Purification, Characterization, and Cloning of a Heme-Binding Protein (23 kDa) in Rat Liver Cytosol[†,‡]

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ABSTRACT: A heme-binding protein (designated HBP23) has been purified from rat liver cytosol using heme-affinity chromatography and either reverse-phase high-performance liquid chromatography or sequential ion-exchange chromatography. The protein (23 kDa) binds heme with an affinity (K_d = 55 nM) higher than that of the abundant cytosolic heme-binding proteins, heme-binding protein (HBP)/liver fatty acid-binding protein (L-FABP) and the glutathione S-transferases (GSTs) (K_d = 100−200 nM). HBP23 is present in the cytosol of liver, kidney, spleen, small intestine, and heart, with the liver showing the highest content. A cDNA coding the 23-kDa protein was cloned using reverse transcription polymerase chain reaction with degenerative oligonucleotides derived from partial amino acid sequences. The cloned cDNA encoded 199 amino acids, and its amino acid sequence showed no homology to HBP/L-FABP, GSTs, or any other heme-binding proteins or hemeproteins. Homology search showed that HBP23 is highly homologous to mouse macrophage 23-kDa stress protein, which is inducible by oxidant stress in peritoneal macrophages [Ishii, T., Yamada, M., Sato, H., Matsue, M., Taketani, S., Nakayama, K., Sugita, Y., and Bannai, S. (1993) *J. Biol.Chem. 268,* 18633−18636]. Thioredoxin peroxidase as well as HBP23 and the mouse macrophage 23-kDa stress protein are members of the peroxiredoxin family, a recently recognized class of antioxidant proteins [Chae, H. Z., Chung, S. J., & Rhee, S. G. (1994) *J. Biol.Chem. 269,* 27670−27678]. An increase in HBP23 mRNA was observed in Hepa 1−6 cells after treatment with heme and cadmium and during liver regeneration after partial hepatectomy. These findings indicate an involvement of HBP23 in heme metabolism.

It is unclear how heme traverses the plasma membranes into cells and is targeted to specific intracellular locations. Cytosolic proteins probably facilitate the intracellular transport of heme (Muller-Eberhard & Vincent, 1985; Muller-Eberhard & Nikkilä, 1989). Candidates for this transport are the major heme-binding protein [HBP[1] = liver fatty acid-binding protein, L-FABP, (Vincent & Muller-Eberhard, 1985; Vincent et al., 1987) constituting 3−5% of total cytosolic proteins], whose affinity for heme is 10 times higher than that for oleic acid (Vincent & Muller-Eberhard, 1985), and the glutathione S-transferases (GSTs) (Ketley et al., 1975; Sugiyama et al., 1984). L-FABP is ascribed a function in fatty acid transport, modulating fatty acid metabolism, and displays multiple binding activities, similar to albumin in plasma (Glatz & Veerkamp, 1985). The GSTs, constituting 3−5% of total cytosolic proteins, catalyze the conjugation of glutathione with xenobiotics but also bind organic anions such as bilirubin and bile acids (Kaplowitz et al., 1973; Boyer, 1989).

Studies on heme transport by these proteins are limited (Senjo et al., 1985; Boyer & Olsen, 1991; Liem et al., 1990) and do not delineate their participation in transport. Heme is also known to regulate protein synthesis: transcriptionally, translationally, and by the modulation of protein transport into mitochondria, i.e., that of δ-aminolevulinic acid synthase (Hayashi et al., 1983; Yamamoto et al., 1988; Ades et al., 1987; Drew & Ades, 1989). Cytochrome *c* heme lyase incorporates heme into apocytochrome *c* synthesized in the cytoplasm and participates in the transport of cytochrome *c* into mitochondria (Dumont et al., 1988; Nicholson et al., 1988). It has been proposed that heme, as well as iron, mediates the interaction of the iron-responsive element binding protein with its target mRNAs (Lin et al., 1990), such as those of ferritin (Theil, 1990), transferrin receptor (Theil, 1990), and erythroid δ-aminolevulinic acid synthase (Cox et al., 1991; Dandekar al., 1991), resulting in the regulation of their translation.

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[‡] The nucleotide sequence reported in this paper has been submitted to the Genbank/EMBL Data Bank with the accession number D30035.

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulphonic acid; bp, base pair; GAP-DH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione S-transferase; HBP, heme-binding protein; HBP23, heme-binding 23 kDa protein; HPLC, high-performance liquid chromatography; L-FABP, liver fatty acid-binding protein; MSP23, macrophage 23-kDa stress protein; NKEF A, natural killer cell enhancing factor A; pag, proliferation associated gene; PAGE, polyacrylamide electrophoresis; PCR, polymerase chain reaction.

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We report here on a heme-binding protein (designated HBP23) in rat liver cytosol, which has a concentration of about 1/50th of that of HBP/L-FABP and GSTs and an affinity for heme higher than that of these two abundant proteins. The mRNA of this cytosolic heme binder increased when heme metabolism changes, i.e., after heme addition to Hepa 1−6 cells and during liver regeneration after partial hepatectomy. HBP23 is expressed not only in liver but also in other organs; it may be related to both heme metabolism and oxidative stress because it is highly homologous to thioredoxin peroxidase (Chae et al., 1994a), a recently described antioxidant protein.

EXPERIMENTAL PROCEDURES

Preparation of Rat Liver Cytosol. The livers of female Sprague-Dawley rats (200−250 g) were blanched by perfusion with ice-cold physiological saline and homogenized with 4 volumes (w/v) of 0.25 M sucrose in 20 mM sodium phosphate buffer (pH 7.5). The homogenate was centrifuged at 105000*g* for 90 min at 4 °C and the clear supernatant (cytosol) was aspirated being careful to avoid contamination by the fat layer.

Purification of Heme-Binding 23 kDa Protein (HBP23). The cytosol, dialyzed against equilibration buffer (20 mM sodium phosphate buffer, pH 7.5), was applied to a heme-agarose column (Tsutsui & Mueller, 1982). To remove unspecifically bound proteins, the column was washed with 0.5 M sodium chloride in equilibration buffer until absorbance at 280 nm became less than 0.05. Bound proteins were eluted with a solution containing 40 mM succinic acid and 40 mM citric acid (pH 2.8) after which fractions were immediately neutralized with 1 M Tris. The eluted proteins were separated by reverse-phase high-performance liquid chromatography (HPLC) using a Vydac C4 column with a linear gradient of 32−58% acetonitrile in 0.1% trifluoroacetatic acid. A Waters HPLC system (Model 720 System Controller, Model 510 HPLC Pumps, and WISP Model 710B) was used, the flow rate being 1.0 mL/min at room temperature.

