# A Novel Approach for Identifying the Heme-Binding Proteins from Mouse Tissues

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**Heme is a key cofactor in aerobic life, both in eukaryotes and prokaryotes. Because of the high reactivity of ferrous protoporphyrin IX, the reactions of heme in cells are often carried out through heme-protein complexes. Traditionally studies of heme-binding proteins have been approached on a case by case basis, thus there is a limited global view of the distribution of heme-binding proteins in different cells or tissues. The procedure described here is aimed at profiling heme-binding proteins in mouse tissues sequentially by 1) purification of heme-binding proteins by heme-agarose, an affinity chromatographic resin; 2) isolation of heme-binding proteins by SDS-PAGE or two-dimensional electrophoresis; 3) identification of heme-binding proteins by mass spectrometry. In five mouse tissues, over 600 protein spots were visualized on 2DE gel stained by Commassie blue and 154 proteins were identified by MALDI-TOF, in which most proteins belong to heme related. This methodology makes it possible to globally characterize the heme-binding proteins in a biological system.**

**Key words: heme, heme-agarose, proteomics, mouse**

## Introduction

In a cellular environment, cofactors can have significant effects on their corresponding proteins' folding status (*1*). Many proteins hence require cofactors, such as metal ions and organic moieties, to perform their biological activities. The studies on cofactor-protein interactions underscore the importance of understanding protein functions.

Heme is essential to aerobic life, both in eukaryotes and prokaryotes (*2*). It is a complex of iron with protoporphyrin IX and serves as the prosthetic group of numerous heme-binding proteins, in which heme plays a versatile and ubiquitous role to form the active centers (*3*). Basically heme interacts with proteins in two different ways. In heme-containing proteins, protoporphyrin IX covalently combines with the amino acid residue(s) in a protein, such as hemoglobin and cytochromes. Another kind heme-related protein is heme-binding in which heme only interacts with proteins non-covalently and reversibly, such as heme binding to aldehyde dehydrogenase. Both heme-related proteins are involved in an extraordinary array of biological functions, ranging from electron transfer, catalysis, oxygen transport and storage, oxygen metabolism, signal transduction, and controlling gene expression. Moreover, recently heme-protein interactions have been found to regulate the cell cycle in part via a specific heme-DNA binding protein, either for differentiation or for antiapoptosis (*4*). Compared to the standard textbook examples of heme-containing proteins in oxygen transport by $\alpha$-helical myoglobin and hemoglobin, today our knowledge regarding the potential and versatile functions of heme and heme-related proteins has reached a new level, at which an insight into the global array of heme-related proteins in a cell or in a tissue would be extremely beneficial.

The organismal heme is mainly synthesized in immature erythroid cells, and the molecular mechanism for heme trafficking and assembly of heme-binding proteins is far less than adequate (*5*). However, many laboratories have confirmed that heme requirements vary significantly among different cells and tissues (*6*). For instance, in rat hepatocytes 20% of newly formed heme is directly converted to bile pigment, whereas 80% is used for the formation of heme-related proteins (*7*). Thus, a logical assumption is the heme-related proteins could be in a wide concentration range in

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various tissues or cells, corresponding to the variable heme concentrations as well as different gene expressions. The integrated information gathered from the distribution of heme-related proteins in tissues constitutes the basis for understanding the biochemical interactions between heme and proteins. To detect and to identify heme-related proteins is not, however, a trivial task due to many technical challenges (*8*). The chemical reactivity of heme in the presence of oxygen affects the stability of heme-protein interactions. Furthermore, heme is an amphipathic molecule, not readily soluble in aqueous solution, whereas the extraction of heme-related proteins by organic solvents complicates in protein isolation as well identification. Moreover, a number of heme-containing proteins are membrane or membrane attached proteins that are difficult to be isolated and purified. With the development of technology-driven high throughput methods in functional genomics and proteomics, it has become possible to analyze expression profiles in a genomic scale (*9*). Currently the strategy, which is composed of optimized enrichment protocols such as fractionation techniques or specific chromatography and sensitive peptides determination by mass spectrometry, is viable with functional proteomics. Proteomic analysis can provide us an insight into heme-protein field.

