REGULAR ARTICLE

A proteomic method for the analysis of changes in protein concentrations in response to systemic perturbations using metabolic incorporation of stable isotopes and mass spectrometry

Niklas Gustavsson, Boris Greber, Thomas Kreitler, Heinz Himmelbauer, Hans Lehrach and Johan Gobom

Max Planck Institute for Molecular Genetics, Berlin, Germany

While several techniques exist for assessing quantitative differences among proteomes representing different cell states, methods for assessing how these differences are mediated are largely missing. We present a method that allows one to differentiate between cellular processes, such as protein synthesis, degradation and PTMs which affect protein concentrations. An induced systemic perturbation of a cell culture was coupled to a replacement of the growth medium to one highly enriched in the stable isotope ^{15}N. The relative abundance of the ^{15}N- and ^{14}N-enriched forms of proteins, isolated from cell cultures harvested at time points following the onset of the perturbation, were determined by MS. Alterations in protein synthesis and degradation were quantified by comparing proteins isolated from perturbed and unperturbed cultures, respectively. The method was evaluated by subjecting HeLa cells to heat stress. As expected, a number of known heat shock proteins (Hsp) increased in concentration during heat stress. For Hsp27, increased *de novo* synthesis accounted for the concentration increase, while for Hsp70, decreased degradation accounted for the increase. A protein that was detected only after prolonged heat stress, vimentin, was not primarily synthesized *de novo*, but appeared rather as a result of PTM.

Received: July 29, 2004
Revised: November 4, 2004
Accepted: December 13, 2004

Keywords:
MS / PTM / Protein turnover / Quantitative proteomics / Stable isotope labeling

1 Introduction

In the last few years, the focus of proteomics has widened from being a research field mainly restricted to cataloguing proteins in cell and tissue types, to one encompassing also the measurement of protein quantity. Reflecting this change is the emergence of new analytical methods for protein quantification. In MS – a key technique in proteome research – the use of stable isotope labeling plays an important role in this development.

For MS quantification, the analyte signal intensity must be related to that of a reference compound of defined concentration. However, differences in the chemical structure between the analyte and the reference compound can lead to different yields during the sample preparation as well as different ionization efficiencies, both of which may result in large errors. The analyte molecule itself, enriched in the stable isotope ^{13}C or ^{15}N, is the ideal reference compound: one that is chemically equivalent to the analyte, but has a distinguishable molecular mass. In proteomics, several analytical strategies have been described using stable isotope-incorporated internal reference compounds (for reviews, see [1, 2]).

Correspondence: Dr. Niklas Gustavsson, Department of Plant Biochemistry, PO Box 124, SE-22100 Lund, Sweden
E-mail: niklas.gustavsson@plantbio.lu.se
Fax: +46-46-222-4116

Abbreviations: FCS, fetal calf serum; **Hsp**, heat shock protein; **nOGP**, *n*-octylglucopyranoside

Oda and coworkers [3] reported on a method for relative quantification of proteins in two separate yeast cultures. One culture was grown in a medium highly enriched in ^{15}N, resulting in metabolic incorporation of the stable isotope into all proteins, while the other culture was grown in a medium with the natural relative abundance of the ^{14}N isotope (99.6%). Protein extracts from the two cell cultures were mixed and, after protein separation and proteolytic digestion, analyzed by MS. Based on the signal intensity ratios of the ^{15}N- and ^{14}N-incorporated tryptic peptides, the relative abundance of each analyzed protein could be determined with high precision.

Pratt and coworkers [4] reported on a different strategy. Instead of comparing protein abundance in two separate cell populations, a single cell culture was studied, which at a defined time point was subjected to a replacement of the growth medium to one of different stable isotope composition. Thus, proteins synthesized after the medium replacement could be quantitatively distinguished from those already existing. By analyzing proteins isolated at defined time points, this method enabled accurate determination of protein turnover rates. In a similar approach, Cargile *et al.* [5] used $^{13}C_6$-glucose for metabolic labeling of proteins in *Escherichia coli* cell cultures.

The analytical strategy reported here extends the use of metabolic stable isotope labeling. By coupling the detection of changes in protein synthesis and degradation to an induced systemic perturbation, a link to protein function is established. HeLa cell cultures were subjected to a systemic perturbation, concomitantly with a growth medium replacement to a medium enriched in ^{15}N. Heat stress was chosen as an example, because the response to this systemic perturbation is well documented and is known to induce significant changes in protein expression [6]. Up- and down-regulation of proteins in cells depend on many different factors, including the rate of synthesis and degradation, post-translational processing as well as subcellular localization. The method described here allows one to distinguish between different processes that affect protein concentrations in response to an induced systemic perturbation.

2 Materials and methods

2.1 Chemicals

Urea, ethanol, and methanol were purchased from Merck (Darmstadt, Germany). ACN was obtained from Roth (Karlsruhe, Germany). Bromophenol blue and acrylamide were purchased from Bio-Rad (Munich, Germany). TFA, *n*-octylglucopyranoside (nOGP), glycerol, and water were obtained from Fluka (Seelze, Germany). SDS was purchased from Serva (Heidelberg, Germany). Recombinant trypsin from *Pichia pastoris* and Complete™ protease inhibitor cocktail were obtained from Roche (Mannheim, Germany). DTT, CHAPS, Tris, thiourea, iodoacetamide, CHCA, and BSA were purchased from Sigma (Seelze, Germany). The peptide calibration standards, angiotensin I and ACTH 18–39 were from Bachem (Heidelberg, Germany).

