

# Fructosamine 3-kinase is involved in an intracellular deglycation pathway in human erythrocytes

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Fructosamine 3-kinase, which phosphorylates low-molecular-mass and protein-bound fructosamines on the third carbon of their deoxyfructose moiety, is quite active in erythrocytes, and was proposed to initiate a process removing fructosamine residues from proteins. In the present study, we show that incubation of human erythrocytes with 200 mM glucose not only caused the progressive formation of glycosylated haemoglobin, but also increased the level of an anionic form of haemoglobin containing alkali-labile phosphate, to approx. 5% of total haemoglobin. 1-Deoxy-1-morpholinofructose (DMF), a substrate and competitive inhibitor of fructosamine 3-kinase, doubled the rate of accumulation of glycosylated haemoglobin, but markedly decreased the amount of haemoglobin containing alkali-labile phosphate. The latter corresponds therefore to haemoglobin bound to a fructosamine 3-phosphate group (FN3P-Hb). Returning erythrocytes incubated with 200 mM glucose and DMF to a low-glucose medium devoid of DMF caused a decrease in the amount

of glycosylated haemoglobin, a transient increase in FN3P-Hb and a net decrease in the sum (glycosylated haemoglobin + FN3P-Hb). These effects were prevented by DMF, indicating that fructosamine 3-kinase is involved in the removal of fructosamine residues. The second step of this 'deglycation' process is most likely a spontaneous decomposition of the fructosamine 3-phosphate residues to a free amine, 3-deoxyglucosone and  $P_i$ . This is consistent with the findings that 2-oxo-3-deoxygluconate, the product of 3-deoxyglucosone oxidation, is formed in erythrocytes incubated for 2 days with 200 mM glucose in a sufficient amount to account for the removal of fructosamine residues from proteins, and that DMF appears to inhibit the formation of 2-oxo-3-deoxygluconate from elevated glucose concentrations.

Key words: fructoselysine, glycation, haemoglobin, HbA<sub>1c</sub>.

## INTRODUCTION

Chronic elevation of the blood glucose concentration in diabetes appears to be responsible for the long-term complications of this disease. It is indeed established that careful control of the blood glucose level decreases the appearance of such complications [1]. The link between the elevated concentration of glucose and the development of these complications is not yet clear. Several theories have been proposed, which include the aldose reductase hypothesis [2], the oxidative stress hypothesis [3–5], the protein kinase C activation theory [6] and the advanced glycation end-products theory [7]. Advanced glycation end-products result, at least partly, from the conversion of protein-bound fructosamines, which are formed through the condensation of glucose with primary amines, followed by an Amadori rearrangement. The formation of fructosamines, known as glycation, typically modifies the N-terminus and the lysine side chains of proteins (reviewed in [3,8,9]).

It is usually taken for granted that the formation of fructosamines is an irreversible process, which is unavoidably followed by their conversion into advanced glycation end-products. However, the recent identification of a fructosamine 3-kinase able to phosphorylate not only fructoselysine [or *N*- $\epsilon$ -(1-deoxyfructose)-lysine] and 1-deoxy-1-morpholinofructose (DMF), but also protein-bound fructosamines with  $K_m$  values in the micromolar range, opened the possibility that intracellular fructosamines can be metabolized [10–12]. The cDNA encoding fructosamine 3-kinase has been cloned, and the recombinant protein was

expressed and shown to have the same properties as the enzyme purified from human erythrocytes [10]. Interestingly, fructosamine 3-kinase is quite active in erythrocytes and in the lens, which are characterized by absent or slow protein turnover, and are therefore more susceptible to protein glycation. In agreement with the hypothesis of intracellular fructosamine metabolism, <sup>31</sup>P-NMR studies have indicated the presence of a small fraction of haemoglobin containing a phosphate group with a chemical shift corresponding to a fructosamine 3-phosphate residue [12]. The purpose of the present study was to test the hypothesis that fructosamine 3-kinase is involved in an intracellular protein 'deglycation' pathway.

## EXPERIMENTAL

### Reagents

Reagents, of analytical grade whenever possible, were from Acros (Geel, Belgium), Biowhittaker Europe (Verviers, Belgium), Merck or Sigma. Radiochemicals and DEAE-Sepharose were from Amersham Biosciences. AG 1X-8 (200–400-mesh) and AG 50W-X4 (100–200-mesh) resins, as well as Biogel P2 fine, were purchased from BioRad. Auxiliary enzymes were from Roche Molecular Biochemicals.

For the synthesis of fructoselysine, glucose (167 mmol) and *N*- $\alpha$ -t-Boc-lysine (where 'Boc' represents 'butoxycarbonyl') (37 mmol; from NovaBiochem) were heated in methanol (450 ml) for 5 h under reflux and N<sub>2</sub>. After freeze-drying, the sample was

Abbreviations used: DMF: 1-deoxy-1-morpholinofructose; FN3P-Hb: haemoglobin bound to a fructosamine 3-phosphate group.

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resuspended in water (150 ml), acidified with HCl to remove the protecting group from lysine, and applied on to a cation-exchange chromatography column (AG 50W-X4, Na<sup>+</sup> form) to separate unreacted glucose from the Amadori compound. The latter was eluted from the column with 150 mM NaCl, decolorized with charcoal, and desalted by gel-filtration on a Biogel P2 column. Yield with respect to lysine was approx. 45% (estimated by the reducing power [13], with fructose as a standard).

