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ABSTRACT

Intact human erythrocytes catalyse the conversion of fructose into fructose 3-phosphate with an apparent $K(m)$ of 30 mM [Petersen, Kappler, Szwergold and Brown (1992) *Biochem. J.* 284, 363-366]. The physiological significance of this process is still unknown. In the present study we report that the formation of fructose 3-phosphate from 50 mM fructose in intact erythrocytes is inhibited by 1-deoxy-1-morpholinofructose (DMF), a synthetic fructosamine, with an apparent $K(i)$ of 100 μ M. (^{31}P) NMR analysis of cell extracts incubated with DMF indicated the presence of an additional phosphorylated compound, which was partially purified and shown to be DMF 3-phosphate by tandem MS. Radiolabelled DMF was phosphorylated by intact erythrocytes with an apparent $K(m)$ (approximately 100 μ M) approx. 300-fold lower than the value reported for fructose phosphorylation on its third carbon. These results indicate that the physiological function of the enzyme that is able to convert fructose into fructose 3-phosphate in intact erythrocytes is probably to phosphorylate fructosamines. This suggests that fructosamines, which are produced non-enzymically from glucose and amino compounds, may be metabolized in human erythrocytes.

CITE THIS VERSION

Delpierre, Ghislain ; Vanstapel, F ; Stroobant, Vincent ; Van Schaftingen, Emile. *Conversion of a synthetic fructosamine into its 3-phospho derivative in human erythrocytes..* In: *The Biochemical journal*, Vol. 352 Pt 3, p. 835-9 (2000) <http://hdl.handle.net/2078.1/21982>

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Conversion of a synthetic fructosamine into its 3-phospho derivative in human erythrocytes

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Intact human erythrocytes catalyse the conversion of fructose into fructose 3-phosphate with an apparent K_m of 30 mM [Petersen, Kappler, Szwegold and Brown (1992) *Biochem. J.* **284**, 363–366]. The physiological significance of this process is still unknown. In the present study we report that the formation of fructose 3-phosphate from 50 mM fructose in intact erythrocytes is inhibited by 1-deoxy-1-morpholinofructose (DMF), a synthetic fructosamine, with an apparent K_i of 100 μ M. ³¹P NMR analysis of cell extracts incubated with DMF indicated the presence of an additional phosphorylated compound, which was partially purified and shown to be DMF 3-phosphate by tandem MS. Radiolabelled DMF was phosphorylated by intact erythro-

cytes with an apparent K_m (\approx 100 μ M) approx. 300-fold lower than the value reported for fructose phosphorylation on its third carbon. These results indicate that the physiological function of the enzyme that is able to convert fructose into fructose 3-phosphate in intact erythrocytes is probably to phosphorylate fructosamines. This suggests that fructosamines, which are produced non-enzymically from glucose and amino compounds, may be metabolized in human erythrocytes.

Key words: erythrocyte, fructose 3-phosphate, glycation, NMR, phosphate esters.

INTRODUCTION

Fructose 3-phosphate is a phosphate ester of unknown function that was first identified by NMR spectroscopy in the lenses of diabetic animals [1,2], and subsequently in human erythrocytes from both control and diabetic subjects [3]. Its formation, to concentrations reaching the millimolar range, can be induced by incubating erythrocytes [4] or lenses [5,6] with fructose. The apparent K_m of this conversion (approx. 30 mM [4]) is much higher than those of the enzymes that physiologically metabolize fructose (i.e. fructokinase and low- K_m hexokinases). A fructose 3-kinase has been reported to be present in human erythrocyte lysates [3], but it has not been further characterized except for a brief mention in an abstract [7] that it also acts on 1-deoxy-1-*N*- ϵ -lysine-fructose. The low affinity for fructose of the enzyme that forms fructose 3-phosphate and the absence of a satisfying role for this phosphate ester suggest that the physiological substrate of fructose 3-kinase may not be fructose itself but structurally related compounds, possibly fructosamines.

