Hemoglobin catalyzes ATP-synthesis in human erythrocytes: A murburn model

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Abstract: Blood hemoglobin (Hb) is the most abundant globular protein in humans, known to transport oxygen. Erythrocytes have $\sim 10^{-3}$ M concentration levels of ATP in steady-state and we estimate that this high cannot be formed from 10^{-4} - 10^{-7} M levels of precursors via substrate-level phosphorylation of glycolysis. To account for this discrepancy, we propose that Hb serves as a ‘murzyme’ (a redox enzyme working along the principles of murburn concept), catalyzing the synthesis of the major amounts of ATP found in erythrocytes. This proposal is along the lines of our earlier works demonstrating DROS (diffusible reactive oxygen species) mediated ATP-synthesis as a thermodynamically and kinetically viable mechanism for physiological oxidative phosphorylation. We support the new hypothesis for Hb with theoretical arguments, experimental findings of reputed peers and in silico explorations. Using in silico methods, we demonstrate that adenoside nucleotide and 2,3-bisphosphoglycerate (2,3-BPG) binding sites are located suitably on the monomer/tetramer, thereby availing facile access to the superoxide emanating from the heme center. Our proposal explains earlier reported in situ experimental findings/suggestions of 2,3-BPG and ADP binding at the same locus on Hb. The binding energy is in the order of 2,3-BPG > NADH > ATP > ADP > AMP and agrees with earlier reports, potentially explaining the bioenergetic physiology of erythrocytes. Also, the newly discovered site for 2,3-BPG shows lower affinity in fetal Hb (as compared to adults) explaining oxygen transfer from mother to embryo. The findings pose significant implications in routine physiology and pathologies like sickle cell anemia and thalassemia.

Keywords: murburn concept; murzyme; hemoglobin; ATP, erythrocytes; RBC; glycolysis; oxygen-binding; reactive oxygen species

Introduction: Energetics of erythrocytes

We have demonstrated the murburn model of redox enzyme function in xenobiotic metabolism (Manoj et al., 2016a; Parashar and Manoj, 2021), aerobic respiration (Manoj et al., 2019a; Manoj et al., 2020; Manoj et al., 2019b) and oxygenic photosynthesis (Gideon et al., 2020; Manoj and Manekkathodi, 2021). We had introduced the term ‘murzyme’ five years ago, to connote those redox enzymes that work along the murburn concept (Manoj et al., 2016b; Venkatachalam et al., 2016). Murburn concept summarizes a ‘*mured burning*’ (closed oxidative process in cells/organelles) or ‘*mild unrestricted redox catalysis*’ occurring via interactive equilibria among component *m*olecules, *u*nbound ions and *r*adicals. Herein, we propose that hemoglobin, the predominant oxygen-transporting protein of red blood corpuscles (RBC or erythrocytes), also serves as a murzyme that synthesizes ATP using diffusible reactive (oxygen) species or DR(O)S.

The source of energy for human erythrocytes is glucose (~5 mM) available from blood fluid (plasma). RBCs depend on glycolysis for the utilization of glucose, as shown in Figure 1. Until the final (10th) step of glycolysis, the 2 ATP molecules that are consumed (at the 6 carbon ‘investment’ stage) and 2 ATP molecules are produced from two molecules of 1,3-bisphosphoglycerate (1,3-BPG, in the 7th step of the 3 carbon ‘payoff’ stage). ATP cannot be generated from NADH (produced in the 6th step of glycolysis) by “ETC-proton pumps-chemiosmosis-Complex V” mechanism of oxidative phosphorylation, as human RBCs lack mitochondria. Therefore, the net ATP productivity of glycolysis depends on the last (10th) step catalyzed by pyruvate kinase, which is the substrate level phosphorylation of ADP from phosphoenolpyruvate (PEP), to give pyruvate (Pyr) and ATP. The mass-charge balanced reaction catalyzed by the enzyme is represented by (Garrett and Grisham, 1999):

$$K_{eq} = ([\text{Pyr}] [\text{ATP}]) / ([\text{PEP}] [\text{ADP}] [\text{H}^+])$$

The equilibrium driven synthesis by pyruvate kinase would give $[\text{ATP}] = K_{eq} \times [\text{PEP}] \times [\text{ADP}] \times [\text{H}^+] / [\text{Pyr}]$. Transposing the equation and substituting the values of K_{eq} (from $\Delta_r G^\circ \sim -31$ kJ/mol), physiological concentrations of the reactants and metabolites (Berg et al., 2002; Garrett and Grisham, 1999), we get the concentration of [ATP]:

$$= \sim 10^5 \times [2.3 \times 10^{-5}] \times [1.4 \times 10^{-4}] \times [\sim 10^{-7}] / [5.1 \times 10^{-5}]$$

Figure 1: The glycolytic production of ATP in erythrocytes is not an energetically efficient process.

The calculation above gives equilibrium driven ATP formation at only $\sim 0.63 \mu\text{M}$, which is 5500-folds lower than the experimental concentrations of $\sim 3.5 \text{ mM}$ ATP found in RBC (Xu et al., 2019). It is to be noted that except for the ATP-involving steps (two each at the six-carbon and three-carbon stages), all reactions are characterized by a positive Gibbs free energy term (Garrett and Grisham, 1999). Therefore, the viability of glycolysis critically depends on ATP/ADP ratio. The 7th step of glycolysis mediated by phosphoglycerate kinase (1,3-bisphosphoglycerate $\rightarrow$ 3-phosphoglycerate) is regulatory, and is favorable when $[\text{ADP}] > [\text{ATP}]$. However, the physiological concentration of ADP is only $\sim 0.14 \text{ mM}$ in RBC (Berg et al., 2002; Garrett and Grisham, 1999), which is $\sim 1/15$ to $1/25$ of ATP concentration. Also, the concentration of 1,3-BPG in erythrocytes is usually very low ($\sim 1 \mu\text{M}$) because most of this metabolite is converted to

