Roles of hemoglobin allostery in hypoxia-induced metabolic alterations in erythrocytes: simulation and its verification by metabolome analysis

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When erythrocytes are exposed to hypoxia, hemoglobin (Hb) stabilizes in the T-state by capturing 2,3-bisphosphoglycerate (2,3-BPG). This process could reduce the intracellular pool of glycolytic substrates, jeopardizing cellular energetics. Recent observations suggest that hypoxia-induced activation of glycolytic enzymes such as phosphofructokinase, aldolase and glyceraldehyde 3-phosphate dehydrogenase is correlated with their release from Band III (BIII) on the cell membrane. Based on these data, we developed a mathematical model of erythrocyte metabolism, and compared hypoxia-induced differences in predicted activities of glycolytic enzymes, their products, and cellular energetics between models with and without the interaction of Hb with BIII. The models predicted that the allostery-dependent Hb interaction with BIII accelerates consumption of upstream glycolytic substrates such as glucose-6-phosphate and fructose-6-phosphate and increases downstream products such as dihydroxyacetone phosphate, 3-phosphoglycerate and phosphoenolpyruvate. This prediction was consistent with metabolomic data obtained from high-throughput capillary electrophoresis mass spectrometry. The hypoxia-induced alterations in the metabolites resulted from acceleration of glycolysis, as judged by increased conversion of $^{13}$C-glucose to $^{13}$C-lactate. The allostery-dependent interaction of Hb with BIII appeared to contribute not only to maintenance of energy charge but also to further synthesis of 2,3-BPG which could help sustain stabilization of T-state Hb during hypoxia. Furthermore, the hypoxia-induced activation of glycolysis was not observed when Hb was stabilized in R-state by treating the cells with carbon monoxide. These results suggest that Hb allostery in erythrocytes serves as an oxygen-sensing trigger that drives glycolytic acceleration to stabilize intracellular energetics and promote the ability to release O$_2$ from the cells.

Erythrocytes deliver molecular oxygen (O$_2$) to tissues through allosteric regulation of hemoglobin (Hb). The ability of Hb to release O$_2$ is determined by a variety of metabolites in the intracellular compartment, such as protons (H$^+$), 2,3-bisphosphoglycerate (2,3-BPG), nitric oxide (NO) and ATP. The decrease in H$^+$ and the increase in the aforementioned compounds (2,3-BPG, NO and ATP) stabilize the T-state of Hb, thereby facilitating O$_2$ dissociation from the cells. In contrast, O$_2$ and carbon monoxide (CO) function as positive allosteric effectors that stabilize Hb in its R-state and downregulate O$_2$ dissociation. When exposed to hypoxic conditions, erythrocytes are known to accelerate glucose consumption (1). This event appears to result from acceleration of glycolysis, as judged by the increase in 2,3-BPG (2), a metabolite stabilizing the T-state of Hb. Since T-state Hb has a higher affinity for 2,3-BPG and ATP than the R-state Hb, stabilization of the former structure would reduce amounts of free ATP available for maintenance of cellular homeostasis. ATP is consumed continuously by various ATPases to maintain ionic homeostasis and by adenylate cyclase to generate cyclic AMP to maintain the deformability of the cells. Upon hypoxia, a fraction of the intracellular ATP is released to the extracellular space to elicit hypoxia-induced vasorelaxation, although the actual amounts of ATP released seem small compared to levels within the cell (3). Furthermore, the cells require ATP in the initial
steps of glycolysis (e.g., used by hexokinase (HK) and phosphofructokinase (PFK)) to trigger ATP synthesis. These features have led researchers to hypothesize that erythrocytes might have mechanisms for responding quickly to hypoxia to upregulate de novo ATP synthesis.

There is a growing body of evidence showing that the interaction of Hb with Band III (BIII), a major transmembrane protein in erythrocytes, plays a role in these compensatory mechanisms to maintain intracellular ATP levels(4-6). BIII, also known as anion exchanger type I, accounts for about 25% of the total erythrocyte membrane protein (7), and its cytoplasmic domain displays a greater affinity for Hb in the T-state rather than the R-state (8). BIII also binds to phosphofructokinase, PFK (9); aldolase, ALD(10); glyceraldehyde-3-phosphate dehydrogenase GAPDH (11) and lactate dehydrogenase, LDH (12). Recent observations have shown that these enzymes reduce their catalytic activities upon binding to BIII, and activity is recovered upon dissociation from BIII as free forms (13,14). These results led us to hypothesize that Hb stabilized in the T-state upon hypoxia serves as a trigger to increase the activity of glycolytic enzymes and to accelerate glucose consumption to increase ATP synthesis. Several reports to date have provided circumstantial evidence showing hypoxia-induced activation of glycolysis by measuring glycolytic intermediates in erythrocytes(15,16). However, the mechanistic features of the coordination and dynamics of sequential glycolytic reactions and the outcomes in terms of alterations in intracellular metabolites are not comprehensively understood.

The aim of this study was to develop a dynamic mathematical model of metabolism in erythrocytes, involving the O2-sensing mechanisms of Hb, to predict temporal alterations in intracellular metabolites and cellular energetics in response to hypoxia, and to verify the predictions derived from the model through metabolome analyses. The differential equations of metabolic reactions used in the model are partially based on previous models, as depicted in detail in the Supplemental data section of this paper. It provides rate equations and the relevant parameters for all processes included in the model.

MATERIALS AND METHODS

Mathematical model of human erythrocyte metabolism - All numerical calculations were performed using the E-Cell 3 simulation environment ([http://ecell.sourceforge.net/download.html](http://ecell.sourceforge.net/download.html)) (27,28). The model can be found in the Supplemental data section of this paper. It provides rate equations and the relevant parameters for all processes included in the model.

