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Analysis of murine S-glutathionyl hemoglobins and beta globin haplotype by dynamic capillary isoelectric focusing☆

James M. Hempe[a,b,*], Amanda M. McGehee[a], Alexandre V. Stoyanov[a]

[a] Research Institute for Children, Children's Hospital, 200 Henry Clay Avenue, New Orleans, LA 70118, United States
[b] Department of Pediatrics, Louisiana State University Health Sciences Center, New Orleans, LA, United States

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ABSTRACT

Genetic variation in the number and reactivity of beta globin sulfhydryl groups causes variation in erythrocyte redox status in mouse populations. These experiments demonstrate the use of capillary isoelectric focusing for measuring endogenous S-glutathionyl hemoglobin and identifying mouse beta globin (Hbb) haplotype in inbred and outbred mouse strains with mono-cysteinyl or di-cysteinyl beta globins. Hbb haplotype can be readily determined in all strains based on characteristic differences in peak profiles or on peak mobility shift induced by thiol exchange with glutathione disulfide in vitro. This method could prove useful for in vivo study of factors that influence thiol protein modification.

1. Introduction

Mammalian hemoglobins are protein tetramers formed from two alpha globin-beta globin (αβ) hetero-dimers [1]. Because hemoglobin has experienced major evolutionary pressure, different species developed different molecular approaches to how hemoglobin is used to fulfill the physiological requirements of each species [2]. Although mice are widely used as models for the study of human biology, diversity in the genetics and chemical reactivity of mouse and human globins significantly reduces the suitability of some mouse strains as models for the study of human physiology and biochemistry.

With relatively few exceptions (e.g. sickle cell disease) all humans produce identical alpha and beta globins because the alleles that produce them are ubiquitous in human populations. In contrast, mouse globin genes are highly polymorphic and commonly produce three structurally different beta globins (β^s, β^{d+} and β^{d-}) and five structurally different alpha globins (α^1 through α^5) [3,4]. Genetic variation in mouse alpha globin is associated with variation in hemoglobin oxygen affinity and altitude adaptation [5,6]. Genetic variation in mouse beta globin is associated with variation in erythrocyte redox status caused by heterogeneity in the number and reactivity of beta globin cysteine sulfhydryl groups [7,8]. Human populations are not subject to this redox variation because with rare exception all human beta globins contain only one reactive cysteine residue (two rare di-cysteinyl exceptions include Hb Rainier (Tyr145Cys) [9] and Hb Porto Alegre (Ser9Cys) [10]). Heterogeneity of functionally different alpha and beta globins in mice but not humans means that mouse globin gene variation can contribute to phenotypic and experimental variation in ways that do not occur in humans.

This fact has significant implications in the selection of inbred and outbred mouse strains, especially in studies where genetic variation in erythrocyte redox status could influence response to different experimental treatments. Experimental variation due to mouse globin gene variation will appear as random experimental error unless accounted for in the experimental design. One alternative is to use only mice that are homozygous for genes that only produce beta globin with a single reactive cysteine residue (β^s, βCys93, Hbb^s haplotype). This mouse beta globin is chemically and functionally more similar to human beta globin than the other two mouse beta globins which both contain two biologically active cysteine sulfhydryl groups (β^{d+} and β^{d-}, βCys93 and βCys13, Hbb^d haplotype). Another alternative, especially when using genetically heterogeneous outbred mice, is to individually determine the Hbb haplotypes of each mouse and account for Hbb genetic variation in the experimental design or analysis of experimental results. If these precautions are not taken, mouse globin gene variation could

Abbreviations: CIEF, capillary isoelectric focusing.

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* Corresponding author. Tel.: +1 504 896 2707; fax: +1 504 896 2722.
E-mail address: jhempe@chnola-research.org (J.M. Hempe).

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confound experimental results and mask real differences in treatment effects.

