Identification of the Hepatic Protein Targets of Reactive Metabolites of Acetaminophen in Vivo in Mice Using Two-dimensional Gel Electrophoresis and Mass Spectrometry*

(Yongchang Qiu, Leslie Z. Benet, and Alma L. Burlingame)

From the Departments of Pharmaceutical Chemistry and Biopharmaceutical Sciences and the Liver Center, University of California, San Francisco, California 94143-0446

Liver toxicity following an overdose of acetaminophen is frequently considered a model for drug-induced hepatotoxicity. Extensive studies over many years have established that such toxicity is well correlated with liver protein arylation by acetaminophen metabolites. Identification of protein targets for covalent modifications is a challenging but necessary step in understanding how covalent binding could lead to liver toxicity. Previous approaches suffered from technical limitations, and thus over the last 10 years heroic efforts were required to determine the identity of only a few target proteins. We present a new mass spectrometry-based strategy for identification of all target proteins that now provides a comprehensive survey of the suite of liver proteins modified. After administration of radiolabeled acetaminophen to mice, the proteins in the liver tissue lysate were separated by two-dimensional polyacrylamide gel electrophoresis. In-gel digestion of the radiolabeled gel spots gave a set of tryptic peptides, which were analyzed by matrix-assisted laser desorption ionization mass spectrometry. Interrogation of data bases based on experimentally determined molecular weights of peptides and product ion tags from postsource decay mass spectra was employed for the determination of the identities of modified liver proteins. Using this method, more than 20 new drug-labeled proteins have been identified.

Acetaminophen is a widely used over-the-counter analgesic and antipyretic. It is therapeutically safe and has a high therapeutic index. It is commonly used as a substitute for aspirin because of its lower incidence of side effects. However, an overdose of acetaminophen may cause acute, often fatal, centrilobular liver necrosis in both humans and animals. For overdosage of acetaminophen may cause acute, often fatal, centrilobular liver necrosis in both humans and animals (1). For overdosage of acetaminophen may cause acute, often fatal, centrilobular liver necrosis in both humans and animals (1). For liver toxicity. Extensive studies over many years have established that such toxicity is well correlated with liver protein arylation by acetaminophen metabolites. Identification of protein targets for covalent modifications is a challenging but necessary step in understanding how covalent binding could lead to liver toxicity. Previous approaches suffered from technical limitations, and thus over the last 10 years heroic efforts were required to determine the identity of only a few target proteins. We present a new mass spectrometry-based strategy for identification of all target proteins that now provides a comprehensive survey of the suite of liver proteins modified. After administration of radiolabeled acetaminophen to mice, the proteins in the liver tissue lysate were separated by two-dimensional polyacrylamide gel electrophoresis. In-gel digestion of the radiolabeled gel spots gave a set of tryptic peptides, which were analyzed by matrix-assisted laser desorption ionization mass spectrometry. Interrogation of data bases based on experimentally determined molecular weights of peptides and product ion tags from postsource decay mass spectra was employed for the determination of the identities of modified liver proteins. Using this method, more than 20 new drug-labeled proteins have been identified.

1 The abbreviations used are: NAPQI, N-acetyl-p-benzoquinone imine; PAGE, polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption ionization; HPLC, high pressure liquid chromatography; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; MS, mass spectrometry; PSD, postsource decay; EST, expressed sequence tag; GST, glutathione S-transferase.

* This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the University of California System-wide Campus Laboratory Collaborations Program (J. Vogel, principal investigator), National Institutes of Health Grants NCRR BRTP RR 01614 (to A. L. B.) and GMS 36636 (to L. Z. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Hepatic Protein Targets of Acetaminophen

that further experiments aimed at elucidation of the biochemical basis of compromised cell homeostasis may proceed.

In this contribution, we wish to report the identities of all major hepatic proteins covalently modified after administration of a toxic dose of acetaminophen in the mouse. Using a combination of techniques including two-dimensional SDS-PAGE, fluorography, and matrix-assisted laser desorption ionization (MALDI) mass spectrometry, we have identified 23 hepatic proteins. Having a knowledge of the entire suite of targets should yield new insights into the role of covalent drug binding in the pathogenesis of toxic doses of acetaminophen.

EXPERIMENTAL PROCEDURES

Materials

Acetaminophen was purchased from Aldrich. [ring-U-14C]Acetaminophen (6.3 mCi/mmol) was obtained from Sigma. The radiochemical purity of [ring-U-14C]acetaminophen was greater than 96%, as determined by HPLC and 'H NMR on a 300-MHz GE instrument. Electrophoresis reagents were obtained either from Sigma or Amer sham Pharmacia Biotech. HPLC grade solvents were obtained from Fisher.

Animals

B6C3F1 mice were obtained from Simonsen (Gilroy, CA). Prior to the administration of acetaminophen, mice were given phenobarbital as a 0.1% solution in their drinking water for 5 days, and food was withheld from all mice for 15 h. Both radiolabeled and nonlabeled acetaminophen were given as aqueous solutions (20 mg/ml) at doses of 350 mg/kg. The mice were killed by cervical dislocation 2 h after dosing. Gallbladders were removed before livers were subjected to the following procedures.

Sample Preparation

Livers were weighed and homogenized with a Duall glass type homogenizer (Kontes Glass Co., Vineland, NJ) in 8 x liver weight (g) ml of a solubilizing solution of 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithiothreitol, and pharmalyte, pH 3–10 (0.36 meq/ml, 1:50 by volume). Thiourea (2 M) was added to the homogenizing buffer to increase the solubility of more hydrophobic proteins (25). The homogenate was centrifuged at either 10,000 or 100,000 x g for 30 min. The supernatant protein was removed and stored at 80 °C as 1-ml aliquots in 1.5-ml microcentrifuge tubes. The protein concentration was determined by 10% light nongradient gel made with 30.8% Duracryl™ solution (Chelmsford, MA). The stacking gel contained 4% acrylamide/bis. The gels were run at 50 mA and 300 V, 1 h; 300–1000 V, 1 h; 1000–3500 V, 1 h; 3500 V, 15 h, using an EPS 3500 XL (Amersham Pharmacia Biotech) electrophoresis power supply. After a standard SDS equilibrium step, proteins were further separated by SDS-PAGE as described by Anderson (Ref. 28). The separating gel was 11% light nongradient gel made with 30.8% Duracryl™ solution (Chelmsford, MA). The stacking gel contained 4% acrylamide/bis. The gels were run at 50 mA and 1 h then and stained for 15 h in 0.1% Coomassie Blue R-250, 45% methanol, 10% acetic acid, 45% water. Gels were destained with 45% methanol, 10% acetic acid, 45% water. Spots containing radioactivity were detected by autoradiography as described in the operations manual (28) with 8 x 10-inch Kodak X-Omat XAR-2 film.

In-gel Digestion

The procedure of Rosenfeld et al. (29), with slight modifications, was used to produce in-gel tryptic digest of all spots of interest.

HPLC and Desalting

HPLC separation was performed according to Hall et al. (30). The salt content was removed by the following procedure. Briefly, samples were injected on a SpeedVac and redissolved in 10 µl of 1% acetic acid, 1% trifluoroacetic acid, water. Using a 10-µl syringe, the reconstituted peptide solution was injected onto a LC Packings µ-guard column (300 µm inner diameter x 1 mm; 5-µm particle size; 300-Å, pore size) at a rate of more than 5 µl/min. After washing off the salt with 3 x 10 µl of 1% acetonitrile, 1% trifluoroacetic acid, water at a rate of no more than 5 µl/min, the peptides were eluted by injecting 10, 30, 50, and 90% acetonitrile in 1% trifluoroacetic acid, water (10 µl each in 1 min), consecutively. Eluents were collected, dried, and finally reconstituted in 10 µl of 2% trifluoroacetic acid, 50% acetonitrile, water.

Mass Spectrometry

Molecular Weight Measurements on Unseparated Tryptic Digests—Molar weights of all peptides were determined by analyzing one-twentieth of the unseparated tryptic digesta employing a matrix-assisted laser desorption (MALDI) delayed extraction reflectron time-of-flight instrument (PerSeptive Biosystems, Voyager Elite mass spectrometer, Framingham, MA) equipped with a nitrogen laser (337 mm), which has a typical mass resolution, M/ΔM, of 6000 (FWHM). Peptides were crystallized in a saturated solution of α-cyano-4-hydroxy-cinnamic acid prepared in 0.1% trifluoroacetic acid, 60% acetonitrile, 50% water. The monoisotopic (31) masses from all spectra recorded for a particular peptide are reported in this work. All MALDI spectra were externally calibrated by using a standard mixture of known peptides.

Mass Spectrometric Determination of Partial Peptide Sequence—After inspection of the MALDI mass spectra of the unseparated mixture of peptides from the spot digest, those components displaying the highest pseudomolecular ion abundance were selected for partial amino acid sequence determination. This was carried out by taking advantage of the inherent metastable fragmentation induced by deprotonation of excess internal energy during the laser desorption process by recording so-called postsource decay (PSD) mass spectra (32). Peptides with similar molecular weights were separated by microwave HPLC before carrying out pseudomolecular ion gating and PSD analysis. Most samples were subjected to a desalting step as described above to obtain higher quality mass spectra (improved signal versus noise ratio can be achieved after such a clean-up step) that facilitate PSD experiments.