2.2 Cell growth conditions and heat stress treatment

Three parallel sets, plus one single culture serving as the control for the whole experiment, each consisting of five separate cultures of HeLa cells, were grown for 2 days in 5 cm petri dishes in Celtone M medium (Spectra Stable Isotopes, Columbia, MD, USA) with the natural relative abundance of ^{14}N and ^{15}N, supplemented with 10% fetal calf serum (FCS; Biochrom, Berlin, Germany). The incubator was set to 5% CO_2 and 95% humidity at 37°C. Upon semiconfluence, the control medium was exchanged for a medium enriched in ^{15}N (>98%) for two of the cell culture sets. In the third set of cell cultures the medium was replaced with the control medium used before. The time point for the medium replacement is referred to as t_0. Concomitantly with the medium replacement, one of the cell culture sets growing in the ^{15}N-enriched medium and the cell culture set still growing in control medium were subjected to 30 min heat stress by transferring the cultures to an incubator kept at 42°C. After this initial heat stress, the cell cultures were transferred back to the 37°C incubator. For the second cell culture set growing in ^{15}N-enriched medium, the temperature was maintained at 37°C throughout the experiment (steady-state control experiment). After 6 h following t_0, the two cell culture sets given the initial heat shock were again transferred to the 42°C incubator where they were incubated for the remainder of the experiment. One cell culture from each of the three sets was harvested 3, 6, 9, 24, and 36 h after t_0. One single cell culture was harvested at t_0 and served as the control for all the other samples in the experiment. The cultures were washed in Ca^{2+}/Mg^{2+}-free PBS (Invitrogen, Karlsruhe, Germany) and cells were harvested by incubation with 0.5 mL of 0.05% trypsin/0.53 mM EDTA (Invitrogen). Upon detachment from the dishes, 50 μL of FCS was added to stop the reaction and cells were pelleted by centrifugation. To remove trypsin and FCS, the cells were then washed with 10 mL of PBS and pelleted again to be subsequently snap-frozen in liquid N_2. Cell morphology was monitored during the course of the experiment. In the heat stress experiment, cell density was somewhat lower in the cultures harvested at the last two time points compared with the control experiment, most likely due to initiating cell death.

2.3 2-DE

Each of the cell pellets was resuspended in 500 μL of 25 mM NH_4HCO_3 buffer supplied with a protease inhibitor cocktail according to the manufacturer (one tablet/50 mL buffer), and cell lysis was performed by subjecting the cell suspensions to five cycles of freezing (liquid N_2) and thawing. The protein concentrations of the soluble fraction of the extracts were determined by the Bradford method using BSA as

standard. Aliquots containing 150 μg of protein from each sample were prepared for 2-DE by acetone precipitation and resuspension in 450 μL of sample buffer (5 M urea, 2 M thiourea, 4% w/v CHAPS, 0.002% w/v bromophenol blue, 0.5% IPG buffer, 18 mM DTT) each. Non-soluble material was pelleted by a quick centrifugation step. The samples were loaded onto non-linear IPG strips (pH range 4–7, Amersham, Uppsala, Sweden) for 30 min with no voltage applied, and IEF was performed on an IPGphor (Amersham,) using the following program: 30 V for 6 h, 60 V for 6 h, gradient to 500 V for 1 h, gradient to 1000 V for 1 h, gradient to 8000 V for 1 h, and constant 8000 V for 7.5 h. The maximum current was limited to 50 mA/strip, and the temperature was kept at 20°C throughout the procedure. Equilibration of the IPG strips was performed in two steps of 30 min each by incubation in equilibration buffer (50 mM Tris/HCl, pH 8.8, 6 M urea, 2% w/v SDS, 30% v/v glycerol, 0.002% w/v bromophenol blue) on a shaking platform. In the first of these two steps, the equilibration buffer was mixed with DTT to 15 mM, and in the second step with iodoacetamide to 55 mM. The second-dimension separation on 12% polyacrylamide gels was performed essentially according to Laemmli [7], using an Ettan DALT*twelve* electrophoresis chamber (Amersham). The cathodic running buffer concentration was double compared to the anodic running buffer to prevent buffer depletion during the electrophoretic separation. The gels were stained with colloidal CBB [8]. After reaching a desired staining intensity, the gels were rinsed in water prior to scanning and image analysis. Protein extracts prepared from both stressed and non-stressed cell cultures not subjected to medium replacement were used for protein identification by PMF and database searching.

2.4 Gel image analysis and gel spot excision

The scanned gel images were analyzed using the software Proteomeweaver (Definiens, Munich, Germany). A pair-match-based normalization function, implemented in the gel image analysis software, was applied to the gel spot intensities. In brief, the algorithm first calculates a normalization factor between all pairs of gels for which protein spots have been matched. Based on the normalization factors, an intensity factor is calculated for each gel with which the measured intensity of each spot on the gel is multiplied for comparisons to spots on other gels. After spot detection and matching of the protein patterns of the different gel images, the x- and y-coordinates for selected protein spots were transferred to a Proteineer SP Spot Picker (Bruker, Bremen, Germany) for excision of protein gel pieces. The gel pieces were deposited in 96-well microtiter plates for *in situ* proteolytic cleavage.