For the synthesis of radiolabelled fructoselysine, D-[U-<sup>14</sup>C]-glucose (50  $\mu$ Ci, 0.15  $\mu$ mol) and *N*- $\alpha$ -t-Boc-lysine (32  $\mu$ mol) were heated overnight in 0.8 ml of methanol at 50 °C and under N<sub>2</sub>. The sample was processed further as for the non-labelled compound, except that no decolorization with charcoal was required. The yield with respect to radioactive glucose was approx. 75%. Radiolabelled DMF was synthesized as described previously [11].

### Incubations of erythrocytes

Human heparinized blood from healthy volunteers was centrifuged for 10 min at 2000 *g* (at 4 °C). The plasma and the buffy coat were discarded, and the erythrocytes were washed three times in autoclaved 150 mM NaCl. Packed erythrocytes (1.25 ml) were resuspended in 20 volumes of a sterile Krebs–Henseleit bicarbonate buffer [14] equilibrated with a gaseous mixture of O<sub>2</sub>/CO<sub>2</sub> (19:1) and containing 1 mg/ml BSA, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and the indicated concentrations of glucose and DMF. Suspensions were incubated at 37 °C in an orbital shaker, and the buffer was changed after 48 and 96 h of incubation to avoid glucose depletion in the low-glucose suspensions. All washings were performed with 12 volumes of the corresponding ice-cold solution, and all centrifugations were performed at 4 °C. For the 5 mM glucose suspensions, erythrocytes were essentially washed three times with solution A (150 mM NaCl/1 mg/ml BSA) and then resuspended in fresh Krebs–Henseleit bicarbonate buffer. In the case of the 200 mM glucose suspensions, the following procedure was adopted to avoid cell breakage during the washings and to eliminate the intracellular glucose. The cells were isolated by a 10 min centrifugation at 1000 *g*, resuspended in solution B (200 mM NaCl/1 mg/ml BSA) pre-warmed at 37 °C, and then incubated for 1 h at the same temperature. After centrifugation, the cell pellet was washed first with 175 mM NaCl/1 mg/ml BSA, and then with solution A, before being resuspended in fresh Krebs–Henseleit bicarbonate buffer.

Incubations were stopped by centrifuging the suspensions for 10 min at 1000 *g*. The extracellular medium was mixed with 0.5 volume of 10% (w/v) HClO<sub>4</sub>, centrifuged for 10 min at 2000 *g*, and the supernatant was neutralized with 3 M KHCO<sub>3</sub>. The erythrocytes were washed twice with either solution A (5 mM glucose suspensions) or B (200 mM glucose suspensions), and a third time with the same NaCl concentration, but without BSA. An aliquot of the final cell pellet was mixed with 3 volumes of 10% (w/v) HClO<sub>4</sub>, and the extract was centrifuged for 10 min at 2000 *g*. The supernatant was neutralized with 3 M KHCO<sub>3</sub>. The remainder of the cell pellet was kept at –70 °C until use. Haemoglobin was measured in washed erythrocytes as described by Drabkin and Austie [15].

### Measurement of total glycosylated haemoglobin

Total glycosylated haemoglobin was measured by retention on affinity columns (boronate columns; Sigma Diagnostics) and elution with salt, according to the manufacturer's instructions,

except that 25  $\mu$ l of erythrocytes was used instead of 50  $\mu$ l of fresh blood.

### Separation of an anionic form of haemoglobin and measurement of protein-bound, alkali-labile phosphate

Packed erythrocytes (60  $\mu$ l) were diluted 20-fold in a buffer containing 10 mM Hepes, pH 7.1, 1 mM dithiothreitol and 1  $\mu$ g/ml leupeptin/antipain, and centrifuged for 15 min at 16000 *g* and at 4 °C. The supernatant (1 ml) was loaded on to a DEAE-Sepharose column (0.5 cm<sup>3</sup>, packed in disposable polystyrene columns; from Pierce) equilibrated with 10 mM Hepes, pH 7.1/1 mM dithiothreitol. The column was washed with 2 ml of the same buffer containing the protease inhibitors, and the flow-through and washing were collected in a single fraction. The retained haemoglobin (monitored by measuring A<sub>415</sub>) was eluted with the buffer containing 100 mM NaCl in a single 2 ml fraction. The latter fraction (1 ml) was mixed with 760  $\mu$ l of water and 140  $\mu$ l of 70% (w/v) HClO<sub>4</sub>, and then centrifuged for 15 min at 16000 *g* and at 4 °C. The supernatant was discarded, and 1 ml of 5% (w/v) HClO<sub>4</sub> was added to the pellet, which was left on ice for 20 min and then centrifuged for 5 min. The same washing step was repeated. The protein pellet was resuspended in 0.5 ml of 50 mM NaOH, and incubated for 40 min at 80 °C. After cooling on ice, 70% (w/v) HClO<sub>4</sub> was added to reach a final concentration of 5% (w/v). Haemoglobin was eliminated by centrifugation, and P<sub>i</sub> was measured as described by Itaya and Ui [16]. P<sub>i</sub> standards were measured in the presence of the same concentration of perchloric acid as that in the samples.