2,3-BPG (which is maintained at ~4 to 6 mM) by the enzyme bisphosphoglycerate mutase. Recycling of 2,3-BPG into the glycolytic intermediate of 3-phosphoglycerate is mediated via a phosphatase enzyme (giving Pi and protons). Therefore, the amount of ATP produced in the 7th step would only be a fraction of the ATP consumed in the investment step. This implies that even the tenth step would be needed to make up for the energy deficit, if glycolysis were the only pathway for ATP-synthesis. Further, anaerobic production of lactate from pyruvate is needed/viable for redox cycling in myocytes and other cells that are conditionally deprived of oxygen supply. This non-spontaneous reaction with $\Delta_r G^\circ \sim 25$ kJ/mol (Cox and Nelson, 2008) cannot be a part of the routine metabolic cycle of RBC, which has a highly oxidic environment. Also, it does not make sense to have a pathway make NADH and then degrade it for futile reasons (as NADH is needed in ample amounts to keep the large number of Hb molecules reduced within the RBCs). Even if we disregard steady-state or equilibrium considerations, reaction stoichiometry (from the highlighted equation) would afford maximally ~ 140 μ M ATP (equivalent to the ~140 μ M ADP found in RBC) or minimally ~50 nM (equivalent to proton concentration) or moderately 23 μ M (equivalent to phosphoenolpyruvate concentration). These values are several folds or orders lower than the physiological concentrations of ATP in RBC. (Each step of the serial scheme of glycolysis would be dependent on the experimental order-terms of all reaction components; and each step's rate constants would be required to calculate the accurate amounts of ATP generated in the tenth step. However, a rough approximation is possible with the stoichiometry approach adopted here.) It is to be noted that ATP expended continually by RBCs (say, for homeostasis) has not been considered herein. Therefore, the source of vast majority of ATP found in RBC needs to be accounted from another pathway.

In order to explain the source of the excess ATP observed in RBC, we apply the murburn mechanistic perspective, which advocates constructive electron/moiety transfer roles for DROS. We propose (and provide ample evidence to argue) that Hb also serves as an enzyme (murzyme) that utilizes DROS dynamics to facilitate ATP production in RBC. We also provide data from reputed peers' publications and our own in silico studies to support our proposal and correlate it to clinically relevant pathologies like sickle cell anemia and thalassemia.

Methods: Exploration of structure-function correlation of human respiratory globins

The alpha monomer of human Hb-alpha (1SI4) (Sen et al., 2004) was first taken as a sample protein and its docking profile checked with a variety of ligands. Thereafter, monomers and tetramers of Hb-beta (6FQF) (Mazzarella et al., 2018), Hb-gamma (4MQJ) (Strader et al., 2014) and other relevant proteins' (1GZX, 3D17, 5E83, 1NQP, and 3RGK) deposited crystal structure coordinates were availed from Protein Data Bank. The structures were visualized with software such as Chimera 1.12 (Pettersen et al., 2004) and PyMol 1.3 (DeLano, 2002). Active site cavity was studied by POCASA 1.1 (Yu et al., 2010), keeping a probe radius of 2 Å, with a grid size of 1 Å. Small flexible molecules were docked to the large rigid proteins using AUTODOCK 4.2 (Forli et al., 2012), as per procedures reported in our earlier works (Manoj et al., 2019a). Literature search was conducted to collect data on binding affinities of relevant small molecules to Hb/Mb. We declare at the outset that we do not disclaim any experimental evidence reported for any other hypotheses in the field, and we approach the system with an *ab initio* parsimonious perspective, as enabled by computation-aided visual explorations.

Results: Binding of adenosine nucleotides and phosphoglycerates to respiratory globins

Figure 2 shows the structures of various molecules explored in this study, in particular: adult human hemoglobin-apha protein (1SI4), with the orientation of heme and the location of active site channel/cavity. Analyses of the active site volumes of the human respiratory globins with POCASA gave the values (in Å³): HbA (1SI4) = 252, HbB (3D17) = 239, HbF (4MQJ) = 263, HbSA (1NQP) = 281, HbSB (1NQP) = 239, HbEA (5E83) = 237, HbEB (5E83) = 252, Mb (3RGK) = 196. Table 1 presents the summary of in silico binding studies conducted herein. Significant depictions of high or low affinity interactions (for select tests and controls) are presented in the figures and discussed in the text.

Figure 3 shows that HbA has a low-affinity adenosine nucleotide docking site at the heme cavity aperture, with a binding energy term of ~ -5 kcal/mol, translating to a K_d value of to ~200 μM (~10⁻⁴ M). It can be seen that ADP presents itself quite favorably for phosphorylation, with the phosphate group enabling a facile presentation to the superoxide that could potentially release from distal end of heme center. From Table 1 and Figures 3 & 4, it can be seen that the ADP &

NADH/ATP binding is relatively significant for the adult variants (α and β) of Hb in humans. However, sickle-cell and thalassemic Hb proteins showed relatively poor ADP-binding, although exhibiting significant affinities for ATP and NADH. The human fetal HbF and myoglobin (Mb) also showed significantly lower ADP-binding affinity, and Mb exhibited approximately three orders higher K_d value than that of blood Hb α .

Figure 5 shows the docking of phosphoglycerates with hemoglobins. Quite interestingly, the small polar molecule of 2,3-bisphosphoglycerate or diphosphoglycerate (DPG/BPG) gave high affinity for binding at the heme pocket, and the energy terms were in the order HbA ~ HbB > HbF > Mb. Using effective controls (1,3 BPG and other phosphoglycerates), it was discerned that this binding was not an artifact. The allosteric site for 2,3-BPG is known to bind via 'H2-K82-H143' triad on the Hb β surface. This site gave a binding energy of -9.35 kcal/mol ($K_d \sim 140$ nM) in the normal Hb α , which was not higher than the active site affinity. In contrast, the sickle cell and thalassemic proteins presented relatively low active site binding (with respect to normal Hb) and/or the beta monomers exhibited high allosteric affinity for 2,3-BPG.