Description of the metabolic model - We developed a mathematical model of human erythrocyte metabolism (Fig. 1) with kinetic descriptions of reaction rates derived from previously published experimental data, and partially from those based on published models, as depicted in detail in the Supplemental data. The metabolic reactions included in the developed model are listed in Table 1, and the abbreviations of enzymes and compounds are shown in Tab. 1 and Tab. 2, respectively. Our mathematical model not only included the glycolytic pathway but also comprised other metabolic pathways that are directly or indirectly related to regulatory mechanisms for glycolysis. The pentose monophosphate shunt, adenine nucleotide metabolism and the salvage pathway including hypoxanthine-guanine phosphoribosyltransferase (HGPRt) were included, since these components determine levels of AMP and Pi, which are known regulators of PFK and GAPDH, respectively (asterisks in Fig. 1). Another important component we included is the de novo synthesis and transport of glutathione. This compound occurs at mM levels in the cell and its synthesis and transport are significant energy-consuming processes. Furthermore, GSH serves as a regulator of HK. The Na/K pump consumes large amounts of MgATP, and is modeled using precise kinetic equations described elsewhere. We also considered effects of magnesium ion (Mg2+) binding to ATP, ADP, AMP, 1,3-BPG, 2,3-BPG, F-1,6BP and GDP, using the respective binding affinities shown in the Supporting Table 1. Because it is now assumed that enzymes employ the complexed form of ATP (MgATP or MgADP) as substrates, these binding processes are important to be considered. Transport of metabolites such as ADE, ADO, INO, HX, and LAC were modeled under the assumption that they move across the membrane simply according to their concentration gradient between intra- and extra-cellular spaces. Thus, the
diagram shown in Fig. 1 constitutes a mathematical model including 83 different chemical reactions and 11 transporter functions which covers a majority of the metabolic pathways influencing the concentrations of allosteric effectors and/or substrates for glycolysis.

**Modeling BIII-mediated interactions between Hb and glycolytic enzymes** - Reversible binding of the glycolytic enzymes (PFK, ALD, GAPDH) and two allosteric forms of Hb (R- and T-states) to BIII on the membrane were modeled in the current study on the basis of the individual association constants listed in Table 3. T-state Hb has 100-fold greater affinity to BIII and is much more likely to associate with this anion transporter than R-state Hb. The catalytic activities of PFK, ALD, GAPDH are inhibited through their specific binding to BIII, but formation of such complexes appeared to be reversible (13). Based on these findings, in the model, these enzymes were assumed to be completely inactivated upon formation of the complex and activated reversibly upon dissociation. Under these circumstances, the competitive association of Hb and the glycolytic enzymes with BIII and the resultant changes in glycolysis could occur in response to alterations in partial O2 tension (pO2 mmHg). Thus, in this mathematical model, we were able to manipulate pO2, an independent variable of the model, as a parameter, and to predict glycolytic metabolism as a dependent variable. Another important assumption is that pO2 alters the T-R transition of Hb according to a reversible Hill-type equation (29) that is also dictated by pCO2 levels, intracellular pH, concentrations of 2,3-BPG and ATP, and temperature (Supplemental data, page 8-9).

**Perturbations mimicking hypoxia** - The aforementioned mathematical model was able to stably achieve individual steady-states under varied pO2 conditions over the range of 0-100 mmHg. Since we aimed to predict metabolic alterations in response to hypoxia with this model, pO2 was initially set to 100 mmHg, a physiological value in alveoli (30). Under these conditions, the basal and initial steady-state concentrations were calculated by this model, as depicted in Table 2 and Supporting Tab. 2. The external parameters, including environmental concentrations of metabolites and initial settings in the model, are listed in Supporting Tab. 3. To adapt the model to hypoxic conditions, pO2 was reduced to 30 mmHg, a value measured in capillary microvessels in vivo (30,31), for desired lengths of time. As seen in previous studies (30,31), circulating erythrocytes may be exposed to such a pO2 value when they travel through capillaries under physiological conditions (30) or when they traverse low-flow or static microvessels belonging to post-ischemic damaged regions in the liver (31).

This protocol allowed us to remodel glycolytic metabolism in erythrocytes for the aforementioned circumstances.

**Preparation of human erythrocytes for CE-MS analysis** - Erythrocytes were isolated from heparinized venous blood samples collected from healthy male volunteers according to our previous methods (31). Briefly, the samples were centrifuged at 2,000g at 4 °C for 10 min, and the cells were washed three times and suspended in Tris buffer (pH 7.4) to adjust hematocrit values to 15%. The cell suspension was divided into hypoxic and normoxic groups. For the hypoxic group, the cells were incubated in a gas-tight glass bottle and followed by a gentle purge with highly purified argon gas for 45 min at 4 °C. Cell samples (2 mL) were transferred to glass tubes on ice in an O2-excluding chamber, and finally incubated at 37 °C for 0, 0.5, 1 and 3 min. The reactions were terminated by cooling the sample-containing glass tubes with ice-cold water at 4 °C. The cells for normoxic controls were incubated similarly to the hypoxic group, but treated by purging for 45 min with air instead of argon gas, and finally incubated at 37 °C for 0, 0.5, 1 and 3 min. Likewise, the reactions were terminated at 4 °C by cooling in ice-cold water. For measurements at 0 min, the cells were incubated at 37 °C and immediately treated on ice to terminate the reaction. The present protocol for treating erythrocytes with argon gas allowed us to set up reproducible hypoxic conditions where the pO2 was approximately 30 mmHg.