Data Base Searching

MS-Fit and MS-Tag were used to perform data base searching. Both programs were developed by Clauser et al. in our group (University of California San Francisco Mass Spectrometry Facility) (33). MS-Fit is a typical peptide mass fingerprinting program, which compares the experimentally determined masses of tryptic peptides with the theoretical masses of all tryptic peptides that can be calculated from sequences of all proteins in the genomic data bases. All of the proteins (data base entries) matching the input data/parameters were listed in a simple ranking system in which data base entries with the least number of unmatched masses are ranked higher. MS-Fit is a sequence data base searching tool used to match fragment ion tag data contained in a user's tandem mass spectrum to a peptide sequence in an existing data base. Ideally, one would prefer to obtain a tandem mass spectrum with enough fragment ions from which a complete peptide sequence can be determined. In practice, samples such as peptides from in-gel digestion of a two-dimensional PAGE gel spot usually only yield a limited number of fragment ions by PSD, which prevents manual interpretation of these tandem mass spectra. However, each fragment ion is characteristic of the sequence of the peptide under analysis and adds a constraint to data base searching. Therefore, all ions present provide very high discriminating power for searching genomic data bases. MS-Tag integrates all of these constraints by considering each fragment ion and parent mass independently to match a single peptide sequence in a genomic

2 Available on the World Wide Web at http://rafael.ucsf.edu.
data base, so the identity of the protein on a gel spot of interest is also
determined. A variety of fragment ion types, as well as immonium ions
and internal sequence ions, can be used by the MS-Tag algorithm. This
not only facilitates the identification of peptides that are identical to
sequences in the data base but also enables homology-tolerant search-
ing to allow for a single mutation, cross-species substitution, sequence
polymorphism, modified amino acid, or data base error. The NCBInr
protein data base was searched first, and if no match was found, the
dbEST DNA data base was further searched to determine the identity
of the target protein or a protein homologous to the target protein.

RESULTS

Two-dimensional Electrophoresis and Autoradiography

Sample application and rehydration of Immobiline DryStrips
were combined in one step to ease the operation and, more
importantly, to increase loading capacity without horizontal
streaking and protein precipitation at the sample application
point (27). Gels prepared from a single batch were found to be
virtually identical, thus readily permitting inter-gel spot cor-
relation. An example of a two-dimensional preparative gel of a
whole liver homogenate stained with Coomassie Blue is de-
picted in Fig. 1A. This homogenate was derived from the ex-
cised whole liver of a phenobarbital-induced B6C3F1 mouse
after treatment with a toxic dose of 14C-labeled acetaminophen.
This gel was then exposed to film for 2 weeks, and the resulting
autoradiogram is shown in Fig. 1B. Twenty-seven major 14C-
containing spots were revealed. Spot-to-spot comparison al-
lowed in-gel digestions to be performed on analogous gels with-
out radioactivity but after administration of the same amount
of drug.
Acetaminophen employing two-dimensional PAGE, MALDI-MS. In the lower mass region are abundant. Internal matched. theoretical masses of tryptic peptides from the protein remaining peptide masses measured by MALDI-MS match the identification can be verified by checking how many of the additional peptides were obtained for one gel spot. Finally, the unambiguous identification, often PSD spectra of several MS-Tag searching based on one PSD spectrum is usually specified. Data base searching with MS-Tag uses all of this information together with the mass of the peptide to match the theoretical counterparts calculated from all protein sequences. To circumvent these known difficulties, partial peptide sequence information was obtained based on recording PSD mass spectra, and MS-Tag was used for data base interrogation using the sequence and composition information obtained. A typical PSD spectrum does not provide complete sequence ions (a, b, c, x, y, and z ions) of a particular peptide, although a, b, and y ions in the lower mass region are abundant. Internal ions with lower masses (<600 Da) and immonium ions are also evident. Data base searching with MS-Tag uses all of this information together with the mass of the peptide to match the theoretical counterparts calculated from all protein sequences in the data base and to determine the identity of the protein. MS-Tag searching based on one PSD spectrum is usually specific enough to identify a unique protein if the protein is in the data base. Therefore, multiple proteins in mixtures may be identified with confidence. To further increase the confidence of an unambiguous identification, often PSD spectra of several additional peptides were obtained for one gel spot. Finally, the identification can be verified by checking how many of the remaining peptide masses measured by MALDI-MS match the theoretical masses of tryptic peptides from the protein matched.

Table I summarizes peptide mass data, sequences determined or attributed by mass, and data base searching results for all protein spots studied. Peptide masses not analyzed by PSD were attributed to a unique sequence by matching the mass to a tryptic peptide from the matched protein (masses of peptides from nonspecific cleavages are not considered). Since the peptide mass measurements were externally calibrated, the mass differences between experimental and theoretical values are different for peptides from different proteins. However, they are very similar for peptides from the same protein (spot) and proportional to the masses of peptides.

Identification of Target Proteins

Proteins Not in the Current Protein Data Bases—In the MALDI spectrum of in-gel digest from spot 1, only one peak at m/z 992.37 gave a sufficiently strong signal for PSD analysis (Fig. 3). However, the PSD mass spectrum of this component was one of the few that provided sufficient sequence fragment ions for manual interpretation. All of the y sequence ion series, except y6, are present in this spectrum. Based on this information, the sequence of this peptide was established as EPFPFFPVR. Using only the molecular weight values from the unseparated digest, MS-Fit was employed to interrogate the NCBI protein data base. No matching entry was found. Then MS-Tag was employed using the peptide sequence deduced above, which also gave a negative result. Finally, MS-Tag was employed again to interrogate the EST DNA data base (36), and that provided a match to one unique sequence from a Life Technologies, Inc., mouse embryo cDNA clone (accession number dbEST: 2049563). In addition, three of the other peptide mass values obtained can be attributed to sequences derived from translation of this partial cDNA clone as shown in Table I. Once this identification was obtained, the data bases were once again interrogated for the existence of homologous protein entries, and none were found in the current protein data bases.

A similar situation was encountered in analyzing the protein in spot 5. Interpretation of the PSD mass spectrum obtained from a peptide in the digest having a mass value of m/z 1464.41 provided the sequence of this component. This sequence was used for interrogation of the EST data base revealing a match to a mouse cDNA clone (accession number 1875803) that is homologous to rat aryl sulfotransferase. In this case, 15 additional peptide mass values in the spot digest can be attributed to sequences of tryptic peptides from this putative mouse aryl sulfotransferase.

Thirteen mass values observed from spot 1 could not be assigned to any theoretical tryptic peptide belonging to this EST-derived protein sequence. This situation may be due to the fact that this EST is only a segment of the complete sequence from a cDNA clone that corresponds to an mRNA (36). The modified mouse protein observed in spot 1 has a molecular mass of approximately 35 kDa, more than twice the molecular mass of the translated EST (12107.3 Da) match from the data base. For this reason, the calculated peptide sequence coverage for both of these proteins is higher than others in Table I (46% for spot 1 and 72% for spot 5) because these percentages could not be calculated against the complete protein sequences.