### Phosphorylation of haemoglobin with recombinant human fructosamine 3-kinase, and SDS/PAGE analysis

Fructosamine 3-kinase (5  $\mu$ g or 0.06 m-unit, prepared as described in [10]) was incubated at 30 °C in a medium containing 25 mM Tris/HCl, pH 7.8, 1 mM EGTA, 0.15 mM MgATP<sup>2-</sup>, 0.01  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP and 0.85 nmol of haemoglobin from the flow-through and washing fraction of the DEAE-Sepharose columns. The final volume was 37.5  $\mu$ l. After 30 min, the reaction was stopped by adding 12.5  $\mu$ l of sample loading buffer [165 mM Tris/HCl (pH 7.8)/8.25% (w/v) SDS/40% (w/v) sucrose/0.03% Bromophenol Blue/40 mM dithiothreitol] for SDS/PAGE. The samples were loaded on to a 13% (w/v) polyacrylamide gel, and allowed to migrate at 4 °C. The gel was dried under a vacuum and exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA, U.S.A.) for the detection of radioactivity.

### Measurement of 2-oxo-3-deoxygluconate in perchloric acid extracts, in erythrocytes and incubation media of erythrocytes

2-Oxo-3-deoxygluconate was measured through the production of ADP in the presence of *Escherichia coli* 2-oxo-3-deoxygluconate kinase. The latter was produced as an N-terminal fusion protein with a hexahistidine tag at 37 °C in the BL21(DE3)pLysS bacterial strain, grown in Luria–Bertani medium [17]. The enzyme was then purified to homogeneity on Ni<sup>2+</sup>-nitrilotriacetate agarose (Qiagen), from which it was eluted with 100 mM imidazole, pH 7.9. The assay mixture for 2-oxo-3-deoxygluconate contained 25 mM Hepes, pH 7.1, 25 mM KCl, 1 mM dithiothreitol, 0.1 mM NADH, 0.25 mM phosphoenolpyruvate, 0.5 mM MgATP<sup>2-</sup>, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mg/ml BSA, 10  $\mu$ g/ml lactate dehydrogenase and 10  $\mu$ g/ml

pyruvate kinase. After stabilization of  $A_{340}$ , 5  $\mu\text{g}$  of 2-oxo-3-deoxygluconate kinase was added and  $A_{340}$  was measured further until a new plateau was reached. The difference between the two readings was used to calculate the 2-oxo-3-deoxygluconate concentration. Measurements with standards (prepared as described in [18]) indicated that  $\Delta A_{340}$  was proportional to the 2-oxo-3-deoxygluconate concentration. It was verified that a series of compounds, including gluconate and 2-oxogluconate, did not interfere with the assay.

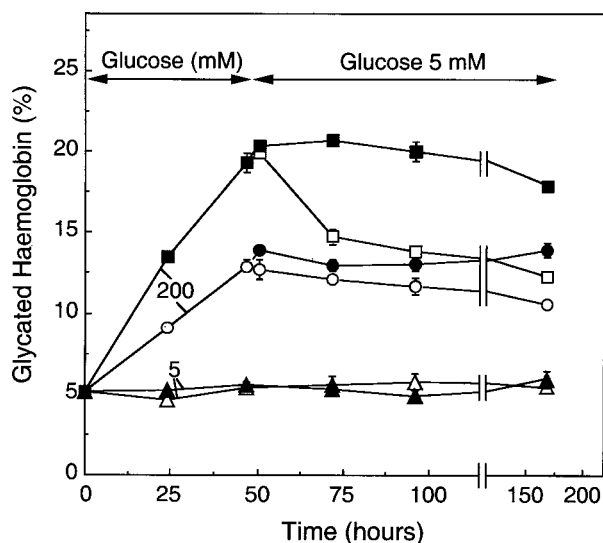
## RESULTS

### Utilization of [ $^{14}\text{C}$ ]fructoselysine by erythrocytes

Since fructoselysine is a good substrate for fructosamine 3-kinase, we measured its utilization by erythrocytes. Cells, at a dilution of 1:20, were incubated with 50  $\mu\text{M}$  fructoselysine (labelled on its deoxyfructose moiety) for 22 h, at which time extracts were prepared from the cell pellet and from the medium. Extracts from the cell pellet were chromatographed on anion-exchange columns (AG 1-X8;  $\text{Cl}^-$  form) to separate fructoselysine from fructoselysine 3-phosphate, which was eluted with 150 mM NaCl. The latter accounted at most for 5% of the total radioactivity at this time (results not shown). Chromatography of the medium on a cation-exchanger (AG 50W-X4,  $\text{Na}^+$  form) showed that one single peak of radioactivity was eluted from the column with 500 mM NaCl, with no radioactivity in the flow-through fractions (results not shown), indicating that no neutral or anionic metabolite was released into the medium.