The cell samples for these two groups were purified by centrifuging at 2,000g at 4 °C for 10 min, and the pellets were treated with 0.16 mL cold methanol containing 300 μM L-methionine sulfate (MES) for deproteination. MES was used as the internal standard to validate the recovery or loss of metabolites during sample preparation and CE-MS analysis (25). Next, 0.16 mL of chloroform and 0.08 mL of distilled water was added and thoroughly mixed. The solution was centrifuged at 12,000g at 4 °C for 15 min, and the upper aqueous layer was filtered through a centrifugal filter (Millipore 5-kDa NMWL) to remove proteins. The filtrate was analyzed by CE-MS. Metabolome data collected from erythrocytes exposed to hypoxia for the aforementioned times were plotted as relative concentrations versus the 3-min normoxic controls (see below). In some experiments, erythrocytes were pretreated by an incubation with buffer saturated with CO, a stabilizer of R-state Hb. This incubation was performed for 20 min prior to the 45-min normoxic incubation time at 4 °C.

In a separate sets of experiments, we examined the effects of the aforementioned procedure of hypoxia on the conversion of 13C(6)-glucose to 13C(3)-lactate in human erythrocytes. In these
experiments, erythrocytes isolated from venous blood samples were suspended in buffer to adjust hematocrit values to 30%. The cell suspension was divided into hypoxic and normoxic samples to be treated by purging for 45 min at 4 °C with argon and air, respectively. Cell samples (1 mL) were then transferred to glass tubes containing 1 mL of buffer containing 5 mM $^{13}$C(6)-glucose, giving a final hematocrit value of 15%. Immediately after mixing at 4 °C, the samples were incubated at 37 °C for 3 min and the reactions were terminated at 4 °C by immersion in ice-cold water. Preparation of cell pellets and samples to determine the amount of $^{13}$C(3)-labeled lactate was identical to that for conventional metabolome analyses, as described below, and elsewhere (26). The flux indicating the conversion of $^{13}$C(6)-glucose to $^{13}$C(3)-lactate was determined as values relative to the amount of MES in the samples added as an internal control. Using the same samples, $^{13}$C(3)-2,3-BPG was also determined when necessary.

Sample recovery of metabolome analysis by CE-MS -
Hb has the ability to capture intracellular metabolites such as 2,3-BPG and ATP. Additionally, deproteinization with cold methanol prior to processing for CE-MS analysis could cause loss of some of these metabolites through binding to denatured Hb. To quantify the putative loss of such metabolites, 2,3-BPG, ATP, glucose-6-phosphate (G6P), lactate or α-ketoglutarate were incubated for 3 min at a final concentration of 300 µM (2,3-BPG) or 100 µM (other metabolites) in buffer containing 300 µM purified human Hb. To examine differences in the loss of these metabolites between T- and R-state Hb, the sample mixture was purged for 60 min with purified argon gas or with air, respectively, in the presence of 1 mg/mL sodium dithionite. Purified Hb solution was generously provided by Oxygenix Co. Ltd., Tokyo, Japan. The samples for CE-MS analysis were prepared as described above.

Instrumentation - All CE-MS experiments were performed using an Agilent CE Capillary Electrophoresis System equipped with an air pressure pump, an Agilent 1100 series MSD mass spectrometer and an Agilent 1100 series isocratic HPLC pump, a G1603A Agilent CE-MS adapter kit and a G1607A Agilent CE-MS sprayer kit (Agilent Technologies). System control, data acquisition and MSD data evaluation were performed using G2201AA Agilent ChemStation software for CE-MSD.

CE-MS conditions for anionic metabolites - A cationic polymer coated SMILE (+) capillary was obtained from Nacalai Tesque (Kyoto, Japan) and used as the separation capillary (50 µm i.d. x 100 cm total length). The electrolyte for the CE separation was 50 mM ammonium acetate solution, pH 8.5. Samples were injected with a pressure injection of 50 mbar for 30 sec (30 nl). The applied voltage was set at -30 kV. ESI-MS was conducted in the negative ion mode and the capillary voltage was set at 3500 V. For MS using the selective ion monitoring (SIM) mode, deprotonated [M-H]- ions were monitored for anionic metabolites of interest (32).

Calculation of energy charge in the mathematical model - Based on the definition proposed by Atkinson and Walton, an energy charge was calculated as an index of contents of high-energy phosphate bonds in adenylate in the BIII(+) and BIII(-) models (33,34). Energy charge is defined as one-half the number of anhydride-bound phosphates per adenylate moiety: Energy Charge = [(ATP)+1/2(ADP)]/[(AMP)+(ADP)+(ATP)]. With this parameter, the energy potential of an erythrocyte may vary from 0 to 1.

Statistical analyses - Differences in mean values among groups in the metabolome analyses were examined statistically using a one-way ANOVA and Fisher’s multiple comparison test.

RESULTS

Modeling the BIII-Hb interaction enhances hypoxia-induced activation of glycolysis – Fig. 2A illustrates the differences in temporal alterations in the enzyme activities between the model involving BIII interactions with Hb and the glycolytic enzymes (BIII(+) model; red solid line) and the model without these interactions (BIII(-) model; blue dotted line) during the 3-min virtual hypoxia. Overall, the activities of glycolytic enzymes in the BIII(+) model were stimulated to greater extents than those in the BIII(-) model (Fig. 2A). The activity of HK, which mediates the initial reaction in glycolysis, was initially equally elevated upon hypoxia in the two models. Putative mechanisms for this increase will be mentioned in the Discussion. HK activity in the BIII(+) model exhibited further activation compared to the BIII(-) model; this difference appeared to result from a decrease in G6P (uppermost panel in Fig. 2B)