Alternatively, HBP23 was purified by sequential ion-exchange chromatography to obtain native protein for binding studies. Proteins eluted from the heme-agarose column were loaded onto a column packed with DEAE Sepharose CL-6B (Pharmacia, Uppsala Sweden), equilibrated with 20 mM Tris (pH 9.0). Unbound fractions were separated by a column packed with SP Sepharose Fast Flow (Pharmacia), using a linear gradient of 50−300 mM sodium chloride in 20 mM sodium phosphate buffer (pH 6.5). HBP23 was eluted in 180−200 mM sodium chloride.

Binding Studies. For binding studies, a slight modification of the protocol used for HBP/L-FABP (Vincent & Muller-Eberhard, 1985) was utilized. In brief, fluorescence spectroscopy was used to determine K_ds of the interaction of purified HBP23 with heme, protoporphyrin IX, tin-protoporphyrin IX, and bilirubin. Ligand binding to HBP23 was monitored by fluorescence quenching measurements of tryptophan residues. The excitation wavelength was 280 nm, and the emission spectrum exhibited a maximum at 333.5 nm. The K_d of the interaction with oleic acid, which does not quench fluorescence, was determined using 8-anilino-1-naphthalene sulfonic acid (ANS) binding competition. Protein fluorescence spectra were recorded on a Perkin-Elmer MPF-44A fluorometer. Interaction of HBP23 with heme was also measured by difference absorption spectroscopy in the Soret region by adding aliquots of heme in both sample and reference cuvettes. Concentrations of heme, protoporphyrin IX, tin-protoporphyrin IX, and bilirubin were determined by spectrophotometry using published values for molar absorption (Collier et al., 1979; Blauer & King, 1970; Falk, 1964; McDonagh & Palma, 1985). The K_d was calculated by computer fitting the binding data employing the binding isotherm (Hulme & Brisdall, 1990).

Preparation of Antisera. Purified 23-kDa protein from HPLC was conjugated with Keyhole Lympets hemocyanin using glutaraldehyde (Cheng et al., 1988). Aliquots (100 μg) of conjugated protein in complete Freund's adjuvant were injected intradermally into a rabbit, followed by two subcutaneous injections of conjugated protein (50 μg) with incomplete Freund's adjuvant at monthly intervals.

Immunoblot Analyses. Proteins were separated by SDS−PAGE using the method of Laemmli (1970) and transferred to nitrocellulose membrane using a Multiphor II Nova blot unit (Pharmacia). Immunodetection was carried out by standard procedures (Harlow & Lane, 1988). Anti-HBP23 antiserum and anti-rabbit IgG antibody were diluted 1000- and 9000-fold, respectively.

Determination of Protein Concentration. Protein concentrations were determined by the method of Bradford (1976). Bovine serum albumin was used as a standard.

Amino Acid Sequence Determination. HBP23 purified by reverse-phase HPLC was alkylated by 4-vinyl pyridine and digested by TPCK-trypsin in 50 mM sodium bicarbonate at 37 °C for 16 h. Digested peptides were separated by reverse-phase HPLC using a Vydac C18 column with a linear gradient of 0−60% acetonitrile in 0.1% trifluoroacetic acid. The amino acid sequence of the peptides of the three major peaks was determined using a gas-phase sequencer (Applied Biosystems 470A). Further sequence determination and tryptic mass mapping of the protein were carried out by mass spectrometry (*vida infra*).

Mass Spectrometry. Mass measurement of the tryptic peptides was accomplished by analyzing approximately $^1/_{25}$ of each HPLC fraction by matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry. A VG TofSpec (VG Analytical, Fisions) mass spectrometer operating in the linear, positive-ion mode equipped with a nitrogen laser emitting 4 ns pulses at a wavelength of 337 nm was used. Samples were prepared by mixing 2 μL of each fraction with 2 μL of the matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/0.1% aqueous trifluoroacetic acid, 1:2). One microliter of sample solution was applied to the sample well and allowed to air dry in the dark. Spectra were generated by averaging 20−30 laser shots.

High-energy positive ion collision-induced dissociation (CID) mass spectra were acquired on a Kratos Analytical Instruments Concept IIHH four-sector tandem mass spectrometer equipped with a continuous flow, liquid inlet probe and a rapid-scanning, charge-coupled, multichannel, array detector (Walls et al., 1993; Burlingame, 1994). HPLC fractions were concentrated on a Speed Vac to approximately 5 μL, diluted to 20 μL with matrix solution (5% thioglycerol/5% acetonitrile/89.9% water/0.1% trifluoroacetatic acid), and then introduced into the source at a flow rate of 3 μL/min. A Cs^+ beam at an energy of 10 kV served as the liquid

secondary ionization source. The collision gas was helium, and the collision cell was floated at 4 kV. Spectra were produced by selecting the C^{12} isobar from the isotope cluster of the protonated molecular ion on MS-1 and then scanning MS-2 from 2000 to 40 Da at 10 s per decade.

Cloning of HBP23 cDNA. First strand cDNA was synthesized with 1 μg of total RNA from liver of a Sprague-Dawley rat using SuperScript reverse transcriptase (GIBCO-BRL, Gaithersburg MD) and random hexamer primers. A PCR experiment was performed using two primers:

5′-AAAATTGGGCACCCTGCTCCCAGCTTCAA-3′

5′-CTTGATGGTATCACTGCCAGGTTTCCAG-
CCAGCTGGGCACACTTC-3′

the sequences of which correspond to the amino acid sequences of two tryptic peptides of HBP23, KIGHPAPSFK, and EVCPAGWKPGSDTIK, respectively. Amplification was carried out by 33 cycles at 94 °C for 45 s, 57 °C for 1 min, and 72 °C for 1 min. The PCR product was purified by 2% agarose gel electrophoresis and cloned into pBluescript KS (Stratagene, La Jolla) at a *Sma*I site. About 5× 104 plaques from a rat liver cDNA library (Clontech RL 1023b, Palo Alto, CA) were screened with the isotope-labeled cloned PCR product. After isolation of two positive plaques, a 0.9 kbp insert was cleaved and subcloned into pBluescript KS vector at an *Eco*RI site. Nucleotide sequences of the fragments were determined by the dideoxynucleotide chain termination method using a 7-deaza Sequenase kit (United States Biochemical Co., Cleveland, OH).