Fructosamines are the products of non-enzymic reactions between glucose and primary amines, followed by Amadori rearrangements. These reactions, known as glycation (to be distinguished from glycosylation, which is an enzymically-catalysed process), can typically modify the amino terminus and the lysine and arginine side chains of proteins (reviewed in [8–10]), as well as a variety of low-molecular-mass compounds, including aminophospholipids [11]. Due to the fact that their formation is slow and proportional to the blood glucose concentration, serum fructosamines (glycated serum proteins) and haemoglobin HbA_{1c} (a form of haemoglobin with a fructosamine group at the N-terminus of one of its β subunits) are assayed to monitor the treatment of diabetes [12–14]. They provide an estimate of the mean blood glucose concentration during the weeks (serum fructosamines) or months (HbA_{1c}) before the sample is taken from a patient, due to the shorter half-life of serum proteins

compared with haemoglobin. Presently no reactions are known that could potentially metabolize fructosamines in mammals. This is in contrast with micro-organisms, where specific oxidases, called amadoriases, have been shown to degrade low-molecular-mass fructosamines [15–17].

The purpose of this work was to test the hypothesis that the enzyme that forms fructose 3-phosphate in erythrocytes is a fructosamine kinase. For this we have made use of a commercially available fructosamine, 1-deoxy-1-morpholinofructose (DMF). We show that this compound not only powerfully inhibits fructose 3-phosphate formation but that it is phosphorylated to its 3-phospho derivative.

EXPERIMENTAL

Materials

AG 1-X8 resin (200–400 mesh; Cl⁻ form) and Biogel P2 fine, a support used for gel filtration, were purchased from Bio-Rad. Radiolabelled DMF was synthesized as described by Hodge and Rist [18], starting from 500 μ Ci (1.5 μ mol) of D-[U-¹⁴C]glucose (purchased from Amersham Pharmacia Biotech), 250 μ mol of anhydrous D-glucose and 340 μ moles of morpholine, with a yield of 23%, as estimated from the radioactivity recovered in the final crystalline product. The absence of glucose in the latter was checked by a spectrophotometric method using hexokinase and glucose 6-phosphate dehydrogenase [19]. Auxiliary enzymes for enzymic assays were obtained from Roche Diagnostics (Indianapolis, IN, U.S.A.). Chemicals were of the best available grades and were purchased from Acros, Merck or Sigma.

Incubation of human erythrocytes

Human heparinized blood, from healthy volunteers, was centrifuged for 10 min at 2000 *g* (at 4 °C), and the buffy coat was

Abbreviations used: DMF, 1-deoxy-1-morpholinofructose.

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discarded. The erythrocytes were washed three times in a Krebs–Henseleit bicarbonate buffer equilibrated with an O₂/CO₂ (19:1) gas mixture [20], resuspended in 20 vol. of the same buffer and incubated at 37 °C in an orbital shaker in the presence of 5 mM glucose and of the indicated substrates. Incubations were stopped by centrifuging the suspension for 10 min at 1000 g (at 4 °C). The supernatant was discarded and the pellet of erythrocytes was washed twice with ice-cold 150 mM NaCl. The final cell pellet was mixed with 3 vol. of ice-cold 10% (w/v) HClO₄, and the extract was centrifuged for 10 min at 2000 g (at 4 °C). The supernatant was neutralized with 3 M KHCO₃ or with a tri-n-octylamine/chloroform mixture (1:3.6) [21] in the case of samples prepared for NMR analysis or MS.

Measurement of fructose 3-phosphate

As fructose 3-phosphate is by far the main phosphate ester of fructose in erythrocytes incubated with this ketose [4], fructose 3-phosphate was determined to be the fructose liberated by alkaline phosphatase. In practice, 1 ml of the neutralized perchloric extract was diluted with 2 ml of water and applied on to an anion-exchange column (AG 1-X8 Cl⁻ resin; approx. 1 ml of wet gel in Pasteur pipettes). The column was washed with 3 ml of water, and the sugar monophosphates were eluted with 6 ml of 150 mM NaCl. A 1 ml sample of this eluate was incubated for 60 min at 30 °C in the presence of 25 mM Tris (pH 8), 5 mM MgCl₂, 50 μM ZnCl₂, 50 μg/ml BSA and 2.3 units/ml alkaline phosphatase, in a final volume of 1.2 ml. The phosphatase was denatured by heating for 5 min at 75 °C, and the liberated fructose was measured enzymically [19], providing an estimate of the total fructose-monophosphate concentration. Fructose 3-phosphate concentration was calculated by subtracting the fructose 6-phosphate concentration (≈ 10 nmol/ml of packed erythrocytes; measured as in [19]) from this value. No fructose 1-phosphate [22] could be detected.

