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In-Gel ^{18}O Labeling for Improved Identification of Proteins from 2-DE Gel Spots in Comparative Proteomic Experiments

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Abstract: The reliability of 2-DE gel-based comparative proteomics is severely impaired by the potential presence of overlapping proteins. We describe a methodological procedure which may solve this problem. Corresponding protein spots from two experimental groups are digested in the presence of ^{16}O and ^{18}O, respectively. Samples are pooled and proteins identified by MS. The $^{18}O/^{16}O$-ratios of the different proteins found in the same spot distinguish proteins with altered from those whose intensity is unchanged.

Keywords: ^{18}O • comparative proteomics • mass spectrometry • quantification • two-dimensional gel electrophoresis

Introduction

In recent years, numerous study groups have unanimously reported that a significant percentage of spots from two-dimentional electrophoresis (2-DE) gels represents more than one protein.[1−5] Several proteins per spot have even been identified after the use of IPG strips with narrow pH ranges[1,2] and after the application of amounts of protein as small as 100 μg.[4]

The identification of proteins from spots showing differences in intensity between different experimental groups is therefore unreliable. This may pose a problem, particularly in experiments which are designed to identify marker proteins for diseases or for the development of new drugs. Some useful suggestions as to how this problem can be solved have recently been published. Yang et al.[5] used an "exponentially modified protein abundance index" to determine the abundance of the individual component proteins from the spots containing multiple proteins. They reported that the top-hit proteins from 40 out of 43 spots identified by MALDI matched the most abundant proteins determined by LC-MS/MS. They also demonstrated that the top-hit proteins in 44 identified spots contributed on average 81% of the spots' staining intensity. The average contribution from the minor protein components is therefore less than 20%. These data indicate that in most cases the first MS hit may represent the altered protein.

Asara et al. recently presented an elegant solution to this problem.[6,7] They performed standard SDS gel electrophoresis and separately derivatized proteins of interest from two different experimental groups with light vs heavy-labeled isotope reagents. The gel slices were then mixed and digested and the peptides were analyzed by LC-MS to determine the relative abundance of light/heavy isotope pairs. Asara et al. have not yet evaluated the applicability and reliability of their method with reference to 2-DE gel-based comparative proteome studies in detail.

A reasonable alternative to the chemical labeling method is enzymatic peptide labeling with oxygen-18 (^{18}O). The use of ^{18}O was first described in 1951 by Sprinson and Rittenberg.[8] Mirgorodskaya et al.[9] suggested this technique for quantification of peptides and proteins. Fenselau et al. introduced this method to quantitative proteomic analyses.[10] This group also further improved the methodological details and areas of application in numerous elegant experiments,[11−14] (for a review, see ref 15). The method is based on the incorporation of ^{18}O into peptides during the enzyme-catalyzed digestion procedure. Tryptic digestion in the presence of ^{18}O water exchanges the oxygen atoms from the carboxyl group of lysine and arginine with one or two ^{18}O atoms, leading to a mass difference of 2 or 4 Da. In recent years, this method has become more popular as it is almost universally applicable, highly specific and relatively inexpensive, and almost all tryptic peptides are accessible for labeling (except C-terminal peptides). An increasing number of authors have suggested useful methodological improvements and successfully applied the ^{18}O technique in comparative proteomic studies.[16−24] Here, we describe a method which employs ^{18}O labeling in order to improve the identification of proteins from those gel spots which show differences in staining intensity between different experimental groups.

Experimental Section

Theoretically, any pair of spots whose intensities differ by a certain amount (e.g., 50%, 100%, etc.) and which had been excised from gels from two different experimental groups could

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have been used for this study. We chose an experiment designed to evaluate the effects of postmortem conditions on the proteome. Tissue from adult male Sprague−Dawley rats weighing approximately 300 g was used throughout. The bodies of one group of six rats were stored at 4 °C for 24 h after death by asphyxiation before the brain areas were dissected on ice and stored at −80 °C. A further group of six rats whose brain areas were immediately dissected on ice and frozen at −80 °C served as controls. The parieto-occipital cortices were used for the quantitative comparisons described here and only spots with significant differences in mean intensity between the two experimental groups were chosen.