Proteins in the Current Protein Data Bases—Characterization of spots 2 and 8 revealed the presence of two different forms of glutathione peroxidases. Data base searching based on the molecular weights of tryptic peptides observed in these spot digests could not distinguish between them. However, one component in the digest of spot 2 had a molecular weight of m/z 1807.51 that did not correspond to an anticipated tryptic component but easily could be distinguished and identified by the unique stable isotopic pattern observed for the pseudomolecu-
| Spot No. | MALDI mass | Δν | Peptide sequence determined by PSD and consistent with mass | Protein identified (NCBI accession no.; percentage of the protein covered molecular mass) Subcellular location |
|----------|------------|----|----------------------------------------------------------|---------------------------------------------------------------------------------------------------------|
| 1        | 992.37     | −0.15 | K(EPPFTPFPVR(G) | Life Technologies mouse embryo 8 Unknown |
|          | 791.29     | −0.09 | R(GiCimGQRTR(P) | |
|          | 1681.80    | −0.19 | R(LAEVGVPLVPNK(K) | |
|          | 2292.79    | −0.31 | K(GLTDNFDVQSVDC(D) | Da |
|          | 932.32     | 1313.44 | 817.30, 1114.48, 1128.44, 1153.41, 1327.44, 1371.54, 1393.42, 1593.71, 2221.82, 2343.95, 2586.05 |
| 2        | 1101.38    | −0.24 | K(ADJPELTFLR(N) | Glutathione peroxidase (984747); 28%; 22,292.5 Da Cytoplasmic |
|          | 1382.42    | −0.34 | R(PLTEGEQPSLSR(G) | |
|          | 1542.43    | −0.36 | R(LASAADSQTVYAPFASRP) | |
|          | 1667.60    | −0.37 | K(VLTLTVASL(GTTTR(D) | |
|          | 1957.57    | −0.41 | K(VYRPPGGFEPNFLFEK(C) | |
|          | 1807.51    | −0.42 | K(VLTLTVASL(GTTTR(D) | |
|          | 1155.39    | −0.28 | K(FVLDGPQVPR(Y) | |
|          | 1311.46    | −0.29 | K(FVLDGPQVPR(Y) | |
| 3        | 1206.43    | −0.23 | K(HLXVYDLPVGR(S) | Housekeeping protein (126896); 15%; 28,127.2 Da Mitochondrial |
|          | 1476.33    | −0.48 | K(DYVLESAIGR(G) | |
|          | 1943.89    | −0.17 | K(pyro-GluISRDYGLLESAIGR(G) | |
|          | 1906.75    | 0.33 | K(QISRDYGLLESAIGR(G) | |
|          | 2458.90    | −0.48 | R(GFLFIDPNKVKEVSXNLPLFVGR(S) | |
|          | 1794.55    | 2720.72 | 2748.33 |
| 4        | 1858.39    | +0.23 | R(LAGLKLPGHNGTTLVTLR(F) | Thioether S-methyltransferase Cytoplasmic or microsomal |
|          | 1106.71    | +0.16 | K(FQHMYQPK(K) | |
|          | 1122.67    | +0.13 | K(FQHGYmet-oxVPKPK) | |
|          | 2032.30    | +0.24 | R(EIVTTYDPQNLQELQK(W) | |
|          | 2447.48    | +0.30 | K(IYLYTTPSFSFGPVAEIVKF( | |
|          | 1262.88    | 2208.24 | 2210.83 |
| 5        | 1446.41    | −0.22 | K(EEMDHSYSPFMKR(K) | Mouse NML M. musculus cDNA Cytoplasmic |
|          | 891.44     | −0.01 | K(ISWQWRK(R) | |
|          | 929.35     | −0.08 | K(FEEHYVK(K) | |
|          | 1070.57    | −0.00 | K(RIDPELNLK) | |
|          | 1227.59    | −0.01 | K(NQFTVAPYKV(F) | |
|          | 1311.64    | −0.04 | K(IYLYHSSFSVMKE) | |
|          | 1327.66    | −0.01 | K(IYLYHSSFSVMKE) | |
|          | 1334.60    | −0.01 | K(IYLYFEDIMK(E) | |
|          | 1418.16    | −0.43 | K(ENPSANYTMTmet-oxMTK(E) | |
|          | 1480.41    | −0.21 | K(EEMDHSYSPFMKR(K)-1Met-ox | |
|          | 1802.85    | −0.05 | R(LFYMAYDMKENPK(C) | |
|          | 1985.86    | −0.02 | K(FMAGQVSFGWPDVK(S) | |
|          | 1984.85    | −0.02 | K(FMet-oxAQGVSFGWPDVK(S) | |
|          | 2137.95    | −0.06 | K(NQFTVAPYKVFEEDYVK(K) | |
|          | 2266.05    | −0.05 | K(NQFTVAPYKVFEEDYVK(K) | |
|          | 2832.13    | −0.00 | K(ENPSANYTMTMKEEMDHSYSPFMKR(K) | |
|          | 734.45     | 1652.89 | 1711.80, 1725.73, 1775.80, 2325.15 |
| 6        | 1067.21    | −0.34 | R(AAPFTLEYR(V) | Homologous to bovine inorganic |
|          | 1327.23    | −0.45 | K(DPVHMWVPEVR(W) | pyrophosphatase (585322); 18%; 92,644.4 Da |
|          | 1694.33    | −0.56 | R(LKPGLETVDWFR(R) | |
|          | 1114.24    | −0.35 | K(YVANPVFK) | |
|          | 1849.31    | −1.68 | R(LKLPYLETVDWFR(R) | |
|          | 1938.27    | −0.65 | K(VPDPKPEHFAFNAEFD(D) | |
|          | 2229.37    | −0.71 | R(KVYKPDGPNPJEAFNAEFD(D) | |
|          | 940.07     | 1281.10 | 1288.27, 2431.38 |
| 7        | 1156.41    | −0.25 | K(LVIEGDLERT) | Tropomyosin 5, cytoskeletal type Cytoskeletal |
|          | 1243.39    | −0.26 | K(LQVEEELDA) | (156907); 27%; 29,220.8 Da |
|          | 1284.48    | −0.27 | K(LIVLIEGDLERT) | |
|          | 1624.47    | −0.33 | K(IQVYQQADDQADIE(A) | |
|          | 894.29     | −0.18 | K(YYEEVAR(K) or (K)YYEEVAR(L) | |
|          | 940.25     | −0.24 | K(HIAEADKR) | |
|          | 1316.37    | −0.27 | R(QAAEAEVEASLNR) | |
|          | 1399.41    | −0.34 | R(QIQLVEEELDRA) | |
|          | 1472.37    | −0.51 | R(QAAEAEVEASLNR(I) | |
|          | 1543.43    | −0.35 | R(QAAEAEVEASLNR) | |
|          | 1770.53    | −0.37 | R(QIQLVEEELDRA) | |
|          | 1998.54    | −0.44 | K(IQVYQQADDQADIEER(L) | |
| 8        | 1101.45    | −0.17 | K(AHPLFTFLR(N) | Glutathione peroxidase (121666); 29%; 22,292.5 Da Cytoplasmic |
|          | 1193.40    | −0.23 | K(IVYWSVCMGRN) | |
|          | 1311.56    | −0.19 | K(FVLDGPQVPR(Y) | |
|          | 1957.71    | −0.27 | K(VYRPGRGGFEPNFLFEK(C) | |
### Hepatic Protein Targets of Acetaminophen