CE-MS conditions for nucleotides and coenzyme A compounds - Separations were carried out on a GC capillary, polydimethylsiloxane (DB-1) (50 µm i.d. x 100 cm total length) (Agilent Technologies). The electrolyte for the CE separation was 50 mM ammonium acetate, pH 7.5. The applied voltage was set at -30 kV and a pressure of 50 mbar was added to the inlet capillary during the run, in order to maintain a conductive liquid junction at the capillary outlet. All other conditions were the same as in the anionic metabolite analysis (32).
Demonstration of hypoxia-induced alterations in glycolytic products by CE-MS - The aforementioned results from the model raise the possibility that hypoxia-driven activation of GAPDH drives activation of LDH as a downstream target through NADH generation, and thereby facilitates the second half of the glycolytic reactions. Consequently, stimulation of the hypoxia-dependent BIII interaction of the intermediate enzymes (PFK, ALD, GAPDH) contributed to activation of HK as the initial step and that of PK and LDH as the final step in glycolysis in the model as a whole. To examine whether temporal alterations in the glycolytic metabolites of human erythrocytes occur in agreement with those predicted by the BIII(+) model, erythrocytes were exposed to hypoxia for 3 min and the amounts of various metabolites were determined by CE-MS. As seen in Fig. 2C, G6P and F6P were significantly lower than those measured as steady-state controls under normoxic conditions. In contrast, levels of F1,6BP, DHAP, 3PG, and PEP were significantly greater than normoxic steady-state controls. These results are entirely consistent with those predicted by the BIII(+) model. The absolute concentrations of glycolytic metabolites measured by CE-MS at each time point are shown in Supporting Tab. 4.

Adsorption of 2,3-BPG and ATP to Hb during protein denaturation in preparation for CE-MS - CE-MS validation of the data predicted by the dynamic mathematical model supported the hypothesis that hypoxia-dependent BIII interactions with PFK, GAPDH, and ALD play a crucial role in systematic activation of glycolytic reactions to support the demand for ATP in erythrocytes. As seen in Fig. 3A, the ATP content of the cells exposed to hypoxia at 4°C was decreased by 30% versus that measured under normoxic steady-state conditions. This level of ATP was maintained and modestly elevated during the subsequent 3-min hypoxic conditions. Likewise, the content of 2,3-BPG did not decrease in hypoxia, but rather was elevated during the 3-min hypoxic period. Since these metabolites are known to adsorb to T-state Hb, we examined whether the current protocol for measuring metabolites reflected only the amount of free metabolite (i.e., that not bound to Hb) or the total amount in the cells. As seen in Fig. 3B, the presence of free T-state Hb, stabilized by hypoxia plus dithionite, but not free R-state Hb, stabilized by normoxia plus dithionite, caused apparent decreases in ATP and 2,3-BPG contents, but not in G6P, lactate and 2-oxoglutarate contents. Approximately 30% of the ATP and 90% of the 2,3-BPG were lost during the protein denaturation step prior to CE-MS.

These results suggest that, in the cell-free protein solution, the ATP and 2,3-BPG data obtained from CE-MS could be underestimated, as CE-MS only takes into account free metabolite contents and neglects the Hb-bound forms of these metabolites. On the other hand, in case of the cell-containing system, the T-state of Hb in the hypoxia-exposed erythrocytes could be easily stabilized into the R-state (35) during centrifugation at low temperatures prior to protein denaturation; such a process prior to sample processing could stimulate the release of 2,3-BPG adsorbed to Hb under hypoxic conditions. Considering the above, we compared the CE-MS data...
for ATP and 2,3-BPG with the predicted data for free and total amounts of these metabolites obtained from the model. As seen in Fig. 3C, the temporal profile of free ATP in the model was in good agreement with the CE-MS results for ATP shown in Fig. 3A. The amounts of total ATP in the model were modestly elevated. On the other hand, the prediction of the temporal profile of free 2,3-BPG, whose adsorption is dictated by the T-state of Hb, displayed an abrupt drop compared to the steady-state baseline value. This difference with respect to the CE-MS data (Fig. 3A) is not surprising when the aforementioned effect of low temperature on 2,3-BPG release from the T-state of Hb is taken into consideration. In the model prediction, the amount of total 2,3-BPG gradually increased with time, suggesting that the drop in free 2,3-BPG predicted in the model does not result from downregulation of its synthesis, but from its adsorption to Hb. This was demonstrated by the $^{13}$C(6)-glucose pulse-chase measurements described below.

**Hypoxia induces acceleration of glycolysis in human erythrocytes** - The results of the metabolome analyses (Fig. 2 and Fig. 3) suggest that hypoxia causes activation of glycolysis. To examine whether the actual flux of glycolysis could be accelerated in response to hypoxia, we determined the rate of conversion of $^{13}$C-glucose into $^{13}$C-lactate in human erythrocytes. As seen in Fig. 4, the rate of production of $^{13}$C-lactate was accelerated 1.8-fold within 1 min after exposure to hypoxia. In contrast, pretreatment of the cells with CO to stabilize Hb in the R-state attenuated the hypoxia-induced acceleration of lactate production almost completely. These results suggest that the hypoxia-induced stabilization of T-state Hb plays a crucial role in triggering glycolytic activation in erythrocytes.

The model including BIII-Hb interaction predicts sustained energy charge and accelerated O$_2$ release - Using the dynamic mathematical model validated by CE-MS analysis, we examined the roles of the BIII-Hb interaction in regulation of cellular energetics and O$_2$ delivery. As an index of the content of high-energy phosphate bonds of adenylate nucleotides, an energy charge was calculated for the BIII(+) and BIII(-) models. As seen in the upper panel of Fig. 5A, the basal energy charge under normoxic steady-state conditions was 0.91. This value, obtained by the model prediction, was comparable to that reported in previous studies ranging from 0.86 (36) to 0.935 (37), supporting the hypothesis that the BIII(+) model is consistent with the real metabolic behavior of human erythrocytes. Upon exposure to hypoxia, the BIII(+) model exhibited further increases in the energy charge and in the amounts of the deoxyHb-2,3-BPG complex (Fig. 5A). Under these circumstances, the amounts of O$_2$ released from the cell is greater in the BIII(+) model than in the BIII(-) model.