Blot Hybrydization of RNA. A rat multiple tissue northern blot was obtained from Clontech. Each lane of the blot contains 2 μg of poly(A)$^+$ RNAs from heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis of rats. Hybridization was carried out according to the manufacturer's protocol using an isotope-labeled cDNA insert for HBP23. The filter was reprobed with an isotope-labeled β-actin cDNA as a control.

Induction of HBP23. Hepa 1−6 cells were grown in Dulbecco's modified Eagle's medium with 2% fetal bovine serum. After confluency cells were washed, and medium was changed to serum-free medium with 10 mM Hepes (pH 7.2) to which either heme or cadmium chloride was added at a final concentration of 10 μM. Total RNA was collected using RNAzol (Biotecx, Houston, TX) according to the manufacturer's instructions. Fifteen micrograms of total RNA was fractionated on a denaturing formaldehyde agarose and transferred to nitrocellulose membrane.

Poly(A)$^+$-enriched RNA was prepared from the livers after partial hepatectomy or sham surgery. Asialoglycoprotein receptor mRNA was used to normalize the results (Kren et al., 1994).

RESULTS

Purification of HBP23. After heme-agarose chromatography of cytosolic proteins, two major bands, 23 and 50 kDa, and several minor bands were detected on SDS−PAGE (Figure 1, lane 2). A small amount of GSTs was bound by the heme-agarose column, but most of the GSTs were in the washing fractions. No HBP/L-FABP was detected, tested by immunoblotting, in any of the fractions eluted. The 23-kDa protein was separated as a major peak, eluted in 45% acetonitrile by HPLC, and no contaminant was detected on SDS−PAGE (Figure 1, lane 3). HBP23 was also purified using sequential ion-exchange chromatography. The purified protein was a basic protein with a pI of 8.5, as determined by isoelectrofocusing, and was estimated to represent about 0.1% of total cytosolic proteins.

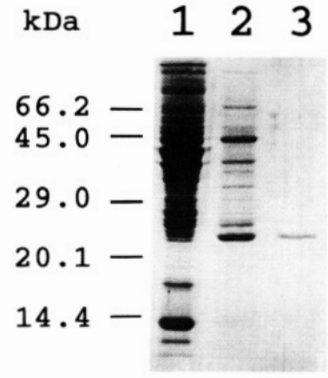

FIGURE 1: Purification steps of HBP23. Fractions of each purification step were analyzed by SDS−PAGE using a 13% polyacrylamide gel. (Lane 1) Rat liver cytosol (20 μg). (Lane 2) Eluted proteins from heme-agarose column (5 μg). (Lane 3) Purified HBP23 obtained by reverse-phase HPLC (1 μg).

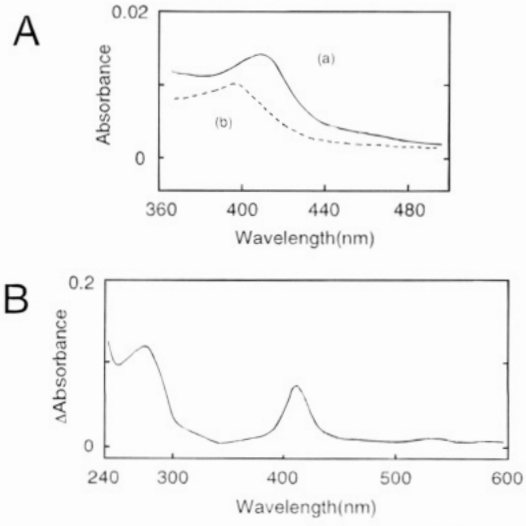

FIGURE 2: Absorption spectrum of the heme−HBP23 complex. (A) Absorption of heme-HBP23 in the Soret region. (Curve a) 0.1 μM heme in 3.4 μM of HBP23 in 20 mM sodium phosphate and 150 mM sodium chloride (pH 7.0); (curve b) 0.1 μM heme by itself. (B) Difference-absorption spectrum for the interaction of heme with HBP23.

Binding Studies. Adding a small amount of heme to the protein solution shifted the peak in the Soret region from that of unbound to heme, from 397 to 408 nm (Figure 2A). Such a spectrum resembles the spectra of heme complexed to GSTs, HBP/L-FABP (Tipping et al., 1976), and albumin (Beaven et al., 1974), which is a major heme-binding protein. The difference-absorption spectrum showed the typical pattern of a heme−protein complex, exhibiting a peak in the Soret region at 412 nm (Figure 2B). Figure 3 shows the result of the titration of heme bound to HBP23 by difference spectrometry; it indicates that HBP23 interacts with heme in a 1:1 molar ratio of heme/protein. Since HBP23 was eluted from Sephadex G-75 column in the same position as that of ovalbumin, its molecular weight appears to be 45000 (data not shown). This finding suggests that HBP23 forms a homodimer in its native condition, and that 1 mol of the dimer binds 2 mol of heme.

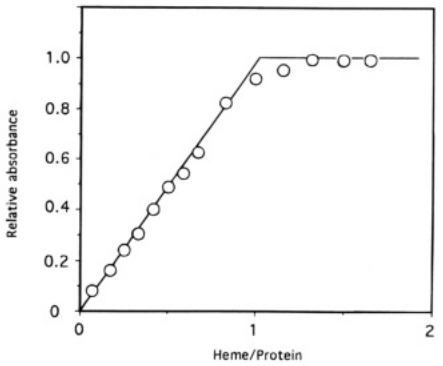

FIGURE 3: Differential spectroscopic titration of heme with HBP23. Effect of increments of heme on the association with HBP23 measured by difference absorption spectroscopy. Absorption was monitored at 415 nm, using 3.1 μM HBP23 in 20 mM sodium phosphate (pH 7.0) and 10% glycerol.