2-DE. Gel electrophoresis was performed as previously described.[25] We used equipment developed by our own group for all analyses, which enabled us to process 24 gels simultaneously throughout all methodological steps of the 2-DE procedure (see ref 25). In brief, the tissue was homogenized with solubilization buffer (9 M urea, 2.5 M thiourea, 5% CHAPS, 12.5 mM DTT, 0.5% Pharmalyte 3−10, 0.1% Pefabloc SC and 150 U/mL Benzonase) and centrifuged for 60 min at 20 000*g* to pellet any debris. The protein concentration of the supernatant was determined with the EZQ quantitation kit, as described elsewhere.[26] In all experiments, 200 μg of protein per gel was loaded. The IPG dry strips (GE Healthcare, Germany, 18 cm (linear), pH 4−7 throughout) were inserted into the slots of the rehydration chamber with the gel side up and rehydrated overnight with the rehydration solution containing the sample. Isoelectric focusing was carried out for 65 kVh, using the Multiphor II unit (GE Healthcare Biosciences). Equilibration and SDS-PAGE were performed as previously described.[25] The gels were stained with ruthenium II tris(bathophenanthroline disulfonate) and scanned on a Fuji FLA-3000 fluorescence scanner using the scanning software BAS-Reader also as described in detail elsewhere.[25] Image analysis was carried out using Delta2D (Decodon, Greifswald, Germany). Prior to statistical analysis, the data were normalized as previously described.[27]

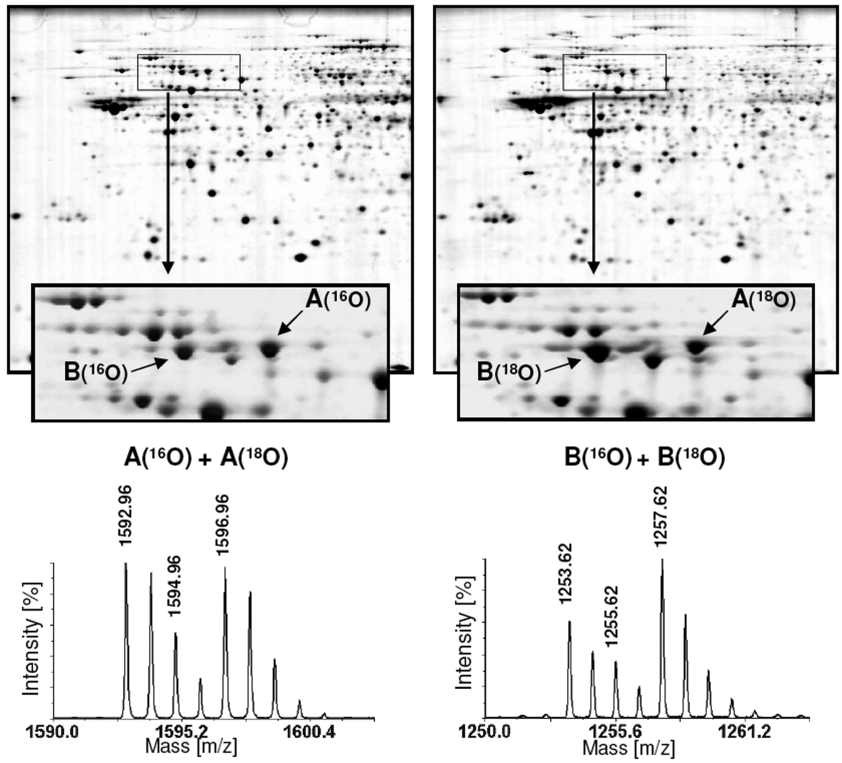
We performed between-group comparisons using the Welch test (a version of Student's *t* test which allows for different variances in the two groups) for independent samples (two-tailed), followed by *p*-value correction as described by Benjamini-Hochberg.[28] Adjusted *p*-values <0.05 (Benjamini-Hochberg) were considered significant. Since for the methodological study presented here we needed only differentially expressed spots and the detailed results of our postmortem study are of no relevance, they will be published in a separate report (Baumgartner et al., in preparation).