In this communication we have developed a novel approach to study a large number of heme-binding proteins from mouse tissues. To avoid the technical problems, in this project we focused at studying heme-binding but not heme-containing proteins, and soluble but not insoluble hem-binding proteins. Using a novel combination of sample preparation with heme affinity chromatography, and two-dimensional electrophoresis (2DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), we were able to detect more than 600 protein spots on 2DE gel stained by Commassie blue and to specifically identify 154 heme-binding proteins. Therefore, our methodology makes it possible to extensively investigate heme-binding proteins in mammalian tissues.

# Results and Discussion

## Purification of heme-binding proteins by heme-agarose resin

Several resins have been developed to purify heme-binding proteins (*10*, *11*). The preparation of heme-agarose using 1,1'-carbonyldiimidazole (CDI) is an efficient affinity resin with high capacity as well as selectivity for heme-binding proteins (*12*). Heme-agarose has been widely used for purifying heme-binding proteins (*13*, *14*). Also, this resin can be employed for estimation of heme-binding sites on a protein (*12*). Although this affinity material was invented many years ago and a number of proteins have been purified by this resin either in column chromatography or batch approach, an investigation into the global profile of heme-binding proteins has not yet been reported yet, partially due to the technique barriers in protein identification. Proteomic analysis, especially equipped with the high accuracy of mass spectrometry for protein identification, has led protein studies to a genomic scale (*15*). An emerging field so called "functional proteomics" aims to create tools and assays for the characterization of protein samples of high complexity by utilizing synthetic chemistry. These approaches include the development of chemical affinity tags to measure the relative expression level and post-translational modifications of proteins in cell and tissue proteomes (*16*). In this study we attempt to develop a proteomic strategy in the studies of heme-binding proteins.

The volumes of the soluble proteins extracted from some mouse tissues were limited for a column chromatography. Hence a batch approach was employed to purify the heme-binding proteins. The contents of soluble proteins as well as heme-binding proteins in six different mouse tissues are shown in Fig. 1, indicating that the yield of soluble proteins correlates with the concentration of heme-binding proteins. The values of distributions of heme-binding protein in total soluble protein range from 0.2% to 0.54% among these tissues. It is not surprising that, compared to other tissues, mouse liver contains the highest concentrations of soluble and heme-binding proteins, due to many metabolic and detoxifying enzymes involved in this organ. In contrast to liver, mouse muscle and stomach show much less heme-binding proteins. The concentration data in Fig. 1 demonstrate that mouse tissues may have a number of proteins with heme affinity, but different tissues may favor certain heme-binding proteins, either in quantity or in quality.

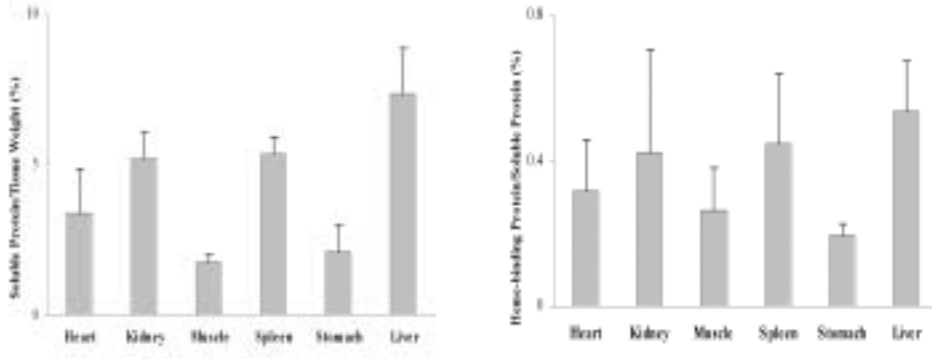



**Fig. 1.** Distribution of the protein yields in relation to extraction procedures. A) The yields of soluble poroteins extracted from mouse tissues; B) The yield of heme-binidng proteins purified from the soluble proteins of mouse tissues by heme-agarose.