Table 1: Dissociation Constants of the Complexes of Heme, and Other Organic Anions with HBP23[a]

ligand	K_d (μM)
heme	0.055 (0.057)
protoporphyrin IX	0.21
tin-protoporphyrin IX	0.69
bilirubin	1.0
oleic acid	4.4
ANS	6.1

[a] Dissociation constants were measured by fluorometric and difference-absorptimetric (heme only = in parentheses) titrations.

The K_d for the interaction of heme with HBP23 was 57 and 55 nM by difference absorption and fluorescence quenching, respectively. This K_d for heme was lower than those for known heme-binding proteins, HBP/L-FABP and GSTs, which are 120−150 nM (Vincent & Muller-Eberhard, 1985) and 100−200 nM (Muller-Eberhard & Nikkilä, 1989), respectively. Dissociation constants of the interaction with other organic anions were higher than those with heme. K_ds for protoporphyrin IX, tin-protoporphyrin IX, bilirubin, and oleic acid were 210 nM, 690 nM, 1.0 μM and 4.4 μM, respectively (Table 1). The K_d for heme was more than one order of magnitude lower than those for bilirubin and oleic acid. Thus, although HBP23 binds other organic anions, it does bind heme preferentially.

Immunological Studies. HBP23 was detected by immunoblot almost exclusively in the cytosolic fraction of the liver. It was not associated with nuclear or mitochondrial fractions but interacted weakly with the microsomal fraction (data not shown).

Figure 4 shows the tissue distribution of HBP23 by immunoblot. HBP23 was detected in the cytosol of all tissues tested, i.e., small intestine, kidney, heart, and spleen. As compared to liver, the amount of HBP23 was about half in small intestine, kidney, and spleen and less than one-fourth in heart. Since HBP23 was not detected in plasma and red cell hemolysate, the existence of HBP23 in all tissues could not be attributed to the contamination with plasma or erythrocytes.

Amino Acid Sequencing. N-terminal amino acid blockage prevented direct amino acid sequencing of HBP23. After HPLC separation of the tryptic peptides, three internal tryptic sequences were determined by Edman degradation. These sequences were confirmed by high energy CID mass spectra. These sequences had no homology to HBP/L-FABP, GSTs, or any other heme proteins.

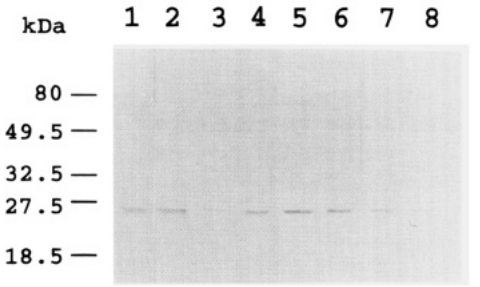

FIGURE 4: Tissue distribution of HBP23 by immunoblot. Cytosolic fractions of rat tissues were separated by SDS−PAGE and blotted onto a nitrocellulose membrane. (Lane 1) Cytosol of small intestine (20 μg). (Lane 2) Cytosol of heart (20 μg). (Lane 3) Cytosol of kidney (20 μg). (Lane 4) Cytosol of spleen (20 μg). (Lanes 5−8) Cytosol of liver (20, 10, 5, and 2.5 μg, respectively).

Mass Spectrometry. HPLC fractions from the tryptic digest of HBP23 were first analyzed by MALDI mass spectrometry. MALDI mass spectrometry is a convenient method for rapidly measuring peptide masses in large numbers of samples. Those fractions that contained peptides were further analyzed by high-energy CID for amino acid sequence determination (Table 2).

Cloning of HBP23 cDNA. To amplify the mRNA for HBP23, two primers were synthesized corresponding to partial sequences of HBP23. The PCR reaction produced a single 550 bp product (data not shown) that was cloned and used for screening of a rat liver cDNA library. Two cDNA clones, whose insert lengths were 0.6 and 0.9 kbp, were isolated using the isotope-labeled probe. The nucleotide and deduced amino acid sequences of the clone contained a longer insert, designated cRHB23kp-2, which is depicted in Figure 5. The clone carried an open reading frame coding 199 amino acids. The amino acid sequences of the tryptic peptides of HBP23 from both Edman analysis and tandem mass spectrometry completely matched the deduced amino acid sequence of the cDNA clone (Figure 5: Edman analysis, double underlined; CID sequences, single underlined). In addition, the estimated (SDS−PAGE) molecular weight (23 000) of HBP23 was compatible with the calculated molecular weight (22 108) of the protein deduced from the cDNA sequence. The clone contained 882 bp of nucleotides, made up of 55 bp of the 5′-untranslated region, 597 bp of the protein coding region, and 230 bp of the 3′-untranslated region. The cDNA is incomplete at its 3′ end and did not contain a poly(A) tail. The putative poly(A) signal is indicated by asterisks in Figure 5. A computer homology search, performed according to the NCBI peptide sequence data base, showed no homology to GSTs, HBP/L-FABP, or any other heme-binding protein or hemeprotein. Instead, HBP23 is a member of the peroxiredoxin family (Chae et al., 1994a,b); HBP23 is highly homologous to three proteins, i.e., human proliferation associated gene (pag) product (Prosperi et al., 1993), mouse macrophage 23-kDa stress protein (MSP23) (Ishii et al., 1993), and human natural killer cell enhancing factor A (NKEF A) (Shau et al., 1994).

In Figure 6 the alignment of the deduced amino acid sequence of HBP23 is compared to those of MSP23, pag, and NKEF A. The sequence of HBP23 shows 96−97% identity with these three proteins, deviating only by six, five, and eight amino acids from MSP23, pag, and NKEF A, respectively.

Table 2: Summary of Data Obtained From Mass Spectrometry

HPLC peak no.	measured MALDI MH^+mass[a]	MH^+ mass selected for CID[b]	calculated mass[c]	sequence[d]	fragment
15	952.2	953.5	953.5	IGHPASFK	8−16
16	893.0	894.4	894.4	ADEGISFR	121−128
17	1163.4	1164.6	1164.6	ATAVMPDGQFK	17−27
18	830.8	831.5	831.5	SVDEILR	152−158
18	1887.6		1887.9	HGEVBPAGWKPGSDTIK	169−185
20	1108.4	1107.6	1107.6	TIAQDYGVLK	111−120
22		1196.7	1196.6	LVQAFQFTDK	159−168
22	1205.5	1208.6	1208.6	JITINDLPVGR	141−151
22		1225.6	1225.6	QITINDLPVGR	141−151
26	1206.3	1208.6	1208.6	JITINDLPVGR	141−151
28	1624.7	1622.9	1622.9	QGGLGPMNIPLVSDPK	94−109

[a] Measured weighted isotopic average MH^+ mass. [b] Measured monoisotopic MH^+ mass, based on $^{12}C = 12.000$. [c] Monoisotopic MH^+ mass calculated from sequence determined by CID. [d] J = pyroglutamic acid.