Protein Digestion and In-Gel ^{18}O Labeling. The non-normalized spot intensity reflects the total abundance of protein in a given spot. After normalization of the data, the spot intensities are corrected according to the differences in overall protein load between all gels. The normalized spot intensities therefore no longer correctly reflect the "true" protein abundance of spots and spot intensity ratios cannot be expected to be correlated to the corresponding ^{18}O/^{16}O-ratios. For this reason, we chose and excised spots of interest on the basis of non-normalized data. The gels were placed on a UV table and spots were excised manually with pipet tips whose diameters were adapted to the different spot sizes (1.5, 2, 2.5, 3, and 3.5 mm). Tryptic in-gel digestion and ^{18}O labeling of proteins was performed as previously described.[19] In brief, the protein spots were excised and washed, first with 50% (v/v) acetonitrile (ACN) in 25 mM ammonium bicarbonate (ABC) and then with 50 mM ABC. Thereafter they were dehydrated in ACN and dried in a vacuum centrifuge. Digestion was performed overnight at 37 °C in 10 μL of 50 mM ammonium bicarbonate in either $H_2{}^{16}O$ or $H_2{}^{18}O$ (Campro Scientific, Berlin, Germany, 97% $H_2{}^{18}O$) containing 90 ng of modified trypsin (Promega, Mannheim, Germany). Incubating the pieces of gel overnight at elevated temperature guarantees efficient digestion of proteins and satisfactory isotopic labeling if the tryptic digestion is performed in the presence of ^{18}O water. The reaction was stopped by adding 10 μL of 0.5% trifluoroacetic acid (TFA) in ACN and the supernatant was collected. The pieces of gel were dehydrated with 20 μL of ACN and the two supernatants were unified and dried in a vacuum centrifuge. Immediately prior to the MS measurement, the peptides were dissolved in 0.1% (v/v) TFA and 6% (v/v) ACN in water in a volume of 10 or 5 μL for MALDI or ESI. The ^{16}O and ^{18}O labeled samples of each spot were mixed. To verify the stability of the labeling, that is, that no back exchange takes place, a control spot was labeled solely with ^{18}O, diluted, dehydrated, redissolved and measured by MALDI-MS. The results showed that ^{18}O had been completely incorporated (data not shown). This demonstrates that ^{18}O/^{16}O back-exchange can be avoided by redissolving and mixing the samples shortly before MS measurement as described.[19]

Protein Identification. MALDI mass spectrometry was performed as previously described.[29] In brief, the peptides were purified on a C18 RP minicolumn (ZipTip C18, Millipore, Bedford, MA) and eluted directly onto the MALDI target plate using α-cyano-4-hydroxycinnamic acid matrix solution. MS and MS/MS measurements were performed using a MALDI-TOF/TOF instrument (4700 Proteomics analyzer, Applied Biosystems, Framingham, MA). MS spectra were acquired in positive ion reflector mode by accumulating 5000 consecutive laser shots. A maximum of 5 precursor ions for MS/MS fragmentation were selected automatically. The GPS Explorer (version 3.5, Applied Biosystems) was used to process the spectra. After exclusion of contaminant ions (known matrix and human keratin peaks), the data were submitted to the MASCOT server (version 2.0, Matrix Science, London, U.K.) for in-house search against the mammalian subset of the NCBInr database (version 011106; 4 076 784 sequences; 1 405 124 249 residues).