The purpose of the present experiments was to demonstrate the use of capillary isoelectric focusing (CIEF) for (1) identification and quantification of endogenous mouse S-glutathionyl hemoglobins, and (2) determination of Hbb haplotypes in inbred and outbred mouse strains. Results obtained with CIEF are compared to those obtained with conventional gel IEF.

2. Experimental

All reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

2.1. Mice and blood samples

Inbred C57BL/6J (C57) and 129S1/SvImj (129) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Outbred CD1 mice were obtained from Charles River Laboratories (Wilmington, MA). All mice were over eight weeks of age. Blood was collected by cardiac puncture in heparinized tubes after carbon dioxide asphyxiation as approved by the Children's Hospital Institutional Animal Care and Use Committee. Blood samples were centrifuged to isolate intact erythrocytes and 1:20 (v/v) hemolysates were prepared by adding 10 μl of erythrocytes to 200 μl of aqueous solution containing 10 mM KCN and 5 mM EDTA that suppresses the formation of oxidation products. Crude hemolysates (containing cell membranes) were stored in aliquots at −70 °C until use.

2.2. S-glutathionyl hemoglobin synthesis in vitro

Synthetic S-glutathionyl hemoglobin was produced in vitro by thiol exchange with glutathione disulfide (GSSG) as previously described [7] with minor modifications. A 100 mM stock solution was prepared by adding 0.3283 g of GSSG to 5 ml of deionized water. The stock solution was stored in aliquots at −70 °C until use. Protein S-glutathionylation was initiated by adding 5 μl of GSSG stock solution to 95 μl of 1:20 (v/v) hemolysate. The hemolysates were then incubated for 2 h at 37 °C and either analyzed immediately or stored at −70 °C until use.

2.3. Dynamic capillary isoelectric focusing

Capillary isoelectric focusing of hemoglobin variants was performed as previously described [7,11–13] with significant modifications. We use the term dynamic to indicate that analytes are separated by zonal electrophoresis in a pH gradient but have not yet reached their isoelectric points (p*I*) at the time of detection. Separations were performed using a 50 μm (i.d.) × 30 cm dimethylpolysiloxane coated capillary (J&W DB-1, 0.05 μm coating thickness, Agilent Technologies, Santa Clara, CA) and a P/ACE MDQ capillary electrophoresis system equipped with UV detector and 32 Karat Software (ver. 8.0, Beckman Instruments, Fullerton, CA). The instrument was operated with the anode and cathode at the inlet and outlet reservoirs, respectively, with the inlet 10 cm from the detector window and with capillary cooling at 20 °C. Cathode solution was 80 mM borate in deionized water adjusted to pH 10.25. Anode solution was 100 mM H_3PO_4 in methylcellulose (0.375% (w/v) in water) solution. Ampholyte solution was 2% (v/v) Pharmalyte pH 6.7–7.7 in methylcellulose solution. Prior to each day's analytical run the capillary was conditioned for 5 min with 20 mM NaOH then 5 min with 50% (v/v) methanol in deionized water. For each analysis, the capillary was filled with ampholyte solution for 1 min at 40 psi, sample was injected for 10 s at 1 psi, and proteins were separated with the instrument operated in reverse polarity (20 kV, 1 min ramp) for up to 20 min. Between analyses, the capillary was rinsed for 1 min with 20 mM NaOH then 2 min with 50% methanol. Peaks were quantified based on peak area of absorbance at 415 nm using perpendicular drop integration. Values are expressed as percent of total hemoglobin. Compared to the original method, analysis by dynamic CIEF takes approximately half as much time per run with no loss in peak resolution.

Table 1
Examples of Hba and Hbb heterogeneity in common inbred mouse strains.