2.5 *In situ* proteolytic cleavage of proteins

The excised gel pieces were subjected to proteolytic cleavage using trypsin. In brief, the gel pieces were washed twice with 75 μL of 50% ethanol followed by reduction and alkylation with DTT and iodoacetamide, respectively. After washing away residual iodoacetamide from the gel pieces, trypsin was added and proteolytic cleavage was performed at 37°C for 4 h in a humidity chamber. The proteolytic cleavage was stopped by acidification by adding 16 μL of 0.5% TFA, 2 mM nOGP.

2.6 MS

Prestructured MALDI sample supports (AnchorChip 384/600; Bruker Daltonics), prepared with thin layers of the MALDI matrix CHCA were used. The two peptides (Angiotensin I, MH^+ (monoisotopic): 1 296.6848, and ACTH (18–39), MH^+ (monoisotopic) 2 465.193) were included in the MALDI matrix solution as reference masses. Sample preparation was performed according to a previously described method [9] by pipetting 1 μL from each of the tryptic peptide extracts onto the sample spots prepared with CHCA. After drying, the samples were washed by three cycles of pipetting 3 μL of 0.1% TFA, 1 mM nOGP onto each sample spot followed by aspiration of the washing solution after 3 s. Sample preparation and washing of the samples were performed using a TECAN Temo-96 pipetting robot (TECAN, Munich, Germany). After washing, the samples were re-crystallized by applying 0.5 μL of 70% ACN, 20% methanol, and 0.01% TFA to each of the sample spots. All mass spectrometric analyses were performed on a MALDI-TOF/TOF instrument (Ultraflex LIFT; Bruker Daltonics) equipped with Panorama™ pulsed ion extraction technology and operated in positive ion mode. Recorded mass spectra were the sums of 1500 single-shot spectra.

2.7 Data processing

The spectra were calibrated as described previously [10]. Protein identification by PMF was performed using the MASCOT software (Matrix Science, London, UK) [11] to search the NCBI non-redundant protein sequence database. Proteins that obtained MOWSE scores over 66 ($p < 0.05$) were considered identified.

Using software developed in-house, the calibrated mass spectra were reduced to isotopic stick spectra, containing one mass/intensity value pair per each mass unit interval. For each ^{14}N-peptide signal matching an identified protein, the signal cluster representing its partially ^{15}N-incorporated counterpart was located by matching features in the stick spectrum to theoretical isotopic signal distributions, pre-calculated in 100 Da steps based on averaged isotopic compositions for tryptic peptides, using least-square fitting. The intensities of the ^{14}N- and ^{15}N-peptide signals were calculated as the sum of the intensities of their isotopic signals. Correction for chemical noise was performed by subtracting the average intensity of the isotopic background noise in the spectra, calculated for the local region around each peak pair. Ongoing work includes development of software for automation of large-scale data processing.