NMR analysis

Perchloric acid extracts were concentrated 4-fold by freeze-drying, and treated with 20 mg of Na₃EDTA/ml. Partially saturated ³¹P NMR spectra were acquired in an AMX 360 spectrometer (Bruker Spectrospin) at 145.79 MHz. Blocks of 24 576 scans were accumulated using a 60° pulse with a repetition time of 1.5 s, with or without Waltz broad-band ¹H decoupling. Time-domain signals were Fourier-transformed with a 1 Hz line broadening exponential filter. Chemical shifts are expressed with reference to the frequency of the β-phosphate of NTP.

Purification and MS of the phosphorylated compound formed from DMF

Suspensions of erythrocytes (150 ml) prepared as described above were incubated for 20 h with 5 mM glucose in the presence or absence of 2 mM DMF, and were subsequently extracted. Samples (20 ml) of the resulting extracts were diluted 2-fold with water and loaded on to an anion-exchange column (AG 1-X8 Cl⁻ resin; 1.6 cm × 15 cm). The columns were washed with 40 ml of water, and a linear gradient of NaCl (0–500 mM in 300 ml) was applied. Fractions of 5 ml were collected and P_i [23] was measured before and after incubation with alkaline phosphatase. Comparison of the elution profiles of the two columns indicated the presence of an additional phosphate ester (9.2 μmol), eluting before the P_i peak from the column loaded with the sample derived from cells incubated with DMF. The corresponding fractions (total volume = 60 ml) were pooled and concentrated

by freeze-drying to approx. 1 ml. This sample was desalted by gel-filtration on a Biogel P2 column (1 cm × 45 cm) equilibrated with water. The fractions containing phosphoric esters and less than 1 mM NaCl (as estimated by conductivity) were pooled, freeze-dried and analysed by positive and negative electrospray ionization MS.

Mass spectra were obtained on a Finnigan LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA, U.S.A.) equipped with an electrospray ionization source. A sample dissolved in methanol/water (1:1) was introduced directly into the source of the mass spectrometer at a flow rate of 3 μl/min. The spray was obtained by applying a potential difference of 5 kV, and with the help of N₂ as a sheath gas. The temperature of the heated capillary tube was 210 °C. The LCQ was operated under manual control in the Tune Plus view with default parameters and active Automatic Gain Control.

Measurement of the phosphorylation of radiolabelled DMF

For the measurement of the conversion of radiolabelled DMF into its phosphate ester in erythrocytes, the cells were incubated with 5 mM glucose, 100 000 c.p.m. of [¹⁴C]DMF and unlabelled DMF at the indicated final concentration in a final volume of 1.5 ml. After extraction, with perchloric acid, and neutralization, 350 μl of the extract was diluted with 2.65 ml of water, and applied on to an AG1-X8 Cl⁻ column as described above. The column was washed with water, and phosphorylated DMF was eluted with 6 ml of 150 mM NaCl. The eluate was mixed with 15 ml of OptimaGold (Packard) scintillation mixture and counted for radioactivity.

RESULTS

When incubated in the presence of fructose, erythrocytes form large amounts of fructose 3-phosphate, which becomes the predominant phosphate derivative of fructose [4]. This finding was confirmed in the experiment shown in Figure 1, where the

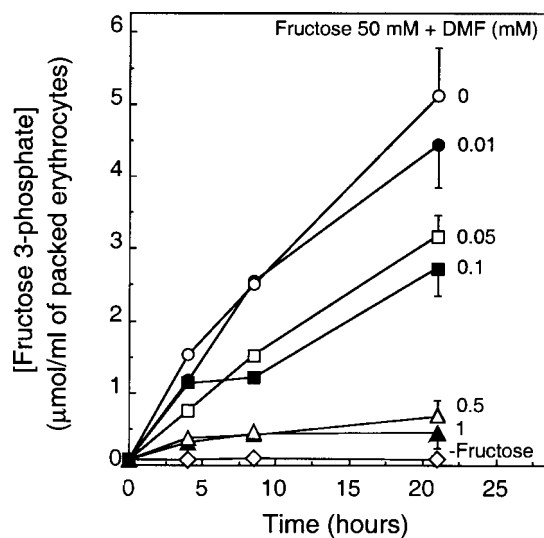


Figure 1 Effect of DMF on the formation of fructose 3-phosphate in erythrocytes

Samples (25 ml) of a suspension of erythrocytes were incubated without fructose or with 50 mM fructose and the indicated concentrations of DMF. Fructose 3-phosphate was measured enzymically. Data are means ± S.E.M. for three separate experiments.