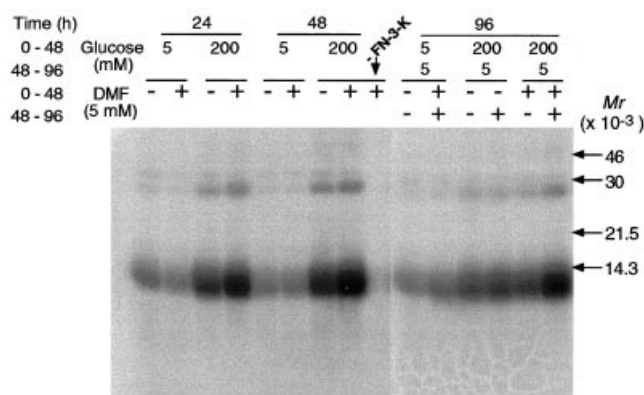
### Effect of DMF on the level of glycosylated haemoglobin

If fructosamine 3-kinase was involved in an intracellular deglycation process, an inhibitor of this enzyme should increase



**Figure 1** Effect of glucose concentration and DMF on the level of glycosylated haemoglobin in suspensions of human erythrocytes

Erythrocyte suspensions were incubated for 48 h with 200 mM glucose with (■) or without (○) 5 mM DMF. They were then subdivided and transferred to media containing 5 mM glucose with (●, ■) or without (○, □) 5 mM DMF. Other samples were incubated for the whole experimental period in the presence of 5 mM glucose with (▲) or without (△) 5 mM DMF. Incubations were arrested at the indicated times and glycosylated haemoglobin was measured by affinity chromatography on boronate columns. Results shown are means  $\pm$  S.E.M. for four independent experiments.



**Figure 2** Phosphorylation of glycosylated haemoglobin by recombinant fructosamine 3-kinase

Erythrocyte lysates derived from the experiment shown in Figure 1 were chromatographed on DEAE-Sephacrose. The fractions containing non-retained proteins were incubated with human recombinant fructosamine 3-kinase and [ $\gamma$ - $^{32}\text{P}$ ]ATP for 30 min. Phosphorylation products were separated by SDS/PAGE, and autoradiographed. A negative control without fructosamine 3-kinase (—FN-3-K) is also shown. One representative experiment is shown. The positions of molecular-mass markers are indicated on the right.

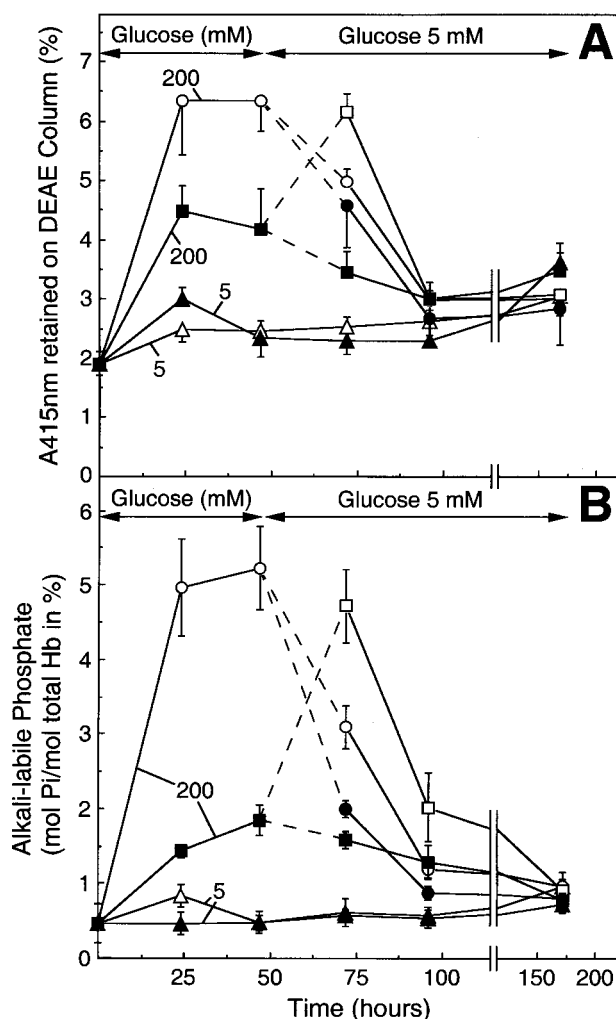
the amount of glycosylated haemoglobin in erythrocytes, most particularly in cells incubated in the presence of an elevated glucose concentration. To this end, we tested the effect of DMF, a substrate of fructosamine 3-kinase also known to act as a competitive inhibitor of this enzyme in intact erythrocytes [11].

Figure 1 shows that when human erythrocytes from control, non-diabetic subjects were incubated with 200 mM glucose, the proportion of glycosylated haemoglobin increased from 5% to approx. 13% in 2 days, and that it did not change significantly in cells incubated with 5 mM glucose. DMF doubled the rate of formation of glycosylated haemoglobin in the presence of 200 mM glucose, but had no detectable effect at the low glucose concentration.

Figure 1 also shows that when cells pre-incubated with 200 mM glucose were returned to a medium containing 5 mM glucose, the level of glycosylated haemoglobin slowly decreased in the absence of DMF, but remained unchanged in its presence. This difference was more marked in cells that had been pre-incubated with 200 mM glucose together with DMF. Returning the cells to a low-glucose, DMF-free medium was followed by a 'rapid' decrease in the level of glycosylated haemoglobin (from 20 to 15% in 24 h), followed by a slower decrease in the next 4 days. The rapid decrease was not observed in the cells returned to the low glucose concentration in the presence of DMF.