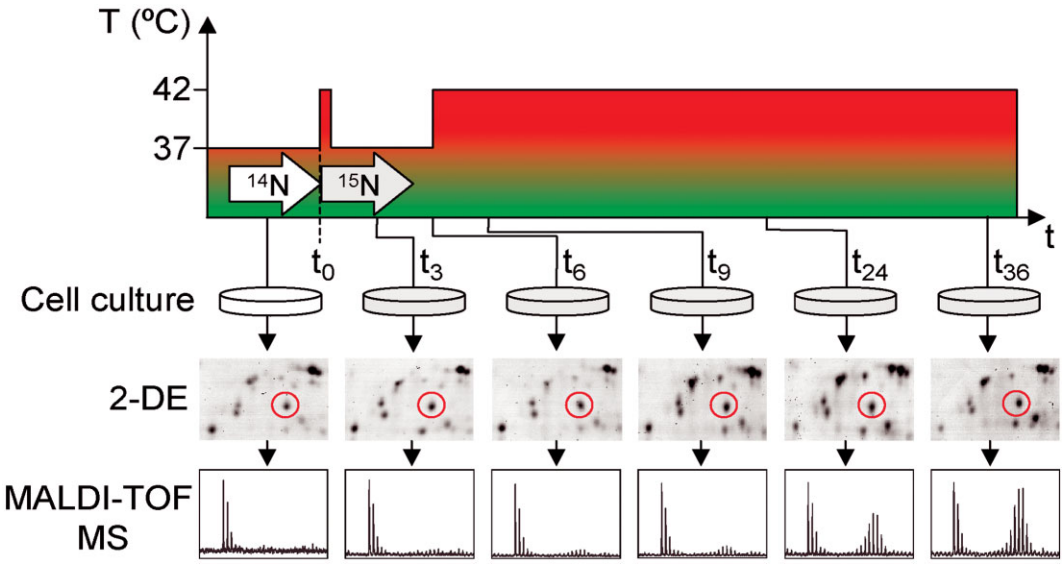

Figure 1. Experiment outline. HeLa cell cultures, grown in a medium containing a natural abundance of the nitrogen isotope ^{14}N (99.6%), were at a time point, t_0, subjected to a replacement of the growth medium to one highly enriched in the stable isotope ^{15}N (>98%). Concomitantly with this change, one set of cell cultures was subjected to a heat stress regime, as shown in the upper part of the figure, whereas another set was maintained at 37°C. Cell cultures from both sets were harvested 3, 6, 9, 24, and 36 h after t_0, and protein extracts prepared thereof. The contained proteins were isolated by 2-DE and, following tryptic digestion, analyzed by MALDI-TOF MS. In the mass spectra corresponding to cells harvested at later time points, each peptide is represented by two isotopic signal distributions: one with lower *m/z* values, corresponding to the peptide containing mostly ^{14}N (natural N isotopic distribution), and one with higher *m/z* values, corresponding to the same peptide enriched in ^{15}N. From their relative signal intensities, the portion of the protein synthesized after t_0 can be calculated. By comparing the results for the different cell cultures, changes in protein concentration caused by the heat stress can be measured.

3 Results

3.1 Experiment design

Our analytical strategy is outlined in Fig. 1. At a certain time point, t_0, individual HeLa cell cultures were subjected to a growth medium replacement to a medium highly enriched in the stable isotope ^{15}N (>98%). Concomitantly with the replacement, the cells were subjected to a heat stress regime. At selected time points (3, 6, 9, 24, and 36 h) after t_0, the cells were harvested and their soluble proteins extracted. These were separated by 2-DE. Isolated proteins were digested with trypsin, and the resulting peptide mixtures analyzed by MALDI-TOF MS. For each protein analyzed, the portions synthesized before and after t_0 are represented by two sets of signals for each of its tryptic peptides (Fig. 2). From left to right, the first set represents synthesis before t_0 and its intensity distribution in first order reflects the number of carbon atoms contained by the corresponding peptide (natural abundance of ^{13}C and ^{14}N). The second set represents synthesis after t_0 and its intensity distribution reflects both the number of carbon (natural abundance) and nitrogen atoms (remaining abundance of ^{14}N after growth medium replacement) contained by the corresponding peptide. The fraction, q, of a protein that was synthesized after the medium replacement is given by:

$$q = \frac{I(^{15}N)}{I(^{14}N) + I(^{15}N)} \quad (1)$$

where $I(^{15}\mathrm{N})$ is the sum of the signal intensities of the ^{15}N-enriched peptide in the mass spectra and $I(^{14}\mathrm{N})$ is the sum of the signal intensities of its ^{14}N-enriched counterpart. The value of q for a protein at any given time point, t, thus depends on the amount of the protein both synthesized and removed since t_0. By comparing values of q obtained for a protein from heat stressed cell cultures with values for q obtained for the same protein from parallel cell cultures not subjected to the heat stress, alterations in the protein synthesis and removal rates as a result of the heat stress can be detected.

3.2 Determination of protein half-life at steady state

The incorporation of ^{15}N into proteins in unperturbed cell cultures over the time course of the experiment is illustrated in Fig. 3 for the heat shock protein (Hsp) Hsp70. At the chosen growth conditions, steady state prevails for most protein concentrations, under which their synthesis and degradation are balanced. This is indicated by the largely unchanged spot densities for Hsp70 isolated from cell cultures harvested at

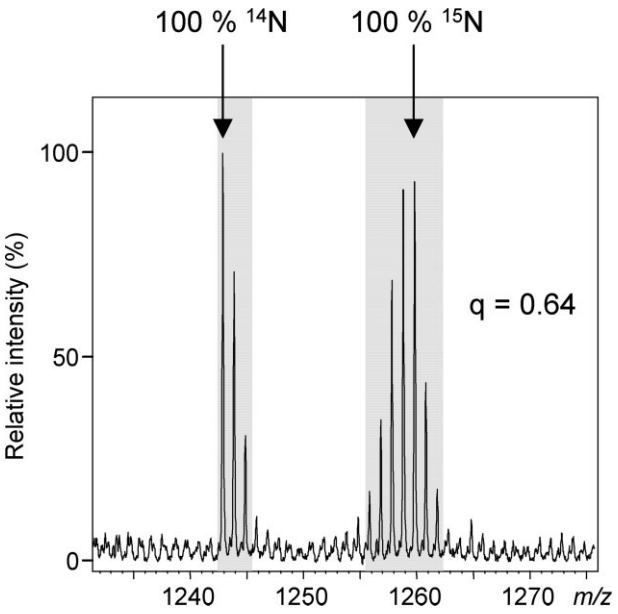

Figure 2. Determination of the relative abundance of a protein synthesized after growth medium replacement. MALDI-TOF mass spectrum, containing both the ^{14}N- and the ^{15}N-enriched isotopic signal distributions of the Hsp70 tryptic peptide DAGQISGLNVLR (amino acid 207–218), 36 h after medium replacement under steady-state growth conditions. The nominal mass difference between the monoisotopic molecular ions containing 100% ^{14}N and 100% ^{15}N corresponds to the number of nitrogen atoms (17). The sum of the intensities of the signals corresponding to the ^{14}N- and ^{15}N-enriched populations of the same peptide, as indicated by the shadowed areas, was used for determination of the relative abundance of Hsp70 expressed before and after the medium replacement. The *q* value of 0.64, calculated according to Eq. 1, indicates that 64% of the protein has been synthesized after the medium replacement.