| Spot No. | MALDI mass | Δm | Peptide sequence determined by PSD and consistent with mass<sup>a</sup> | Protein identified (NCBI accession no.; percentage of the protein covered molecular mass) | Subcellular location |
|----------|------------|----|---------------------------------------------------------------|-----------------------------------------------------------------------------------|---------------------|
| 9        | 1149.35    | -0.23 | IPYVWDQRAH                                                   | Selenium-binding liver protein                                                   | Cytoplasmic         |
|          | 1330.49    | -0.29 | LQPGFLLGGSIVRG                                               |                                                    |                     |
|          | 1454.28    | -0.46 | RIEIVLYCIMYR                                               | Selenium-binding liver protein                                                   |                     |
|          | 1689.46    | -0.34 | KDGFNPAVEAGLYGR                                              |                                                    |                     |
|          | 1034.32    | -0.21 | KIPyro-GluFYDPILR                                            | Acetaminophen-binding protein                                                    |                     |
|          | 1051.34    | -0.22 | EQFYDPILR                                                   |                                                    |                     |
|          | 1068.46    | -0.22 | LLLPGISSR                                                   |                                                    |                     |
|          | 1214.38    | -0.25 | ISQYQSVHH                                                   |                                                    |                     |
|          | 1233.39    | -0.26 | YYVDGSEP                                                   |                                                    |                     |
|          | 1345.40    | -0.29 | QYDINQPQKR                                                  |                                                    |                     |
|          | 1687.50    | -0.33 | FLYPSNWLGDIB                                               |                                                    |                     |
|          | 1709.58    | -0.36 | RIGPGGQMILQSLDGRK                                           |                                                    |                     |
|          | 1871.49    | -0.50 | KNAEGTWSVEKVYPSK                                           |                                                    |                     |
|          | 2219.77    | -0.44 | RHEIQTLMTDGILPIRF                                           |                                                    |                     |
|          | 2235.81    | -0.29 | RHEIQTLMet-oxTDGILPIRF                                       |                                                    |                     |
|          | 2471.67    | -0.44 | GTWKEPKGDPADAPMet-oxGYDFWYQQPR                              |                                                    |                     |
|          | 2487.71    | -0.39 | KGTWKEPSKDAAPMet-oxGYDFWYQQPR                               |                                                    |                     |
|          | 1302.45    | 149.38 | 2172.80, 2404.73, 2546.89                                    |                                                    |                     |
| 10       | 1360.33    | -0.47 | LITGQFLLGGSIVRG                                             | Acetaminophen-binding protein                                                    | Cytoplasmic         |
|          | 1689.26    | -0.54 | KDGFNPAVEAGLYGR                                              |                                                    |                     |
|          | 1034.21    | -0.32 | KIPyro-GluFYDPILR                                            | Selenium-binding liver protein                                                   |                     |
|          | 1051.22    | -0.34 | EQFYDPILR                                                   |                                                    |                     |
|          | 1086.28    | -0.35 | KILPGILMSR                                                  |                                                    |                     |
|          | 1149.22    | -0.36 | RIFWWDQQR                                                  |                                                    |                     |
|          | 1214.23    | -0.40 | ISPQYQSVHH                                                  |                                                    |                     |
|          | 1233.23    | -0.42 | YYVDGSEP                                                   |                                                    |                     |
|          | 1320.30    | -0.42 | KEPGLPALHELRY                                              |                                                    |                     |
|          | 1328.24    | -0.42 | Rpyro-GluYDISNPQKR                                           |                                                    |                     |
|          | 1345.26    | -0.43 | RQYDINQPQKR                                                |                                                    |                     |
|          | 1454.09    | -0.65 | RIEIVLYCIMYR                                               |                                                    |                     |
|          | 1465.08    | -0.67 | RIEIVLYCIMYR                                               |                                                    |                     |
|          | 1687.30    | -0.53 | FLYPSNWLGDIB                                               |                                                    |                     |
|          | 1709.38    | -0.56 | RIGPGGQMILQSLDGRK                                           |                                                    |                     |
|          | 1725.40    | -0.53 | RIGPGGQMet-oxQLSLDGRK(L)                                    |                                                    |                     |
|          | 1829.26    | -0.64 | RHNVMVSTWWAPANVFKE                                          |                                                    |                     |
|          | 2192.41    | -0.64 | (-)MATKCamTKCamGPGSTPLEAMK(G)                               |                                                    |                     |
|          | 2219.52    | -0.69 | RHEIQTLMTDGILPIRF                                           |                                                    |                     |
|          | 2235.52    | -0.68 | RHEIQTLMet-oxTDGILPIRF                                       |                                                    |                     |
|          | 2418.40    | -0.78 | RFLHDPSATQFVGCAMALSSNNQRF                                  |                                                    |                     |
|          | 2429.52    | -0.78 | KGTWKEPSKGAPEMGYDFWYQQPR                                    |                                                    |                     |
|          | 2445.42    | -0.67 | KGTWKEPSKGAPEMGYDFWYQQPR                                    |                                                    |                     |
|          | 2546.57    | -0.80 | KLNPLNVLDFKFKEPLPALHELRY                                     |                                                    |                     |
|          | 2303.37    | 149.38 | 2344.49, 2404.49                                          |                                                    |                     |
| 11       | 1959.49    | -0.40 | (-)acetSSITGYSLALSASTFSIDGR(V)                              | Homologous to proteasome subunit                                                  | Cytoplasmic         |
|          | 763.25     | 0.16  | LFNVDRH                                                     |                                                    |                     |
|          | 1380.42    | -0.32 | HVGMAVAGLADAR(S)                                            |                                                    |                     |
|          | 852.25     | 0.14  | EEASFRS(I)                                                  |                                                    |                     |
|          | 1095.33    | 0.21  | KLYEEGSSRK(R)                                               |                                                    |                     |
|          | 1396.46    | 0.28  | RHGVMet-oxAVAGLADAR(S)                                      |                                                    |                     |
|          | 1744.51    | 0.40  | RSNFGYNIPKHLADR(V)                                          |                                                    |                     |
|          | 798.17     | 101.25 | 948.25, 1128.38, 1290.40, 1354.15, 1524.40, 1541.43, 1588.42, 1781.56, 1837.53, 1863.57 |                                                    |                     |
| 12       | 1068.30    | 0.17  | KTGACimYHFGS(R)                                             | Methionine adenosyltransferase                                                    | Cytoplasmic         |
|          | 1295.53    | -0.21 | KNFILFLEPGVVRD(I)                                           |                                                    |                     |
|          | 1354.50    | -0.25 | RSQVLWLPRDSK(T)                                             |                                                    |                     |
|          | 1430.41    | -0.33 | RFVGGPQCDAGTVGRK                                            |                                                    |                     |
|          | 1792.59    | -0.26 | RYLDETHVYLQPSGKR                                            |                                                    |                     |
|          | 1082.30    | -0.18 | KTGACimYHFGS(S)                                             |                                                    |                     |
|          | 1949.64    | -0.31 | KIIIVTVGGQWAGHGGAGSPKGD(K)                                  |                                                    |                     |
|          | 2018.69    | -0.34 | KTVQTVQYMQDNGAIVPRV(I)                                      |                                                    |                     |
|          | 2034.73    | -0.30 | KTVQTVQYMet-oxQDNGAIVPRV(I)                                 |                                                    |                     |
|          | 2685.90    | -0.40 | KTGACimNLVLYALEQQSPDIAQcemVHLDR(N)                          |                                                    |                     |
|          | 1268.80    |        |                                                             |                                                    |                     |
| 13       | 1068.33    | -0.22 | EQFYINVER(E)                                                | Protein synthesis initiation factor 4A                                            | Ribosomal           |
|          | 1827.59    | 0.35  | RGYIAQFPKSPQAQQB(A)                                         |                                                    |                     |
|          | 2143.74    | 0.40  | HQIDYQRQVSLNYDLFTNR(E)                                      |                                                    |                     |
|          | 831.24     | -0.17 | REINHRYR(I)                                                 |                                                    |                     |
|          | 894.28     | -0.17 | RVFDMLNKR(R)                                                |                                                    |                     |
|          | 910.26     | -0.19 | RFVDMet-oxLNKR(R)                                           |                                                    |                     |
|          | 904.29     | -0.20 | KFMGIPv(I)                                                  |                                                    |                     |
|          | 950.26     | -0.23 | KFMet-oxRGIPv(I)                                            |                                                    |                     |
|          | 1051.31    | -0.21 | Rpyro-GluFYINVER(E)                                         |                                                    |                     |
| Spot No. | MALDI mass | $\Delta\nu$ | Peptide sequence determined by PSD and consistent with mass | Protein identified (NCBI accession no.; percentage of the protein covered molecular mass) | Subcellular location |
|---------|------------|-------------|---------------------------------------------------------------|--------------------------------------------------------------------------------|---------------------|
| 1114.48| -0.20      | (R)VLITTDLLAR(G) | Acetaminophen-binding protein                                 | Cytoplasmic                                |
| 1140.45| -0.22      | (K)ATQALVLAPTR(E) |                                                                            |                                  |
| 1157.41| -0.25      | (K)KEELTLEGIR(Q) |                                                                            |                                  |
| 1501.33| -0.44      | (R)GFDPQYDIPFKQ(S) |                                                                            |                                  |
| 1555.40| -0.31      | (K)MFVLEADEMLSR(G) |                                                                            |                                  |
| 1571.41| -0.30      | (K)MFVLEADEMLSR(G)-1Met-ox |                                                                            |                                  |
| 1588.49| -0.48      | (R)GSSRVLITTDDLAR(G) |                                                                            |                                  |
| 1605.52| -0.31      | (K)KVGINmet-oxTVTEDKR(T) |                                                                            |                                  |
| 1618.55| -0.32      | (K)LQMEAPIHVT6PTGR(V) |                                                                            |                                  |
| 1644.56| -0.31      | (K)LQMet-oxEAPHIVCTPGR(V) |                                                                            |                                  |
| 1515.38| 1544.54$^c$ |                                                                         |                                                                            |                                  |
| 1051.37| -0.19      | (K)QFYPFDLR(E) |                                                                            |                                  |
| 1149.38| -0.20      | (R)IFFWDWR(H) |                                                                            |                                  |
| 1034.35| -0.18      | (K)Rpyro-GluFYPFDLR(E) |                                                                            |                                  |
| 1068.47| -0.21      | (K)LILPGLISSR(I) (only for SBP and SBLP) |                                                                            |                                  |
| 1086.44| -0.19      | (K)LILPGLISSR(I) (AP56 only) |                                                                            |                                  |
| 1102.41| -0.22      | (K)LILPGLmet-SSR(I) (AP56 only) |                                                                            |                                  |
| 1214.42| -0.21      | (K)SPQYSQSV(L) |                                                                            |                                  |
| 1233.42| -0.22      | (R)FVVVDGSEPRA |                                                                            |                                  |
| 1302.49| -0.23      | (K)EPLGPALAHREL(Y) (AP56 only) |                                                                            |                                  |
| 1228.42| -0.24      | (R)Rpyro-GluYSNPQKPR(L) |                                                                            |                                  |
| 1330.50| -0.27      | (K)LAGQFGLGSGIVR(G) (SBP and SBLP only) |                                                                            |                                  |
| 1345.44| -0.25      | (R)QYDISNPQKPR(L) |                                                                            |                                  |
| 1360.53| -0.27      | (R)LILQIFLGGSIVR(G) (AP56 only) |                                                                            |                                  |
| 1454.30| -0.43      | (R)EEIVYLPCimYR(N) |                                                                            |                                  |
| 1468.29| -0.46      | (R)EEIVYLPcamYR(N) |                                                                            |                                  |
| 1667.50| -0.30      | (R)FLYSWNLHDIR(Q) |                                                                            |                                  |
| 1689.51| -0.29      | (K)IDGFNPAMVEAGLYSRI |                                                                            |                                  |
| 1709.63| -0.31      | (R)IPGGPQMQILSDGKR(L) and/or |                                                                            |                                  |
| 1829.56| -0.34      | (K)MATCamTkCamGPGSTPLEAMK(G) |                                                                            |                                  |
| 2219.83| -0.38      | (R)NHIQTLMGTDGLIEIR(F) |                                                                            |                                  |
| 2235.84| -0.36      | (R)NHIQTLMet-oxTDGLIEIR(F) |                                                                            |                                  |
| 2418.75| -0.44      | (R)FLHDPSATQGFGVcamALSNNIQR(F) |                                                                            |                                  |
| 2429.66| -0.44      | (K)GTWKEPQGAPLMGDFWYQPR(H) (AP56 only) |                                                                            |                                  |
| 2546.89| -0.48      | (K)LINPNFLVDKGELPALAHREL(Y) (AP56 only) |                                                                            |                                  |
| 2108.78|            | 2173.05 2404.82, 2721.97 |                                                                            |                                  |
| 1132.47| -0.12      | (K)RAAFQKLGPWRR(R) |                                                                            | Mitochondrial                                   |
| 1177.53| -0.13      | (K)FKTGEVYVGR(A) |                                                                            |                                  |
| 1288.54| -0.15      | (K)RAAFQKLGPWRR(M) |                                                                            |                                  |
| 1351.54| -0.20      | (K)ITPIDGDFFSYR(H) |                                                                            |                                  |
| 1774.63| -0.20      | (R)TPVQENYDEPFVER(S) |                                                                            |                                  |
| 829.37 | -0.11      | (R)ILADLIER(D) |                                                                            |                                  |
| 990.39 | -0.11      | (R)VGNPFDSR(T) |                                                                            |                                  |
| 1233.47| -0.16      | (K)SRVVGPFDSR(T) |                                                                            |                                  |
| 2174.80| -0.24      | (K)MSGSGRELGEYGGLAQYTEVK(T) |                                                                            |                                  |
| 2442.01| -0.30      | (K)VAFTGSTEVHGLIQVAAGSNLKR(V) |                                                                            |                                  |
| 2511.95| -0.33      | (K)ILCimGGAADGRGYFIQPTVFDVK(D) |                                                                            |                                  |
| 2606.00| -0.29      | (R)VGVPFSDSITSEQGPQVDTQFKK(I) |                                                                            |                                  |
| 2748.99|            |          |                                                                            |                                  |