The setting for maximal missed cleavage was set to 1. The mass tolerances for the precursor and sequence ions were set to 30 ppm and 0.3 Da, respectively. Allowed variable modifications were carbamidomethylation on cysteine and methionine oxidation, N-acetylation, pyroglutamic acid on N-terminal Gln, and the C-terminal ^{16}O/^{18}O exchange. The proteins were identified by a combined search using the mass fingerprint (PMF) data and MS/MS spectra. Proteins were considered as identified if the following criteria were fulfilled: (i) a probability based Mowse score greater than 70 ($p < 0.05$); (ii) a PMF with more than 30% sequence coverage (SC) *or* a PMF with 15−30% SC and one MS/MS spectrum with a MASCOT score for identity *or* a PMF with less than 15% SC and two MS/MS spectra with a MASCOT score for identity or one MS/MS spectrum with additional information, for example, from neighboring spots. These criteria were applied for proteins with a mass greater than 10 kDa.

Nano-LC-ESI-MS/MS experiments were performed as previously described[19] using a Q-Tof Ultima quadrupole orthogonal acceleration time-of-flight mass spectrometer (Micromass, Manchester, U.K.) equipped with a Z-spray nanoelectrospray source. A Micromass CapLC liquid chromatography system was



spot	protein name	accession number	Mowse score	sequence coverage [%]	ratio $^{18}O/^{16}O$	ratio intensity (gel)
A	pre-mtHSP70	gi|896232	209	27	1,04	1,00
B	heat shock protein 8	gi|13242237	219	36	1,93	2,12

Figure 1. Comparison of the staining intensity ratios with the $^{18}O/^{16}O$-ratios of pairs of corresponding spots excised from gels from two different experimental groups.

used to deliver the peptide solution to the electrospray source. The peptides were eluted onto an analytical column (PepMap C18, 3 μm, 100 Å, 150 mm $\times$ 75 μm i.d., LC Packings) and separations were performed at an eluent flow rate of 200 nL/min. Mobile phase A was 0.1% formic acid (v/v) in ACN/water (5:95, v/v) and B was 0.1% formic acid in ACN/water (8:2, v/v). Runs were performed using a gradient of 3−64% B in 60 min. To perform MS/MS experiments, automatic function switching (survey scanning) was employed. The MS survey range was m/z 300−1990 and the scan duration was 1.0 s. Doubly and triply charged ions were selected for MS/MS and the MS/MS range was m/z 50−1990. The collision gas was argon at a pressure of 6.0×10^{-5} mbar. The MS/MS ion search option of MASCOT was used to search against the NCBI nonredundant protein database (version 150508; 6 530 794 sequences; 2 229 583 460 residues). The mass tolerance of precursor and sequence ions was set to 0.1 and 0.2 Da, respectively. The database search includes variable modifications of cysteine with acrylamide, methionine oxidation, and the C-terminal $^{16}O/^{18}O$ exchange. A protein was accepted as identified if the total MASCOT score was greater than the significance threshold and at least two peptides appeared the first time in the report and were the first ranking peptides.

Quantification of Proteins. Relative quantification of proteins was performed using an algorithm described previously.[19] Briefly, relative protein amounts were calculated from relative amounts of at least two different ^{18}O labeled peptides that had been identified by MS/MS with a score above the MASCOT homology threshold. With the use of signal intensities of tryptic peptides containing zero, one, or two ^{18}O at m/z, $(m + 2)/z$, and at $(m + 4)/z$, respectively, the method considers that either one or both oxygen atoms of the carboxyl group could be exchanged during in-gel digestion of the protein. The contribution of naturally occurring isotopes at $(m + 2)/z$ and at $(m + 4)/z$ as well as the isotopic purity of ^{18}O water (97%) were considered.