Figure 6 and Table 2 show the results of 2,3-BPG and ADP docking to the tensed and relaxed tetrameric forms of Hb. The relaxed form has an ultra-high affinity 2,3-BPG site that fits the central slot between the four monomers whereas the tensed form has a moderate-affinity ADP site (which is positioned adjacent to a 2,3-BPG high-affinity site) suitably posed to be attacked by DROS that could potentially emanate from beta-monomers' secondary apertures.

The survey of overall binding affinities across various monomers/tetramers gives a relative trend in the order of 2,3-BPG > NADH > ATP ~ ADP > AMP.

Figure 2: Structural features of hemoglobin Hb A (1SI4) The orientation and rotation of heme (for the monomeric depictions in bottom panels) is shown in the top-left panel, with the proximal histidine jutting out of the plane of paper. The top center panel depicts the hemoglobin monomer showing the solvent-accessible heme, with oxygen binding site of Fe atom shown in black (onto which oxygen binds, within the distal space) at the heme-center. Unlike the relatively superficial proximal thiolate ligand of liver microsomal cytochrome P450s (Parashar & Manoj, 2021), it can be seen that the proximal histidylate ligand of Hb is buried within the protein. The active site cavity per se is marked out (with POCASA) in the top right panel. The heme-ensconcing pouch is $\sim 12 \text{ \AA}$ deep. The association of monomers into the tetramer (with the location of the active site cavity) is presented in the bottom-left panel. The structures of three key small molecules (explored in this study for interaction with Hb) are shown in the bottom right panel.

Figure 3: Docking of AMP/ADP/ATP to Hb-alpha (1SI4).

Figure 4: Docking of NADH to various monomers of human hemoglobin.

Figure 5: Docking of various phosphoglycerates to human Hb.

Figure 6: Interaction of tetramer with 2,3-BPG and/or ADP: Monomers of α -hemoglobin are represented in pink and of β -hemoglobin in turquoise. Relaxed tetramer (PDB ID: 3D17) exhibits very strong affinity towards 2,3-BPG (green) at the junction of the β -dimer but does not allow a strong bond with ADP, as shown in the left panel. The right panel shows the tensed state of hemoglobin with a fairly high affinity towards 2,3-BPG (green) and moderate affinity towards ADP (yellow). β -monomer has a secondary opening (circumference indicated in black) towards the O_2 /DROS channel between the tetrameric junctions.

Table 1: Docking of respiratory globin monomers with adenosine nucleotides and phosphoglycerates

No.	Monomer	Description	Ligand [#]	ΔG (kcal/mol) [*]	K_d [*]	Interacting Amino Acids
1	HbA (1SI4)	$\alpha 2\delta 2$ in R2 state bound to CN	2,3-BPG	-10.16	35 nM	H58, K61
2	HbA (1SI4)	Ditto	NADH	-5.27 (-5.43)	138 μ M (104 μ M)	Y42, H45, H58, K90
3	HbA (1SI4)	Ditto	ATP	-5.01	213 μ M	H58, K61
4	HbA (1SI4)	Ditto	ADP (1)	-5.04	202 μ M	Q54, H58, K61
5	HbA (1SI4)	Ditto	AMP	-4.84	285 μ M	Y42, H45
6	HbA (1SI4)	Ditto	1,3-BPG	-3.40 (-3.76)	3.24 mM (1.75 mM)	Q54, H58, K61
7	HbA (1SI4)	Ditto	3-PG	-3.63	2.20 mM	Q54, H58, K61
8	HbA (1SI4)	Ditto	2-PG	-2.96 (-3.51)	6.76 mM (2.68 mM)	Q54, H58, K61
9	HbA (1SI4)	Ditto	NAD^+	-0.81	254 mM	NA
10	HbA (1SI4)	Ditto	NADPH	-3.68 (-4.64)	1.99 mM (397 μ M)	T41, H45, K90, L91
11	HbA (1SI4)	Ditto	$NADP^+$	+1.25	NA	NA
12	HbA (1GZX)	$\alpha 2\beta 2$ Tetra ligated with O_2 (tensed)	2,3-BPG	-9.88	57 nM	Q54, H58, K61
13	HbA (1GZX)	Ditto	NADH	-6.66	13 μ M	A82, K90
14	HbA (1GZX)	Ditto	ATP	-5.16	165 μ M	K61
15	HbA (1GZX)	Ditto	ADP (1)	-4.31	695 μ M	Q54, H58, K61

