea and SDS seemed to provide a synergistic denaturation process that efficiently exposed all the cysteine residues, leading to optimal conditions for the nucleophilic attack of these residues by the ICAT-reactive group.

In conclusion, these results open the possibility of applying the ICAT strategy to complex samples containing very hydrophobic proteins solubilized in urea-SDS buffers. An example is given here with the study of membrane-enriched fractions

prepared from murine embryonic stem cells. We could confirm on this sample that the use of our solubilization and labeling buffer allowed better recovery of membrane proteins, as exemplified by ANT-1 protein, than when using classical ICAT labeling conditions. Results obtained on these fractions indicate that the labeling procedure developed using a high concentration of SDS allows the study of the relative expression of membrane proteins in a complex biological sample. Finally the specific recovery of membrane proteins and their accurate quantification indicates that this approach is very promising for measuring the relative expression of hydrophobic proteins and thus identifying specific developmental markers.

However, two bottlenecks remain to be overcome. One is the isolation of ICAT peptides by avidin affinity chromatography, which is both time consuming, as it must be performed on each fraction of peptides eluted from one-dimensional gel bands, and leads to loss of material as with all chromatographic steps. This could be improved by miniaturization of this step as described recently (31). The other bottleneck of the sample process resides in the bioinformatic analysis of quantitative data obtained by this strategy. The recent advances made in this domain with the release of several software packages dedicated to the validation of nano-LC-MS/MS data and to quantitative analysis of nano-LC-MS data (ProICAT, Applied Biosystems; Decyder MS, Amersham Biosciences; MSight, www.expasy.org/MSight) together with the methodological developments described in this work suggest that the c-ICAT labeling approach may become a method of choice for the differential study of membrane proteomes.

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