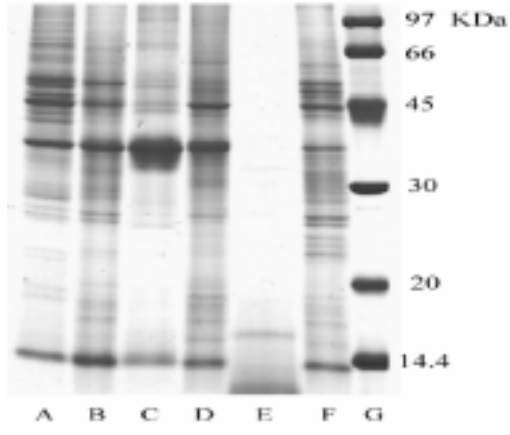
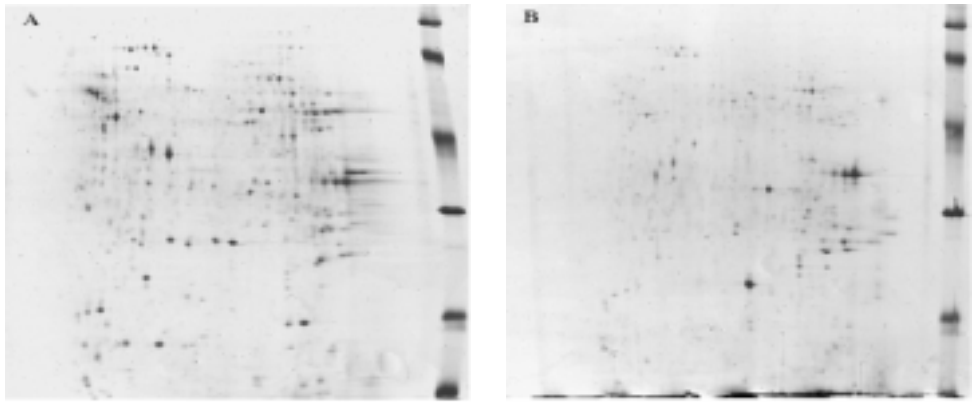



**Fig. 2.** SDS-PAGE analysis of heme-binding proteins obtained from six mouse tissues and stained with Coomassie Brilliant Blue. Lanes: A, heart; B, kidney; C, muscle; D, stomach; E, liver; and F, protein ladder.