16	HbA (1GZX)	Ditto	NAD ⁺	-1.80	48 mM	K61
17	HbA (3D17)	$\alpha 2\beta 2$ Triply ligated with CO (relaxed)	2,3-BPG	-10.04	44 nM	H45, Q54, H58, K61
18	HbA (3D17)	Ditto	NADH	-7.08	6.5 μ M	H45, H58, K61
19	HbA (3D17)	Ditto	ATP	-5.55	85 μ M	H45, H58, K61
20	HbA (3D17)	Ditto	ADP	-3.31	3.73 mM	H45, K61
21	HbA (3D17)	Ditto	NAD ⁺	-1.95	38 mM	K61
22	HbB (6FQF)	$\beta 4$ Met Hb	2,3 BPG	-10.15 [-9.35]	36 nM [140 nM]	K59, H63, K66
23	HbB (6FQF)	Ditto	NADH	-6.70	12 μ M	K59, H63, K66, K95
24	HbB (6FQF)	Ditto	ATP	-4.93	244 μ M	S44, H63, K66, K95
25	HbB (6FQF)	Ditto	ADP (1)	-4.73	343 μ M	K59, H63, K66
26	HbB (6FQF)	Ditto	NAD ⁺	-2.19	25 mM	S44, K95
27	HbB (1GZX)	$\alpha 2\beta 2$ Tetra ligated with O ₂ (tensed)	2,3-BPG	-9.31 (-11.24)	150 nM (5.73 nM)	S187, K202, H206, K209
28	HbB (1GZX)	Ditto	NADH	-7.47	3.37 mM	S187, K202, H206, K209
29	HbB (1GZX)	Ditto	ATP	-5.68	68.74 μ M	K209
30	HbB (1GZX)	Ditto	ADP (2)	-4.16	895 μ M	K238, L239
31	HbB (1GZX)	Ditto	NAD ⁺	-1.62	65 mM	K238
32	HbB (3D17)	$\alpha 2\beta 2$ Triply ligated with CO (relaxed)	2,3 BPG	-9.28 (-10.04)	159 nM (44 nM)	A62, K65, K66
33	HbB (3D17)	Ditto	NADH	-7.11	6.1 μ M	R40, S44, K95
34	HbB (3D17)	Ditto	ATP	-4.21	826 μ M	A62, K65, K66
35	HbB (3D17)	Ditto	ADP (1)	-2.84 (-4.0)	8.2 mM (1.2 mM)	S44, K59, H63
36	HbB (3D17)	Ditto	NAD ⁺	-1.8	48 mM	S44, K59, S63
37	HbF (4MQJ)	$\alpha 4\gamma 4$ O ₂ and CO bound	2,3 BPG	-8.37	735 nM	S44, K59, H63, K66
38	HbF (4MQJ)	Ditto	NADH	-7.07	6.6 μ M	S44, H63
39	HbF (4MQJ)	Ditto	ATP	-4.44	561 μ M	K66
40	HbF (4MQJ)	Ditto	ADP (1)	-3.82	1.58 mM	H63, K66, K95
41	HbF (4MQJ)	Ditto	NAD ⁺	-1.84	45 mM	H63, K66, K95
42	HbSA (5E83)	$\alpha 2\beta 2$ CO bound tetrameric sickle cell	2,3 BPG	-10.95	9.3 nM	H45, Q54, H58, K61
43	HbSA (5E83)	Ditto	NADH	-6.30	24 μ M	Q54, H58, K61
44	HbSA (5E83)	Ditto	ATP	-6.63	13.9 μ M	Q54, K61
45	HbSA (5E83)	Ditto	ADP (1)	-3.97	1.22 mM	Q54, H58, K61
46	HbSB (5E83)	Ditto	2,3 BPG	-10.18 (-10.92)	35 nM (10 nM)	K59, H63, K66
47	HbSB (5E83)	Ditto	NADH	-8.89	305 nM	S44, K59, H63, K66, T87
48	HbSB (5E83)	Ditto	ATP	-5.72	64.4 μ M	S44, K66
49	HbSB (5E83)	Ditto	ADP (2)	-4.36	638 μ M	K66, D73, T84, T87
50	HbEA (1NQP)	$\alpha 2\beta 2$ CN bound tetrameric thalassemic cell	2,3 BPG	-9.66	83 nM	H45, H58, K61, K90

51	HbEA (1NQP)	Ditto	NADH	-6.08	<i>34.9 μM</i>	H45, K61, A82
52	HbEA (1NQP)	Ditto	ATP	-5.53	<i>89 μM</i>	H58, K61
53	HbEA (1NQP)	Ditto	ADP (2)	-4.08 (-4.61)	<i>1.02 mM</i> (417 μ M)	H58, K61, K90
54	HbEB (1NQP)	Ditto	2,3 BPG	-9.47 (-12.09)	<i>114 nM</i> (1.4 nM)	S44, K59, H63, K66
55	HbEB (1NQP)	Ditto	NADH	-7.44	<i>3.5 μM</i>	S44, H63, K66
56	HbEB (1NQP)	Ditto	ATP	-4.22 (-5.31)	<i>802 μM</i> (128 μ M)	S44, K66
57	HbEB (1NQP)	Ditto	ADP (1)	-3.62 (-3.71)	<i>2.23 mM</i> (1.91 mM)	K59, H63, K66
58	Mb (3RGK)	Aquomet form	2,3 BPG	-7.91	<i>1.6 μM</i>	R45, K63, H64, T67
59	Mb (3RGK)	Ditto	NADH	-5.66 (-6.14)	<i>71.5 μM</i> (32 μ M)	R45, H64, T67
60	Mb (3RGK)	Ditto	ATP	-3.77 (-4.17)	<i>1.74 mM</i> (870.5 μ M)	R45, K63, T67
61	Mb (3RGK)	Ditto	ADP	-2.38 (-2.90)	<i>17.9 mM</i> (7.42 mM)	R45, H64, T67
62	Mb (3RGK)	Ditto	1,3 BPG	-3.20 (-4.02)	<i>4.52 mM</i> (1.12 mM)	R45, H64, T67
63	Mb (3RGK)	Ditto	NAD ⁺	+0.56	NA	NA
64	Mb (3RGK)	Ditto	NADPH	-4.45 (-5.27)	<i>543 μM</i> (138 μ M)	R45, H64, T67
65	Mb (3RGK)	Ditto	NADP ⁺	Nil	Nil	Nil

Values given in braces for ADP are the number of unique binding sites with $K_d < 2$ mM. * Values given in small braces are the cases when an alternative binding site (outside heme-cavity) has higher affinity. For the non-active site binding of beta monomer with 2,3-BPG, the values are given in large parenthesis. K_d values with physiological significance are italicized. HbS and HbE stand for sickle cell and thalassemia hemoglobins. A and B are alpha and beta monomers of normal Hb.