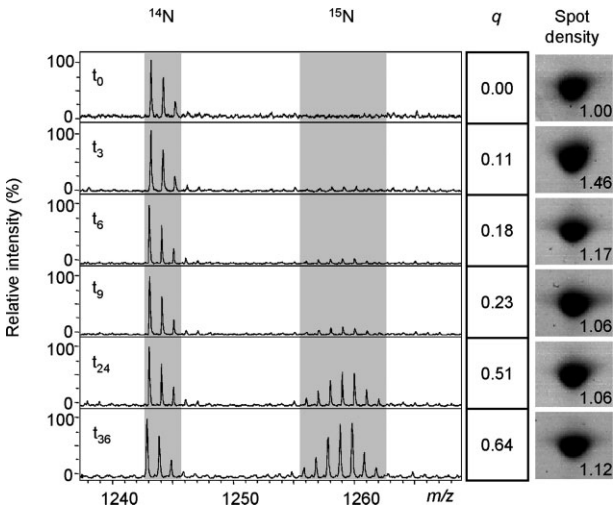

Figure 3. Gradual increase of the ^{15}N-enriched population of Hsp70 at steady state, as monitored by MALDI-TOF MS. The relative abundance of the ^{15}N-enriched portion of the Hsp70 tryptic peptide DAGQISGLNVLR (amino acid 207–218) synthesized after medium replacement increases as compared with the pool of protein that was synthesized before the replacement (left panel). The *q* values, calculated according to Eq. 1, show that the ^{15}N-enriched population has reached 64% of the total concentration 36 h after medium replacement. The total concentration of Hsp70 was virtually constant over the time course of the experiment as judged by the protein spot densities indicated in the right part of the figure (arbitrary units).

the indicated time points. Although the overall protein amount in the cell culture increases as an effect of cell proliferation, and thus the rate of synthesis clearly exceeds the rate of degradation, the concentration of a specific protein in a single cell is indeed maintained constant. The figure also shows the corresponding isotopic signals of the tryptic peptide DAGQISGLNVLR from the peptide mass fingerprints of Hsp70. The *q* values are shown for each time point, and indicate an increase in the relative abundance of ^{15}N-enriched Hsp70 from 0 to 64% over the time course of the experiment.

The *q* values for Hsp70 are plotted *versus* time in the graph in Fig. 4a. Under these steady-state conditions, the first-order rate constant, *k*, can be calculated according to:

$$k = -\frac{\ln(1-q)}{t} \quad (2)$$

In Fig. 4b, values for *k* calculated for different Hsp70 tryptic peptides are plotted *versus* time. As can be seen, *k* values calculated for early sampling time points display a higher degree of scattering compared to later time points. This is attributed to low S/N ratios of the ^{15}N signals for the early time points. Therefore, throughout this report we have exclusively used t_{36} for determination of *k*. For Hsp70, *k* was determined as 0.0295 h^{-1} ($\pm$0.0011 h^{-1}).

The time point where 50% incorporation of ^{15}N was reached ($q = 0.5$) equals the protein's half-life, *t*, and is obtained by:

$$t_{1/2} = \ln 2/k \quad (3)$$

Accordingly, the half-life for Hsp70 was determined as 23.5 h ($\pm$0.83 h). The half-lives for 11 proteins determined using this method are listed in Table 1.

3.3 Detection of changes in protein synthesis and degradation rates in response to heat stress

As expected, the heat stress led to significant changes in the abundance of many proteins, as was evident from the 2-DE protein spot patterns (data not shown). Not surprisingly, a number of Hsps were identified among the proteins that increased in concentration. For Hsp27, a more than five-fold increase in the total concentration, as determined by the spot densities (Fig. 5, right panel), was accompanied by significantly higher *q* values during the heat stress treatment (left panel). This indicates that under these conditions the increased abundance of Hsp27 is mainly due to an increased synthesis rate.