| 1189.27| -0.34      | (K)RAQFOQKGPWRR(T) |                                                                            | Cytoplasmic                                   |
| 1920.42| -0.53      | (K)HIGTHTPSDDGIDPFTYR(R) |                                                                            |                                  |
| 1973.41| -0.53      | (K)GFDFQVQPTVPSNVTDEMR(I) |                                                                            |                                  |
| 1172.27| -0.32      | (R)Rpyro-AFQKGPWRR(T) |                                                                            |                                  |
| 1261.29| -0.34      | (K)KEGAKLEcmGGR(W) |                                                                            |                                  |
| 1645.34| +0.53      | (K)IFINremoteSVSNGK(K) |                                                                            |                                  |
| 1759.41| -0.48      | (R)IFVEESVYDFKR(S) |                                                                            |                                  |
| 1994.90| -0.54      | (K)GFDFQVQPTVPSNVTDEMet-ox(R) |                                                                            |                                  |
| 2661.26| -0.10      | (K)KYLGLNPIPTGIGNQGPQDKHDK(I) |                                                                            |                                  |
| 1553.51| -0.43      | (R)EAYPDGVFLYLHSLR(L) |                                                                            |                                  |
| 1624.43| -0.46      | (R)TGAVIDVPGVEELLGR(V) |                                                                            |                                  |
| 2337.51| -0.66      | (K)REVAAFAQGFDSDLATATQLLQSLR(G) |                                                                            |                                  |
| 1433.51| -0.66      | (K)GIRPAINVGLSSR(V) |                                                                            |                                  |
| 1926.85| -0.18      | (R)EPMet-oxQTGHTKAVDSLVPGR(G) |                                                                            |                                  |
| 2381.55| -0.58      | (K)Rpyro-GluGQSMPAIEEKNQTAIVYAVGIR(V) |                                                                            |                                  |
| 2308.51| -0.65      | (R)QQGYSMPAIEEKNQTAIVYAVGIR(V) |                                                                            |                                  |
| 2409.62| -0.64      | (K)KFENAFLHVISQHQLSLGIR(S) |                                                                            |                                  |
| 2226.55|            |          |                                                                            |                                  |
| 1189.54| -0.07      | (K)RAQFOQKGPWRR(T) |                                                                            | Cytoplasmic                                   |
| 1920.84| -0.11      | (K)HIGTHTPSDDGIDPFTYR(R) |                                                                            |                                  |
| 1973.84| -0.10      | (K)GFDFQVQPTVPSNVTDEMR(I) |                                                                            |                                  |
| 1172.50| -0.09      | (R)IFVEESVYDFKR(S) |                                                                            |                                  |
| 1759.73| -0.16      | (R)Rpyro-GluAFQKGPWRR(T) |                                                                            |                                  |
| Spot Number | MALDI Mass | Δν | Peptide sequence determined by PSD and consistent with massa | Protein identified/NCBI accession no.; percentage of the protein covered molecular mass | Subcellular location |
|------------|------------|----|----------------------------------------------------------|-------------------------------------------------------------------------------------|---------------------|
| 17         | 1377.47    | 0.21 | (RDVDFAEIAGWAVR(D)                                       | ATP synthetase α-subunit (416677); 23%; 59,752.9 Da                               | Mitochondrial       |
|            | 2134.76    | 0.20 | (−Ac-AHVDNYKGKDNENFVR(T)                                 |                                                   | Peroxisomal          |
|            | 765.40     | 0.07 | (KVLHHQIR(D)                                            |                                                   |                     |
|            | 1007.36    | 0.12 | (KNDVEQFVR(T)                                           |                                                   |                     |
|            | 1216.56    | 0.13 | (KEVATSVQLTLR(S)                                        |                                                   |                     |
|            | 1448.44    | 0.30 | (KIDQFTTPLEPKDRC)                                       |                                                   |                     |
|            | 1512.55    | 0.25 | (R)AHYYVEEPVPKR(F)                                      |                                                   |                     |
|            | 1533.61    | 0.18 | (R)RDVDFAEIAGWAVR(D)                                    |                                                   |                     |
|            | 1944.80    | 0.22 | (KYHSHIKVAEVTQVTLT(R)                                   |                                                   |                     |
|            | 2143.06    | 0.09 | (KMGLINKKVEYVLPLDNPY(K(I                                  |                                                   |                     |
|            | 2353.95    | 0.25 | (KHVAHFINHPTGFICMEVEQMRN)                               |                                                   |                     |
|            | 2550.00    | 0.18 | (KHVAHFINHFTPFGICMEVEQMet-ox(N)                         |                                                   |                     |
|            | 2666.94    | 0.30 | (R)NIEFTAMNIECHFLSSFNHVT(A)                             |                                                   |                     |
|            | 1013.34    | 0.19 | (R)NIEFTAMNIECHFLSSFNHVT(A)                             |                                                   |                     |
|            | 1798.75    | 0.26 | (R)NIEFTAMNIECHFLSSFNHVT(A)                             |                                                   |                     |
|            | 2487.77    | 0.38 | (R)NIEFTAMNIECHFLSSFNHVT(A)                             |                                                   |                     |
| 18         | 2264.06    | 0.08 | (KRVAGHDPDVINNAAGNFISPESL)                               | Homologous to 2,4-dienoyl-CoA reductase precursor from rat (220732); 36,204.0 Da | Mitochondrial, peroxisomal |
|            | 845.48     | 0.28 | (K RVAGHDPDVINNAAGNFISPESL)                              |                                                   |                     |
|            | 1225.58    | 0.11 | (Q)RTINDLPFGVR(S)                                       | Osteoblast-specific factor 3 (macrophase)                                             |                     |
|            | 831.35     | 0.11 | (R)SVDVHEIR(L)                                           |                                                   |                     |
|            | 894.32     | 0.10 | (K)ADEGISFRG(Q)                                         |                                                   |                     |
|            | 1208.57    | 0.11 | (R)pyro-GluTIINDLPFGVR(S)                                |                                                   |                     |
|            | 1356.66    | 0.14 | (R)GLFIDIKDKIRG(Q)                                      |                                                   |                     |
|            | 1775.95    | 0.14 | (R)pyro-GluGLGPFMNIPLISDPKR(T)                           |                                                   |                     |
|            | 1784.85    | 0.04 | (R)pyro-GluGLGPFMet-oxNIPLISDPKR(T)                     |                                                   |                     |
|            | 1792.83    | 0.14 | (R)QGGGLPMNIPLISDPKR(T)                                 |                                                   |                     |
|            | 1808.72    | 0.25 | (R)QGGGLPMet-oxNIPLISDPKR(T)                            |                                                   |                     |
|            | 1937.84    | 0.38 | (R)QGGGLPMet-oxNIPLISDPKR(T)                            |                                                   |                     |
| 20         | 1351.63    | 0.11 | (−)PPPITYYPFPVR(G)                                      | Glutathione S-transferase Pi (576133); 23%; 24,781.1 Da                             | Cytoplasmic, endoplasmic reticulum     |
|            | 1564.76    | 0.11 | (−)PPPITYYPFPVRG(R)                                     |                                                   |                     |
|            | 1852.80    | 0.12 | (K)EFGQFIIILDLDKIK(R)                                   |                                                   |                     |
|            | 1936.87    | 0.13 | (R)KFLLSSPEHVRNPINGNKQ(G)                               |                                                   |                     |
|            | 2064.93    | 0.16 | (R)KFLLSSPEHVRNPINGNKQ(G)                               |                                                   |                     |
|            | 2306.09    | 0.09 | (R)KFLLSSPEHVRNPINGNKQ(G)                               |                                                   |                     |
|            | 2374.25    | 0.17 | (R)SFSSAENEPVVPLVGNWRPPQVK(G)                           | Carbonic anhydrase III (226778); 29%; 29,347.5 Da (115463); 29%; 29,528.7 Da | Cytoplasmic         |
|            | 1129.44    | 0.08 | (R)VVFDFTYDK(R)                                          |                                                   |                     |
|            | 1602.85    | 0.06 | (K)GEFQFIIILDLDKIK(T)                                   |                                                   |                     |
|            | 1616.71    | 0.06 | (R)VVFDFTYDRSLMR(L)                                     |                                                   |                     |
|            | 1632.66    | 0.10 | (R)VVFDFTYDSDMet-oxLR(G)                                |                                                   |                     |
|            | 1859.97    | 0.08 | (R)EKGFQFIIILDLDKIK(T)                                  |                                                   |                     |
|            | 1944.95    | 0.13 | (KIGRKEFKQIIILDLDK(I)                                   |                                                   |                     |
|            | 2051.78    | 0.14 | (K)EAPFTHFDPScmLFACim(RD)                               |                                                   |                     |
|            | 2236.84    | 0.19 | (K)EAPFTHFDPScmLFACim(RD)                               |                                                   |                     |
|            | 903.34     | 1.15 | (K)EAPFTHFDPScmLFACim(RD)                               |                                                   |                     |
| 22         | 2051.91    | 0.01 | (K)EAPFTHFDPScmLFACim(RD)                               | Carbonic anhydrase III (226778); 29%; 29,347.5 Da (115463); 29%; 29,528.7 Da | Cytoplasmic         |
|            | 1129.35    | 0.17 | (R)VVFDFTYDK(R)                                          |                                                   |                     |
|            | 2745.12    | 0.32 | (R)SFSSAENEPVVPLVGNWRPPQVK(G)                           |                                                   |                     |
|            | 2775.41    | 0.06 | (−)Ac-AKEWGYAHRHGPDHWDHLEYPIAK(G)                       |                                                   |                     |
|            | 2817.15    | 0.22 | (−)Ac-AKEWGYAHRHGPDHWDHLEYPIAK(G)                       |                                                   |                     |
|            | 903.34     | 1.15 | (K)EAPFTHFDPScmLFACim(RD)                               |                                                   |                     |
|            | 1105.44    | 1.76 | (K)EAPFTHFDPScmLFACim(RD)                               |                                                   |                     |
| 23         | 1582.76    | 0.07 | (K)GENSLVHVHGPDGIR(L)                                   | Sorbitol dehydrogenase precursor (2137466); 29%; 40,091.6 Da                     | Cytoplasmic         |
|            | 822.41     | 0.05 | (K)HLKPDVDR(V)                                           |                                                   |                     |
|            | 937.50     | 0.05 | (R)VAIEPGVP(E)                                           |                                                   |                     |
|            | 1002.46    | 0.05 | (K)ETQPEQIAK(V)                                          |                                                   |                     |
|            | 1277.71    | 0.06 | (K)TNVYKLPLTVHR(F)                                      |                                                   |                     |
|            | 1575.75    | 0.07 | (K)AMGAAQVVVTDLSASRL(L)                                 |                                                   |                     |
|            | 1591.77    | 0.04 | (K)Gmet-pGAAQVVVTDLSASRL(L)                             |                                                   |                     |
|            | 2472.07    | 0.11 | (R)YNLTPITFCATPDDIGNLcim(RF)                            |                                                   |                     |
|            | 2636.24    | 0.09 | (K)LPDSVFTEEGAILIEPLSVGYACim(RR)                        |                                                   |                     |
|            | 786.31     | 0.40 | (K)LPDSVFTEEGAILIEPLSVGYACim(RR)                        |                                                   |                     |
| Spot No. | MALDI mass / Da | Δ / Da | Peptide sequence determined by PSD and consistent with mass<sup>α</sup> | Protein identified (NCBI accession no.); percentage of the protein covered molecular mass | Subcellular location |
|---------|----------------|--------|-------------------------------------------------|-----------------------------------------------------------------------------------|---------------------|
| 24      | 941.47         | −0.12  | (KIAWLGLLRR(Q)                                  | Homologous to glycine N-methyltransferase (rat) (121328); 27%; 32,549.2 Da        | Cytoplasmic         |
|         | 1989.72         | −0.23  | (RSLGVAAEHPDQYDAEAGEAR(V)                       | Homologous to glycine N-methyltransferase (rat) (121328)                         | Cytoplasmic         |
|         | 780.29          | −0.10  | (~Ac-VDSYVR(T)                                  | Homologous to 3-amide-modified Cys; Ac-, acetylated N terminus                    |                    |
|         | 1250.49         | −0.16  | (R/VQWLYGIDTR(S)                                |                                                                                  |                    |
|         | 1847.80         | −0.23  | (KNIASVPRPGGLVIDHR(N)                          |                                                                                  |                    |
|         | 1863.78         | −0.24  | (KNIASMet-oxVPRPGGLVIDHR(N)                    |                                                                                  |                    |
|         | 1013.49         | 1076.47| 1140.43, 1801.66, 1955.74, 2267.82             |                                                                                  |                    |
| 25      | 941.60          | +0.01  | (KIAWLGLLRR(Q)                                  | Homologous to glycine N-methyltransferase (rat) (121328)                         | Cytoplasmic         |
|         | 1989.99         | +0.04  | (RSLGVAAEHPDQYDAEAGEAR(V)                       | Homologous to glycine N-methyltransferase (rat) (121328)                         | Cytoplasmic         |
|         | 780.39          | 0.00   | (~Ac-VDSYVR(T)                                  | Homologous to 3-amide-modified Cys; Ac-, acetylated N terminus                    |                    |
|         | 1250.67         | +0.02  | (R/VQWLYGIDTR(S)                                |                                                                                  |                    |
|         | 1864.03         | +0.01  | (KNIASMet-oxVPRPGGLVIDHR(N)                    |                                                                                  |                    |
|         | 913.59          | 1013.63| 1076.63, 1262.64, 1322.68, 1801.57, 1956.00*   |                                                                                  |                    |
| 28      | 1143.62         | −0.03  | (RQQGEFILLPAR(V)                                | Homologous to 3-hydroxynitrile 3,4-dioxygenase (rat) (1040694); 30%; 32,582.3 Da| Cytoplasmic         |
|         | 2392.12         | −0.07  | (KIDLGTLAPIQEFPHSEQYR(T)                        | Homologous to 3-hydroxynitrile 3,4-dioxygenase (rat) (1040694); 30%; 32,582.3 Da|                    |
|         | 820.41          | −0.03  | (K/RVPHSPQR(F)                                  |                                                                                  |                    |
|         | 1091.54         | −0.03  | (KIMFVGPPNTR(K)                                |                                                                                  |                    |
|         | 1107.51         | −0.05  | (KIMet-oxFVGGPNTR(K)                            |                                                                                  |                    |
|         | 1126.60         | −0.03  | (R/p-water-GluGEFILLPAR(V)                     |                                                                                  |                    |
|         | 1250.63         | 230.03 | (R/FANTMGLVIEER)                                |                                                                                  |                    |
|         | 1266.61         | −0.04  | (R/FANTMet-oxGLVIEER)                          |                                                                                  |                    |
|         | 2558.14         | −0.11  | (K/SWVEENRASFQPVCimNKLMHR(E)                    |                                                                                  |                    |
|         | 2601.17         | −0.08  | (K/SWVEENRASFQPVCimNKLMet-oxHR(E)               |                                                                                  |                    |
|         | 2910.39         | −0.02  | (R/ELAQGTSLSLFDSYETQVIAHGQGSSR(K)              |                                                                                  |                    |
|         | 1086.57         | 1358.62| 1499.59, 1509.75, 2538.40, 2713.23, 2729.24*   |                                                                                  |                    |
| 29      | 1091.55         | −0.02  | (KIMFVGPPNTR(K)                                | Homologous to 3-hydroxynitrile 3,4-dioxygenase (rat) (1040694); 17%; 32,582.3 Da| Cytoplasmic         |
|         | 1107.55         | −0.01  | (KIMet-oxFVGGPNTR(K)                            |                                                                                  |                    |
|         | 1126.61         | −0.02  | (R/p-water-GluGEFILLPAR(V)                     |                                                                                  |                    |
|         | 1143.63         | −0.02  | (R/QGEFILLPAR(V)                                |                                                                                  |                    |
|         | 1250.65         | −0.01  | (R/FANTMGLVIEER)                                |                                                                                  |                    |
|         | 1266.62         | −0.03  | (R/FANTMet-oxGLVIEER)                          |                                                                                  |                    |
|         | 2391.97         | −0.22  | (KIDLGTLAPIQEFPHSEQYR(T)                        |                                                                                  |                    |
|         | 1086.58         | 1261.60| 1322.73, 1358.62, 1499.59, 1509.75, 1581.90, 1833.96, 1892.85* |