Table 2: Extra-heme pocket binding of select ligands to tetrameric Hb.

No.	Protein	Description	Ligand	ΔG (kcal/mol) [#]	K_d [#]	Interacting Amino Acids
1	Hb (1GZX)	$\alpha_2\beta_2$ Tetra ligated with O ₂ (tensed)	2,3-BPG (4+1)	-16.12	<i>1.53 pM</i>	V544, H545, K625, G679, N682
2	Hb (1GZX)	Ditto	ADP (4+1)	-5.65	<i>72 μM</i>	V544, L621, K625
3	Hb (3D17)	$\alpha_2\beta_2$ Triply ligated with CO (relaxed)	2,3-BPG (4+2)	-20.75, -12.54	<i>619 aM,</i> <i>638 pM</i>	K82, H143, H146
4	Hb (3D17)	Ditto	ADP (4)	Nil	Nil	Nil

[#]Bold values in parentheses represent heme pocket binding.

Discussion: Placing our findings/proposals with respect to available literature

Several works have explored the implications or DROS in hemoglobin/RBC physiology (Ellsworth et al., 2016; Ellsworth et al., 2006; Sprague et al., 2009; Ellsworth and Sprague, 2012; Sprague et al., 2010; Turpin et al., 2020). There is also speculation that human skeletal muscle blood flow as well as tissue oxygen delivery is dependent on circulating ATP concentrations (González-Alonso et al., 2002). In the light of our earlier works, the literature such as above and the findings herein could be amalgamated with a fresh perspective, to give greater insights to better understand several physiological scenarios and pathological states.

Over the last several decades, experimental evidence for adenosine nucleotides' (like ADP and NADH) binding to hemoglobin (using equilibrium dialysis, UV-Vis and NMR spectroscopy, etc.) have been reported by several groups (Banerjee et al., 2012; Cashon et al., 1986; Gupta et al., 1978; Lo and Schimmel, 1969; Ogo et al., 1989; Peng et al., 2003). The order of affinity, as determined by equilibrium dialysis (a suitable technique to study association-dissociation phenomena) is BPG>ATP>ADP>AMP, and the pioneering researchers had suggested that they bind at the same locus (Lo and Schimmel, 1969). The in silico binding energy values determined in this study for the heme cavity gave the order of affinity as BPG>ADP $\geq$ ATP>AMP. Since there were 3 distinct clusters for ATP binding (compared to one of ADP), the net outcome obtained with in silico explanation is quite similar to the one observed with equilibrium dialysis. Therefore, our in silico exploration results are valid and also justify the pioneers' deduction that the small molecule ligands bind at the same site. In most cases, the binding affinity of ADP/ATP/NADH/2,3-BPG to the proteins of interest is relevant with respect to the physiological concentrations of the small molecules. Although the binding of adenosine nucleotides to Hb or Mb (Inohara et al., 2013) was known, their effects were attributed to allostery and the 'cause-consequence correlations' were not drawn with adequate physiological relevance. This is when other salient protagonists like $H^+ + CO_2$ (Bohr Effect) were well-explored with respect to Hb function.

I. Murburn model for transport/metabolism mediated/catalyzed by hemoglobin

Murburn concept advocates that certain redox enzymes can mediate catalysis via DR(O)S stabilized and released from one site, on a substrate bound at another site on/around the enzyme.

We posit that hemoglobin is a murzyme, working along murburn concept. Since the majority of ATP formed in RBC must stem from alternative metabolic routes (that relies on the conversion of glucose), we propose that it is synthesized from the NADH formed in the 6th step, from glyceraldehyde-3-phosphate (catalyzed by the enzyme glyceraldehyde phosphate dehydrogenase). As per the murburn perspective, Methb reductase, also known as diaphorase or cytochrome *b*₅ reductase (Kitao et al., 1974), could activate oxygen to give superoxide, along the lines established for cytochrome-reducing flavoenzymes (Manoj et al., 2010). The superoxide thus formed could directly bind hemoglobin. Else, DR(O)S may relay the electron to the hemeFeIII (as the heme is accessible to solvent) and reduce it to hemeFeII, after which an oxygen molecule can access and bind it at the distal side. The two probabilistic events give the same outcomes. The association of oxygen to hemeFeII ($k_{on} = 6.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) and dissociation thereof ($k_{off} = 2 \times 10^1 \text{ s}^{-1}$) are well characterized (Wilbur et al., 2012). It implies that the coordinate-ligated complex has a K_d of ~313 nM and dissociates ~20 times per second. Quite similar to the association event, the dissociation event can occur either via “FeIII + superoxide” or “FeII + oxygen”. In case of the former outcome, the hemeFe needs to be re-reduced; and the superoxide released would set off a murburn phosphorylation cascade near the Hb protein, leading to DROS/oxygen, ATP and water as products. (DROS like peroxide or superoxide would auto or catalytically dismutate or cross-react, to regenerate oxygen, which can be used by cells. This would facilitate oxygen-superoxide binding in the other Hb molecules within vicinity, explaining the cooperativity. In case the latter dissociation occurs, oxygen can be availed by tissues and the hemeFe is ready to bind another molecule of oxygen yet again. In fact, the nature of the oxy-Hb adduct is a mixture of both, with Fe(III)-^{*}O₂⁻ superseding Fe(II)-O₂ populations in the ratio of 3:1 to 4:1, as indicated by DFT calculations, reactivity profiles and spectroscopic studies (Jensen & Ryde, 2004). Therefore, our proposal that superoxide could emanate from the heme center is probabilistically/statistically viable. At low acidity and low 2,3-BPG concentrations, the high affinity 2,3-BPG sites (with pM level K_d) within the tetramer are occupied in the oxygenated Hb (with relaxed monomers). In this state, the escape of oxygen/superoxide via the secondary aperture of beta-monomers can be minimized and the primary cavities of the monomers are also bound to ATP/NADH, preventing oxygen loss via dissociation. This serves Hb well in its O₂-transport function. At higher acidity and higher concentrations of 2,3-BPG (which is correlated to lower concentration of NADH/ATP), the