Results and Discussion

Results of the MALDI-TOF/TOF Analyses. We first quantified 27 spatially matched protein spots with almost the *same* staining intensities from two 2-DE gels each taken from one of the two experimental groups. The spots from the gels of the first experimental group were digested in the presence of ^{18}O and those of the other group in ^{16}O-water. The mean $^{18}O/^{16}O$-ratio of all spots was 1.07, with a standard deviation (SD) of 0.15. The 95% confidence interval of this quantity is thus 0.78−1.36, assuming normal distribution under the null hypothesis. If, on the other hand, $^{18}O/^{16}O$-ratios of >1.36 or <0.78 are obtained, then the expression of the respective protein will differ between the two gels at a 95% confidence level.

Figure 1 shows two pairs of corresponding spots excised from two gels from different experimental groups. Spot A had the same staining intensities on two different gels; the intensity ratio was therefore 1.0. MS analysis yielded an $^{18}O/^{16}O$-ratio of 1.04, correctly reflecting that the expression level of the *pre-mtHSP70* was unaltered. In contrast, for spot B, the staining

Table 1. MALDI-MS/MS Analyses: Validation of Differences in Spot Intensity between Two Experimental Groups by Means of the $^{18}O/^{16}O$-Ratios (Representative Examples)

spot no.	name of protein	accession number	Mowse score	sequence coverage [%]	peptide mass for $^{18}O/^{16}O$	ratio $^{18}O/^{16}O$	mean ratio $^{18}O/^{16}O$	ratio spot intensity (gel)	design	result	sample size
1	formyltetrahydrofolate dehydrogenase	gi|57921067	191	32	950.53 1281.72 1363.68 1394.69 1540.77	2.09 2.50 2.85 2.06 2.21	2.34	2.31	Staining intensity more than doubled	$^{18}O/^{16}O$ ratio agrees with spot intensity ratio	53
2	formyltetrahydrofolate dehydrogenase	gi|57921067	186	29	950.53 1281.72 1363.68 1394.69 1540.77	0.42 0.50 0.46 0.40 0.44	0.44	0.42	Staining intensity more than halved	$^{18}O/^{16}O$ -ratio agrees with spot intensity ratio	20
3	chaperonin-containing TCP1, subunit 5	gi|51890219	159	38	818.48 1093.53 1290.67 1427.68 1610.91	0.99 1.37 0.91 0.54 0.61	0.88	2.23	Staining intensity more than doubled	$^{18}O/^{16}O$ ratio too low, as one spot was not completely picked	5
4	dynamin 1	gi|18093102	143	31	1042.48 1065.63 1091.53 1272.67 1375.70 1609.85 1709.89	0.78 1.14 1.67 1.54 1.18 1.23 1.19	1.25	1.69	Change in staining intensity of between 50% and 100%	$^{18}O/^{16}O$ ratio does not reflect spot intensity ratio, as differences in the spot intensities are not large enough	4
5	thimet oligopeptidase	gi|417293	103	28	1086.56 1103.59 1508.77 1551.79	1.12 1.10 1.12 0.99	1.08	2.30	Staining intensity more than doubled	$^{18}O/^{16}O$ ratio does not reflect spot intensity ratio, no reason evident	3
6	dnaK-type molecular chaperone hsp72-ps1	gi|347019	255	39	1199.67 1253.61 1487.7 1659.89 1691.73	1.16 0.96 1.04 1.01 0.73	0.98	1.02	Same staining intensity in both samples	$^{18}O/^{16}O$ ratio agrees with spot intensity ratio	25

intensity ratio was 2.12. The $^{18}O/^{16}O$-ratio was 1.93, confirming that the expression level of the *heat shock protein 8* was increased.