<sup>α</sup> The difference between the measured mass and calculated mass (monoisotopic).

<sup>β</sup> Sequence determined by PSD is underlined. Residues before/after peptide are in parentheses. Cam, acylamide-modified Cys; Cit, idioacetamide-modified Cys; Ac-, acetylated N terminus; Met-ox, methionine sulfoxide; pyro-Glu, pyroglutamine at the N terminus; u, selenocysteine; X, carbamidomethylated selenocysteine; SBP, selenium-binding protein; SBLP, selenium-binding liver protein; AP56, acetaminophen-binding protein.

<sup>γ</sup> Masses neither identified nor attributed.

<sup>δ</sup> Glu<sup>1</sup> to Aep<sup>7</sup>.

lar ion as a selenium-containing component (Fig. 4). Measurement of the PSD mass spectrum established the identity of this active site selenocysteine-containing peptide (VLLIENVASLYGGTTIR, where X represents carbamidomethylated selenocysteine). The same peptide was not observed in the MALDI spectrum of the digest from spot 8, nor was the homologous tryptic peptide containing cysteine 47 observed. This may be due to the fact that spot 8 contained a much lower amount of protein compared with spot 2, as is obvious by inspection of Fig. 1A. Hence, these two forms are almost identical in sequence, except selenocysteine (residue 47) is present at the active site in spot 2 and cysteine is residue 47 in spot 8.

Mitochondrial housekeeping protein was unambiguously identified in spot 3 by MS-Tag searching based on the PSD mass spectra obtained from two tryptic peptides gated from the digest mixture at m/z 1206.43 and 1476.33. From similar mass spectral data, thioether S-methyltransferase was found in spot 4. For spot 7, data base interrogations based on four different PSD mass spectra revealed a single common entry, tropomyosin 5, cytoskeleton type.

Selenium-binding liver protein (and/or selenium-binding protein) and acetaminophen-binding protein were found in spots 9 and 10, respectively, and both of them together were found in spot 14. These proteins are isomers (37, 38), and most of their tryptic peptide sequences are identical. The known sequence differences between selenium-binding liver protein and selenium-binding protein (only 3 residues are different) could not be discerned based on the digest peptide mass values observed for each spot, nor could the partial sequences derived from the PSD measurements undertaken. However, several subtle differences were found for acetaminophen-binding protein compared with the other two selenium-binding proteins (Table I). For example, peptide LAQQIFLGGSIVR with a mass of m/z 1330.69 was found in spots 9 and 14 but not in spot 10; rather peptide LTQQIFLGGSIVR with a mass of m/z 1360.80, which is specific for acetaminophen-binding protein, was found in spot 10. The sequences of both of these tryptic peptides were determined by interpretation of their PSD mass spectra. Other sequence differences that were observed are specified in Table I (spot 14) and are also reflected in different percentages of coverage for these isomers.