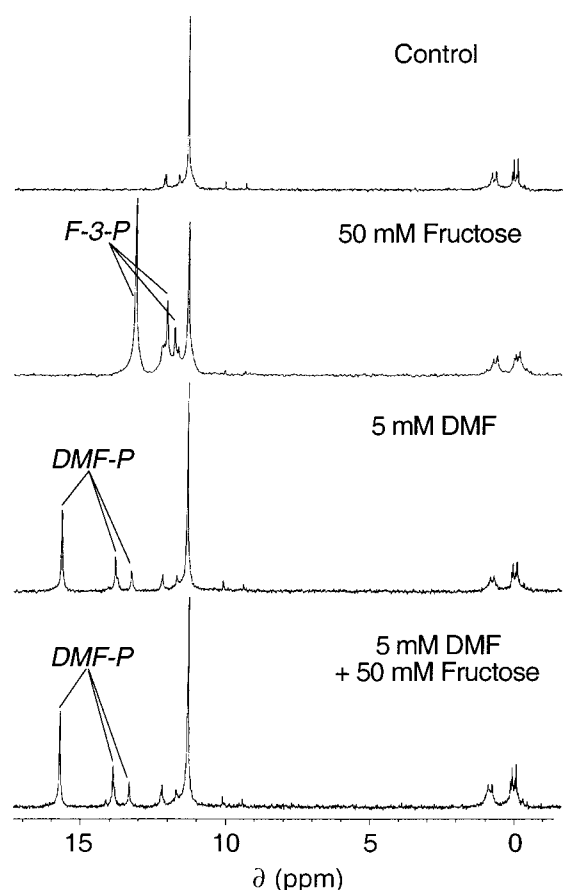


Figure 2 ^{31}P NMR analysis of extracts of erythrocytes incubated with or without fructose or DMF

The erythrocytes were incubated for 20 h in the presence of the indicated concentrations of fructose and/or DMF. DMF-P, DMF-phosphate; F-3-P: fructose 3-phosphate.

accumulation of fructose 3-phosphate proceeded at a rate of $0.25 \mu\text{mol/h}$ per ml of packed cells in erythrocytes incubated in the presence of 50 mM fructose. Several fructose or glucose analogues were tested to check if they could modulate this rate of fructose 3-phosphate synthesis. No effect was observed with 50 mM D-tagatose, L-sorbose, D-arabinose, D-mannose, D-glucose or D-galactose or with 20 mM D-glucosamine or N-acetyl-D-glucosamine (results not shown). In marked contrast, DMF, a synthetic fructosamine, exerted strong inhibition, which was half-maximal at approx. 0.1 mM.

^{31}P NMR analysis of extracts of erythrocytes incubated with fructose for 20 h indicated the presence of three additional peaks as compared to the control extracts (Figure 2). These peaks, which split into doublets in ^1H -coupled spectra, correspond to the three most important conformations of fructose 3-phosphate, i.e. (in order of decreasing abundance and from left to right in the spectrum) β -fructopyranose, β -fructofuranose and α -fructofuranose [2,24]. Incubation with DMF also caused the appearance of three peaks, which, as in the case of fructose 3-phosphate, split into doublets in ^1H -coupled spectra. However, these peaks were 1–2 p.p.m. more downfield than those of fructose 3-phosphate, and presumably corresponded to phosphorylated DMF. Only the latter were observed in the combined presence of fructose and DMF, confirming that the fructosamine strongly inhibited fructose phosphorylation on its third carbon.