Hba haplotype	Alpha globins present	Hbb^s	Hbb^d
Hba^a	α^1	C57BL/6J	129S1/SvImJ
Hba^b	α^2, α^3	C58/J	Balb/cJ
Hba^c	α^1, α^4	SJL/J	C3H/HeJ
Hba^d	α^1, α^2	SM/J	CBA/J
Hba^f	α^5	CE/J	AKR/J
Hba^g	α^1, α^5		DBA/2J
	Beta globins present	β^s	β^{d-}, β^{d+}

Hbb haplotypes based on Genetic Quality Control Annual Report (12-1-99 to 11-30-00), Jackson Laboratory, Bar Harbor, ME. Hba haplotypes based on reports by Whitney et al. [3,4] and Lyon et al. [14].

2.4. Gel IEF

Gel IEF was performed using commercially available precast Novex® pH 3–10 gels (8 cm × 8 cm, 1 mm, 10 well) and reagents as described by the manufacturer (Invitrogen, Carlsbad, CA). Hemolysates were prepared as described above, mixed 1:1 (v/v) with sample buffer, then 10 μl of the mixture was applied to each lane. Proteins were separated at constant voltage (100 V, 1 h; 200 V, 1 h; 500 V, 0.5 h), stained with 0.1% Coomassie blue (Gradipure, NuSep, French's Forest, AU) and destained in a solution of 10% acetic acid and 20% methanol.

3. Results and discussion

Unlike humans, mice inherit a pair of tightly linked alpha globin (Hba) genes and a pair of tightly linked beta globin (Hbb) genes from each parent [14]. Among common inbred strains of laboratory mice (Table 1) there are six common Hba haplotypes ($Hba^{a,b,c,d,f,g}$) where the two loci each produce one of five different common alpha globins with neutral amino acid substitutions (α^{1-5}) [3]. There are only two common mouse Hbb haplotypes, Hbb^s and Hbb^d (Hbb^p haplotype also exists but is uncommon). Mice with the Hbb^s haplotype produce identical mono-cysteinyl (βCys93) beta globins (β^s) at each beta globin locus; βCys93 is conserved in all mouse and human beta globins. In contrast, mice with the Hbb^d haplotype produce two structurally different di-cysteinyl (βCys93 and βCys13) beta globins at each locus. One of these (β^{d+}) has charge properties similar to that of β^s. The other (β^{d-}) is more positively charged and is characteristically present at ∼20% of total hemoglobin in homozygous Hbb^d mice and at 10% in heterozygous Hbb^{sd} mice [7].

Dynamic CIEF separates proteins based on surface charge [11]. The capillary is first filled with ampholytes (pH 6.7–7.7) then a small plug of hemolysate is pressure-injected into the end of the capillary. When voltage is applied, the ampholytes move quickly to establish a pH gradient that is lowest near the inlet and highest near the outlet. The more positively charged hemoglobin isoforms move more quickly towards the cathode/outlet and thus pass through the detector first. More negatively charged isoforms, i.e. singly or doubly glutathionylated hemoglobins, move more slowly and thus have longer migration times. To interpret CIEF peak profiles, it is important to note that on most electrophoretic and chromatographic separation techniques hemoglobin tetramers dissociate into αβ dimers that are resolved based on differences in p*I* [1]. Under these separation conditions, more positively charged αβ dimers migrate faster towards the cathode and pass the detector