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                                5' GGCTCACGGTTGGTTCTGTTTGTGAGACCTGTAGCTCGACTCTG      44

CTGATAGCAAG ATG TCT TCA GGA AAT GCA AAA ATT GGG CAT CCT GCT CCC AGC TTC      100
            Met Ser Ser Gly Asn Ala Lys Ile Gly His Pro Ala Pro Ser Phe       15

AAA GCC ACG GCT GTT ATG CCG GAT GGA CAA TTC AAA GAT ATC AGC CTA AGT GAT      154
Lys Ala Thr Ala Val Met Pro Asp Gly Gln Phe Lys Asp Ile Ser Leu Ser Asp       33

TAC AAA GGA AAA TAT GTT GTA TTC TTT TTT TAC CCT CTT GAC TTT ACT TTT GTG      208
Tyr Lys Gly Lys Tyr Val Val Phe Phe Phe Tyr Pro Leu Asp Phe Thr Phe Val       51

TGT CCC ACG GAG ATC ATT GCT TTC AGT GAT AGA GCA GAA GAA TTT AAG AAA CTC      262
Cys Pro Thr Glu Ile Ile Ala Phe Ser Asp Arg Ala Glu Glu Phe Lys Lys Leu       69

AAC TGC CAA GTG ATT GGA GCT TCT GTG GAT TCT CAC TTC TGT CAT CTG GCA TGG      316
Asn Cys Gln Val Ile Gly Ala Ser Val Asp Ser His Phe Cys His Leu Ala Trp       87

ATT AAC ACA CCC AAG AAA CAA GGA GGA TTG GGA CCC ATG AAC ATT CCC TTG GTA      370
Ile Asn Thr Pro Lys Lys Gln Gly Gly Leu Gly Pro Met Asn Ile Pro Leu Val      105

TCA GAT CCC AAG CGC ACC ATT GCT CAG GAT TAT GGA GTC TTA AAA GCT GAT GAA      424
Ser Asp Pro Lys Arg Thr Ile Ala Gln Asp Tyr Gly Val Leu Lys Ala Asp Glu      123

GGT ATC TCT TTC AGG GGC CTC TTT ATT ATT GAT GAT AAA GGT ATC CTT CGC CAG      478
Gly Ile Ser Phe Arg Gly Leu Phe Ile Ile Asp Asp Lys Gly Ile Leu Arg Gln      141

ATA ACA ATA AAT GAT CTT CCT GTT GGC CGC TCT GTG GAT GAG ATT CTG AGA CTA      532
Ile Thr Ile Asn Asp Leu Pro Val Gly Arg Ser Val Asp Glu Ile Leu Arg Leu      159

GTC CAG GCC TTC CAG TTC ACT GAC AAA CAT GGT GAA GTG TGC CCA GCT GGC TGG      586
Val Gln Ala Phe Gln Phe Thr Asp Lys His Gly Glu Val Cys Pro Ala Gly Trp      177

AAA CCT GGC AGT GAT ACC ATC AAG CCT GAT GTC AAT AAG AGT AAA GAG TAT TTC      640
Lys Pro Gly Ser Asp Thr Ile Lys Pro Asp Val Asn Lys Ser Lys Glu Tyr Phe      195

TCT AAG CAG AAG TGA GCACTGGACCAGTTTTCTGGCAGACAGCTTTGAGCAGCCAGAAGAAATTTG      706
Ser Lys Gln Lys End                                                          199

TACTCTACACATGACGTGGTGTGATTCCAGATAAGCCTTTCCTACAAGGGCTAGGGGTGGTTAGCCTTTCT      777

TCCACTATTGGTAAGGGGCAGACCATCTTATATCAGTCACAGAAACCAACCTGTTAATTCTCTCTCTCTCT      848

TTTTTTTTTTTAAGTATCTATTAAACGTGAATTC 3'                                        882
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FIGURE 5: Nucleotide and deduced amino acid sequences of HBP23 cDNA. The sequence contains a single open reading frame that encodes a protein of 199 amino acids as shown below the nucleotide sequence. The consensus polyadenylation sequence is indicated by asterisk. The three tryptic peptides for which amino acid sequences were determined are double underlined. The tryptic peptides determined by mass spectrometry are single underlined.

Northern Blot Analysis. The tissue distribution of HBP23 mRNA, about 1.2 kbp in size, was detected by Northern blot analysis in every organ tested (Figure 7). However, the mRNA expression of the HBP23 gene, normalized with

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                                                                  ***          67
HBP23    MSSGNAKIGHPAPSFKATAVMPDGQFKDISLSDYKGKYVVFFFYPLDFTFVCPTEIIAFSDRAEEFK
MSP23    MSSGNAKIGYPAPNFKATAVMPDGQFKDISLSEYKGKYVVFFFYPLDFTFVCPTEIIAFSDRADEFK
PAG      MSSGNAKIGHPAPNFKATAVMPDGQFKDISLSDYKGKYVVFFFYPLDFTFVCPTEIIAFSDRAEEFK
NKEFA    MSSGNAKIGHPAPNFKATAVMPDGQFKDISLSDYKGKYVVFFFYPLDFTFVCPTEIIAFSDRAEEFK
ratTxP   MASGNAHIGKPAPDFTGTAVV-DGAFKEIGKPDYRGKYVVLFFYPLDFTFVCPTEIIAFSDHAEDFR