primary heme active sites' access of 2,3-BPG becomes more facile and relevant; and Hb's transportation of oxygen ends and metabolic role is discharged. Now, oxygen/DROS are displaced from the active site, and DROS dynamics is facilitated around the Hb monomers. The moderate affinity ADP bound at the central slot of the tensed tetramer serves as a highly efficient modality for attack by the DROS that could potentially emerge from the secondary aperture of the beta-monomers. Therefore, the secondary aperture of beta-monomer (Figure 7) enhances efficient oxygen/DROS delivery and inter-monomer communication (which is the same as the erstwhile concept of co-operativity). The overall murburn scheme is depicted in Figure 8 and supported by the following features of Hb/RBC:

1. When superoxide was presented to RBC (via xanthine oxidase activity in situ) enhanced ATP production/release was observed alongside increased DROS production (Ellsworth et al., 2006). This is direct and corroborative evidence for our proposal, which is identical to the ATP synthesis afforded by in situ provisions of xanthine oxidase activity in the mitochondrial system (Mailer, 1990). We had earlier demonstrated that superoxide can aid ATP synthesis in vitro (with just ADP and phosphate), and provided luciferase activity-based (Manoj et al., 2019a) and HPLC-based (Manoj et al., 2019b) confirmation for the feasibility of DROS-mediated ATP synthesis under physiological ambiances.

Figure 7: Structure of the beta monomer of human hemoglobin shows a secondary heme cavity aperture.

Overview

Transport of oxygen by Hb

Metabolism of oxygen by Hb

Figure 8: The murburn perspective for Hb-sponsored physiology (roles of proton and water not shown). For enabling facile depiction, the two alpha and two beta monomers are considered equivalent to the opposing faces of a cube. (This has no realistic bearing with the actual structure!) For clarity, the heme pouches of beta monomer are not shown. The tetramer may shift shapes to intermediary structures, with varying affinities for the ligands. The schematic depiction shows effective ligand/structure aided confinement of oxygen for transport (left) and ligand/aperture aided O_2 /DROS delivery (right).

2. The thermodynamic/kinetic and mechanistic aspects of the overall murburn reaction schemes have been discussed in our earlier works (Manoj et al., 2019a,b; Manoj & Bazhin, 2019). The murburn equation for ATP synthesis from NADH can be minimally represented theoretically as:

$$\text{For } n = 1, \Delta_r G'^{\circ}_{\text{aq}} = -546 \text{ kJ/mol, or } K_{\text{eq}} = \sim 10^9$$

It can be envisaged that the depletion of DROS in milieu due to catalase/SOD activity in RBC and the continuous ATP-consumption by various life-activities would drive the equilibrium to the right. Though the depletion of catalytically essential DROS would lower ATP yields, this strategy would be essential to minimize collateral cellular damage, to afford longer life-spans. Given the scenario above, we can envisage a fractional yield of the useful DROS reactions from the theoretical maximum. Let's say we get $K_{\text{eq}} \sim 10^9 \text{ M}^{-1}$ for the equation/equilibrium above. Since $K_{\text{eq}} (\text{M}^{-1}) = ([\text{NAD}^+] \times [\text{ATP}] \times [\text{H}_2\text{O}] \times [\text{H}_2\text{O}_2]) / ([\text{NADH}] \times [\text{H}^+] \times [\text{O}_2] \times [\text{ADP}] \times [\text{Pi}])$, we can solve the equation for the equilibrium theoretical yield of [ATP] by substituting the ascertained values of reactants/metabolites in erythrocytes ($[\text{NADH} \approx \text{NAD}^+] = [\text{O}_2] = [\text{ADP}] = 10^{-4} \text{ M}$; $[\text{Pi}] = 10^{-3} \text{ M}$) as below:

$$\begin{aligned} [\text{ATP}] &= K_{\text{eq}} \times ([\text{NADH}] \times [\text{H}^+] \times [\text{O}_2] \times [\text{ADP}] \times [\text{Pi}]) / ([\text{NAD}^+] \times [\text{H}_2\text{O}] \times [\text{H}_2\text{O}_2]) \\ &= \sim 10^9 \times ([\sim 10^{-4}] [\sim 10^{-7}] [10^{-4}] [10^{-4}] [10^{-3}]) / ([\sim 10^{-4}] [10^1] [10^{-10}]) \\ &= \sim 10^9 \times (\sim 10^{-22}) / (\sim 10^{-13}) = 10^0 = 1 \text{ M!!} \end{aligned}$$

It can be seen that even with a fraction of the overall yield (and with water and peroxide concentrations respectively taken as 10 M and 0.1 nM), the ATP formation overshoots the expectations by three orders of magnitude! Therefore, the murburn phosphorylation equation involving the intermediacy of DROS is highly efficient, and the product concentrations can be comfortably explained in thermodynamic/equilibrium treatments. Also, since the reactions involve bimolecular collisions (involving radicals that pose low activation energy barriers), the overall reactions are also probabilistically, kinetically and mechanistically facile.

3. Direct EPR-spectroscopic evidence is available for the release of superoxide from hemoglobin (Balagopalakrishna et al., 1996). Owing to the high concentrations of hemeFe, catalase, peroxiredoxins, SOD, etc. (which is expected to efficiently deplete DROS found away from Hb, to levels below temporal and measurable detection-ability), the online detected peroxide

concentrations would be low. In spite of the high concentrations of such enzymes, DROS have been detected in RBCs (Giulivi et al., 1994; Novak et al., 1998; Scarpa et al., 2011; Schacter, 1986; Song et al., 2015). It is important to note that catalases and peroxidases produce ROS depending on the reaction conditions and these enzymes do not have strictly antioxidant functions. For example: catalase also serves as an oxidase (Vetrano et al., 2005). Similarly, hemoglobin also performs oxidative reactions (Winterbourn, 1990).