Based on the predictions of the mathematical model shown in Fig. 5A as well as in Fig. 3C, we also examined differences in the conversion of glucose to 2,3-BPG between normoxic and hypoxic cells under the same experimental conditions as in Fig. 4. The ratios of $^{13}$C(3)-2,3-BPG/methionine sulfone were 2.1 +/- 0.3% and 5.4 +/- 0.2% (mean +/- SE of 1-min incubations in 4 separate experiments) under normoxic and hypoxic conditions, respectively, indicating a significant acceleration in de novo 2,3-BPG synthesis upon hypoxia. Considering the CE-MS data in Fig. 3A indicating maintenance of free 2,3-BPG in erythrocytes, the results achieved by the model were consistent with the hypothesis that the BIII-Hb interaction contributes greatly to the delivery of 2,3-BPG to Hb to stabilize the T-state and, thereby, to sustain the release of O$_2$ in hypoxic conditions.

In Fig. 5B, we conducted sensitivity analysis to determine whether or not the amount of any particular enzyme making up the glycolytic pathway could alter the effectiveness of the sustained increase in energy charge and 2,3-BPG generation during hypoxia in the model. We therefore increased the amount of each glycolytic enzyme in the pathway by 2-fold simultaneously with hypoxia. As shown in Fig. 5B, HK activation (green line in Panel B) resulted in a decrease in energy charge and an increase in 2,3-BPG, while PK activation (black in Panel B) increased energy charge without stimulating 2,3-BPG generation. On the other hand, activation of PFK (dotted red in Panel B) or PFK+ALD+GAPDH (red in Panel B) led to simultaneous elevation of energy charge and 2,3-BPG generation in the model. These analyses suggest that activation of enzymes situated midway through the glycolytic pathway positively regulate energy charge and 2,3-BPG generation simultaneously in erythrocytes. The results lend further support to the notion that sustained elevation of 2,3-BPG and the resultant increase in O$_2$ release predicted by the model are likely to be metabolically relevant.

**DISCUSSION**

Glycolysis involves a series of enzymatic reactions to couple glucose oxidation to generation of ATP. On the basis of previous studies (for metabolism (21,24), and for BIII-enzymes/Hb interactions, (13,14)), the BIII(+) model simulated dynamic alterations in enzyme activities and the corresponding metabolites using the assumption that the intermediate glycolytic enzymes, such as PFK, GAPDH and ALD, bind to R-state Hb in their quiescent form and become activated upon their release from T-state Hb in response to hypoxia. The model predictions developed in this study clearly...
indicate that such an activation of the intermediate enzymes triggered by hypoxia-induced stabilization of T-state Hb not only induces acceleration of the flux of metabolites through the glycolytic reactions, but also triggers activation of the initial and final steps of glycolysis as a result of alterations in the corresponding allosteric effectors (e.g. 2,3-BPG for HK) and levels of substrates and products (e.g. ATP and G6P for HK, NADPH for LDH), respectively.

As a consequence, PGK and PK, the glycolytic enzymes for ATP synthesis, are predicted to be accelerated upon hypoxia. The predicted hypoxia-induced remodeling of glycolytic enzyme activities results in decreases in upstream products such as G6P and F6P and increases in the intermediate products such as F1,6BP, DHAP, 3PG, 2,3-BPG and PEP. Furthermore, these predicted alterations in glycolytic products were actually demonstrated by CE-MS analysis in human erythrocytes. These results indicate that the mathematical model described in this study is capable of predicting hypoxia-induced remodeling of energy metabolism, suggesting a physiological significance of the BIII-Hb interaction in accelerating glycolysis in erythrocytes upon exposure to hypoxia.

The sensitivity analysis, showing that the activation of these glycolytic enzymes altered hypoxia-induced responses of metabolites, led us to suggest that the alterations in metabolites determined by CE-MS are comparable to those elicited by about a 2- to 5-fold increase in the amounts of PFK (Supporting Fig. 1A). On the other hand, the increases in ALD and GAPDH apparently had little or no effect on glycolytic activation. However, careful analysis of the sensitivity data (Supporting Fig. 1A-C) indicates that increases in the latter 2 enzymes obviously contributed to the spike in the activation of LDH. It is worthwhile noting that such an initial LDH spike is observed in Fig. 2A, but not in Supporting Fig. 1A where only PFK is activated. Thus, these simulations collectively suggest that PFK activation plays a major role, while activation of ALD and/or GAPDH per se plays a minimal role. This does not exclude, however, a cooperative effect of these two latter enzymes in PFK activation for the stimulation of downstream reactions in the glycolytic pathway, which may be of physiologic and metabolic importance.

Our results show the predicted time-dependent alterations in concentrations of metabolites and the demonstration by CE-MS of these changes. The changes are physiologically relevant to previous observations of hypoxia-elicited metabolic responses in human erythrocytes. For instance, the change in G6P could not only influence glycolysis but also other metabolic pathways. In the BIII(+) model, but not in the BIII(-) model, G6P decreased rapidly after the onset of hypoxia. Since this metabolite serves as a substrate for G6PDH, the rate-limiting step of the pentose phosphate pathway (PPP), such a change in G6P could rapidly limit the flux of metabolites through this pathway. We were unable to detect a decrease in the flux of 13C-6-glucuronolactone, the product of G6PDH, presumably because the changes were insufficient to be detected by CE-MS. However, the hypoxia is well supported by previous observations that hypoxia causes down-regulation of the PPP in parallel with an acceleration in glycolysis in erythrocytes (38). Secondly, erythrocytes exposed to hypoxia have been known to release ATP into the extracellular space, which results in endothelium-dependent vasorelaxation of arterioles (39-41). Considering that the Km of the purinergic receptor for ATP is in the micromolar range, the amount of ATP released from erythrocytes appears to be far smaller than levels normally found in the intracellular spaces. Despite the fact that the time needed to transit through the organ microvascular systems is thought to be in the range of several seconds, erythrocytes could undergo hypoxia frequently during their passage through the capillaries. In some organs (e.g. liver), erythrocytes exhibit transitory stasis among the capillary networks (42,43). Under these circumstances, glycolytic acceleration triggered by the BIII-Hb interaction could greatly enhance the maintenance of intracellular ATP levels.