                                                                         134
HBP23    KLNCQVIGASVDSHFCHLAWINTPKKQGGLGPMNIPLVSDPKRTIAQDYGVLKADEGISFRGLFIID
MSP23    KLNCQVIGASVDSHFCHLAWINTPKKQGGLGPMNIPLISDPKRTIAQDYGVLKADEGISFRGLFIID
PAG      KLNCQVIGASVDSHFCHLAWVNTPKKQGGLGPMNIPLVSDPKRTIAQDYGVLKADEGISFRGLFIID
NKEFA    KLNCQVIGASVDSHFCHLAWVNTPKKQGGLGPMNIPLVSDPKRTIAQDYGVLKADEGISFRGLFIID
ratTxP   KLGCEVLGVSVDSQFTHLAWINTPRKEGGLGPLNIPLLADVTKSLSQNYGVLKNDEGIAYRGLFIID

                                                  ***                    199
HBP23    DKGILRQITINDLPVGRSVDEILRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVNKSKEYFSKQK
MSP23    DKGILRQITINDLPVGRSVDEIIRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVNKSKEYFSKQK
PAG      DKGILRQIVINDLPVGRSVDETLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVQKSKEYFSKQK
NKEFA    DKGILRQITVNDPPCCRSVDETLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVPKTKEYFSKQK
ratTxP   AKGVLRQITVNDLPVGRSVDEALRLVQAFQYTDEHGEVCPAGWKPGSDTIKPNVDDSKEYFSKHN
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FIGURE 6: Comparison of primary structures of HBP23, MSP23, pag, NKEF A, and rat thioredoxin peroxidase. Alignment of the deduced amino acid sequences of rat HBP23, mouse MSP23 (Ishii et al., 1993), human pag (Prosperi et al., 1993), human NKEF A (Shau et al., 1994), and rat thioredoxin reductase (TxP) (Chae et al., 1994b). The places of amino acid differences from rat HBP23 are denoted by dotted boxes. Conserved VCP regions in a peroxiredoxin family are indicated by asterisks.

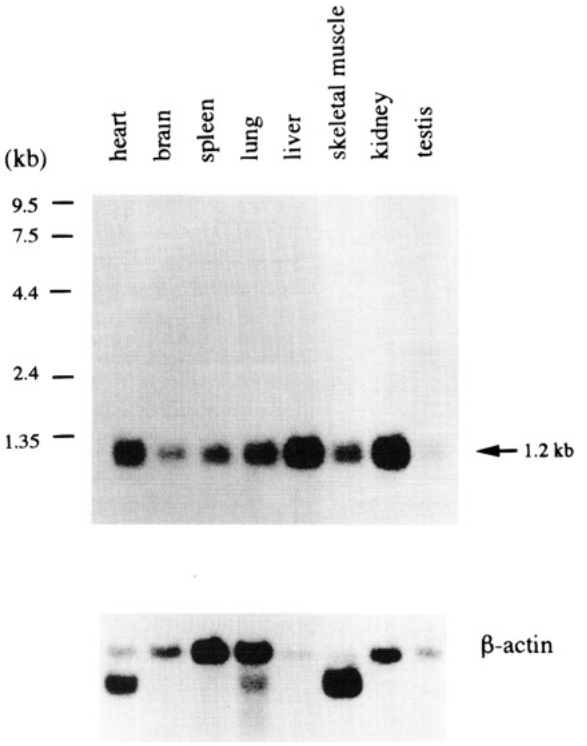

FIGURE 7: Northern blot analysis of the mRNA from several tissues. A multiple tissue Northern blot containing equal amounts of poly-(A) RNA (≃2 μg) (Clontech Laboratories) was hybridized using a full-length cDNA clone. The membrane was washed and reprobed with β-actin cDNA.

β-actin mRNA, was higher in liver and kidney than in testis and brain. The Northern blot pattern correlated well with that displayed by Western blot analysis (Figure 4), suggesting that the expression of HBP23 in the different tissues is regulated on the pretranslational level.

Induction of HBP23 mRNA Levels. Treatment of mouse Hepa 1−6 cells with heme or $CdCl_2$, each at a concentration of 10 μM, increased HBP23 mRNA after 3 h (Figure 8). In addition, the level of HBP23 mRNA was already increased 3 h after partial hepatectomy with peak expression at 24 h

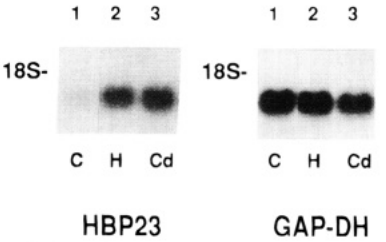

FIGURE 8: Regulation of HBP23 mRNA levels in Hepa 1−6 cells treated with heme and cadmium. Hepa cells were incubated for 3 h in either serum-free control medium (lane 1) or medium containing heme (lane 2) or cadmium chloride (lane 3) at a concentration of 10 μM. Total RNA was isolated, and 15 μg samples were electrophoresed and transferred to a nitrocellulose membrane. The filter was hybridized successively with HBP23 cDNA and GAP-DH cDNA.

and sustained expression until 48 h. By comparison, the mRNA level, with the exception of a slight decrease after 18 h, did not change after sham surgery (Figure 9).

DISCUSSION

HBP23, a high affinity heme-binding protein, does not share amino acid sequences with any of the other heme-binding proteins in liver cytosol. The other major heme-binding proteins are 14-kDa HBP/L-FABP and the GSTs, the latter occur naturally as dimers (subunits are 25−28 kDa) as does HBP23. The role of none of these proteins in heme metabolism in hepatocytes has been clarified.

Alterations in the level of cellular heme pools cause changes in the amounts of heme-metabolizing enzymes and in cell differentiation and growth (Sassa, 1988). The concentration of the regulatory "free" heme pool has been suggested to be between 10 and 100 nM (Granick et al., 1975). Thus, it has been estimated that a K_d of 100 nM would be most suitable for a postulated heme−apoprotein complex which regulates the synthesis of δ-aminolevulinic acid synthase, the first enzyme of heme synthesis (Badawy, 1978; Bonkovsky et al., 1991). This postulated regulatory protein is still unknown. Candidates are HBP23 and the previously recognized heme-binding proteins, HBP/L-FABP and the GSTs. In addition, one cytosolic hemeprotein,