### Phosphorylation of glycosylated haemoglobin by fructosamine 3-kinase

The experiments mentioned above suggested that glycosylated haemoglobin is a substrate for fructosamine 3-kinase. In order to measure incorporation of [ $^{32}\text{P}$ ]P<sub>i</sub> from [ $\gamma$ - $^{32}\text{P}$ ]ATP catalysed by purified fructosamine 3-kinase, it was important to separate haemoglobin from enzymes that could interfere in the kinase assay (e.g. hexokinase, which could exhaust ATP in the presence of glucose; nucleoside diphosphate kinase, which could form a phosphoenzyme with almost the same subunit size as haemoglobin [19]). Erythrocyte lysates were therefore applied on to DEAE-Sephacrose, and the resulting flow-through fractions, which contained > 90% of the haemoglobin, were incubated



**Figure 3** Effect of glucose concentration and DMF on the proportion of haemoglobin retained on DEAE-Sepharose and on FN3P-Hb

The same experiment is shown as described in the legend to Figure 1. (A) The fraction of haemoglobin retained on the DEAE-Sepharose column; (B) the content of this fraction in alkali-labile phosphate. Results are expressed in relation to total haemoglobin in the sample applied on to the DEAE-Sepharose column.

with fructosamine 3-kinase and [ $\gamma$ - $^{32}$ P]ATP. Preliminary experiments indicated that near-maximal incorporation was obtained after 30 min of incubation. Phosphorylation products were separated by SDS/PAGE and autoradiographed.

As shown in Figure 2, the amount of haemoglobin that could be phosphorylated by fructosamine 3-kinase was higher in cells incubated with 200 mM glucose than with 5 mM glucose. DMF increased this level in the cells incubated with the elevated glucose concentration, but not with 5 mM glucose. The amount of phosphorylatable haemoglobin decreased when cells incubated with 200 mM glucose (with or without DMF) were returned to a low-glucose medium devoid of DMF, but remained stable in the presence of DMF. We ensured that no protein phosphorylation occurred when the cell extracts were incubated in the absence of recombinant fructosamine 3-kinase. From measurement of [ $^{32}$ P]P<sub>i</sub> incorporation, we calculated that, in samples incubated with 200 mM glucose and DMF, up to 0.03 mol of phosphate could be incorporated per mol of total haemoglobin.

These results showed therefore that glycated haemoglobin is a substrate for fructosamine 3-kinase, and that inhibition of this enzyme in intact erythrocytes leads to the accumulation of glycated haemoglobin.

#### Identification of haemoglobin bound to a fructosamine 3-phosphate group (FN3P-Hb) in intact erythrocytes

While separating haemoglobin on DEAE-Sepharose columns as described above, we noted that a small proportion of haemoglobin (approx. 2%) was retained on the columns in samples derived from cells incubated with 5 mM glucose, and that this proportion increased to approx. 6% in cells incubated with 200 mM glucose alone, but only to approx. 4% in cells incubated with 200 mM glucose together with DMF (Figure 3A). This result suggested that the anionic form of haemoglobin, which was retained on the column, had been phosphorylated by fructosamine 3-kinase.

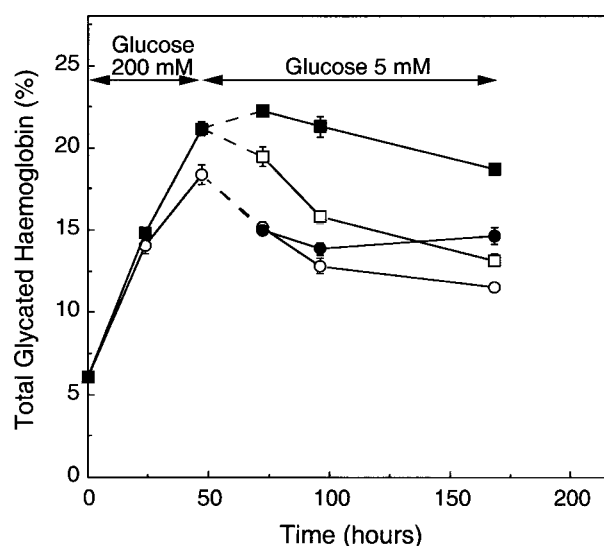
As fructosamine 3-phosphates are alkali-labile, we measured the protein-bound, alkali-labile phosphate in this fraction. As shown in Figure 3(B), alkali-labile phosphate nearly matched the amount of haemoglobin present in the salt-eluted fraction. In contrast, measurement of the alkali-labile phosphate in the flow-through fraction, which contained > 90% of the haemoglobin, yielded a value of 0.5% mol of phosphate/mol of haemoglobin, irrespective of the conditions under which the cells had been incubated (results not shown). These results indicated that protein-bound, alkali-labile phosphate in the salt-eluted fraction of the anion-exchange column was an estimate of FN3P-Hb.

Figure 3(B) shows that incubation of erythrocytes with 200 mM glucose increased the level of FN3P-Hb by more than 10-fold to approx. 5% of total haemoglobin. This effect was inhibited, although not completely suppressed, by DMF. Transfer of cells incubated with 200 mM glucose to a low-glucose medium caused a more rapid decrease in FN3P-Hb in the presence, rather than in the absence, of DMF. This decrease amounted to approx. 60% in 1 day in the presence of the inhibitor of fructosamine 3-kinase. Transfer of cells incubated for 2 days with 200 mM glucose together with DMF to a medium with low glucose, but no DMF, caused a transient increase in the amount of FN3P-Hb, which was followed by a rapid decrease, indicating that fructosamine 3-phosphate residues undergo further conversion.

