Short Communication

Differential redox potential between the human cytosolic and mitochondrial branched-chain aminotransferase

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The human branched-chain aminotransferase (hBCAT) isoenzymes are CXXC motif redox sensitive homodimers central to glutamate metabolism in the central nervous system. These proteins respond differently to oxidation by H₂O₂, NO, and S-glutathionylation, suggesting that the redox potential is distinct between isoenzymes. Using various reduced to oxidized glutathione ratios (GSH:GSSG) to alter the redox environment, we demonstrate that hBCATc (cytosolic) has an overall redox potential that is 30 mV lower than hBCATm (mitochondrial). Furthermore, the CXXC motif of hBCATc was estimated to be 80 mV lower, suggesting that hBCATm is more oxidizing in nature. Western blot analysis revealed close correlations between hBCAT S-glutathionylation and the redox status of the assay environment, offering the hBCAT isoenzymes as novel biomarkers for cytosolic and mitochondrial oxidative stress.

Keywords glutathione; Nernst equation; redox potential; branched-chain aminotransferase

Manipulating the reduced (GSH) to oxidized (GSSG) glutathione ratio (GSH:GSSG) in these assays could also promote Cys oxidation through S-glutathionylation and highlight distinct redox sensitivities between the hBCAT isoenzymes.

The GSH:GSSG system plays a central role in redox homeostasis through maintaining the intracellular redox environment (Eₗ), where changes can affect normal cell growth [5]. For example, in mammals, alterations from ‘reducing’ (< -270 mV) to ‘oxidizing’ intracellular environments (> -160 mV) correspond to the transition between cell proliferation and apoptosis [5]. In our system, such alterations in Eₗ may be critical in neuronal physiology/pathology [6]. As such, calculating the redox potential of the hBCAT proteins will establish the susceptibility to oxidation of each isoenzyme during Eₗ fluctuations. Given that glutathione represents the major low-molecular-weight intracellular thiol (1–5 mM in the healthy human CNS) [7,8] measuring the GSH:GSSH ratio in cells allows for a reliable estimation of Eₗ in cells [5]. Accordingly, robust Eₗ estimations can be made by adapting the Nernst equation provided the total [glutathione] is known (Equation 1).

\[ Eₗ = Eₘ - \frac{RT}{nF} \log\left(\frac{GSH}{GSSG}\right) \]  

where \( Eₘ \) is the reduction midpoint potential of the redox couple (−240 mV for glutathione at pH 7.0 and 25°C) [9], \( R \) is the gas constant (8.314 J K⁻¹ mol⁻¹), \( F \) is Faraday’s constant (9.6485 × 10⁴ C mol⁻¹), \( T \) is the temperature in Kelvin, and \( n \) is the number of electrons involved in the redox couple [5].

Here, we illustrate a distinct difference in the redox potential between the hBCAT isoenzymes, offering functional consequences and drawing on comparisons between other redox-sensitive proteins with both cytosolic and mitochondrial counterparts.
Materials and Methods

Overexpression and purification of hBCAT proteins
Overexpression and purification of hBCATm and hBCATc were carried out in *Escherichia coli* BL21-DE3 cells from pET28a plasmids as previously described previously [10]. Briefly, following 4 h of induction, when optical density value = 0.8, the His-tagged hBCAT fusion proteins were extracted and purified using nickel-NTA resin according to the manufacturer’s instruction (Qiagen, Chatsworth, USA). Thrombin (100 NIH units per extraction) was added to cleave the His-tag. Anion-exchange chromatography using a Mono-Q HR column (GE Healthcare, Bucks, UK) was performed as a final step. Final purified hBCAT concentration was determined from the absorbance at $\lambda_{280}$ nm using the extinction coefficient of 67,000 M$^{-1}$ cm$^{-1}$ per monomer for hBCATm or 86,300 M$^{-1}$ cm$^{-1}$ for hBCATc [10]. Electrospray ionization mass spectrometry confirmed the respective molecular mass and the purity of each hBCAT protein was determined to be >98%.

Analysis of Cys thiol groups by spectrophotometry
Solvent-accessible Cys thiol groups in hBCATc were assayed by 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) titration. Briefly, 10 nmol hBCAT was exchanged into assay buffer (50 mM HEPES at pH 7.2 and 1 mM EDTA) using a PD10 column (GE Healthcare). And 2 nmol of hBCAT was titrated with 100-fold excess of DTNB at room temperature for 15 min. The absorbance change at $\lambda_{412}$ nm was monitored and the concentration of free thiol groups was calculated from the liberated 2-nitro-5-thiobenzoate dianion using a molar extinction coefficient of 13,600 M$^{-1}$ cm$^{-1}$ [10].