4. Superoxide mediated phosphorylation of ADP would be facile in the vicinity of Hb, as shown by molecular docking (Figure 3). In this regard, binding of NADH (present at relatively lower concentrations) at the heme-aperture could also facilitate DROS-dynamics in milieu, enabling efficient ATP synthesis and sequential/concerted oxygen or superoxide binding/utilization by the Hb tetramer. In this regard, the binding of ADP (Lo & Schimmel, 1969) and NADH (Cashon et al., 1986) to Hb have been substantiated with earlier research reports from reputed labs.

5. Experimental/theoretical evidence for the predominance and relevance of heme Fe(III)-superoxide type adduct population is available in literature (Jensen & Ryde, 2004). If we consider that Hb is present at ~5 mM ($\sim 2 \times 10^8$ copies) in RBC, and account that the naturally found ~1% MetMb are formed by dissociation via the former modality (FeIII-superoxide), we have a working Hb-murzyme concentration of 50 μ M ($\sim 2 \times 10^6$ copies). For a comparison, this is twice the concentration of the most abundant of glycolytic enzymes (which form only ~2% of the total protein content) of RBC, glyceraldehyde-3-phosphate dehydrogenase, which makes NADH at the 6th step (Bryk and Wiśniewski, 2017). Also, the same authors in their exhaustive proteomics study above had reported that redox enzymes- catalase, peroxiredoxins, and superoxide dismutase (SOD) were the other proteins present in copy numbers ranging in millions. The presence of these enzymes signifies the high relevance of DROS production and involvement in RBCs. It must be remembered that the presence of cutting boards, knife-racks and gloves (analogous to SOD/catalase/peroxidase, glutathione, peroxiredoxins, etc.) in the kitchen (analogous to RBCs) do not signify the irrelevance of knives (analogous to DROS), but it is an indication of the routine relevance/utility of knives (Jacob and Manoj, 2019).

6. Hb-Fe needs to be reduced by a flavoenzyme (which must be supported by NADH, in turn) for binding oxygen and both NADH and flavoenzyme cytochrome reductase are present in RBCs. (If hemeFeIII or MetHb could directly bind and transport oxygen, then it would have made sense to argue that murburn mechanism would not be physiologically relevant.). Further, the concentrations of components (proteins, NADH, ADP, Pi, O₂, etc.) in RBCs (Garrett and Grisham, 1999) are conducive for murburn chemistry (Manoj et al., 2016; Parashar and Manoj, 2021); (Manoj et al., 2019a; Manoj et al., 2020; Manoj et al., 2019b), in spite of the water-filled and larger dimensions of the RBC. Particularly, since the oxidative phosphorylation route depends on mM levels of inorganic phosphate (which is available in RBC), the formation of mM levels of ATP by this route seems more probable (when contrasted to the highly regulated substrate-level phosphorylations whose intermediates are in μ M ranges).

II. What are some salient facts/observations that murburn proposal explains better?

Visualizations, data and interpretations from the current in silico explorations (in conjunction with murburn concept) allow for several novel explanatory paradigms in the field, pertaining to the energy metabolism in RBCs and oxygen delivery by Hb.

1. DROS-assisted formation of ATP in RBC can potentially explain the mM concentration ranges of ATP when ADP is less than one tenth of the same (Garrett & Grisham, 1999). Also, the long life of circulating erythrocytes (~4 months) carrying out various functions (Lu et al., 2008; Pandey and Rizvi, 2011), without nucleus or mitochondria, is explained with Hb serving as an agent of ATP synthesis. The current binding energy analyses suggest that Mb has far lower functional relevance for interaction with ADP, when compared to Hb. This could be explained by considering the fact that myocytes have mitochondria to generate high concentrations of ATP.

2. How does hemoglobin get reduced? The concentration of MetHb/Cyt. *b*₅ reductase is very low, which precludes the protein-protein complexation based long-range electron transfer process (Yubisui et al., 1980). We have already explained the rationale for murburn model of electron transfers in several heme-flavin protein systems (Manoj et al., 2016a-c). The positively

charged amino acid residues at the heme pouch entrance serve to attract negatively charged species like superoxide and phosphate ions.

3. Tetrameric nature of Hb would favor radical scavenging by the neighboring monomer, which could also explain the sigmoid binding of oxygen that could result from the superoxide binding dynamics, as seen in P450 systems (Harlow and Halpert, 1998; Manoj et al., 2016a). The radical chemistry had also explained the respirasome supercomplex associations in the mitochondrial system (Manoj et al., 2019a). Myoglobin, although a hemeprotein involved in oxygen-binding, is a monomer, because it has little roles in oxidative phosphorylation of ADP. From another perspective, Mb also shows sigmoid oxygen binding curves (Nerimetla et al., 2017), although they have been interpreted as hyperbolic. This signifies that the profiles obtained in Hb are due to the involvement of DROS equilibriums (FeIII-superoxide is also involved, not just FeII-oxygen!). The fact that Hb- β monomer has two channels (Figure 7) for oxygen/superoxide access/exit to/from hemeFe (which is conserved even in the sickle and thalassemic forms) supports the murburn perspective. We have not seen this aspect of Hb- β being attributed any physiological functions in our survey of literature. We project that the larger active site pocket volumes of the beta monomer in sickle cell and thalassemic states (in comparison to the normal beta monomer) could lead to deleterious DROS dynamics in physiology.