The $^{18}O/^{16}O$-ratios obtained by MALDI-MS for selected spots whose intensities changed and which are representative of a specific type of result are presented in Table 1. The staining intensity ratio of spot No. 1 was 2.31, indicating that abundance of protein in this spot was more than twice as high in one experimental group as in the other. The mean $^{18}O/^{16}O$-ratio was 2.34, demonstrating that it was in fact the identified protein (*formyltetrahydrofolate dehydrogenase*) whose expression was more than doubled. We were able to identify specific proteins with increased expression in a further 52 spots.

To determine a potential labeling bias, crossover experiments were performed. Twenty corresponding spots from other gels from the same biological samples were excised and spots with the lower intensity were now labeled with ^{18}O and those with higher intensity were labeled with ^{16}O, yielding $^{18}O/^{16}O$-ratios ≤ 1. For all 20 proteins, the increase in expression found after labeling the lower intensity spots with ^{16}O was confirmed (Table 1, spot No. 2). These results show that the way labels are combined has no impact on the results.

Spot No. 3 showed an intensity ratio of 2.23, but an $^{18}O/^{16}O$-ratio of only 0.88. Inspection of the gel scans after spot-picking revealed that the spot which was later labeled with ^{18}O was not completely excised, leaving part of the stained area in the gel ("half moon"). We observed an error of this type five times among the first 25 spots analyzed. We therefore conducted intensive tests to determine whether using different numbers of technical replicates or digesting pools from more than one spot would help to avoid this problem. Forty different spots whose intensities had at least doubled between the two experimental groups were chosen. From the first group of 10 spots, only one pair of spots was excised from the gels of the two experiments. Its $^{18}O/^{16}O$-ratio was analyzed by MS. From the second group of 10 spots, two pairs of spots were excised, separately labeled and analyzed in duplicate. From the third group of 10 spots, three pairs of spots were excised, separately labeled and analyzed in triplicate. From the fourth group of 10 spots, two pairs of spots were excised, pooled and analyzed. The $^{18}O/^{16}O$-ratios from the first group correctly indicated an increase in spot intensity in 8 out of the 10 samples. The $^{18}O/^{16}O$-ratios of the other three groups correctly indicated increased spot intensities in all 10 samples. As using more technical replicates instead of a pool of two samples did not improve the quality of the results, we chose the less labor-intensive variant for all further experiments, that is, two corresponding spots from two gels each were always pooled and compared with two pooled spots from the second experimental group. Thereafter, an erroneous $^{18}O/^{16}O$-ratio due to incomplete excision of a spot as demonstrated for spot No. 3 (Table 1) no longer occurred.

Another source of error was demonstrated for spot No. 4. The spot intensity ratio was 1.69, whereas the $^{18}O/^{16}O$-ratio was only 1.25, incorrectly indicating that there was no statistically different change in protein level. It is therefore advisable to choose only those spots from two experimental groups whose staining intensity ratio is high enough to prevent an overlap with the upper limit of the range of spots with unchanged spot intensities (1.37), for example, $^{18}O/^{16}O$-ratios larger than 2.0.

Three incorrect $^{18}O/^{16}O$-ratios occurred for which we found no explanation (Table 1, spot No. 5). The error rate of our methodological approach is therefore 3.9% (3 out of 76). In those three cases, *all* labeled $^{18}O/^{16}O$-peptide ratios deviated

Table 2. LC-ESI-MS/MS Analyses: Validation of Differences in Spot Intensity between Two Experimental Groups by the $^{18}O/^{16}O$-Ratios (Representative Examples)