Determination of hBCAT redox potential
A final amount of 10 nmol hBCAT protein was incubated in various redox buffers for 30 min at 37°C, with constant agitation. Redox buffers were generated by decreasing GSH:GSSG (10 mM total glutathione) in assay buffer. The Nernst equation was used to determine the $E_h$ of each redox buffer (Equation 1) relating to: $−322$ mV (0.1% GSSG), $−288$ mV (25% GSSG), $−269$ mV (50% GSSG), $−246$ mV (75% GSSG), and $−160$ mV (99.9% GSSG), using $−253.3$ mV as $E_m$ for the GSH:GSSG redox by factoring in pH 7.2 and 37°C [9]. Control samples did not contain any GSH or GSSG. Non-linear regression analysis was carried out to generate the equation $f = y_0 + a/[1 + \exp(-(x-x_0)/b)]$, from which respective hBCAT redox potentials were calculated. Analysis of hBCAT Cys thiol was performed following desalting using a PD10 column, after which respective redox potentials were determined from $E_m$ calculations (mV at which one Cys is oxidized for hBCATm and three Cys oxidized for hBCATc) [5]. Aliquots were taken for western blot analysis using anti-GSH, clone D8 (Virogen, Watertown, USA) to detect S-glutathionylated adducts as previously described [3]. Pixel density analysis was performed using ImageQuant 5.2 followed by linear regression to establish the relationship with $E_h$.

Results

**hBCATc has a lower redox potential compared with hBCATm**
We used a modified Nernst equation (Equation 1) to calculate respective redox potentials for the hBCAT proteins. Incubation of the hBCAT proteins in the various redox buffers resulted in progressive Cys oxidation for both isoenzymes [Fig. 1(A,B)], demonstrating that alteration of $E_h$ alone has the capacity to oxidize the hBCAT proteins irrespective of the presence of free radical. Using non-linear regression comparing oxidized Cys against the mV for each redox buffer, results show that the overall redox potential of hBCATc was lower compared to hBCATm.

Figure 1 Calculation of $E_m$ for the hBCAT proteins DTNB titration reduced Cys estimation for (A) hBCATc and (B) hBCATm, following incubation in each redox buffer (mediated by decreasing GSH:GSSG). Control (closed circle), $−322$ mV (open circle), $−288$ mV (closed triangle), $−269$ mV (open triangle), $−246$ mV (closed square), and $−160$ mV (open square). (C) Non-linear regression analysis was carried out to generate a robust curve that was used to calculate respective hBCAT protein $E_m$, $f = y_0 + a/[1 + \exp(-(x-x_0)/b)]$. $P < 0.001$ (Sigma Plot v10).
potential for hBCATc is \(-260\) mV, compared with \(-225\) mV for hBCATm [Fig. 1(C)], illustrating that hBCATc is more sensitive. The redox curves for each hBCAT protein show that the greatest proportion of oxidation occurs between \(-300\) and \(-225\) mV for each isoenzyme, suggesting that both hBCATc and hBCATm are reducing in nature. Taken together, these data highlight that alterations in \(E_h\) alone can promote hBCAT oxidation; however, hBCATc appears more sensitive compared with hBCATm.

**Pixel density from \(E_h\)-mediated hBCAT S-glutathionylation directly correlates with [GSSG] and hBCAT Cys oxidation**

Previously we have demonstrated using quadrupole-time-of-flight mass spectrometry and western blotting analysis that the hBCAT proteins form S-glutathionylated adducts through Cys-oxidation under similar conditions used in this study [4]. Here, we performed pixel density analysis on western blots for hBCAT S-glutathionylation and asked whether the data correlate with different parameters within each redox buffer. A representative western blot illustrates progressive hBCAT S-glutathionylation at increasing \(E_h\) [Fig. 2(A)]. The data show a close correlation between pixel density and [GSSG] [Fig. 2(B)]. Because of this and our above observation showing a non-linear correlation between \(E_h\) and hBCAT Cys-oxidation, we asked if hBCAT S-glutathionylation pixel density correlated with Cys-oxidation. The data show a direct correlation between pixel density and Cys-oxidation for hBCATc [Fig. 2(C)] and hBCATm [Fig. 2(D)]. In summary, these data illustrate strong linear correlations among hBCAT S-glutathionylation, [GSSG], and oxidized-Cys, suggesting that S-glutathionylated hBCAT pixel density analysis may be used to estimate [GSSG] in biological systems.

**Discussion**

Previous studies have demonstrated that the hBCAT proteins may be inactivated by free radicals through Cys oxidation; however, these studies failed to establish the sensitivity of these isoenzymes with respect to the redox environment \(E_h\). This is an important question that needs addressing, particularly in our system where persistent oxidative stress (as seen in neurological disorders) can raise \(E_h\) [11]. Therefore, this study was carried out to establish the sensitivity of the hBCAT isoenzymes to fluctuations in \(E_h\). The findings presented herein show for the first time that the optimal \(E_h\) for hBCAT Cys oxidation occurs at \(-300\) to \(-225\) mV for both isoenzymes, which is typical of cells in a proliferative state [5]. Thus, hBCAT oxidation is likely to occur during normal cell function.