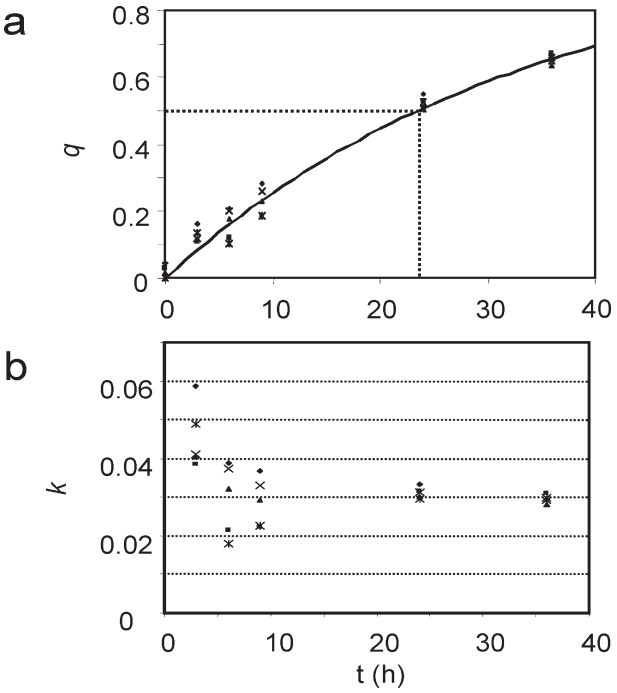

Figure 4. Determination of the relative abundance of ^{15}N-enriched protein, the synthesis rate constant, and protein half-life for Hsp70 at steady state. (a) The relative abundance of ^{15}N-enriched Hsp70 at five time points after growth medium replacement is represented by the *q* values for each of five tryptic peptides (▲, *m/z* 1242.5, DAGQISGLNVLR; ×, *m/z* 1290.5, VQQTVQDLFGR; ◆, *m/z* 1361.8, AQFEGIVTDLIR; ✳, *m/z* 1518.0, AQFEGIVTDLIRR; ■, *m/z* 2056.3, STNGDTFLGGEDFDQALLR). The dashed line indicates the time point where 50% of the total protein concentration consists of newly synthesized ^{15}N-enriched protein. Superimposed in panel (a) is a theoretical synthesis-degradation curve, based on the average synthesis rate constant 0.0295, determined from the *q* values of the five peptides at t_{36}. (b) The synthesis rate constant *k* was calculated (Eq. 2) for the five peptides from the *q* values in panel (a).

Table 1. Protein half-lives at steady state

Protein spot ID	Protein (accession number)	Half-life at steady state (h)
8	Hsp27 (gi 662841)	17.1 ± 0.002
9	Glucose-6-phosphate dehydrogenase (gi 182890)	23.2 ± 1.8
20	Alpha-tubulin (gi 32015)	33.9 ± 1.0
21	Hsp70 (gi 4758570)	23.7 ± 0.7
44	H+-transporting ATP synthase (gi 92350)	30.5 ± 0.8
45	Hsp60 (gi 14603309)	37.5 ± 2.7
49	Protein disulfide isomerase (gi 135267)	24.5 ± 1.0
51	Hsp27 (gi 662841)	16.8 ± 0.002
65	Actin (gi 14250401)	32.7 ± 1.5
172	Nuclear chloride ion channel protein (gi 2073569)	32.6 ± 2.1
175	Keratin 18 (gi 12653819)	26.7 ± 2.2

Along with the up-regulation of proteins needed for the cell to survive, heat stress also leads to down-regulation of other proteins [12]. While the total concentration of actin had decreased by approximately 20% at t_{36} (Fig. 5, right panel), the *q* values decreased from 0.54 to 0.35 (left panel); a decrease of 35%. Thus, while the synthesis rate of actin decreased significantly, the degradation rate remained virtually constant (Fig. 5, right panel, green part of the bars).

An unexpected result came from the analysis of Hsp70. While the total concentration of Hsp70 increased as expected upon heat stress (Fig. 5, right panel), the *q* values decreased (left panel). This indicates that the increase is not achieved by up-regulation of gene expression, but rather by decreased degradation of Hsp70 (Fig. 5, right panel, green part of the bars).

3.4 Characterization of changes in protein concentration as a result of PTM

The series of 2-DE gel image regions in Fig. 6a represents two samples from the steady-state control experiment and five samples from the heat stress experiment. Protein spots in which the protein vimentin was identified are indicated with an arrow. Vimentin is a major component of the cytoskeletal intermediate filaments, involved in maintaining cellular mechanical stability [13]. Although vimentin appears first at t_{24}, its *q* value at t_{36} was 0.38, indicating that 62% of the protein amount was not synthesized after onset of the heat stress, but instead was recruited from an already existing pool, possibly by intermediate filament disassembly (Fig. 6b).

4 Discussion

The examples shown in Fig. 5 were chosen to illustrate the ability of the presented method to differentiate between the different means of the cell to regulate protein concentrations. For Hsp27 and actin, the observed changes in protein concentration during heat stress were due to increased and decreased synthesis rates, respectively, while the protein removal rates remained unchanged. For Hsp70, the observed increased concentration was due to a decreased degradation rate, whereas the synthesis rate remained virtually constant. An alternative explanation for this observation could involve PTM of a different form of Hsp70. Using radiolabeling and fluorescence detection of proteins separated by 2-DE, the synthesis rate of Hsp70 was, in contradiction to our result, found to increase and to correlate with the accumulating amount of Hsp70 after a short heat shock treatment of U937 cells [14]. These seemingly contradictory results are most likely due to the significantly different experimental protocols used for heat stress treatment between these two studies. In addition, as the same protein is often identified in different spots on 2-DE gels, it cannot be ruled out that different forms of Hsp70, potentially with different *q* values, are reported in these two studies.