Interpretation of the PSD mass spectra of five of the tryptic peptides extracted from spot 12 easily established the presence of methionine adenosyltransferase. Similarly, spot 13 was found to contain protein initiation factor 4A.

Two different aldehyde dehydrogenases were identified in the 56-kDa region. The isomer found in spot 15 can be assigned as mitochondrial aldehyde dehydrogenase based upon partial sequence information contained in five PSD mass spectra. The other is cytosolic class I aldehyde dehydrogenase located in both spots 16 and 16’. In addition, spots 16 and 16’ contain co-migrating ATP synthetase α-subunit (in spot 16’, carbonic
anhydrase III was also present). Spots 16 and 16' are the only spots observed in this study where the presence of more than one protein was determined. These two spots appear very close to each other but in fact are completely separated by two-dimensional PAGE (see Fig. 1A). Except for peptides from carbonic anhydrase III in spot 16', the peptide patterns from these two spots were virtually identical.

Peroxisomal urate oxidase was identified in spot 17. PSD sequence analysis of a component with a mass value of m/z 2134.76 established that the N terminus of this protein was acetylated (see Table I).

Similar analysis of the protein in spot 19 established the presence of osteoblast-specific factor 3, also named macrophage 23-kDa stress protein. This spot is very close to spot 20 on the two-dimensional map but distinct in that spot 20 was shown to contain glutathione S-transferase (GST) Pi. For glutathione S-transferase Pi, both N- and C-terminal peptides were detected. Two N-terminal tryptic peptides were detected with cleavage at Arg11 and Arg13, respectively (PPYTIVYFPVR and PPYTIVYFPVRGR). Subsequent partial sequence analysis established these assignments unambiguously (Table I).

Mass spectral characterization of spots 21 and 22 revealed the presence of carbonic anhydrase III in both spots. These forms of this protein have the same pI value but slightly different molecular weights (Fig. 1A). Based on similar mass spectral analysis, sorbitol dehydrogenase precursor was identified in spot 23.

Proteins Found Homologous to Proteins of Other Species in the Current Protein Data Bases—Mass spectral sequence information of the tryptic peptides from spot 6 establish that this protein is highly homologous to bovine inorganic pyrophosphatase. MS-Tag searching also identified a cross-species substitution (Glu1 to Asp1), in which the peptide with mass at m/z 1237.23 has a sequence of DVFHMVVEVPR instead of EVFHVMEVPR found in bovine inorganic pyrophosphatase.

The protein characterized in spot 11 is highly homologous to both human and rat proteasome subunit C8. As usual, evaluation of the fragment ions in the PSD mass spectrum of one tryptic peptide from this spot shown in Fig. 5 are not sufficient to allow manual interpretation. However, these fragment ions are adequate for use in data base interrogation with MS-Tag to match a single peptide entry. Consideration of both the molecular weight measured and sequence ions present reveals that this peptide must be N-terminally acetylated (Ac-SSIGTGYDLSASTFSPDGR).

In similar studies of spot 18, five PSD mass spectra were determined and evaluated. Only one of these obtained from the component at m/z 2264.06 matches a tryptic peptide (VAGHPDVVINNAAGNFSPEIR) from rat 2,4-dienoyl-CoA reductase precursor. This result indicates that the mouse protein at spot 18 is homologous to rat 2,4-dienoyl-CoA reductase precursor. MS-Tag searching was carried out based on the other four PSD mass spectra including the EST data base, but further matching was not obtained.

Proteins in both spots 24 and 25 were found homologous to rat glycine N-methyltransferase. The peptide mass at m/z 780.39 in the MALDI spectrum may indicate that this protein was N-terminally acetylated. Proteins in both spots 28 and 29 have the same molecular weight but different pI values. Eleven measured mass values from spot 28 and seven values from spot 29 can be attributed to tryptic peptides from rat 3-hydroxyanthranilate 3,4-dioxygenase. This apparent high homology was further verified using sequence information derived from two PSD mass spectra of components at m/z 1143.62 and 2392.12.

In addition to the posttranslational N-terminal acetylation discussed above, other chemical modifications were also identified as shown in Table I. These are artifacts that occur during the experimental processes. For example, carbamidomethylation and acrylamide conjugation on cysteine residues can occur during gel electrophoresis (30); methionine oxidation may be due to exposure to oxygen in the air and was found to increase over time (data not shown); and N-terminal cyclization of glutamine (pyro-Glu) may occur during the in-gel digestion where the tryptic peptides undergo both basic (pH 8 during trypsin digestion) and acidic (pH 1 after peptide extraction step) conditions.
DISCUSSION

Over the last several decades, an understanding of the relationship between the covalent binding of chemical carcinogens or their metabolically formed reactive intermediates to DNA and ensuing genotoxicity (mutagenesis and carcinogenesis) has been developed (39). However, despite the accumulation of very extensive circumstantial evidence, a molecular description of the events underlying the correlation between organ toxicity and covalent binding of reactive drug intermediates to tissue proteins has yet to be rigorously established (40–44). In the case of carcinogen DNA adducts, structural studies have provided a rather detailed description of the implications of specific chemical modifications of DNA to the processes of mutagenesis and carcinogenesis (45). However, analogous research on the identification of those proteins (and drug protein adducts) implicated by virtue of drug modification in vivo has lagged far behind, as exemplified by the slow progress in the case of acetaminophen and hepatic toxicity (46). Unfortunately, previous approaches even to protein identification have been labor-intensive, of relatively low sensitivity, and poorly suited to dealing with the numbers of modified proteins involved. They have involved subcellular prefractionation followed by several large scale chromatographic steps to isolate a single pure protein for Edman amino acid sequence determination. Without the knowledge of the complete suite of proteins implicated, there are clearly considerable difficulties in the formulation of a robust mechanistic hypothesis that would explain the nature of the compromised protein function and cell homeostasis responsible for the observed toxicity.

In this study, we have carried out the identification of the entire suite of hepatic proteins involved as targets of covalent modification in vivo. This undertaking was carried out with relative ease by taking advantage of a new analytical strategy developed in our laboratory during the past few years. This approach combines the power of two-dimensional SDS-PAGE of tissue lysates and advanced versions of MALDI-time-of-flight MS for the identification of suites of proteins (35, 47, 48). From an analytical point of view, two-dimensional PAGE is unmatched in its ability to simultaneously resolve hundreds of cellular proteins (49). Recently, with the maturation of technologies for immobilized pH gradient in the first dimension (isoelectric focusing), its resolution, loading capacity, and especially reproducibility have all been improved significantly (50). Similarly, MALDI-time-of-flight MS has advanced to the point of providing high sensitivity and mass resolution for analysis of unseparated digests as well as providing partial amino acid sequences for components in the low fmol range from in-gel digestion of protein spots on two-dimensional PAGE (35, 47, 48, 52), particularly after the introduction of delayed extraction technology (53). Partial peptide sequence may be obtained in many cases by interpretation of the fragment ion mass spectrum (so-called PSD spectrum) formed by dissociation of metastable pseudomolecular ions after acceleration during flight (32). The amount of energy deposition taking place during the MALDI process varies with several parameters in an unpredictable manner, so the degree of sequence information contained in a PSD mass spectrum varies similarly. Thus, the complete de novo peptide sequence that may be obtained by MALDI high energy collision-induced dissociation is not possible with this method (54–56). While typical PSD spectra are incomplete views of complete sequence per se, they usually contain sufficient information on amino acid composition and amino acid ordering, so that data base searching programs based on these results are easily able to match and retrieve those proteins having identical or homologous characteristics. Such algorithms have been developed in our laboratory to match tandem mass spectral data to existing sequences in data base entries, thus suggesting the possible identity of the protein being analyzed (33). Combining these technologies, we

3 K. R. Clauser, P. Baker, and A. L. Burlingame, manuscript in preparation.
were able to identify 23 protein targets for acetaminophen-reactive metabolites (for a list, see Table I). As a general tool, the methods developed here should also prove valuable in the identification of modified proteins after the administration of any drug to animals (or humans).

Of the suite of protein targets identified, most are of cytosolic origin, but a number are from mitochondria as well. Among them a few have been previously identified, including three isomers of selenium-binding proteins in spots 9, 10, and 14 and mitochondrial aldehyde dehydrogenase in spot 15 (17, 18, 22). However, five additional proteins, which were identified previously by other methods, have not been found among our set, including glutamine synthetase subunit (19), glutamate dehydrogenase (19), N-10-formyltetrahydrofolate dehydrogenase (20), lamin-A (23), and carbamyl phosphate synthetase I (24).

Very few microsomal proteins were found, and this is probably due to the loss of membrane protein during sample preparation and the fact that water-soluble proteins can enter the gel much more easily than hydrophobic membrane proteins (57). However, even after consideration of these points, one cannot fully explain why only two of seven previously found protein targets were detected in our autoradiogram, because the missing proteins are all cytosolically soluble. This discrepancy may be due to the differences in experimental conditions such as phenobarbital induction used in our case, different liver harvest time points, and even different detection methods, because immunochemical detection procedures using antibodies prepared from different haptens were used in all previous studies of protein target identification (17–24). Immunochemical methods may be generally more sensitive than fluorography (58). However, the specific sensitivity will vary considerably among different protein adducts due to different affinities and accessibilities of the in vivo adducts to the antibody employed. It is possible that a protein adduct of low relative abundance could have been previously classified as high due to its high accessibility and affinity for the antibody used. However, such a particular adduct may not be detected by autoradiography due to possible low stoichiometry of modification.