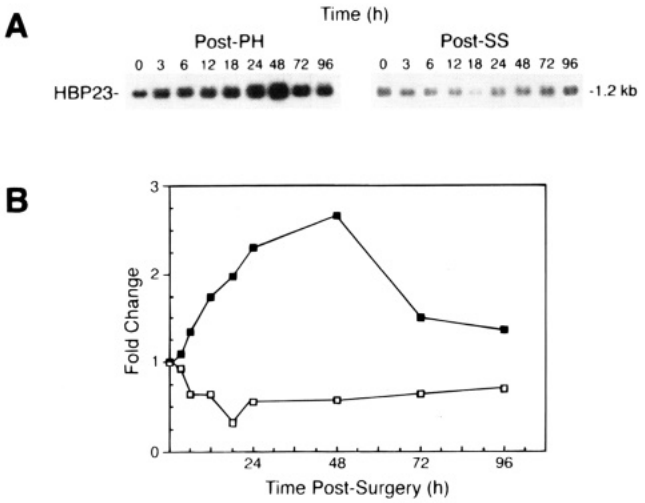

FIGURE 9: Regulation of HBP23 mRNA levels in rat liver after partial hepatectomy. RNA samples were obtained 0, 3, 6, 12, 18, 48, and 72 h after partial hepatectomy or sham surgery. (A) Five micrograms of poly(A)$^+$-enriched RNA was loaded, electrophoresed, transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled HBP23 cDNA. (B) Graphic illustration of the levels of HBP23 mRNA for the data shown in panel A. HBP23 mRNA levels for each time point were normalized by using the asialoglycoprotein receptor cDNA as a control. Closed squares indicate post partial hepatectomy (Post-PH), and open squares indicate post sham surgery (Post-SS).

tryptophan pyrrolase, has been considered as a marker of the "free" or "readily exchangeable" heme pool. Its concentration in liver and K_m for heme are estimated to be 0.1 and 6 nM, respectively (Greengard & Feigelson, 1962). Assuming that the majority of the heme in the regulatory pool is in the cytosol and "free", i.e., not constitutively in hemeproteins, we compared the percentages of how much of each of the four proteins would contain heme and how much of the cytosolic heme could be expected to be in each of them for a cellular concentration of heme at 10 or 100 nM. We took into consideration the relative affinities of the proteins for heme, taking an average of 150 nM for the heme/protein interaction of HBP/L-FABP as well as for that of the GSTs [see Table 1 in Muller-Eberhard and Nikkilä, (1989)], and their concentrations (Vincent et al., 1987; Fleischner et al., 1972). For the latter parameter our estimates were based on a total protein concentration of 25 mg/mL in cytosol of a 25% homogenate. For a 10 nM regulatory "free" heme pool each protein would contain the following percentage of the cytosolic heme: HBP23, 4.7; HBP/L-FABP, 1.6; the GSTs, 1.6; and tryptophan pyrrolase, 29.1. The percentage of the amount of heme in HBP23 would be 2.0, in HBP/L-FABP, 58.1, in the GSTs, 15.0, and tryptophan pyrrolase, 0.3. The corresponding percentages for a 100 nM regulatory "free" heme pool would be for HBP23, 35.7 and 1.5, for HBP/L-FABP, 15.6 and 56.2, for the GSTs, 15.6 and 14.5, and for tryptophan pyrrolase, 82.2 and 0.1. The "free" heme concentration for 10 nM would be 24.6%, and for 100 nM it would be 27.72%. At both concentrations, the major amount of heme would be found in HBP/L-FABP and the least amount in tryptophan pyrrolase. In view of the low concentration of HBP23, in comparison to HBP/L-FABP and the GSTs, an increase in hepatic heme concentration would cause a larger change in the heme/protein ratio for HBP23 than in that for the other proteins. Therefore, HBP23 may have a more specialized function, e.g., a regulatory function. It is of interest to note that HBP23 also binds protoporphyrin IX and tin-protoporphyrin IX with a lower K_d than those of the other proteins, suggesting that HBP23 may mediate intracellular effects caused by porphyrins and metalloporphyrins in addition to heme.

We assessed whether HBP23 is responsive to alterations in heme metabolism by using two experimental conditions. First, the mRNA levels of HBP23 were monitored after addition of heme to cultured cells. This changes the cellular heme pool and induces the heme catabolizing enzyme heme oxygenase (Alam et al., 1989; Cable et al., 1993). Secondly, HBP23 mRNA levels were determined in the regenerating liver after partial hepatectomy. During regeneration the level of δ-aminolevulinic acid synthase decreases and that of heme oxygenase increases (Srivastava et al., 1982; Stout & Becker, 1986). Under both conditions HBP23 mRNA levels increased while control proteins (GAP-DH or asialoglycoprotein receptor) and HBP/L-FABP did not (preliminary observation). These findings suggest that the changes in HBP23 mRNA levels are related to heme metabolism, a suggestion compatible with the occurrence of HBP23, unlike that of HBP/L-FABP, in many tissues.

The N-terminal amino acid of the mature protein is blocked. The amino acid analysis of HBP23 indicates that HBP23 contains two methionines. Three methionines are present at positions 1, 21, and 100 in the deduced amino acid sequence (Figure 5), the first methionine of which might have been cleaved. The N-terminal amino acid may be acetylated serine, acetylation being the most common blocking motif in soluble proteins.

High-energy CID mass spectrometry is now established as a powerful and versatile method for determination of protein primary sequence and structure with a sensitivity at least comparable to that of Edman degradation. It has advantages over Edman degradation in its inherent high speed and ability to deal with covalently modified peptides and mixtures directly (Burlingame, 1994). This technique was employed to deduce independently the sequence of a significant amount of the entire protein (40%, see Table 2) to establish unambiguously that the cDNA sequence did not contain errors. During this work two nonadjacent HPLC fractions were found to contain a peptide of molecular weight 1208.6 which did not agree with the molecular weight of any peptide anticipated on the basis of the cDNA sequence. The CID mass spectrum of this component is shown in Figure 10A. From interpretation of this spectrum it became clear that it represented the amino acids 141−151 with pyroglutamic acid as an N-terminal residue of the peptide. This and the fact that fraction 22 also contained a peptide of MW 1225.6 possessing the exact sequence (compare with Figure 10B) corresponding to the cDNA, including an N-terminal glutamine residue, readily resolved the observation of these two components.