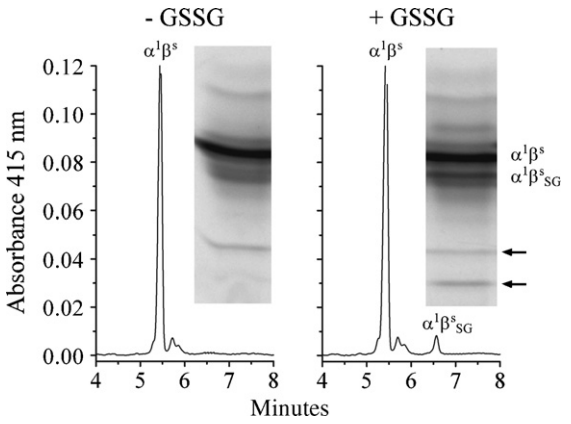

Fig. 1. Homozygous Hbbs haplotype: inbred C57 mouse. CIEF electropherograms (pH 6.7–7.7) and Coomassie stained IEF gels (pH 3–10) of untreated (−GSSG) and GSSG-treated (+GSSG) hemolysates. The cathode is oriented towards the left in the electropherograms and at the top of the gels in this and all other figures. The arrows highlight anodal mobility shift in a protein besides hemoglobin that was susceptible to S-glutathionylation in vitro.

sooner than dimers with lower p*I* and longer migration times. S-glutathionylation of beta globin sulfhydryl groups adds negative charge to αβ dimers due to the unbalanced addition of the extra negative charge from the glutamyl carboxyl group of glutathione. This lowers the p*I* of the modified dimer causing a significant anodal peak shift and longer migration times for S-glutathionyl αβ dimers. Although mouse alpha globins contain one cysteine residue (αCys104) we have no evidence that this cysteine binds glutathione.

3.1. Inbred mice

Inbred C57 and 129 mice have the same alpha globin haplotype (Hba^a) but different beta globin haplotypes (Hbb^s and Hbb^d, respectively). Since mice with the Hba^a haplotype produce the same alpha globin (α^1) at each alpha globin locus, C57 and 129 mice produce αβ dimers with different beta globins but identical alpha globins. Consequently, differences in the CIEF profiles of C57 (Fig. 1) and 129 (Fig. 2) mice are solely attributable to differences in Hbb haplotype and beta globin composition.

Hemolysates from C57 mice (Fig. 1, left panel) contained a single major peak ($\alpha^1\beta^s$). This CIEF peak profile is similar to that observed in human hemolysate. In contrast, hemolysates from 129 mice (Fig. 2, left panel) contained two major peaks, one dominant peak ($\alpha^1\beta^{d+}$) and a more positively charged, less abundant peak ($\alpha^1\beta^{d-}$). The proportion of $\alpha^1\beta^{d-}$ (~20%) reflects the characteristically lower proportion of this beta globin in hemolysate from Hbb^d mice. Minor peaks immediately to the right (anodal) of the major peaks in each figure are glycated isoforms of the major peaks [15]. Endogenous S-glutathionyl $\alpha^1\beta^s$ ($\alpha^1\beta^s{}_{SG}$) was not typically detected in hemolysate from C57 mice (Fig. 1, left panel). In vitro incubation of hemolysate from the C57 mouse with 5 mM GSSG for 2 h (Fig. 1, right panel) produced only 5.5% of $\alpha^1\beta^s{}_{SG}$ which is modified by glutathione on βCys93. In contrast, endogenous S-glutathionyl hemoglobins were routinely observed in hemolysates from 129 mice (Fig. 2, left panel). As previously reported these peaks disappear when hemolysates are treated with reducing agents like mercaptoethanol or DTT [7]. Hemolysate from the mouse in Fig. 2 contained 3% $\alpha^1\beta^{d-}{}_{SG}$ and 12% $\alpha^1\beta^{d+}{}_{SG}$. Furthermore, treatment of hemolysate from the same 129 mouse with GSSG in vitro resulted in the near complete conversion of $\alpha^1\beta^{d-}$ and $\alpha^1\beta^{d+}$ to $\alpha^1\beta^{d-}{}_{SG}$ and $\alpha^1\beta^{d+}{}_{SG}$, both of which are modified by glutathione on βCys13. A small proportion (6.2%) was converted to $\alpha^1\beta^{d+}{}_{SG(2)}$ which contains two negatively charged glutathione molecules, one bound to βCys13 and one to the conserved βCys93. The p*I* of the different unmodified and S-glutathionyl modified αβ dimers were sufficiently different that no peak interference was observed after separation.