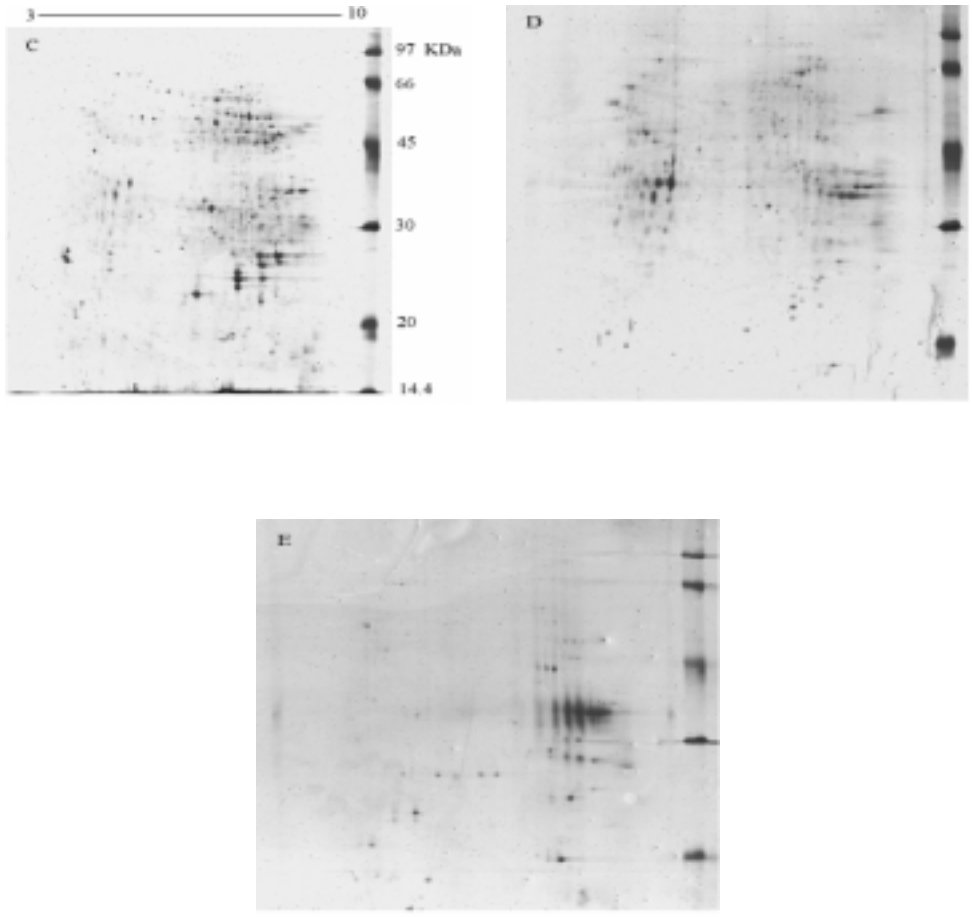



**Fig. 3.** Two-dimensional electrophoretic analysis of heme-binding proteins from mouse tissues. The proteins were separated on a pH 3-10 IPG strip, followed by a 12% SDS-polyacrylamide gel. The gels were stained with Coomassie Brilliant Blue. Gels: A, heart; B, kidney; C, liver; D, spleen; and E, muscle.

## Isolation of heme-binding proteins by electrophoresis

To further compare the distributions of heme-binding proteins in mouse tissues, the purified proteins were analyzed by 12% SDS-PAGE. In Fig. 2, it is clear that each tissue has a unique pattern of heme-binding proteins. Surprisingly, even though with equal protein loading in all lanes, few protein bands in the extraction of mouse stomach were visualized on this SDS-PAGE gel. This appears to be due to proteolysis and protein size. To examine the degradation of stomach proteins during the sample preparation, we conducted the experiments under strict control in proteolysis and reduced the operation time from sample preparation to electrophoresis. Four repeated experiments came to the same observation, suggesting proteolysis is unlikely a key factor for the bias distribution of heme-binding proteins in mouse stomach. Another interesting phenomenon is that on the gel the smear staining region below 14 kDa in the sample of mouse stomach indicates that this protein extract may contain a number of polypeptides in small sizes, but with affinity to heme. On SDS-PAGE gel we observed several proteins exist in all five mouse tissues, such as 14, 40, 45 and 50 kDa bands. Except liver, the protein around 40 kDa appears to be a major component in the purified proteins, indicating it is a high abundance heme-binding protein.

Shown in Fig. 3 are the proteome 2DE profiles of heme-binding proteins extracted from mouse tissues. Since mouse stomach doesn't contain the detectable heme-binding proteins above 14 kDa, we have only analyzed the protein extracts from five mouse tissue

in 2DE. To monitor a broad range of these proteins, either in pI or in molecular weight, the first dimensional electrophoresis was conducted in nonlinear pH 3-10, and the second electrophoresis was run at 12% polyacrylamide separating molecules from 10 to 100 kDa. In Fig. 3 these 2DE profiles display good resolution with only minimal streaking. Although the loading proteins in each gel were in equal amount, the stained spots by Coomassie blue are variable on these gels. The image dada analysis is summarized in Table 1, which indicates the number of stained spots in heart, kidney, muscle, spleen, and liver is 155, 118, 56, 135 and 202, respectively. Similar to the pattern of heme-binding proteins distribution in mouse tissues, of five the protein extracts, mouse liver has the highest number of protein spots. Interestingly, the 2DE profiles display two different patterns regarding the distribution of protein spots. In kidney, muscle and liver, the major heme-binding protein spots are located in at alkalic pI region; whereas in heart and spleen, the heme-binding proteins spread through entire pH range. Analysis of data in Table 1 reveals that these heme-binding proteins found in two tissues less overlap. For instance, a total of 202 protein spots were visualized in mouse liver, but these spots only overlap with 28 in heart, 15 in muscle, and 22 in spleen, respectively. Except the proteins in mouse muscle, where a few protein spots were detected, most overlapping rates in other tissues are not more than 30%, indicating mouse may have a number of tissue specific heme-binding proteins. However, there are a few of exceptions. For instance, the heme-binding proteins in kidney overlap 45% of those proteins found in liver.