#### Evidence for a net decrease in the total amount of glycated haemoglobin

To ensure that fructosamine 3-kinase initiated a process leading to a net decrease in the amount of glycated haemoglobin, it was of interest to determine the evolution of the sum of glycated (non-phosphorylated) haemoglobin and FN3P-Hb. Boronate columns, used to retain glycated haemoglobin, bind compounds that contain two neighbouring alcohol groups. Fructosamines, in their predominant pyranose form, have three such pairs (on C2/C3, C3/C4 and C4/C5), whereas fructosamine 3-phosphates have only one pair of neighbouring alcohol groups (C4/C5). It was therefore important to evaluate retention of FN3P-Hb on the boronate columns.

To this end, extracts of cells incubated with 200 mM glucose in the absence of DMF were chromatographed on boronate columns, and protein-bound, alkali-labile phosphate was measured in the flow-through fraction and in the salt eluate. Compared with the DEAE-Sepharose columns, only approx.



**Figure 4** Sum of glycated haemoglobin and FN3P-Hb

The same experiment is shown as described in the legend to Figure 1. FN3P-Hb was estimated from the alkali-labile phosphate.

70% of the protein-bound, alkali-labile phosphate was recovered. This lower yield is probably due to the fact that the boronate chromatography is performed at alkaline pH and room temperature, favouring decomposition of fructosamine 3-phosphate, whereas the anion-exchange chromatography is run at 4 °C and neutral pH. A minor amount (only approx. 10%, corresponding to 0.6% mol/mol of total haemoglobin) of the alkali-labile, protein-bound phosphate was recovered, together with glycated haemoglobin, in fractions of the boronate columns eluted with salt, the remainder (60%) being eluted with non-

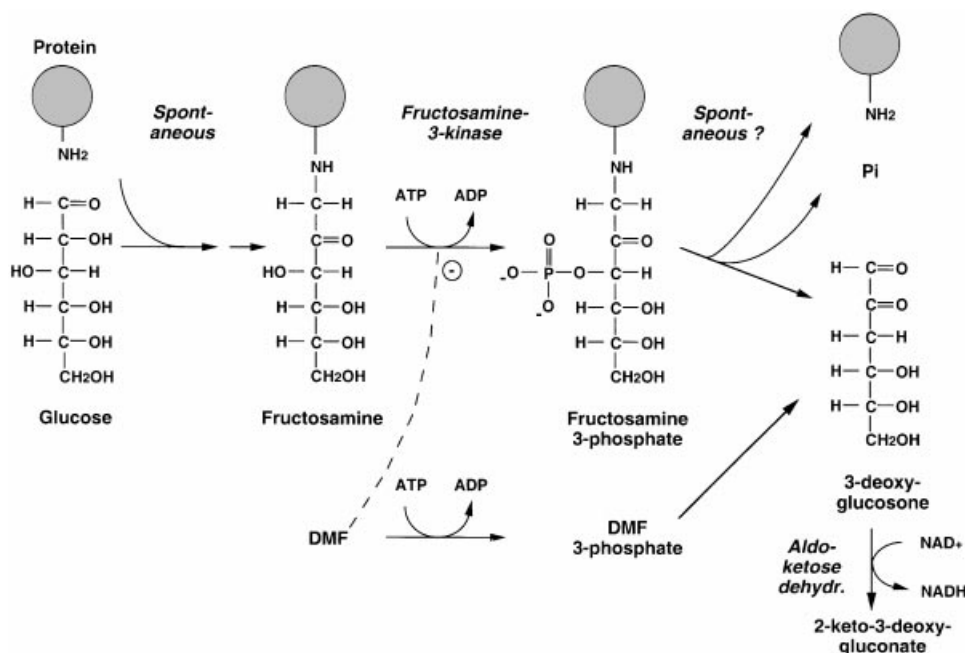
glycated haemoglobin in the flow-through fraction. On this basis, we assumed that the fraction eluted with salt from the boronate column represented essentially the glycated, non-phosphorylated form of haemoglobin, with negligible contamination by FN3P-Hb.

Figure 4 shows that the sum of glycated haemoglobin and FN3P-Hb increased from 6% to 18% in the presence of 200 mM glucose, and to 21% in the combined presence of 200 mM glucose and 5 mM DMF. Transfer of the cells to a medium with low glucose and without DMF resulted in a decrease in total glycated haemoglobin, which was most evident if the cells had been pre-incubated with DMF during the first 48 h. DMF prevented to a large extent the decrease in total glycated haemoglobin occurring when the cells had been transferred to the low-glucose medium.

### Formation of 2-oxo-3-deoxygluconate

*In vitro*, fructoselysine 3-phosphate decomposes spontaneously to  $P_i$ , free lysine and 3-deoxyglucosone with a half-life of approx. 6 h at 37 °C and pH 7.5 [12]. 3-Deoxyglucosone is mainly oxidized to 2-oxo-3-deoxygluconate in erythrocytes (Scheme 1) [20]. In order to check the possibility that the product formed from fructosamine 3-phosphate corresponds to 3-deoxyglucosone, we developed a specific enzymic assay for 2-oxo-3-deoxygluconate on the basis of *E. coli* 2-oxo-3-deoxygluconate kinase [21].