3.2. Outbred mice

Unlike homozygous inbred mice, outbred mice may be homozygous or heterozygous for multiple combinations of different Hba or Hbb haplotypes. Identification of Hba haplotypes by CIEF in outbred mice is complicated because (1) each of the four alpha globin loci can potentially produce a different alpha globin, (2) the proportions of alpha globins produced at each locus varies in different Hba haplotypes, and (3) some αβ dimers with different alpha globins have very similar charge properties and cannot be readily differentiated by CIEF when present together in the same sample. Theoretically, an individual mouse genome that was heterozygous at both Hba and Hbb could produce up to 12 different αβ dimer combinations.

To our knowledge, the complexity of the possible αβ dimer combinations in Hba and Hbb heterozygous outbred mice has not been comprehensively evaluated. Highly complex peak profiles could interfere with the identification and quantification of $\alpha^1\beta^{d-}$ dimers whose proportions (0, 10 or 20% of total hemoglobin) are highly characteristic of homozygous Hbb^s, heterozygous Hbb^{sd} and homozygous Hbb^d haplotypes, respectively. Complex peak profiles could also interfere with assessment of endogenous S-glutathionyl hemoglobin levels if any S-glutathionyl αβ dimers and unmodified αβ dimers have similar p*I*. We analyzed hemolysates from 10 outbred CD1 mice to determine the extent to which Hba heterogeneity might hinder interpretation of CIEF peak profiles in genetically heterogeneous mouse strains. We observed eight different peak profile patterns that differed in the number, migration times, and abundance of αβ dimer peaks. Each hemolysate, however, contained only two or four abundant peaks attributable to Hba and/or Hbb heterogeneity. The presence of fewer than the potential 12 different αβ dimer peaks greatly simplifies data interpretation and is evidently due to the fact that several αβ dimers have different alpha globins but nearly identical p*I* and are not resolved by CIEF [3].

Fig. 3 shows a relatively simple profile of a CD1 mouse with two abundant αβ dimer peaks (63 and 37%, respectively). Regardless of the number of alpha globins present, mice that are homozygous or heterozygous for the Hbb^d haplotype will produce $\alpha^x\beta^{d-}$ dimers (where *x* represents any alpha globin) that are more positively charged than the associated $\alpha^x\beta^{d+}$ dimers. When present,

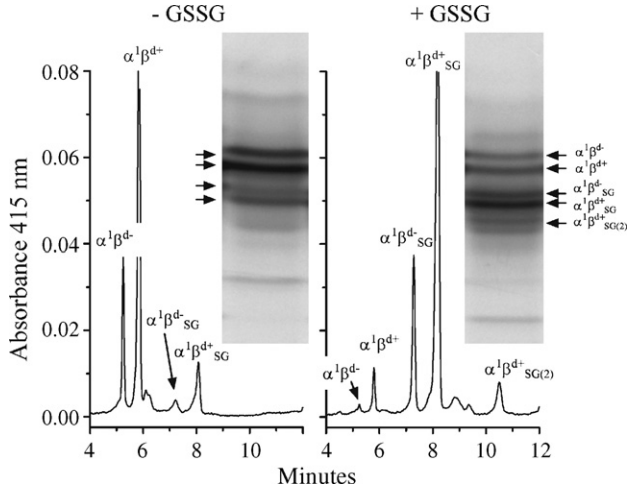

Fig. 2. Homozygous Hbb^d haplotype: inbred 129 mouse. CIEF electropherograms and Coomassie stained IEF gels as in Fig. 1.