In this context, acceleration of glucose oxidation upon exposure to hypoxia is likely to be necessary to maintain the functional integrity of erythrocytes through at least two different aspects, that is, supplementation of ATP and sustained stabilization of T-state Hb by 2,3-BPG, an allosteric effector generated through a side reaction in glycolysis. Judging from the CE-MS measurements shown in Fig. 3A, the free amounts of ATP and 2,3-BPG were not apparently reduced during the 3-min hypoxia. Considering that deoxyHb has a greater ability to absorb these metabolites than oxyHb, these results led us to hypothesize that de novo synthesis of these metabolites is dramatically up-regulated during even short periods of hypoxia. Evidence to support this hypothesis was well documented by the CE-MS measurements indicating accelerated conversion of 13C-labeled glucose into 2,3-BPG and lactate under hypoxic conditions. As predicted by the mathematical model in Fig. 5A, such an acceleration of 2,3-BPG synthesis is likely to contribute to a rapid increase in the Hb-2,3-BPG complex that could consequently lead to release of residual Hb-bound O2 from erythrocytes.

Through further analyses of the model depicted in Fig. 5B, we suggest that PFK activation is a crucial step for the upregulation of both energy charge and 2,3-BPG generation, while activation of the initial (e.g. HK) or final (e.g. PK) steps of the glycolytic pathway fails to satisfy these requirements. Furthermore, activation of ALD or GAPDH with that
of PFK appears to help activate LDH at the onset of hypoxia. Whether the amounts of O₂ released from the cells through these putative mechanisms are sufficient to fulfill tissue O₂ demand should be further examined in vivo.

In this model, several of the components of real erythrocytes are missing. For instance, hypoxia-induced switching between BIII and 3 glycolytic enzymes is simply based on their individual association constants for binding to BIII. On the other hand, a previous report suggested that these enzymes behave as a macromolecular complex for the execution of glycolysis (13). Since pH effects on Hb allostery were not included in the current model, the disparity in O₂ saturation between BIII(+) and BIII(-) models could, in reality, be greater than that calculated in this study. In addition, the initial environmental conditions governing external metabolite concentrations (e.g. lactate in the extracellular space) could be altered under disease conditions. Such an alteration, however, did not change the prediction of hypoxia-induced glycolytic remodeling in silico (Supporting Fig. 2). With regard for these considerations, the model needs to be further refined by including the function of carbonic anhydrase, an enzyme that senses tissue CO₂ (6). This would be very helpful when it comes to applying the current model for the prediction of time-dependent alterations in erythrocyte metabolism under chronically hypoxic conditions.

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FOOTNOTES
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FIGURE LEGENDS

Fig. 1
Metabolic pathways included in the mathematical model. Blue: Glucose-derived carbon flux in glycolysis. Red: Substrates or allosteric effectors of glycolytic enzymes, including adenine nucleotides, inorganic phosphates, and glutathione. Metabolites with asterisks indicate allosteric effectors of glycolytic enzymes. The mathematical model developed in this paper is comprised of 46 enzymatic reactions, 11 membrane transport or ion pump systems, 37 reversible binding processes and 1 allosteric hemoglobin transition. The model includes 61 metabolic intermediates, whose steady state levels are shown schematically in Table 1, and 36 protein-metabolite or protein-protein complexes as variables. In this figure, the reactions indicating the catalytic processes of HK, PFK and PK are described as apparently irreversible ones by a one-headed arrow for the principles of thermodynamics(44), while the kinetic equations used in the model are reversible in reality (See Supplemental data).

Fig. 2
Prediction of hypoxia-induced alterations in activities of glycolytic enzymes and in levels of their products in the mathematical model, and demonstration by CE-MS analysis in human erythrocytes. A: Hypoxia-induced changes in predicted activities of glycolytic enzymes. B: Predicted concentrations of glycolytic products. Red and blue lines indicate results of the simulation with and without inclusion of the BIII-Hb interactions, respectively. C: Hypoxia-induced alterations in the relative concentrations of the products determined by CE-MS analysis. Closed circles indicate ratios of hypoxic metabolite concentrations to normoxic control concentrations which are represented with open circles. Values are mean ± SE of 4 separate experiments. Asterisks: P < 0.05 versus the steady-state baseline values.

Fig. 3
Hypoxia-induced alterations in measured concentrations of ATP and 2,3-BPG and effects of the presence of Hb on measurements of metabolites by CE-MS. Panel A: Hypoxia-induced changes in measured concentration of ATP and 2,3-BPG. Panel B: Influence of the presence of Hb. Labels indicate: C, standard measurements in the Hb-free buffer, R and T, measurements in Hb-containing buffer under ambient and hypoxic conditions, respectively. Data shown are means ± SD of 3 separate experiments. * P < 0.05 vs the R group. Panel C: Prediction indicating temporal alterations in free and total ATP and 2,3-BPG in the model. Red lines: the data collected from the BIII(+) model. Blue dotted lines: the data collected from the BIII(-) model.