Using this assay, we found that 2-oxo-3-deoxygluconate amounted to  $36 \pm 7$  and  $271 \pm 13$  nmol/ml of packed erythrocytes (mean values  $\pm$  S.E.M. for  $n = 3$  experiments) after 48 h of incubation with 5 mM and 200 mM glucose respectively, and to  $877 \pm 96$  and  $669 \pm 18$  nmol/ml in cells incubated with 5 mM DMF in the presence of the same two concentrations of glucose. The starting level, in washed erythrocytes, was barely detectable (approx. 10 nmol/ml). These results indicated that 3-deoxyglucosone is formed from both glucose and DMF, and that the



**Scheme 1** Deglycation pathway initiated by fructosamine 3-kinase

effects of these two precursors are not additive, but rather that the presence of 200 mM glucose decreased the formation of 2-oxo-3-deoxygluconate from DMF. In an attempt to account for this decrease, we measured the formation of DMF 3-phosphate in cells incubated with low or high glucose concentrations, and found values corresponding to 5 and 4.5  $\mu\text{mol/ml}$  packed cells after 48 h of incubation with radiolabelled DMF (at a concentration of 5 mM; results not shown).

## DISCUSSION

### Glycated proteins are the physiological substrate of fructosamine 3-kinase

A previous study has shown that fructosamine 3-kinase is able to phosphorylate both low-molecular-mass and protein-bound fructosamines. Our results indicate that the physiological substrate is most likely not to be free fructoselysine, which is poorly utilized by erythrocytes (probably because it is not, or only slowly, transported across the plasma membrane), but glycated proteins. Incubation of erythrocytes with elevated concentrations of glucose leads indeed to the formation not only of glycated haemoglobin, but also of a form of haemoglobin that contains alkali-labile phosphate. The fact that the formation of this phosphorylated form of haemoglobin is inhibited by DMF, a substrate and competitive inhibitor of fructosamine 3-kinase, indicates that it corresponds to FN3P-Hb. This conclusion is consistent with the lability to alkali of the phosphate group, and with the finding that haemoglobin derived from erythrocytes incubated with 200 mM glucose is a good substrate for fructosamine 3-kinase (Figure 2).

### Evidence for fructosamine 3-kinase-mediated deglycation of haemoglobin

The finding that the sum of glycated haemoglobin and FN3P-Hb declines when cells incubated with 200 mM glucose are returned to a low-glucose medium (see Figure 4) indicates that fructosamine 3-phosphate residues are removed. Inhibition of this decrease by DMF indicates that it involves fructosamine 3-kinase and the conversion of fructosamine residues into their 3-phospho derivative. Removal of fructosamine via this process is already apparent during the first phase of the incubation, since the presence of DMF increases the level of glycated haemoglobin in cells incubated with 200 mM glucose.

The mechanism by which fructosamine 3-phosphates are removed from proteins could be via spontaneous conversion into 3-deoxyglucosone,  $\text{P}_i$  and the original amine (Scheme 1), as indicated by the lability of fructoselysine 3-phosphate at near-physiological pH values and at 37 °C [12]. From the data shown in Figure 3, it appears that the half-life of FN3P-Hb would be between 12 and 24 h in intact erythrocytes, as compared with approx. 6 h for fructoselysine 3-phosphate *in vitro*. These values are in reasonable agreement, considering that the free carboxylic and amino groups of fructoselysine 3-phosphate might modify the rate of the reaction.

Consistent with this spontaneous deglycation, we observed that 2-oxo-3-deoxygluconate is formed when erythrocytes are incubated with elevated concentrations of glucose. The amount that is formed during the first 2 days of incubation with 200 mM glucose (approx. 250 nmol/ml of packed erythrocytes) can certainly account for the amount of haemoglobin that underwent deglycation during this period. The latter corresponds to the difference between the total amount of glycated haemoglobin (see Figure 4) in the absence and in the presence of DMF

(approx. 3%), i.e. 135 nmol/ml of packed cells (assuming a haemoglobin concentration of 4.5  $\mu\text{mol/ml}$ ). This value may be slightly underestimated, since DMF does not completely block the formation of FN3P-Hb (see Figure 3B).

The finding that DMF, which is converted intracellularly into DMF 3-phosphate, gives rise to 2-oxo-3-deoxygluconate is best explained by a spontaneous conversion of DMF 3-phosphate to 3-deoxyglucosone. The fact that the formation of 2-oxo-3-deoxygluconate from 200 mM glucose and 5 mM DMF is not additive suggests that DMF is able to suppress the formation of 3-deoxyglucosone from glucose, and that the latter therefore involves fructosamine 3-kinase. As a matter of fact, significantly less 2-oxo-3-deoxygluconate was formed in cells incubated with 5 mM DMF and 200 mM glucose than in cells incubated with DMF and a low glucose concentration. This is probably because less DMF 3-phosphate accumulated, due to inhibition of fructosamine 3-kinase by protein-bound fructosamines.

The lack of additivity of 2-oxo-3-deoxygluconate formation from DMF and 200 mM glucose is probably not due to saturation of the enzyme converting 3-deoxyglucosone into 2-oxo-3-deoxygluconate. From the data of Fujii et al. [20], it appears that intact erythrocytes convert (0.35 mM) 3-deoxyglucosone into 2-oxo-3-deoxygluconate at a rate (approx. 0.7  $\mu\text{mol/8 h}$  per ml of packed cells) more than 4 times faster than the maximal rate of 2-oxo-3-deoxygluconate accumulation that we observed (0.9  $\mu\text{mol/48 h}$  per ml of packed cells).