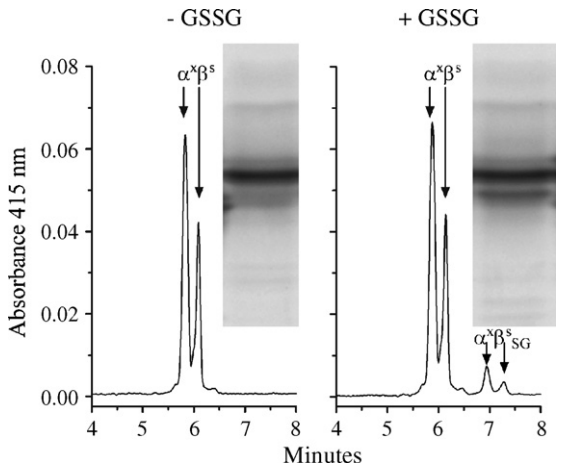

Fig. 3. Homozygous Hbb^s haplotype: outbred CD1 mouse with two major hemoglobin variants. CIEF electropherograms and Coomassie stained IEF gels as in Fig. 1. The identity of the alpha globins in this outbred mouse is unknown thus all dimers with β^s are referred to as α^x.

the $\alpha^x\beta^{d-}$ dimers will collectively represent either 20% of the hemoglobin present in homozygous Hbb^d mice or 10% in heterozygous Hbb^{sd} mice. Although we cannot with certainty identify the Hba haplotype of the mouse in Fig. 3, the lack of peaks attributable to $\alpha^x\beta^{d-}$ dimers clearly identifies the two abundant peaks as different $\alpha^x\beta^s$ dimers and this mouse as homozygous Hbb^s haplotype. Like the homozygous Hbb^s C57 mouse (Fig. 1), S-glutathionyl hemoglobins were not evident in the untreated hemolysate from this CD1 mouse and only small proportions of each of the two abundant peaks (representing 6 and 3% of total hemoglobin, respectively) were converted to $\alpha^x\beta^s{}_{SG}$ (βCys93) in GSSG-treated hemolysate (Fig. 3, right panel).

Fig. 4 is an example of a slightly more complex profile from a CD1 mouse with four abundant peaks in untreated hemolysate (Fig. 4, left panel). We observed two highly abundant peaks (28 and 51%, respectively) and two more positively charged and less abundant peaks (8 and 13%, respectively). Collectively, the two smaller $\alpha^x\beta^{d-}$ dimer peaks represented ~20% of total hemoglobin, which identifies this mouse as homozygous Hbb^d haplotype for reasons described above. This haplotype identification was confirmed by the comprehensive peak mobility shift observed when nearly all $\alpha^x\beta^{d-}$ and $\alpha^x\beta^{d+}$ dimers were converted to $\alpha^x\beta^{d-}{}_{SG}$ and $\alpha^x\beta^{d+}{}_{SG}$ (βCys13) by in vitro exposure to GSSG (Fig. 4, right panel). Very low

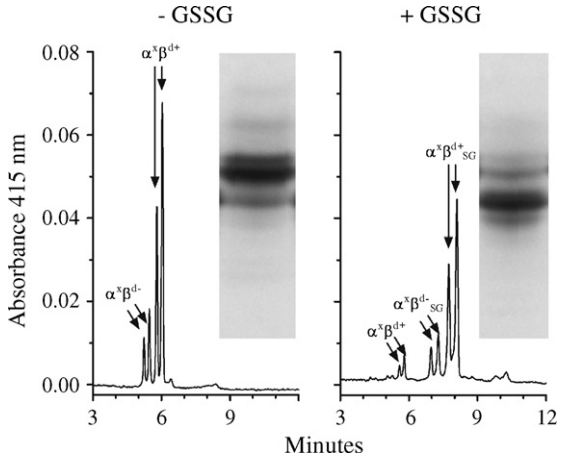

Fig. 4. Homozygous Hbb^d haplotype: outbred CD1 mouse with four major hemoglobin variants. CIEF electropherograms and Coomassie stained IEF gels as in Fig. 1.

levels of αβ dimers with glutathione attached to both βCys13 and βCys93 were also apparent in GSSG-treated hemolysate.