spot no.	name of protein	accession number	protein score	no. of peptides[a]	mean ratio $^{18}O/^{16}O$	ratio spot intensity (gel)	result
7	NADH dehydrogenase (ubiquinone) Fe-S protein 1	gi|53850628	2020	27	**1.94**	**1.77**	**Two proteins identified. For only one protein the $^{18}O/^{16}O$ ratio is increased.**
	Ngef protein	gi|60688157	174	2	**0.32**		
8	isocitrate dehydrogenase 3 (NAD+) alpha	gi|16758446	1120	10	**0.66**	**0.55**	**Two proteins identified. For only one protein the $^{18}O/^{16}O$ ratio is reduced.**
	protein phosphatase 1 delta	gi|227436	148	2	**0.81**		
9	sorting and assembly machinery component 50 homolog	gi|51948454	1297	10	**1.94**	**1.83**	**Eleven proteins identified. The $^{18}O/^{16}O$ ratio is increased for five and decreased for three proteins.**
	seryl-aminoacyl-tRNA synthetase 2	gi|157819737	808	7	**2.49**		
	rCG55098	gi|149045822	735	6	**5.98**		
	N-ethylmaleimide sensitive fusion protein	gi|13489067	496	5	**0.27**		
	synapsin IIb	gi|112350	471	2	**2.13**		
	eukaryotic translation initiation factor 2B, subunit 3 gamma	gi|48675860	444	3	**0.33**		
	calcium/calmodulin-dependent protein kinase II, alpha	gi|6978593	443	5	**1.24**		
	Chain A,rat liver F1-ATPase	gi|6729934	397	3	**0.99**		
	dihydrolipoamide dehydrogenase	gi|40786469	277	3	**1.63**		
	glutamate dehydrogenase 1	gi|6980956	228	3	**0.97**		
	fascin	gi|497775	201	2	**0.76**		

[a] Peptides with MASCOT ion-score indicating homology or identity.

from the expected result. We did not observe erroneous results due to widely varying $^{18}O/^{16}O$-ratios of the different peptides of one protein. The errors are therefore more likely to be due to mistakes occurring during the sample preparation procedures than to erroneous MS analyses. On the other hand, the $^{18}O/^{16}O$-ratios of all three incorrectly classified spots were too low. This may be an indication of ^{18}O back exchange (for further discussion of this problem, see refs 15, 16, and 30). Apart from the results presented in Table 1, we observed decreased $^{18}O/^{16}O$-ratios for almost all spots obtained in two further experiments. Inspection of the methodological details revealed that the samples of only these two experiments had been stored in a 4 °C refrigerator for more than 2 weeks during the period between digestion and MS analysis. This may indicate that longer storage times between digestion and MS analyses lead to a reduction in the $^{18}O/^{16}O$-ratios due to ^{18}O back exchange. All MS analyses were therefore performed not more than 48 h after digestion and the samples were never stored at 4 °C. In the event that an $^{18}O/^{16}O$-ratio does not match the spot staining intensity ratio, we suggest repeating the experiment with corresponding spots excised from other gels from the same experimental groups. As it is highly unlikely that an incorrect $^{18}O/^{16}O$-ratio will occur in two repeated analyses, a repeated run may either yield the expected result or the expression level of the identified protein most likely remained unchanged. LC-MS analysis should then be performed in order to evaluate whether the expression level of a second protein not identified by MALDI-MS may have changed.

Spot No. 6 is an example of correct $^{18}O/^{16}O$-ratios obtained from the comparison of spots with the same staining intensities. Altogether, we obtained correct $^{18}O/^{16}O$-ratios for 25 same-intensity spots. Two spots (7.4%) with the same staining intensities in both experimental groups were incorrectly classified as "increased" ($^{18}O/^{16}O$-ratio = 1.91) and "decreased" ($^{18}O/^{16}O$-ratio = 0.66), respectively. These results confirm that the "error rate" of our methodological approach is below 10%.

We were unable to identify 17 out of 114 excised spots with MALDI-MS. Most of these were spots yielding very low staining intensities. In individual cases, however, spots with a staining intensity of 0.3 were identified, whereas others with a staining intensity of around 1.5 were not. In our experiments, ruthenium staining intensities vary between 0.02 and 40. Thus, a considerable percentage of spots cannot be identified by MALDI-MS. We therefore performed additional experiments using LC-MS.