**Table 1 Summary of Image Data for Analyzing 2DE Profiles of Heme-Binding Proteins Purified from Mouse Tissues**

| | Heart | | Kidney | | Muscle | | Spleen | | Liver | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Shared | Unshared | Shared | Unshared | Shared | Unshared | Shared | Unshared | Shared | Unshared |
| Heart | 155 | - | 33 | 85 | 27 | 29 | 33 | 102 | 28 | 174 |
| Kidney | 33 | 122 | 118 | - | 17 | 39 | 34 | 101 | 53 | 149 |
| Muscle | 27 | 128 | 17 | 101 | 56 | - | 20 | 115 | 15 | 187 |
| Spleen | 33 | 122 | 34 | 84 | 20 | 36 | 135 | - | 22 | 180 |
| Liver | 28 | 127 | 53 | 65 | 15 | 41 | 22 | 113 | 202 | - |

## Identification of heme-binding proteins by mass spectrometry

A total of 666 protein spots were analyzed by ImageMaster software. We further excised 288 spots with high volume values for protein identification using MALDI-TOF. Of these spots, 154 proteins were verified by mass spectrometry. As shown in Table 2, we list the top ten identified proteins from five mouse tissues, ranked according to the unified scores calculated by Mascot.

As described above, SDS-PAGE showed a major protein band around 40 kDa that was observed in all mouse tissues. The same phenomenon was observed on 2DE gel as well. In contrast to Fig. 2, three proteins spots appeared on the 2DE gels of five mouse tissues (Fig. 3) with apparent molecular weight of 40 kDa and pI of 8-9. Furthermore these spots were analyzed by MALDI-TOF, and the resultant identifications are two proteins, heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2/B1) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), both having a close theoretical molecular weight of 36 kDa, and different theoretic pI values, 8.61 and 8.87, respectively. An hnRNP is capable of shuttling between the nucleus and cytoplasm, as an RNA destabilizing protein *in vivo*. During hemin-induced erythroid differentiation, the RNA destabilizing function was impeded because hnRNP was proposed to form a complex with a hemin-induced protein (*17*). GAPDH is a housekeeping gene and distributed high abundance in lot of tissues. Although there is no report related to heme binding to GAPDH, heme is really involved in GAPDH expression and activity. For instance, in chicken erythroblast cell line HD3, the activity of GAPDH activity and the corresponding mRNA level were dramatically increased in the presence of hemin (*18*). These changes decreased and virtually disappeared in the post-induction period.

Based upon the references, we classify these identified proteins in Table 2 into three categories, heme-binding proteins, heme related proteins, and heme un-

related proteins. 1) Heme-binding proteins. In this class these proteins, such as $\alpha$-actin (*19*), $\beta$-actin, GST (*20*), peroxiredoxin (*21*), thioredoxin (*22*), ATP synthase (*23*), aldehyde dehydrogenase (*24*) and ferritin (*25*), were reported to have a direct interaction with heme. The functions of these proteins are significantly affected due to heme-binding. For instance muscle actin binds to hemin with high affinity at $K_a$ value of 5.3 $\mu$M (*19*). When actin was incubated with hemin for 24 h, the amount of actin polymer was markedly reduced, suggesting that high hemin concentration might be toxic to cells by interfering with actin polymerization cycles. 2) Heme related proteins. Several proteins in Table 2, such as heat shock proteins (*26*), glutamate dehydrogenase (*27*), adenylate kinase (*28*), creatine kinase (*29*), GAPDH (*18*), isocitrate dehydrogenase (*30*), tubulin (*31*) and phosphoprotein phosphatase (*32*), have been implicated in the adaptive response to the impact of heme induction or heme oxygenase-1 (HO-1) activation. A typical example is the effects of heme on creatine kinase. Funanage *et al.* found that 20 $\mu$M hemin caused increases in the number, size, and alignment of myotubes of satellite cells isolated from rat skeletal muscle (*29*). Notably, with incubation of hemin, the specific activity of creatine kinase increased over 50%, especially two muscle-specific creatine kinase isoenzymes (MM-CK and MB-CK) with high sensitive to hemin concentration. These results thus demonstrated a physiological significant role for heme in myotube maturation. 3) Heme un-related proteins. A few of proteins such as thiolase, cofilin, mercaptopyruvate sulfurtransferase and dihydrolipoamide dehydrogenase have not been documented their affinities to heme so far.

