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Stimulation of glyceraldehyde-3-phosphate dehydrogenase by oxyhemoglobin

Paul S. Brookes[a,*], John M. Land[a,b], John B. Clark[a], Simon J.R. Heales[a,b]

[a]*Department of Neurochemistry, Institute of Neurology, Queen Square, London WC1N 3BG, UK*
[b]*Department of Clinical Biochemistry, Institute of Neurology, Queen Square, London WC1N 3BG, UK*

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Abstract Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key glycolytic enzyme regulated by many diverse mechanisms. In this study we present evidence that GAPDH activity is stimulated in the presence of oxyhemoglobin (2.3-fold, $P < 0.005$). No stimulation was seen by myoglobin, and only slight stimulation (1.2-fold, not significant) by methemoglobin was observed. Such stimulation may have physiological significance as 1,3-bis-phosphoglycerate, the product of GAPDH, isomerises to 2,3-bis-phosphoglycerate, an allosteric effector that decreases the oxygen affinity of hemoglobin, thus providing a feedback loop. The results suggest that when assaying GAPDH activity in biological samples, hemoglobin content should be taken into account.

Key words: Glyceraldehyde-3-phosphate dehydrogenase; Hemoglobin; 2,3-diphospho-glycerate; Alzheimer's disease

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC1.2.1.12) is a key enzyme in glycolysis, catalysing the conversion of glyceraldehyde-3-phosphate to 1,3-bis-phosphoglycerate. Previous work has indicated that this enzyme may be subject to regulation by multiple mechanisms [1–12]. There is evidence to suggest that GAPDH is inhibited by a nitric oxide dependent ADP ribosylation [1–3] and recent work suggests that this method of regulation may in fact proceed by the covalent linking of an NAD^+ molecule to the enzyme following nitrosylation of a cysteine residue [2]. Oxygen free radicals have been observed to enhance this inhibition in cultured neurones [4]. In erythrocytes, an increased production of free radicals may cause GAPDH to traverse the plasma membrane, leading to up-regulation of erythrocyte glycolysis in times of oxidative stress [5]. It is reported that GAPDH is inhibited by interaction with erythrocyte membranes [6] and tubulin complexes [7], and activated by interaction with α-amino acids, ATP and dithiothreitol [8], although other evidence suggests dithiothreitol may inhibit the enzyme [9]. GAPDH also interacts with the cytosolic domain of the Alzheimer's β-amyloid protein [10], calmodulin [11], and its neighbouring glycolytic enzyme 3-phosphoglycerate phosphokinase (PGK) [12], although these interactions do not appear to affect the enzyme's activity, and may serve to sequester it away from other cellular components.

In the current study a routine series of GAPDH activity assays in liver cytosolic fractions gave a wide range of values. As tissue samples contained differing amounts of blood, and GAPDH activities were expressed per mg protein, it was decided to assay for hemoglobin and correct for its presence. However, upon examining GAPDH activity and hemoglobin content data, a distinct correlation was noticed between them, and it was decided to investigate this further using purified enzyme and hemoglobin.

2. Materials and methods

All enzymes and biochemicals were from Sigma, Poole, Dorset, UK. Mice of the C57/CBA strain were maintained and killed under recognised procedures of the Animals (Scientific Procedures) act 1986. Following sacrifice, liver was removed and frozen at −70°C until use. After thawing, tissue (100 mg) was homogenised (glass:glass, 0.1 mm clearance) in 3 ml ice cold assay buffer (110 mM triethanolamine-hydrochloride, pH 7.6 with Tris at 30°C), and centrifuged at 60 000 rpm (152 500 × *g*) in a fixed angle rotor. The supernatant was retained for enzyme assays and pellet discarded.

GAPDH activity was measured by following the backward reaction converting NADH to NAD^+, at 340 nm [13] in a Uvikon 940 spectrophotometer. The assay buffer contained triethanolamine-hydrochloride (110 mM), β-NADH (200 μM), EDTA (0.9 mM) and $MgSO_4$ (1.7 mM), pH 7.6 at 30°C. 3-phosphoglyceric acid (6 mM) and ATP (1.1 mM) were added as substrates for the auxiliary enzyme PGK (15 U/ml). Cytosolic fraction samples (10 μl) were added neat, and for the in vitro analysis GAPDH of approximately the same final activity (100 nmol/min/ml) was added (10 μl). Hemoglobin, myoglobin, or methemoglobin dissolved in water were added to give final concentrations of 0.01–0.1 mg/ml. Hemoglobin was purchased in the A_0 ferrous form, essentially free of methemoglobin. Scanning wavelength spectrophotometric analysis was used to confirm this (result not shown). To check that any effect of hemoglobin was not an artefact of the assay method, GAPDH activity was also measured in the forward direction, with glyceraldehyde-3-phosphate, β-NAD^+ and pyrophosphate as substrates, with arsenate present to inhibit the back reaction [14].