4. Non-specific glycation and phosphorylations of various biological components are observed in RBCs, and many such outcomes have relevance in several signaling pathways and some present pathophysiological manifestations (Grancara et al., 2015; Nedić et al., 2013; Zhang et al., 2009). Some of these can be explained with the relevance of DROS-based murburn reaction chemistry. (Seen in another way- if proteins can get glycated and phosphorylated by DROS, why can't ADP get phosphorylated?)

5. It is a significant consideration that from the time of Hb-oxygenation in lungs, RBCs take at least a few seconds to reach the various parts of the body (as blood moves at ~3 feet per second). Therefore, significant amount of dissociations occur within this time range. At high oxygen and reducing equivalents (NADH) concentrations, ample ATP is formed. Binding of this

ATP/NADH to heme pocket of Hb (Figures 3 & 4) could serve as a lid preventing the escape of oxygen from the active site, thereby facilitating oxygen transport by Hb.

6. The binding of organic and inorganic phosphates to globins' active site has been reported and characterized earlier (Yoshida & Izuka, 1976). In the classical perspective, it is difficult to rationalize the cause-consequence sequence for the effect of 2,3-BPG on the structural dynamics of Hb tetramer. That is- the effector (2,3-BPG) can bind only within the large cavity of the tense form. However, the cavity will only be formed if the active site ligand (oxygen) is not present! It is unlikely that the small molecule of 2,3-BPG could push against the "inaccessible site" and liberate oxygen and make the Hb monomers tense, so as to get itself a binding slot on the tetramer. The murburn perspective of binding of 2,3-BPG at the active site is a parsimonious and direct explanation for some of the effects posed by the same ligand. It is known that 2,3-BPG binding is associated with the deoxygenated state of Hb, and this is visually justifiable from the active site visualization shown in Figure 5. The data for 2,3-BPG binding at active site is also in the order $\text{HbB} > \text{HbA} > \text{HbF}$, which justifies the fetal physiological requirements (for receiving oxygen from mother's blood supply). Further, the allosteric 2,3-BPG is always picked up by the docking procedure in the beta-monomers (and the process clearly demarcates between other small molecules like 1,3-BPH and 3-phosphoglycerate), signifying the efficiency of the protocol. The combined effects of poor ADP binding, high ATP/NADH binding, low 2,3-BPG binding at the heme-pocket and the energetically favorable 2,3-BPG binding at the allosteric site would render the energy metabolism and oxygen transfer by HbS and HbE inefficient. Such functionality-based argument is more appropriate than a merely structure-based Hb stacking failure in sickle-cell RBCs. (That is- all Hbs in sickle cell individuals have the same structural fault. Then, why do only some of the RBCs of such individuals sickle? In contrast, the murburn perspective mandates that depending on the circumstances, the metabolic activities of some cells could get skewed because of inefficient catalysis, thereby denying the cell the necessary energy requirements, leading to sickling.) Among a host of factors like temperature surrounding RBCs, pH, CO_2 concentration and pO_2 , one of the key determinants of RBCs' shape is the concentration of ATP and 2,3-BPG. ATP concentrations maintain the discocyte shape of RBCs, while depletion of ATP has been shown to lead to formation of echinocytic shaped cells. This is because ATP minimizes the cytoskeletal stiffness and hence, lower ATP concentrations cause

contraction of the cytoskeleton, causing the formation of spicules on RBC surface by pulling the bilayer over a smaller projected area and increased O₂ requirement because of the higher volume: area ratio caused by the shape change. ATP as well as other organic phosphates have higher affinity for deoxyHb, and therefore, maintain RBC in deoxygenated state (Benesch and Benesch, 1967; Chowdhury et al., 2017). In many diseases, the varying shape (poikilocytosis) and morphology (anisocytosis) of the RBCs is a key factor – for example, in thalassemia and splenomegaly, the shape of the RBCs is teardrop shaped (dacrocytes) and in other cases, we have other variations. The biconcave disc shape is ideal for rapid gaseous exchange. ATP and other organic phosphates influence RBC shapes. The shape of the oxygen dissociation curve depends on allosteric modulators such as 2,3-BPG (and ATP/ADP?). Lower O₂ concentrations (such as in metabolic acidosis) trigger release of ATP and this would induce shape changes in RBCs (Sridharan et al., 2010). Glycolysis can occur in the absence of oxygen; however, to achieve very high (mM) concentrations of ATP, the RBCs would need normoxic O₂ concentrations. Each RBC has an estimated 260 million Hb molecules. Any decrease in iron levels (anemia) would mean lesser Fe/heme is available for DROS formation and thereby, lack of ATP would directly affect RBCs' shape and tissue oxygenation, because gaseous exchange is directly correlated to shape of the RBCs. ATP has been proposed to be an extracellular molecule (due to its role in c-AMP formation and signaling) which is released by healthy RBCs in response to hypoxia to trigger vasodilation (as a protective measure) and by oxidatively stressed / malaria infected RBCs (Ramdani and Langsley, 2014). These are some of the implications that could be re-investigated with the insight provided by murburn perspective.

Conclusions

The DROS-based ATP-synthesis and redox cycle stochastic mechanism not only accounts for the mM level of ATP observed in RBCs but also presents a thermodynamically and probabilistically viable proposal. Our hypothesis opens doors to explore further avenues, on the far reaching physiological and pathological implications of modulating reactive oxygen species as a key player (not always the villain) while designing personalized treatment regimes for type 2 diabetes, sickle cell anemia, thalassemia and microvascular dysfunction. Summing up, the new murburn perspective for Hb function affords a “win-win” situation for RBC wherein Hb could

simultaneously serve as an oxygen transportation, ATP generation and NADH recycle modality. Although protons are in plenty within RBCs (enabling facile DROS dismutations and thereby lowering yields), this is offset by the high packing density of Hb. Since erythrocytes lack the mitochondrial Complex V or F_0F_1 ATP(synth)ase, the millimolar amounts of ATP formed in RBC is direct evidence of the murburn model functioning of Hb.

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