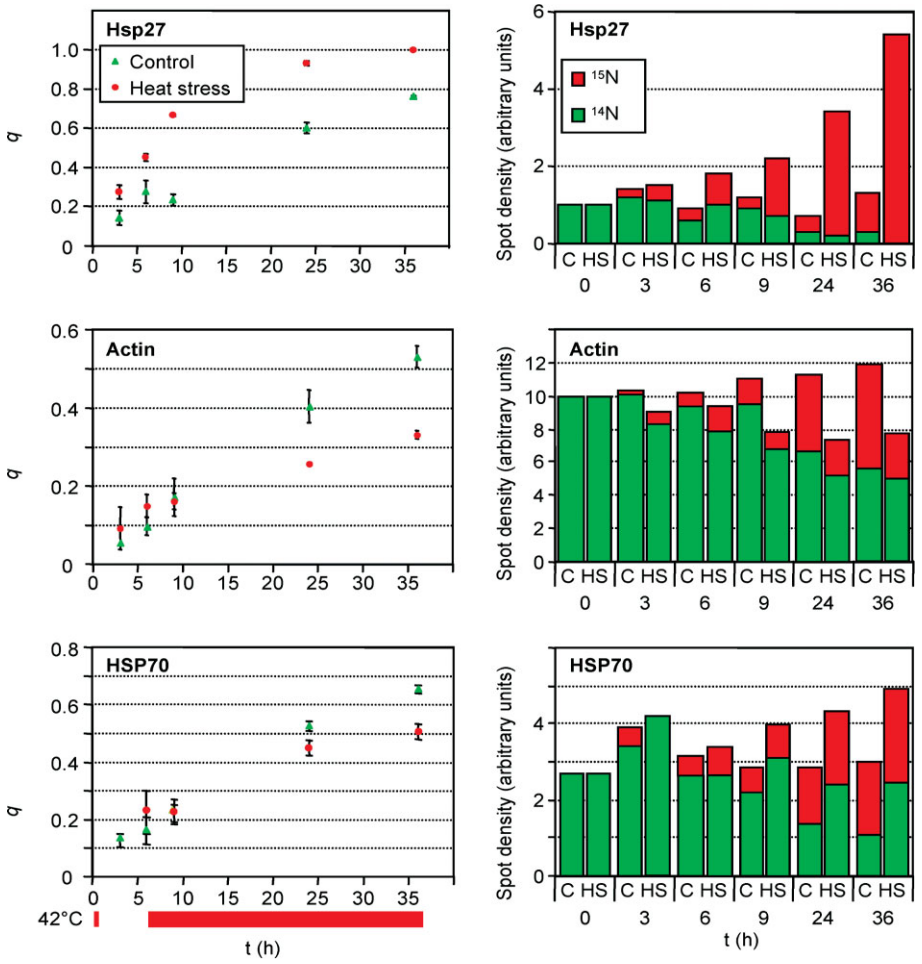

Figure 5. Changes in protein synthesis-degradation profiles in response to heat stress. The left panels show the difference between the synthesis-degradation profiles given by the *q* values at steady state (green triangles) and during heat stress (red circles) for Hsp27, actin, and Hsp70. Each *q* value represents the average of at least three individual peptide pairs with the scattering for the experimental values indicated with error bars. The right panels represent the corresponding protein spot densities, obtained from the 2-DE gel images. Each bar is divided into a lower (green) and an upper (red) region, corresponding to the contribution of the ^{14}N- and ^{15}N-enriched portions to the total amount, respectively. For Hsp27, the increase in concentration was mainly due to up-regulation of its synthesis (red part of the bars). For actin, the observed decrease was predominantly caused by down-regulation of its synthesis (red part of the bars). Note that although the overall Hsp70 concentration increases in response to the heat stress as judged by the increased gel spot densities (right panel), the corresponding *q* values (left panel) indicate a down-regulation of its synthesis during the applied stress.