Results of the ESI-LC-MS Analyses. Altogether, 21 spot pairs digested in the presence of ^{18}O and ^{16}O, respectively, were analyzed by LC-MS. In seven spots, only one protein was identified, and in the other 14 spots, we found between two and 11 proteins. Eight of the spots analyzed with LC-ESI/MS had been analyzed before by MALDI-MS/MS. For seven of these spots, the MALDI-hit corresponded to the first LC-ESI-hit.

Representative results comparing the spot intensity ratios with the $^{18}O/^{16}O$-ratios are presented in Table 2. Two proteins were identified in spot No. 7. Increased expression of the *NADH dehydrogenase* was expected from the spot intensity ratio (1.77) and was confirmed by an increase in the $^{18}O/^{16}O$-ratio (1.94). The second protein found in this spot, the *Ngef protein*, was identified from only two peptides with a MASCOT score of only 174. In contrast, 27 peptides were identified for *NADH dehydrogenase*, with a protein score of 2020. An $^{18}O/^{16}O$-ratio of 0.32 was calculated for the *Ngef protein*. These results clearly identify *NADH dehydrogenase* as the protein with increased expression

level detected in spot No. 7. Whether the expression of the *Ngef protein* was indeed reduced or whether the ratio of 0.32 reflects an artifact caused by a slight between-gel deviation of the position of this protein remains unclear. Repetition of the experiments with corresponding spots from other gels may help to clarify this issue.

Two proteins were identified from spot No. 8. A decreased expression level of *isocitrate dehydrogenase 3* was confirmed by a decrease in the $^{18}O/^{16}O$-ratio of this protein (0.66). The expression of the second protein, the *protein phosphatase 1 delta*, indicates that no statistically significant change took place ($^{18}O/^{16}O$-ratio = 0.81).

Eleven proteins were identified from spot No. 9. The $^{18}O/^{16}O$-ratio of the dominant (first MALDI-hit) protein *sorting and assembly machinery component 50 homolog* was 1.94, which was highly consistent with the spot staining intensity ratio of 1.83. However, according to our criteria, four further proteins had increased and three proteins decreased $^{18}O/^{16}O$-ratios. Therefore, postmortem effects may have altered the expression levels of up to eight out of 11 proteins. This does not seem too unlikely, as we found that more than 40% of all the spot intensities were significantly altered, sometimes several fold, after the rat bodies had been stored at 4 °C for 24 h prior to dissection of the brain areas (Baumgartner et al., in preparation). However, the altered $^{18}O/^{16}O$-ratios of these additional 7 proteins may also reflect an artifact caused by between-gel deviations of the positions of these proteins, whose borders could not be visually differentiated from each other. Likewise, we cannot entirely exclude the possibility that the altered $^{18}O/^{16}O$-ratios of even the *sorting and assembly machinery component 50 homolog* protein may reflect an artifact caused by between-gel deviations of the positions of this protein. In this case, the expression of one of the proteins with the second or third highest protein scores (e.g., *seryl-aminoacethyl-tRNA synthetase 2* or *rCG55098*) had increased. It would therefore only seem possible to identify spots with altered intensity accurately if the analysis is repeated with further pairs of the same spot excised from other gels from the same experimental groups. However, we feel that such a labor-intensive procedure seems justified only if the correct identification of quantitatively altered proteins is crucial, as, for example, in experiments designed to identify marker proteins for diseases or for the development of new drugs.

Conclusion

We have presented a method for the identification and relative quantification of proteins with altered expression levels excised from 2-DE gel spots by digesting corresponding protein spots from two experimental groups in the presence of ^{16}O and ^{18}O, respectively. The application of the $^{18}O/^{16}O$ labeling technique described here is easy to perform, relatively cheap and yields a more reliable quantification of altered proteins than results based on conventional 2-DE spot intensities alone. In sum, the additional ^{18}O labeling should preferably be applied in 2-DE based differential proteomic experiments which require correct identification of quantitatively altered proteins.

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