The hemoglobin content of samples was assayed by following the catalytic action of hemoglobin on the oxidation of tetramethyl benzidine by hydrogen peroxide at 600 nm, using Sigma assay kit #527 [15]. Protein was measured by the Lowry method [16] with bovine serum albumin as standard. For liver homogenates, data were expressed per mg of non-hemoglobin protein, following corrections for amounts of hemoglobin contamination. Statistical analysis was by the Student's *t*-test.

3. Results

Fig. 1 shows mouse liver cytosolic fraction GAPDH activity (expressed per mg non-hemoglobin protein) as a function of hemoglobin concentration. The total protein content of samples was in the range 28.6 to 41.0 mg/ml, and hemoglobin content was in the range 0.64 to 2.87 mg/ml. Thus, hemoglobin made up no more than 10% of total protein, and the shape of the curve in Fig. 1 is not altered if activities are expressed per mg total protein (not shown).

Greater GAPDH activities are associated with greater hemoglobin concentrations, suggesting that hemoglobin may be

*Corresponding author. Fax: +44 (171) 8331016.
E-mail: pbrookes@ion.ucl.ac.uk

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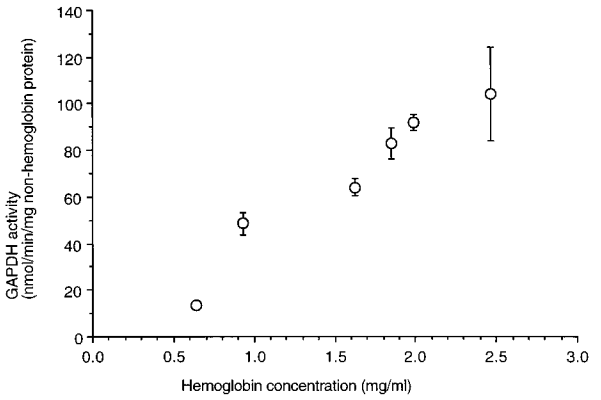

Fig. 1. GAPDH activity and hemoglobin concentration in mouse liver cytosolic fractions. Data were obtained as in Section 2. Activity values are means ± S.E.M. of three determinations. N.b. protein concentrations are corrected for hemoglobin content, so data are expressed per mg non-hemoglobin protein.

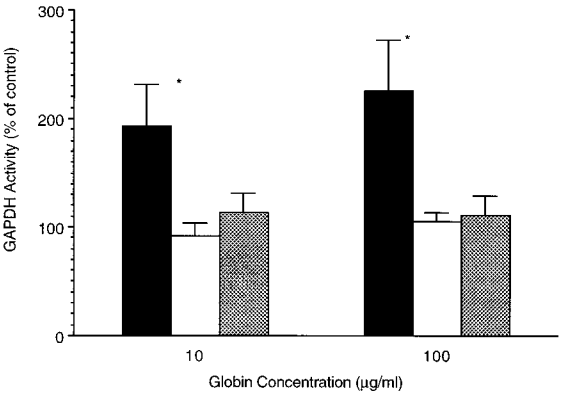

Fig. 2. Effect of added globin proteins on the activity of GAPDH (black bars: hemoglobin, white bars: myoglobin, shaded bars: methemoglobin). Values are expressed as percentages of control (no globin) values, and are means ± S.E.M. of 6 independent experiments. An asterisk indicates $P < 0.005$ relative to controls.

activating GAPDH. Because GAPDH is present in large quantities in erythrocytes, the observation that those samples with greater amounts of blood contamination (indicated by more hemoglobin) have greater GAPDH activity is not surprising. To investigate whether the relationship between GAPDH activity and hemoglobin concentration was of a cause and effect nature, a series of in vitro assays were performed using purified enzyme and hemoglobin.

Fig. 2 shows the effect of globin proteins on the activity of purified rabbit muscle GAPDH. The graph clearly shows that oxyhemoglobin stimulates GAPDH (in solution, hemoglobin rapidly binds oxygen to become oxyhemoglobin; the oxidative state of hemoglobin was checked by scanning wavelength spectrophotometry, monitoring peaks at 420, 540 and 580 nm). Fig. 1 only reports activities at 10 and 100 μg/ml globin, as stimulation by hemoglobin reached a plateau at around 10 μg/ml (not shown). Myoglobin had no significant effect on GAPDH activity, suggesting that the stimulation is not just a protein agglutination effect. Methemoglobin showed slight stimulation, though not as much as oxyhemoglobin. There was no measurable rate in the presence of hemoglobin and absence of enzyme (not shown), indicating that hemoglobin does not catalyse the reaction itself. The effect of deoxyhemoglobin was tested by using dithionite to deplete oxygen in the assay mixture, but dithionite alone decreased the GAPDH activity, presumably through an effect on the thiol groups of GAPDH, or by re-reducing NAD^+ made by the reaction (result not shown). As methemoglobin does not bind oxygen, we assume that it behaves similarly to deoxyhemoglobin. Hemoglobin that had been denatured by boiling gave similar results to methemoglobin (not shown).