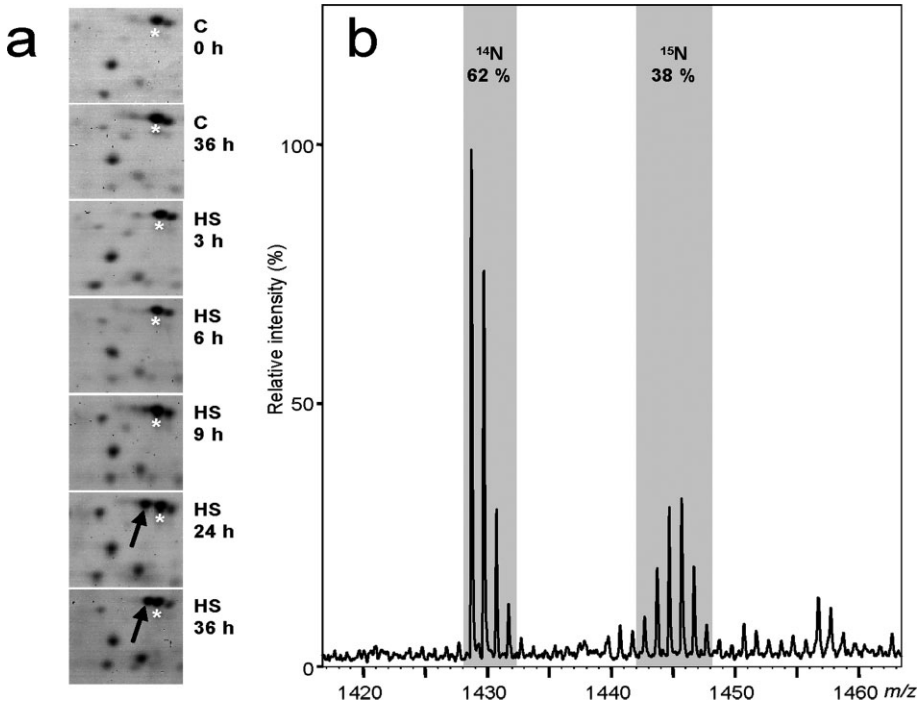

Figure 6. Identification of a vimentin isoform, which arises due to post-translational processing. (a) The 2-DE gel images represent two samples from the control and five samples from the heat stress (HS) experiment. A protein found to contain vimentin appeared only in the samples after 24 and 36 h of heat stress treatment as indicated with an arrow in the bottom two 2-DE gels. To assist orientation, protein spots representing tubulin, present in all the samples, are indicated with asterisks. (b) MALDI-TOF mass spectrum showing the relative abundance of the ^{15}N-enriched portion of the tryptic peptide SLYASSPGGVYATR (amino acid 51–64) of vimentin extracted from cells subjected to heat stress for 36 h. The majority (62%) of the vimentin molecules were synthesized before medium replacement, although the protein was first detected 24 h after this change.

While the described method does not necessarily rely on 2-DE for protein separation, the choice of separation technique affects the results with regard to what information is made available for different forms of the same protein. Because differently modified forms of a protein often appear as separate spots on a 2-DE gel [15], it follows that a change in spot density during the time course of an experiment does not necessarily reflect an altered synthesis or degradation rate, but may well be the result of a protein modification. These scenarios can be distinguished by the presented method, as was demonstrated for vimentin, which appeared as a new spot in the 2-DE gels only after prolonged heat stress, while its determined *q* value indicated that the major part of the protein had in fact been synthesized prior to the heat stress treatment. In addition to the vimentin gel spot indicated in Fig. 6, we also identified vimentin from another gel spot, whose density decreased after prolonged heat stress, concomitantly with the previously described spot density increase. By comparing the MALDI-TOF peptide maps of these two forms of vimentin, we identified four signals present only in the form that increased in concentration upon heat stress (data not shown). The corresponding peptides were all mapped to the *N*-terminal head region of vimentin, in which Asp85 is reported as a caspase cleavage site [16]. Caspase-9 cleavage of vimentin is reported to be an early event in the apoptotic pathway mediated by release of cytochrome-*c* from mitochondria, and is believed to promote the dismantling of intracellular structures.

Heat stress was chosen as a systemic perturbation to illustrate the potential of the presented method to differentiate among processes that influence changes in protein concentrations. Naturally, any perturbation that can be temporally linked to the replacement of growth medium can be assessed for its effect on a proteome using our method. In addition to analyzing perturbation-induced changes in proteomes of one specific cell line, the proteomes of different cell lines can be compared with respect to protein concentration changes, which arise from different synthesis and degradation rates, as well as from post-translational processing.

The authors would like to thank Dorothea Theiss, Beata Lukaszewska-McGreal, and Klaus-Dieter Kloeppel for technical support and scientific discussion. This project was funded by the National Genome Research Network (NGFN) of the German Ministry for Education and Research (BMBF), and the Max Planck Society.

5 References

[1] Moritz, B., Meyer, H. E., *Proteomics* 2003, *3*, 2208–2220.

[2] Ong, S. E., Foster, L. J., Mann, M., *Methods* 2003, *29*, 124–130.

[3] Oda, Y., Huang, K., Cross, F. R., Cowburn, D., Chait, B. T., *Proc. Natl. Acad. Sci. USA* 1999, *96*, 6591–6596.

[4] Pratt, J. M., Petty, J., Riba-Garcia, I., Robertson, D. H. L. *et al.*, *Mol. Cell. Proteomics* 2002, *1*, 579–591.

[5] Cargile, B. J., Bundy, J. L., Grunden, A. M., Stephenson, J. L., *Anal. Chem.* 2004, *76*, 86–97.

[6] Lindquist, S., Craig, E. A., *Annu. Rev. Genet.* 1988, *22*, 631–677.

[7] Laemmli, U. K., *Nature* 1970, *227*, 680–685.

[8] Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W., *Electrophoresis* 1988, *9*, 255–262.

[9] Gobom, J., Schuerenberg, M., Mueller, M., Theiss, D. *et al.*, *Anal. Chem.* 2001, *73*, 434–438.

[10] Gobom, J., Mueller, M., Egelhofer, V., Theiss, D. *et al.*, *Anal. Chem.* 2002, *74*, 3915–3923.

[11] Perkins, D. N., Pappin, D. J. C., Creasy, D. M., Cottrell, J. S., *Electrophoresis* 1999, *20*, 3551–3567.

[12] Parsell, D. A., Lindquist, S., *Annu. Rev. Genet.* 1993, *27*, 437–496.

[13] Fuchs, E., Weber, K., *Annu. Rev. Biochem.* 1994, *63*, 345–382.

[14] Gerner, C., Vejda, S., Gelbmann, D., Bayer, E. *et al.*, *Mol. Cell. Proteomics* 2002, *1*, 528–537.

[15] Jensen, O. N., *Curr. Opin. Chem. Biol.* 2004, *8*, 33–41.

[16] Nakanishi, K., Maruyama, M., Shibata, T., Morishima, N., *J. Biol. Chem.* 2001, *276*, 41237–41244.