**Table 2 Comparison of Identities of Protein Spots from Mouse Tissues**

| Tissue Rank | Heart | Kidney | Muscle | Spleen | Liver |
|---|---|---|---|---|---|
| 1 | ATP synthase | G3PD | Creatine kinase | hnRNP A2/B2 | hnRNP A2/B2 |
| 2 | hnRNP A2/B2 | Glutamate dyehydrogenase | ATP synthase | mercaptopyruvate sulfurtransferase | mercaptopyruvate sulfurtransferase |
| 3 | mercaptopyruvate sulfurtransferase | Peroxiredoxin | Adenylate kinase | $\beta$- actin | Aldehyde dehydrogenase |
| 4 | Isocitrate dehydrogenase | $\beta$- actin | G3PD | $\alpha$- actin | Thiolase |
| 5 | Elongation factor | Adenylate kinase | Heat shock protein 27 | cofilin | Peroxiredoxin |
| 6 | Heat shock protein 70 | $\beta$- thydroxysteroid dehydrogenase | Phosphoprotein phosphatase | Tubulin-$\alpha$ | Glutamate dyehydrogenase |
| 7 | $\alpha$- actin | Phosphoprotein phosphatase | $\alpha$- crystallin | Heat shock protein 71 | Homocysteine methyltransferase |
| 8 | Fumarate dehydrogenase | Isocitrate dehydrogenase | RNase S2 | Peroxiredoxin | Heat shock protein 71 |
| 9 | Glutamate dyehydrogenase | GST- $\pi$ | | G3PD | Thioredoxin peroxidase |
| 10 | G3PD | Ferritin | | Tubulin- $\beta$ | Dihydrolipoamide dehydrogenase |

Note: The identified proteins are ranked according to the unified scores calculated by Mascot.

In summary, we have developed an integrated technique of proteomic analysis which enables an array of heme-binding proteins. Our results clearly show that the heme-binding proteins are widely distributed in a number of mouse tissues. Of these identified proteins, some exist in all five tissues and some

are tissue specific; most of them have been confirmed with heme affinity by the earlier studies and the others may be new members in the family of heme-binding proteins. Therefore, the presented procedure may not only be a helpful tool in an endeavor to globally characterize heme-proteins, but also support the solid data to depict an overall feature of heme's functions in cells or tissues. The nature of several identified proteins and their correlation with affinity to heme are currently under study.

# Materials and Methods

## Materials

Mice strain ICR (body weight around 30 g) raised under pathogen free conditions was obtained from Beijing Medical University (Beijing, China). IPG strips, ampholytes and all chemicals employed for electrophoresis were purchased from Amersham Biosciences (Uppsala, Sweden). Heme-agarose and all chemicals of analytical grade were from Sigma (St. Louis, MO, USA). All HPLC solvents were from J. T. Baker (Phillipsburg, NJ, USA). Modified trypsin (sequence grade) was obtained from Promega (Madison, WI, USA).

## Sample preparation

Phenobarbital was injected to anaesthetize the mouse. The mouse abdomen was opened and the tissues, including liver, spleen, stomach, kidney, heart, and skeletal muscle, were excised carefully to avoid blood contamination. The isolated tissues were being perfused with cold phosphate buffer saline (PBS) to remove excess blood. The washed tissues were stored at -20° C until analysis.

The frozen mouse tissues were placed into liquid nitrogen and were immediately powdered. The aliquots of homogenization buffer, containing 25 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 1 mM DTT, 25 $\mu$g/mL leupeptin, 25 mM NaF, and 1 mM $Na_3VO_4$, was mixed with the powdered tissues according to the ratio of weight (g)/buffer volume (mL) as 0.5:1. The mixtures were further homogenized by sonication with a probe sonicator. The homogenates were then centrifuged at 14,000 *g* for 15 min and the resulting supernatants were collected as cytosolic proteins for affinity purification.