Fig. 4
Hypoxia-induced acceleration of glycolysis assessed by pulse-chase analysis of the conversion of 13C-glucose to 13C-lactate in human erythrocytes and its blockade by CO. Red and blue bars indicate relative amounts of 13C-lactate generated per 1 min after loading with 13C-glucose at 5 mM under normoxic and hypoxic conditions, respectively. CO(-): control erythrocytes not treated with CO. CO(+): CO-pretreated erythrocytes. Methionine sulfone is an internal standard that was added at 100 µM to protein-free samples for CE-MS collected from the erythrocyte lysates. Means + SE of 3-4 separate experiments are shown. *P < 0.05 vs. normoxic controls (CO(-)).

Fig. 5
Beneficial effects of BIII-Hb interactions on cellular energetics and Hb allostery predicted by the mathematical model. A: Hypoxia-induced alterations in the values of energy charge which is defined by: (ATP+0.5ADP)/(AMP+ADP+ATP). B: The sensitivity analysis indicating changes in energy charge (upper) and 2,3-BPG (lower) generation under different hypoxic conditions when the amounts of the following enzymes were increased by 2-fold. Red: PFK+ALD+GAPDH, Blue: none (same as BIII(-) model), Green: HK, Red (broken): PFK and Black : PK. Note that HK activation results in a decrease in energy charge and an increase in 2,3-BPG generation, while PK activation alone increases energy charge without stimulating 2,3-BPG generation, suggesting that activation of PFK or PFK+ALD+GAPDH leads to simultaneous increases in energy charge and 2,3-BPG generation in the model.
### Table 1. Enzymatic reactions included in the model.

| Enzyme / Process | Abbreviation | Substrates | Products | Effectors |
|------------------|--------------|------------|----------|-----------|
| Hexokinase       | HK           | GLC+MgATP  | G6P+MgADP | 2,3-BPG,GDP,GSH |
| Phosphoglucoisomerase | PGI       | G6P        | F6P      |            |
| Phosphofructokinase | PFK       | F6P+MgATP  | F-1,6BP+MgADP | fATP,Mg,2,3-BPG,fAMP,Phos,GDP |
| Aldolase         | ALD          | F-1,6BP    | DHAP+GA3P | 2,3-BPG,GDP,Mg,GA3P |
| Triose phosphate isomerase | TPI       |            | GA3P     |            |
| Glyceraldehyde phosphate dehydrogenase | GAPDH | GA3P+Phos+NAD | 1,3-BPG+NADH |            |
| Diphosphoglycerate mutase | DPGM | 1,3-BPG    | 2,3-BPG  |            |
| Diphosphoglycerate phosphatase | DPase | 2,3-BPG    | P3GA+Phos |            |
| Phosphofructokinase | PFK       | F6P+MgATP  | F-1,6BP+MgADP | fATP,Mg,2,3-BPG,fAMP,Phos,GDP |
| Enolase          | EN           | P2GA       | P2GA     | Mg         |
| Pyruvate kinase  | PK           | PEP+MgADP  | PYR+MgATP | fATP,F-1,6BP,GDP |
| Lactate dehydrogenase (NADPH) | LDH | PYR+GA3P   | LAC+GA3P |            |
| 6-Phosphogluconolactonase | 6PGDH | GL6P       | GO6P     |            |
| 6-Phosphogluconate dehydrogenase | 6PGDH | GO6P+NA+D | RUS+NP+DADH+CO2 |            |
| Transaldolase    | TA           | STP+GA3P   | EQP+4F6P |            |
| Transketolase I  | TK1          | X5P+R5P   | GA3P+STP |            |
| Transketolase II | TK2          | X5P+4F6P  | GA3P+STP |            |
| Ribose 5-phosphate isomerase | R5PI | R5P       | R5P     |            |
| Xylose 5-phosphate isomerase | X5PI | R5P       | X5P     |            |
| Gamma-glutamyl synthetase | L_GCS | MgATP+glutamate+cyt | MgADP+L_GC+Phos | GSH |
| Glutathione synthetase | GH_S | L_GC+glycine+MgATP | GSH+MgADP+Phos |            |
| Glutathione reductase (NADPH) | GSSGR | GSSG+NP+DADH | GSH+NADP |            |
| Adenosine deaminase | ADA | AD0       | INO     |            |
| Adenine phosphoribosyltransferase | ADPRT | ADE+PRPP | fAMP+2Phos |            |
| Adenosine kinase | AK           | ADO+MgATP  | MgADP+fAMP |            |
| Adenosine monophosphate deaminase | AMPDA | fAMP | IMP |            |
| AMP phosphohydrolase | AMPase | fAMP | ADO+Phos |            |
| Adenylate kinase | APK          | fAMP+MgADP | fAMP+MgATP |            |
| Hypoxanthine-guanine phosphoribosyltransferase | HGPRT | PRPP+Hxi | IMP+2Phos |            |
| Inosine monophosphatase | IMPase | IMP | INO+Phos |            |
| Purine nucleotide phosphorylase | PNase | INO+Phos | HX+R1P |            |
| Phosphoribosyl phosphate synthetase | PRM | R1P | R5P |            |
| Phosphoribosyl phosphate synthetase | PRPPsyn | R5P+MgATP | PRPP+fAMP+Mg |            |
| Adenosine triphosphate phosphohydrolase | ATPase | MgATP | MgADP+Phos |            |
| Glutathione turnover | OX | 2GSH | GSSG |            |
| Non-glycolytic NADH consumption process | OXNADH | NADH | NAD |            |
| Adenine transport process | ADetr | ADE | ADEe |            |
| Adenosine transport process | ADotr | ADO | ADOe |            |
| Hypoxanthine transport process | HXtr | HX | HXe | ADEe |
| Inosine transport process | INOtr | INO | INOe |            |
| Leak of Potassium | K_Lek | Ke | Ki |            |
| Lactate transport process | LACtr | LAC | LACe |            |
| Sodium/potassium pump | NaK_Pump | 3Na+2K+MgATP | 3Na+2K+MgADP+Ph |            |
| Leak of Sodium | Na_Lek | Na | Nai |            |
| Pyruvate transport process | PYRtr | PYR | PYRe |            |
| Inorganic phosphate transport process | Phostr | Phos | Phos |            |
| GSSG transport process | GSSGtransport | GSSG+MgATP | GSSG+MgADP+Phos |            |
Table 2. Steady state concentrations of metabolic intermediates in human erythrocytes in normoxia: abbreviation, concentrations predicted by the model and observed in vivo.