The collective $\alpha^x\beta^{d-}$ dimer proportions observed in some of the remaining eight CD1 mice summed to only ~10% of total hemoglobin, rather than the 20% observed in homozygous Hbb^d mice. Furthermore, GSSG-treatment did not completely convert all abundant peaks to lower p*I* S-glutathionyl adducts. Both of these observations indicate that these mice were heterozygous Hbb^{sd} haplotype which has all three types of mouse beta globins, one of which (β^s) is relatively resistant to glutathione modification. Using this analytical approach, we identified five of the 10 CD1 outbred mice as homozygous Hbb^d, three as homozygous Hbb^s, and two as heterozygous Hbb^{sd}.

These results indicate that hemoglobin CIEF peak profiles in heterozygous outbred mice are less complex than might be expected. Hba haplotype did not interfere with measurement of S-glutathionyl hemoglobins because glutathione-modified isoforms had p*I* that were significantly lower than any of the unmodified isoforms. Because the peak profiles in all of the outbred mice were relatively simple, Hba haplotype did not interfere with Hbb haplotype determination either (1) directly based on the peak profiles of untreated hemolysates, or (2) indirectly based on the effect of GSSG treatment on αβ dimer mobility.

3.3. Gel IEF

Because gel IEF is readily available to more laboratories than CIEF, we analyzed each of the inbred and outbred mouse samples to determine if Hbb haplotype could be identified either directly or indirectly by gel IEF. Unlike gel IEF, CIEF profiles are relatively free of interference because detection at 415 nm is specific for proteins containing heme. Interpretation of αβ dimer band profiles in Coomassie stained gels was complicated by the presence of other relatively abundant proteins (Figs. 1–4). In general, however, the peak/band profiles observed in untreated or GSSG-treated samples were similar when analyzed by CIEF or gel IEF. Consequently, the anodal CIEF peak mobility shift observed in GSSG-treated hemolysates from homozygous Hbb^d haplotype mice was apparent as a significant band shift in gel IEF (Figs. 2 and 4). Interpretation of Hbb haplotype in heterozygous Hbb^{sd} outbred mice, however, was inconclusive due to interference, low resolution and lower sensitivity of gel IEF under these conditions (data not shown).

The arrows in Fig. 1 mark the location of a protein other than hemoglobin identified by a glutathione-induced anodal band shift as susceptible to thiol modification. Other proteins also exhibited marked anodal band shifts (Figs. 1–4) indicating that a glutathione mobility shift assay can be used to identify proteins susceptible to thiol modification.

4. Conclusions

Mouse and human hemoglobins differ in the number and chemical reactivity of beta globin sulfhydryl groups and in the relative complexity of peak profiles obtained by isoelectric focusing. Because of this inter-species diversity, mice with beta globins that are chemically and functionally more similar to human beta globins are more suitable models for the study of human biology. CIEF can be used to measure endogenous mouse S-glutathionyl hemoglobins in inbred or outbred mouse strains. Regardless of Hba haplotype, Hbb haplotype can be identified in inbred or outbred mice either directly based on characteristic CIEF peak profiles, or indirectly using a glutathione mobility shift assay. Compared to conventional gel IEF, CIEF is automatable, faster, and far better able to quantify low levels of post-translationally modified hemoglobins due to the relatively greater sensitivity of online UV detection. Compared to

cation exchange HPLC assays [16,17], analysis is faster (~10 min compared to >40 min) and less susceptible to interference because S-glutathionyl hemoglobins elute in a pH range free of other structural or post-translationally modified hemoglobins.

Protein S-glutathionylation plays a complex role in redox signaling pathways that regulate metabolism and are challenging to study at the cellular or organismal level [18]. Since the number and reactivity of mouse beta globin cysteine sulfhydryl groups have variable influences on redox status, mouse hemoglobins and the mouse strains described in this report could be used as models for the study of factors that influence redox status and protein thiol modification in vivo.

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