Another potential compounding factor is the fact that 3-deoxyglucosone is converted into 3-deoxyfructose in several tissues by an NADPH-dependent reductase [22,23]. It is not known at present if this enzyme is present in erythrocytes, and therefore to what extent 3-deoxyglucosone is converted into 3-deoxyfructose in these cells. Our results are therefore compatible with a spontaneous conversion of fructosamine 3-phosphates into 3-deoxyglucosone, but further work is clearly needed to establish firmly the degradation process.

### Presence of deglycation-resistant haemoglobin

It is obvious from Figures 1 and 4 that part of the glycated haemoglobin that has been formed during the 2 days of incubation with 200 mM glucose is 'resistant' to deglycation. This is also true for the background level of glycated haemoglobin found in erythrocytes incubated with 5 mM glucose, since DMF had no significant effect on this level. Furthermore, the background level of glycated haemoglobin appeared to be a poor substrate for fructosamine 3-kinase in *in vitro* assays (see Figure 2). This background level of glycated haemoglobin mainly corresponds to  $\text{HbA}_{1c}$ , a form of haemoglobin with a fructosamine residue at the N-terminal valine residue of one of its  $\beta$ -chains [24].  $\text{HbA}_{1c}$  is not differentiated from haemoglobin glycated elsewhere (mostly on lysine residues) in the assay that we use, which is based on boron-affinity chromatography.

These considerations bring us to the conclusion that deglycation-resistant haemoglobin essentially corresponds to  $\text{HbA}_{1c}$ , and that the latter is a poor substrate for fructosamine 3-kinase. This is consistent with the kinetic properties of this enzyme, which phosphorylates N- $\alpha$ -glycated amino acids (fructosevaline and fructoseglycine; [10,12]) with a much lower affinity than fructoselysine, in which the  $\epsilon$ -amino group is glycated. Furthermore, the N-terminal valine of the  $\beta$ -chain is buried, which may cause a problem of accessibility to fructosamine 3-kinase. Further work is obviously needed to determine whether  $\text{HbA}_{1c}$  is a substrate for this enzyme at all, but our data suggest that it is, at best, a poor substrate.

### Other arguments in support of a deglycation mechanism

Besides the experimental arguments given above, an intracellular deglycation mechanism is supported by several other observations. First, according to some [25] but not all [26,27] authors, there is a wide discrepancy between the observed level of glycation of haemoglobin in erythrocytes *in vivo* and that expected from measurement of the rate of haemoglobin glycation *in vitro*, the first one being approx. 5-fold lower than the latter [25].

A second, possibly more convincing, argument is that some of the sites at which haemoglobin is glycated *in vivo* are different from those at which it is glycated *in vitro* [24]. For instance, Lys<sup>16</sup> of the  $\alpha$ -chain is a predominant site of glycation *in vitro*, but a negligible one *in vivo*. This observation can be easily accounted for by an intracellular deglycation mechanism, if one hypothesizes that Lys<sup>16</sup> of the  $\alpha$ -chain is readily phosphorylated by fructosamine 3-kinase, whereas other residues, especially the glycated valine at the N-terminus of the  $\beta$ -chain, are not.

### Physiological implications

The rate of haemoglobin 'deglycation', estimated from the rate at which FN3P-Hb accumulates in cells incubated with 200 mM glucose, amounts to at least 5% per day. As a first estimate, it is expected to amount to 40 times less, i.e. 0.125% per day at 5 mM glucose, since protein glycation is first-order with respect to glucose concentration. This low rate may account for the reason why there is no significant difference in the level of glycated haemoglobin observed between cells incubated with or without DMF at a physiological glucose concentration (Figure 1).

However, this rate allows us to calculate that approx. 15% of haemoglobin undergoes deglycation during the whole erythrocyte lifetime (approx. 120 days). This value is probably underestimated, because the deglycation process might be more efficient at concentrations of glucose lower than 200 mM. Deglycation might be important to prevent formation of cross-links between haemoglobin molecules due to further reaction of the fructosamine residues, or to prevent inactivation of enzymes that are easily glycated on a lysine residue that has a critical role in catalysis. The fact that some fructosamine residues escape deglycation cannot be taken as an argument against the importance of deglycation; residues that escape deglycation could, for instance, be buried, and therefore not prone to make cross-links with other proteins.

### Is there a need to re-interpret the glycated haemoglobin values?

The level of glycated haemoglobin is used by clinicians to estimate the blood glucose level over the preceding 2 months, which is extremely important to assess the efficacy of diabetes treatment. Different methods are used to estimate total glycated haemoglobin (including the boronate-affinity chromatography used in the present study), or HbA<sub>1c</sub> [28]. The existence of a deglycation mechanism means that, in theory, the level of total glycated haemoglobin is not only dependent on the blood glucose concentration, but that it is also inversely related to the fructosamine 3-kinase activity. If HbA<sub>1c</sub> proves to be a substrate, albeit a poor one, for fructosamine 3-kinase, variations in the activity of this enzyme should also affect the HbA<sub>1c</sub> level, though to a lesser extent than total glycated haemoglobin. This might provide an explanation for the fact that HbA<sub>1c</sub> appears to be determined by genetic factors, independently of the blood glucose concentration, as indicated by a recent study on monozygotic and dizygotic twins [29]. Other factors (differences in either glucose penetration into erythrocytes or the intracellular concentration of phosphate

esters) could, however, also have a role [30]. Further work is obviously needed to appreciate if there are inter-individual differences in the activity of fructosamine 3-kinase, and if this significantly impacts on glycated haemoglobin values.

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