| Metabolite                        | Abbreviation | Concentration (mM) | Model | Literature |
|-----------------------------------|--------------|--------------------|-------|------------|
| Glucose 6-phosphate               | G6P          | 6.0E-02            |       | 3.8E-02, 3.9E-02 |
| Fructose 6-phosphate              | F6P          | 1.9E-02            |       | 1.6E-02  |
| Fructose 1,6-bisphosphate         | F-1,6BP      | 5.6E-03            |       | 7.6E-03, 2.7E-03 |
| Dihydroxy acetone phosphate       | DHAP         | 1.5E-02            |       | 1.4E-02  |
| Glyceraldehyde 3-phosphate        | GA3P         | 3.6E-03            |       | 4.0E-03, 5.7E-03 |
| 1,3-bisphosphoglycerate           | 1,3-BPG      | 2.3E-04            |       | 4.0E-04  |
| 3-Phosphoglycerate                | 3PG          | 4.8E-02            |       | 4.5E-02, 5.4E-02 |
| 2-Phosphoglycerate                | 2PG          | 1.4E-02            |       | 1.4E-02  |
| Phosphoenolpyruvate               | PEP          | 8.1E-03            |       | 1.7E-02  |
| Pyruvate                          | PYR          | 5.2E-02            |       | 5.0E-02, 7.7E-02 |
| Lactate                           | LAC          | 1.3E+00            | 1.1E-03, 1.4E-03 |
| Gluconolactone 6-phosphate        | GL6P         | 5.3E-06            |       |           |
| Gluconate 6-phosphate             | GO6P         | 4.5E-02            |       |           |
| Ribulose 5-phosphate              | RU5P         | 4.9E-03            |       |           |
| Sedoheptulose 7-phosphate         | S7P          | 2.1E-02            |       |           |
| Xylulose 5-phosphate              | X5P          | 9.0E-03            |       |           |
| Erythrose 4-phosphate             | E4P          | 4.5E-04            |       |           |
| Ribose 5-phosphate                | R5P          | 5.8E-03            |       |           |
| Ribose 1-phosphate                | R1P          | 8.1E-02            |       | 6.0E-02  |
| 5-Phosphoribosyl 1-phosphate      | PRPP         | 1.4E-03            |       | 5.0E-03  |
| Inosine monophosphate             | IMP          | 8.1E-03            |       | 5.7E-03  |
| Inosine                           | INO          | 1.5E-04            |       | 1.0E-03  |
| Adenine                           | ADE          | 1.5E-02            |       | 1.3E-02  |
| Adenosine                          | ADO          | 4.5E-05            |       |           |
| Hypoxanthine                      | HX           | 1.6E-03            |       | 2.0E-03  |
| L-glutamyl cysteine               | L_GC         | 4.2E-04            |       |           |
| Glutathione (reduced)             | GSH          | 3.3E+00            | 3.2E+00 |           |
| Glutathione (oxidized)            | GSSG         | 4.7E-03            |       | 6.0E-03  |
| Nicotinamide adenine dehydrogen   | NAD          | 6.5E-02            |       | 6.2E-02  |
| Nicotinamide adenine dinucleotide | NADH         | 2.8E-04            |       |           |
| Nicotinamide adenine dehydrogen   | NADP         | 6.5E-05            |       |           |
| Nicotinamide adenine dehydrogen   | NADPH        | 6.5E-02            |       | 6.6E-02  |
| Potassium ion                     | Ki           | 1.3E+02            | 1.4E+02 | |
| Sodium ion                        | Nai          | 3.4E+01            | 1.0E+01 |           |
| Inorganic phosphate               | Phos         | 1.0E+00            | 1.0E+00 |           |
| Total adenosine diphosphate       | tADP         | 3.3E-01            | 3.1E-01 |           |
| Total adenosine monophosphate     | tAMP         | 3.4E-02            | 3.0E-02 |           |
| Total adenosine triphosphate      | tATP         | 1.9E+00            | 2.1E+00 |           |
| Total 2,3-bisphosphoglycerate     | t2,3-BPG     | 3.7E+00            | 4.5E+00 |           |

Experimental data were taken from the literatures: a reported by Joshi and Palsson (22), b reported by Mulquiney and Kuchel (24), c (45), d (46), e (47).
Table 3. Association constants of Hbs and glycolytic enzymes to BIII.

| Proteins | $K_a$ (M)   |
|----------|-------------|
| deoxyHb  | 10000 $^a$  |
| oxyHb    | 100 $^a$    |
| PFK      | 5000000 $^b$|
| ALD      | 1000000 $^c$|
| GAPDH    | 2000000 $^c$|

Data taken from literatures: $^a$ (48), $^b$ (49) and $^c$ (50).
Figure 2
Figure 3
Figure 4
Figure 5
Roles of hemoglobin allostery in hypoxia-induced metabolic alterations in erythrocytes: simulation and its verification by metabolome analysis
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