GAPDH activity was also measured in the forward direction, to ensure that any stimulation by hemoglobin was not due to interaction with the auxiliary enzyme PGK, or a strengthening of the reported interaction between GAPDH and PGK [12]. Such experiments gave identical results to those in Fig. 2 (not shown), indicating that hemoglobin interacts directly with GAPDH, not via PGK.

4. Discussion

Overall, the results presented here suggest that oxyhemoglobin interacts directly with GAPDH to stimulate it. This is the first reported stimulation of GAPDH by another protein. Interactions with low molecular mass compounds both inhibit [1–3,9] and stimulate [8] the enzyme, and other proteins either inhibit it [7] or have no effect [10–12].

The observation that hemoglobin concentration and GAPDH activity correlate positively is in contrast to the findings of a study in sickle cell anaemia patients [17]. The authors report that sickle cell patients fell into two sub-groups, and that the group with the highest hemoglobin count had the lowest erythrocyte GAPDH activity. However, the abnormal nature of sickle cell hemoglobin, or other factors in the pathology of sickle cell disease may account for this apparent discrepancy.

Stimulation of GAPDH by oxyhemoglobin may be rationalised by examining the scheme depicted in Fig. 3. The product of GAPDH, 1,3-diphospho-glycerate, can isomerise to 2,3-diphospho-glycerate (2,3-DPG) via the enzyme diphosphoglycerate mutase (DPGM). 2,3-DPG is a well established allosteric effector of hemoglobin [18], decreasing its affinity for oxygen by favouring the deoxy form when bound. The oxygen carrying capacity of hemoglobin and the rate of erythrocyte glycolysis are closely linked, as the latter will control the availability of 2,3-DPG [19]. Thus we propose a simple feedback loop mechanism whereby oxyhemoglobin may modulate glycolysis to affect its own oxygen carrying capacity.

At first sight this would appear to be in contrast to the

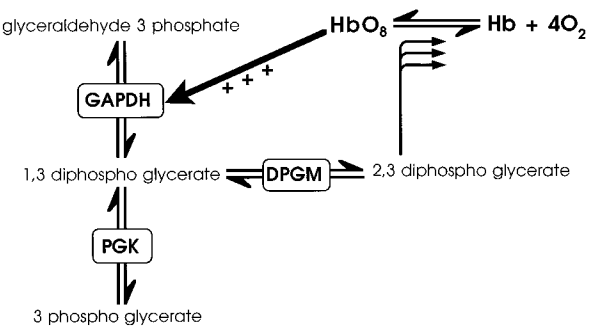

Fig. 3. Schematic representation of a possible physiological function of GAPDH stimulation by oxyhemoglobin in erythrocytes. GAPDH: glyceraldehyde-3-phosphate dehydrogenase, PGK: 3-phosphoglycerate phosphokinase, DPGM: diphosphoglycerate mutase, Hb: deoxyhemoglobin, HbO_8: oxyhemoglobin.

results of Oski et al., who report that deoxyhemoglobin stimulates the synthesis of 2,3-DPG in an in vitro glycolytic system [20]. However, this is due to the binding of 2,3-DPG by deoxyhemoglobin, removing product inhibition of DPGM. The authors also report stimulation of glycolysis by foetal hemoglobin, which has a much lower affinity for 2,3-DPG. This result may be explained by the current proposed mechanism – action at the site of GAPDH and not DPGM, although the use of carboxyhemoglobin in these experiments, not oxyhemoglobin, makes their interpretation difficult.

The current results are also interesting with respect to the effects of nitric oxide (NO) on GAPDH activity. NO binds hemoglobin, converting it to methemoglobin ($Fe^{2+} \rightarrow Fe^{3+}$) [21]. We have shown that methemoglobin only slightly stimulates GAPDH, so some of the observed NO induced inhibition of GAPDH [1–4] may in fact be due to removal of stimulatory oxyhemoglobin rather than direct inhibition. Different amounts of blood contamination in tissue samples may account for the conflicting results of these experiments [1,2].

A characteristic of Alzheimer's disease is senile plaques abundant in abnormally processed proteins such as the β-A4 amyloid peptide [22] and hemoglobin fragments [23]. GAPDH binds to the C-terminal domain of the amyloid precursor protein (APP) [10], and antibodies raised to β-amyloid cross-react with oxyhemoglobin [24]. These observations suggest that GAPDH may bind to oxyhemoglobin, agreeing with the results of the current investigation.

It was recently reported that GAPDH may be involved in age induced neuronal apoptosis [25]. The possible localisation of GAPDH at Alzheimer's plaques by β-A4 amyloid or hemoglobin fragments, and the subsequent stimulation of GAPDH by oxyhemoglobin, may lead to an increased rate of apoptosis in neurones surrounding the plaques, possibly accounting for some of the pathological symptoms of the disease.

As a final cautionary note, we would advise those analysing GAPDH activities in tissue samples to also assay for hemoglobin and correct for its presence, or possibly add a saturating amount to all samples to ensure that different levels of blood contamination do not affect results.

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