Homology search revealed that HBP23 is a member of the peroxiredoxin family, the most extensively studied among which is yeast thioredoxin peroxidase, which is a recently recognized class of antioxidants (via the thiol group of cystein). The structure of these proteins has been conserved from bacteria to human (Chae et al., 1994b). HBP23 contains characteristic sequences of this family which includes three highly homologous proteins: mouse macrophage 23-kDa stress protein (MSP23; Ishii et al., 1993),

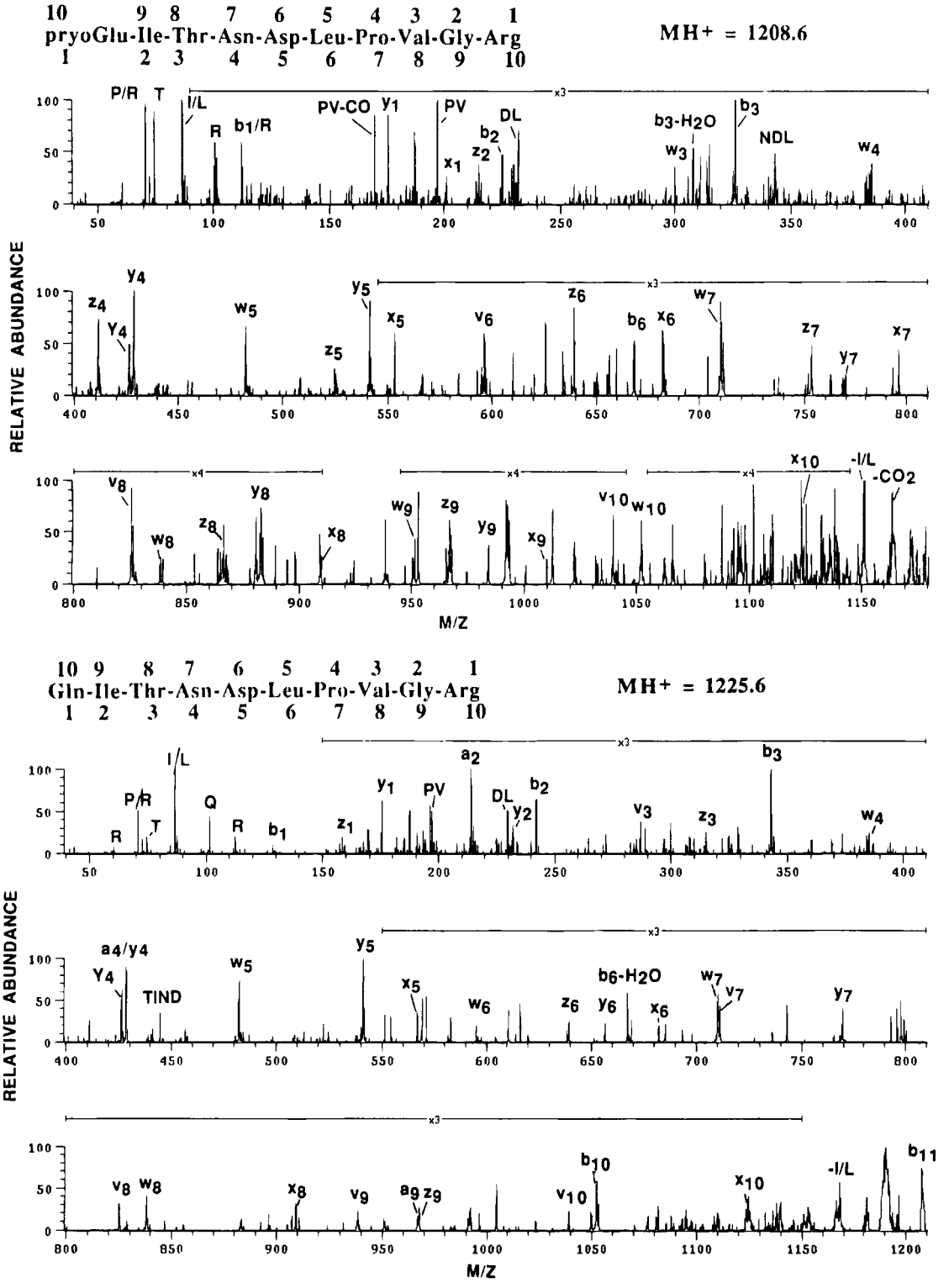

FIGURE 10: High-energy CID spectra of two HBP23 peptides. The tryptic peptides of HBP23 were MH^+= 1208.6 Da (A, top) and 1225.6 Da (B, bottom). Immonium ions corresponding to individual amino acid residues are denoted by the single-letter code. Peptide backbone cleavage ions formed from fragmentation indicative of charge retention at the C terminus are denoted by x, y, and z. N termini cleavages are denoted by a and b, and ions formed by side-chain fragmentation are denoted as v and w. The difference between these two spectra is in the conversion of glutamine to pyroglutamic acid (J). The b ions are shifted by 17 Da. The nomenclature first proposed by Roepstorff and Fohlman (1984), and modified by Biemann (1988), is used for peptide fragment ions.

human proliferation associated gene (pag; Prosperi et al., 1993) product, and human natural killer cell enhancing factor A (NKEF A; Shau et al., 1994). The amino acid identity of these proteins with HBP23 is 96−97% (Figure 6), whereas the amino acid identity of human (Lim et al., 1994a) and rat (Chae et al., 1994b) thioredoxin peroxidase with HBP23 is 74%. Thioredoxin peroxidase is one amino acid shorter than HBP23, and thioredoxin peroxidase is, unlike HBP23 (pI = 8.5), an acidic protein (pI = 5.2, calculated from the deduced sequence). These findings suggest that HBP23 belongs to a subgroup, or is an isozyme, of thioredoxin peroxidase. However, the tissue distribution of these two proteins differs.

Thioredoxin peroxidase occurs mainly in brain, liver (Kim et al., 1989) and red blood cells (Lim et al., 1994b), whereas HBP23 occurs mainly in liver and kidney and is not detected in red blood cells. This finding indicates that the regulation of these proteins may also differ. Nevertheless, HBP23 may be a ubiquitous stress protein inducible by oxidant stress, as is the homologous MSP23 (Ishii et al., 1993), and may protect cells by binding heme, which is a prooxidant molecule. We plan to compare the regulation of HBP23 with that of thioredoxin peroxidase and investigate the function of this heme-binding protein.

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