## Purification of heme binding proteins with hemem agarose

The resin of hemin agarose was fully equilibrated with the binding buffer, 20 mM $NaPO_4$, pH 7.4, 500 mM NaCl. The equilibrated resins were incubated with the prepared mouse tissue cytosolic proteins, according to the ratio of protein (g)/resin volume (mL) as 1:5. To compare the concentrations of heme-binding proteins among the tissues, in all tissues the equal amount of cytosolic proteins was loaded onto the equal volume of affinity resin. The slurries were incubated for 1 h at room temperature with constant rotation followed by centrifugation at 5000 g to remove the unabsorbed supernatant. The resins were washed thoroughly with binding buffer, but finally went through 2×1 mL wash with 20 mM Tris-HCl, pH 8.0 until no protein could be detected in the wash solution. The bound proteins were eluted by adding 500 $\mu$L of elution buffer containing 20 mM Tris-HCl, pH 8.0, 20 mM DTT and 8 M urea. The protein concentration was determined by modified Bradford's method using BSA dissolved in 6 M urea as a standard.

## Electrophoresis

For estimating how many were proteins partially purified by the affinity batch approach, the eluted proteins from heme-agarose were loaded onto a 12% SDS-polyacrylamide gel using a Bio-Rad electrophoresis device (Hercules, CA, USA). The separated proteins were visualized by staining with Coomassie Brilliant Blue.

For studying the purified heme-binding proteins in detail, 2DE was applied to separate the proteins. Commercial IPG strips (Amersham Biosciences, Uppsala, Sweden), 13 cm with a nonlinear range of pH 3-10, were rehydrated overnight with 250 $\mu$L of solution containing 8 M urea, 2% w/v CHAPS, 20 mM DTT, 0.5% v/v IPG buffer, 0.002% bromophenol blue and 150 $\mu$g sample protein. Electrofocusing was carried out for 64 kVh at 20 ° C using a gradually increasing voltage. The electrophoresed strips were placed into 12% SDS-polyacrylamide gels (15 × 20 cm) and were further electrophoresed by an Ettan 2-DE system (Amersham Biosciences, Uppsala, Sweden) with a programmable power control, 0.5 h at 0.5 W per gel, then at 15 W per gel until the dye front reached the gel bottom. The separated proteins were visualized by Coomassie Brilliant Blue staining. Images of the stained gels were acquired with an Amersham Biosciences Image Scanner (Uppsala, Sweden) using

transmitted light. The gel image files were subsequently analyzed by ImageMaster 2D Elite (Amersham Biosciences, Uppsala, Sweden).

### Tryptic digestion and peptides extraction

The 2DE gel pieces were excised, successively destained with 50% acetonitrile and dehydrated with pure acetonitrile. Proteins were reduced with 10 mM DTT at 56 ° C for 1 h and alkylated by 55 mM iodoacetamide in the dark at room temperature for 45 min *in situ*. The gel pieces were thoroughly washed with 25 mM ammonium bicarbonate in 50% acetonitrile solution (percentage) and were completely dried in a Speedvac. Proteins were digested in 20 $\mu$L of modified trypsin solution (5 ng/$\mu$L trypsin in 25 mM ammonium bicarbonate buffer) by incubation overnight at 37 ° C. The peptides were extracted by vigorous shaking in 50 $\mu$L of 50% acetonitrile containing 2.5% TFA.

### Mass spectrometry

The digested peptides from 2DE gel spots were analyzed by MALDI-TOF-MS using an AutoFlex (Bruker Daltonics, Billerica, MA) in a reflectron mode. This instrument employed a 337-nm pulsed nitrogen laser to generate ions. Recrystallized $\alpha$-cyano-4-hydroxycinnamic acid (HCCA) was used as the matrix, which was suspended in the solvent of acetonitrile/water/TFA 50:49.9:0.1. Before mass determination, the tryptic peptides obtained from in-gel digestion were mixed with HCCA suspension in the ratio of 1:1. The matrix mixtures were applied onto the target well and dried at room temperature.

Monoisotopic peptide masses obtained from MALDI-TOF were queried against entries for mouse cDNA and protein databases in NCBI using a protein search program, Mascot (Matrix Science Ltd, London). The mass accuracy of 50 ppm in the parent ion mass and 0.1 Da in the product ion mass was used as search parameters.

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