Our finding that many mitochondrial proteins are modified is consistent with the occurrence of mitochondrial dysfunction reported during the early stage of acetaminophen toxicity. In this study, ATP synthetase α-subunit was present in both spots 16 and 16'. This is an essential subunit of the F₁ unit of ATP synthase. ATP synthase is an enzyme complex consisting of a F₀ unit, a proton channel, and a F₁ unit, which utilize the proton gradient potential to synthesize ATP. F₁ contains the catalytic site for ATP synthesis and consists of five kinds of polypeptide chains with the stoichiometry α₃β₃γδε. Modification of ATP synthetase α-subunit may have abolished the function of ATP synthase and subsequently resulted in ATP depletion.

Inhibition of mitochondria respiration and ATP depletion has been reported earlier based on experiments carried out both in vitro (59–63) and in vivo (64–68). Parmar et al. (68) found an overall 35% decrease in ATPase activity after acetaminophen treatment (650 mg/kg) in rats and suggested this was due to covalent modification on ATPase by NAPQI.

In spot 13, we found protein initiation factor 4A, a protein catalyzing the initiation process, which sets the reading frame required for correct protein translation. During protein synthesis, this protein forms a complex with GTP and mediates the binding of the methionyl initiator tRNA to the small ribosomal subunit, which then binds to the 5'-cap of the mRNA and begins scanning along the mRNA for an AUG codon. This factor is then released by hydrolysis of GTP to GDP before the large ribosomal unit joins the small one to form a complete ribosome that begins protein synthesis. Because the large subunit binds only after the small ribosomal subunit loaded with initiation factors finds the start codon, regulation of the level of initiation factors is an important mechanism for adjusting the rate of protein synthesis. Conceivably, modification on protein initiation factor 4A may have impaired the very first step for synthesis of some proteins. This hypothesis is supported by the fact that total protein synthesis is inhibited by acetaminophen in a
concentration-dependent manner (69).

Ironically, many proteins revealed in this study are classic detoxification enzymes. This includes the aryl sulfotransferase analog found in spot 5, because compromised function would in effect diminish normal detoxification and increase metabolic flux into NAPQI formation. Spot 20 contains GST Pi. GSTs are a family of detoxification enzymes that catalyze the nucleophilic attack of the sulfur atom of glutathione over a wide range of electrophilic compounds such as NAPQI. GSTs consists of four main families including Alpha, Mu, Pi, and Theta (70, 71). The Pi class is expressed at high levels in tumors, and it has become clear that overexpression of GST Pi plays a role in acquired resistance to chemotherapy (72). Elevated GST Pi has been associated with protection of liver cells from the cytotoxicity produced by acetaminophen (73). Covalent modification and inhibition of GSTs by various agents (74–76) including trans-4-hydroxy-2-nonenal (77), a cytotoxic lipid peroxidation product, have been reported. Therefore, in retrospect, it is not surprising that GST is a target of NAPQI after GSH depletion.

Modified selenium-binding protein was found in spots 9, 10, and 14. The function of selenium-binding proteins is not known (37, 38), but they were thought to be involved in a defense mechanism against arylation agents, because selenium-binding proteins are common targets for electrophilic metabolites of many other chemicals including bromobenzene (78), 2,6-dimethyl-acetaminophen (79), 3-hydroxyacetanilide (80), and 3-methyl indole (81).

Glutathione peroxidase found in both spots 2 and 8 is an enzyme that catalyzes the reduction of hydroperoxide into O2 and water, together with the oxidation of GSH to GSSG. This reaction is a part of the mechanism that protects cells from oxidative damage induced by endogenous reactive oxygen species derived from superoxide anion, which may be released from the mitochondrial respiration chain. Our findings are supported by earlier studies indicating that inhibition of gluthathione peroxidase by gold thioglucose increased the susceptibility of hepatocytes to acetaminophen toxicity (82). Tirmenstein and Nelson (43) also demonstrated that, 1 h after acetaminophen administration, glutathione peroxidase activity decreased to about 60% of control values.

Osteoblast-specific factor 3 (macrophage 23-kDa stress protein), found in spot 19, is highly inducible by oxidative and sulfhydryl-reactive agents. It has a domain structure highly homologous to the C22 component of alkyl hydroperoxide reductase of Salmonella typhimurium and was suggested to play a role in an oxidoreductase reaction system in mammalian cells (83). Because osteoblast-specific factor 3 is from macrophage and exhibits especially high content in the liver (83), covalent modification of this protein further supports the concept that macrophages may play a role in the pathogenesis of acetaminophen hepatotoxicity (84–87). It is conceivable that these detoxification enzymes/proteins are in fact early targets for reactive metabolites after glutathione depletion, triggering the onset of compromised homeostasis because of their ready accessibility to reactive metabolites. Although contradictory data have been reported regarding the oxidative stress hypothesis for acetaminophen toxicity (88–90), it is a fact that NAPQI, the major reactive metabolite of acetaminophen, is a potent oxidant as well as an electrophile. Oxidative damage in liver cells has been demonstrated both in vivo (43, 82, 91–96) and in vitro (89, 97–100). The modification of detoxification enzymes discovered in this work may compromise their function and thus potentiate the toxicity of acetaminophen by further breaking down the cell’s normal defense mechanism against oxidative stress caused by NAPQI and also possibly by endogenous reactive oxygen species (101). Overall, it is likely that inhibition of critical protein functions, directly by protein arylation and indirectly by oxidation, are both important in pathogenesis of acetaminophen hepatotoxicity, with the former preceding the latter.

It is well known that not all possible covalent modifications of proteins cause toxicities (102), particularly when metabolites, such as those of acetaminophen, bind to so many proteins. For example, urate oxidase, or uricase, in spot 17 was one of the major targets for acetaminophen metabolites, as seen in Fig. 1B. However, this enzyme is lost during primate evolution (51). Because both humans and mice develop centrilobular liver necrosis with similar per kg doses of acetaminophen, it is unlikely that covalent modification of urate oxidase plays a significant role in acetaminophen toxicity in mice. Furthermore, it is reasonable to assume that other targeted proteins were modified like urate oxidase only because they are accessible and contain nucleophiles (particularly free cysteine residues) reactive toward NAPQI and other reactive metabolites. Therefore, to elucidate the role of covalent binding in this toxicity, it will be essential to single out those protein modifications relevant to acetaminophen toxicity.

As proposed by Anderson and Anderson (34), proteins in the cell as a whole make up the genome operating system, which works as a network responsible for the two-way informational translation and trafficking between genome and environmental input. Drug binding to a protein can lead to complex effects through this network. Therefore, implications of modifications of these proteins are indeed profound and certainly cannot be answered until the effect of formation of these adducts, not only on functions of these proteins directly modified but also on other related cellular functions, are characterized. As shown in Table I, some of the protein targets we have discovered are not in the current protein data bases; in fact, the protein in spot 1 is a major binding target for acetaminophen but it is not homologous to any known proteins. The effects of modifications on proteins with previously undescribed properties or function will certainly be more difficult to elucidate until their normal functions are discovered. Because the percentage of protein modified may be more important than the absolute amount of modification and because the abundance of mammalian proteins varies from a few to hundreds of millions copies per cell, it is likely that some low abundance protein adducts not detected in this study may play important roles in the pathogenesis of acetaminophen hepatotoxicity. After all, it continues to be a challenge to discover a complete set of critical protein targets responsible for toxicity, and as pointed out by Nelson (46), it will be even more of a challenge to determine the importance of the modification in the pathogenesis of hepatoxicity caused by acetaminophen.

REFERENCES

1. Hinson, J. A., Puhl, L. R., Monks, T. J., and Gillette, J. R. (1981) Life Sci. 29, 807–110
2. Harvison, P. J., Guengerich, F. P., Rashed, M. S., and Nelson, S. D. (1988) Chem. Res. Toxicol. 1, 47–52
3. Corcoran, G. B., Mitchell, J. R., Vaishnav, Y. N., and Horning, E. C. (1986) Mol. Pharmacol. 18, 536–542
4. Dahlin, D. C., and Nelson, S. D. (1982) J. Med. Chem. 25, 885–886
5. Dahlin, D. C., Miwa, G. T., Lu, A. Y., and Nelson, S. D. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1327–1331
6. Holme, J. A., Dahlin, D. C., Nelson, S. D., and Dying, E. (1984) Biochem. Pharmacol. 33, 401–406
7. Miner, D. J., and Kissinger, P. T. (1979) Biochem. Pharmacol. 28, 3285–3290
8. Potter, W. Z., Thorgerisson, S. S., Jollow, D. J., and Mitchell, J. B. (1974) Pharmacology 12, 129–143
9. Jollow, D. J., Mitchell, J. R., Putter, W. Z., Davis, D. C., Gillette, J. R., and Bredie, B. D. (1973) J. Pharmacol. Exp. Ther. 187, 195–202
10. Roberts, D. W., Bucci, T. J., Benson, R. W., Warbritton, A. R., McRae, T. A., Pumphord, N. R., and Hinson, J. A. (1991) Am. J. Pathol. 138, 359–371
11. Roberts, D. W., Pumphord, N. R., Putter, D. W., Benson, R. W., and Hinson, J. A. (1987) J. Pharmacol. Exp. Ther. 241, 527–533
12. Bartolone, J. B., Cohen, S. D., and Khairallah, E. A. (1989) Fundam. Appl. Toxicol. 13, 859–862

---

17952 Hepatic Protein Targets of Acetaminophen