We show that hBCATc is more sensitive to increase in \(E_h\) compared with hBCATm as a whole, highlighting that hBCATm is more oxidizing in nature. Since hBCATm only contains two solvent-accessible reactive Cys (CXXC motif) [12], it can be concluded from this study that the CXXC motif of hBCATm has a redox potential very close to \(-225\) mV. Interestingly, our redox potential value for the hBCATm CXXC motif is very similar to the CXXC motif of human thioredoxin-1 (Trx1) \(-230\) mV, which is believed to remain \(\geq 90\%\) reduced in healthy cells [13]. This is in contrast to \(E.\ coli\) Trx which has a redox potential of \(-270\) mV, which is closer to that of overall hBCATc and is likely to remain just 30%–60% reduced [14]. Since oxidation of the CXXC motif is central for regulating hBCAT activity, our findings are consistent with the notion that, at least in healthy proliferating cells, hBCATm is largely reduced and thus in an active state.

Using the same logic, redox potential estimations for the hBCATc CXXC motif are more challenging to formulate since hBCATc contains four additional Cys [2]. However, through site-directed mutagenesis we have previously demonstrated that the hBCATc CXXC motif is the more
sensitive to oxidation compared with the other four Cys [4]. Thus, it can be assumed that oxidation of the initial Cys represents the redox potential for the hBCATc CXXC motif, which related to 310 mV in this study. This finding reveals for the first time that the hBCATc CXXC motif is much more sensitive to increase in $E_h$ compared with hBCATm and is therefore more reducing in nature. The differential redox potential of the CXXC motif between hBCAT isoenzymes is an interesting observation given the degree of sequence homology in this area [2]. Nevertheless, modifying the XX residues can alter the redox potential of the motif by stabilizing the nucleophilic Cys [15,16]. In our system a conserved GTA preceding the redox potential of the motif by stabilizing the nucleophilic Cys [17]. However, the CXXC motif alters from CQVC in hBCATc to CVVC in hBCATm [2]. Although the electrostatic charge between these altered residues is similar, this Q→V substitution is conserved between mammalian cytosolic and mitochondrial hBCAT isoenzymes [12], which may be important for hBCAT CXXC motif redox sensitivity. Conversely, charged residues extra to the CXXC motif such as Asp or Lys have been shown to alter the redox sensitivity of Trx [18]. Therefore, the differential redox potentials observed between hBCAT CXXC motifs may also result from charge differences within the microenvironment of the CXXC motif.

The findings presented in this study may reflect the different sub-cellular locations of the hBCAT isoforms, in which a decreased sensitivity to $E_h$ would be more advantageous for hBCATm, which resides in the oxidizing mitochondrial environment, thus preserving amino-transferase activity. However, previous studies have shown that the hBCAT CXXC motif can interact with distinct proteins in a redox-dependent manner [4,19]. For hBCATm this interaction occurs between the branched-chain alpha-keto acid dehydrogenase enzyme complex and glutamate dehydrogenase, providing optimal substrate channeling conditions for branched-chain amino acid catabolism [20]. Thus, the differential redox potentials between the hBCAT CXXC motifs may reflect different biochemical behaviors as well as their different sub-cellular locations.

Overall, our findings are consistent with the cytosolic glutaredoxin (Grx1) and mitochondrial (Grx2) isoenzymes, for which Grx2 has a higher redox potential [21]. In the Grx system it is likely that Grx1 adopts a redox sensing role and we can postulate a similar role for hBCATc in our system. Interestingly, Grx2 activity has been shown to alter from ‘reducing’ to ‘oxidizing’ in an $E_h$-dependent manner under the same conditions that can inhibit hBCATm activity [22]. Thus, we can speculate similar characteristics for hBCATm, in the sense that during cell rest ($-270$ mV) the CXXC motif of hBCATm will attract electrons (oxidizing in nature), compared with releasing electrons during differentiation ($-200$ mV) (reducing in nature) [5]. Ultimately such fluctuations will impact on aminotransferase activity and the way in which the hBCAT isoenzymes interact with other redox proteins, affecting both biochemical function and behavior.

This study shows for the first time that the proportion of hBCAT S-glutathionylation as determined through western blot pixel density analysis may be used to estimate $E_h$ in biological systems. Given the abundance of neuronal intracellular glutathione, this raises the possibility that the hBCAT proteins may be used as biomarker cytosolic and mitochondrial oxidative stress. Such calculations have intrinsic value for studying disease where redox stress is a pathological feature. In our system such calculations could be made regarding $E_h$ during neuropathology, allowing insight regarding hBCATc and hBCATm activity during this process.

In summary, this study identifies that the overall redox potential for hBCATc is lower than that of hBCATm, demonstrating that hBCATm is more oxidizing in nature.

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**References**

Distinct redox potentials between hBCAT isoenzymes