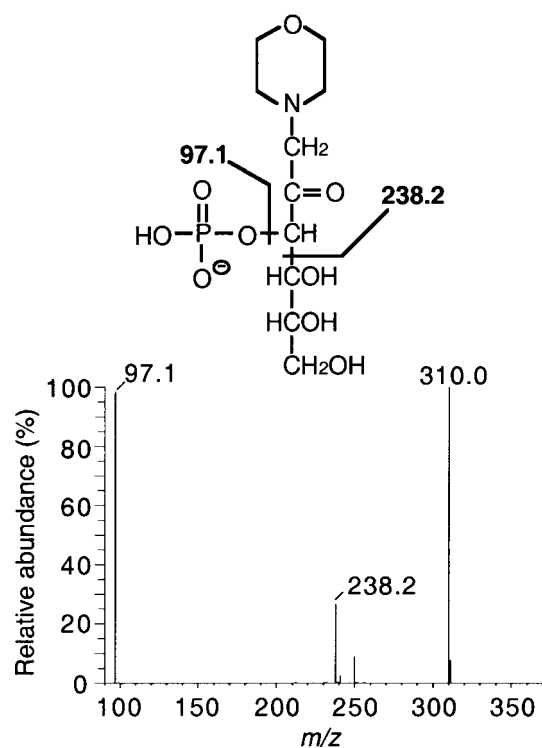


Figure 3 Tandem MS analysis of the phosphorylated compound formed in erythrocytes incubated with DMF

The fragmentation spectrum of the deprotonated molecular ion at m/z 328 is shown. The structure of DMF 3-phosphate and its fragmentation pattern is shown above the spectrum. The ion at m/z 310 probably corresponds to a dehydration product.

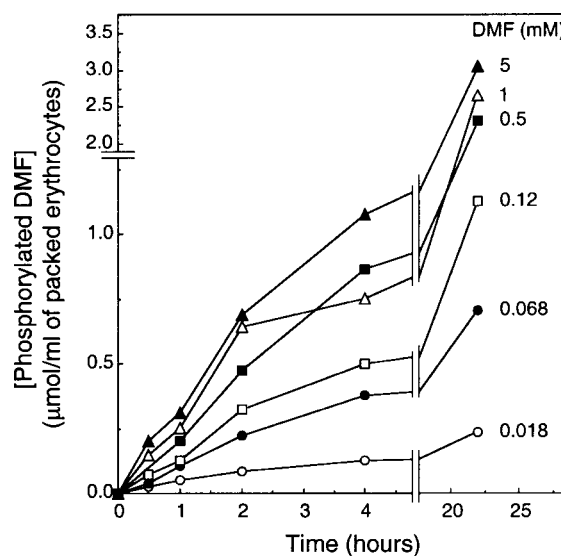


Figure 4 Phosphorylation of radiolabelled DMF in intact erythrocytes

Samples (1.5 ml) of a suspension of erythrocytes were incubated at 37°C with the indicated concentrations of DMF. The means for two experiments are shown.

The phosphorylated compound formed in erythrocytes incubated with 2 mM DMF was partially purified by anion-exchange chromatography and gel-filtration (results not shown). Analysis

by tandem MS disclosed the presence of a major anion at m/z 328, as expected for phosphorylated DMF. Upon fragmentation, this deprotonated molecular ion yielded negatively charged fragments of m/z 97.1 and 238.2, corresponding to $H_2PO_4^-$ and to a phosphorylated DMF species that had lost carbons C4–C6 of the 1-deoxyfructose moiety respectively (Figure 3). These results suggested that the phosphorylated carbon was either C2 or C3 of the 1-deoxyfructose moiety.

Radiolabelled DMF was synthesized from [^{14}C]glucose and morpholine to facilitate further investigations into the phosphorylation of this fructosamine. With this compound the dose dependency and time course of DMF phosphorylation in intact erythrocytes could be examined (Figure 4). A maximal rate of phosphorylation of approx. $0.38 \mu\text{mol/h}$ per ml of packed erythrocytes was observed, with an apparent K_m of approx. 0.1 mM .

DISCUSSION

Since the discovery of fructose 3-phosphate, the function of this phosphate ester has been quite mysterious. Indeed no enzyme is known to catalyse the conversion of fructose 3-phosphate into other compounds. Furthermore, the K_m ($\approx 30 \text{ mM}$) of the enzyme that forms fructose 3-phosphate is very high in comparison with the fructose concentration ($\approx 2 \text{ mM}$) that can be found in blood after a fructose-rich meal [25], and its capacity to phosphorylate fructose in erythrocytes is at least three orders of magnitude lower than the V_{max} of fructokinase in the liver [26]. These considerations indicated that the physiological substrate of fructose 3-kinase may not be fructose itself but a structural analogue.

We found that DMF, a synthetic fructosamine, is not only a powerful inhibitor of fructose 3-phosphate formation but that it is also phosphorylated in intact erythrocytes. With respect to the position of the phosphorylated carbon in DMF-phosphate, tandem MS allowed us to exclude carbons C4–C6, suggesting that either C2 or C3 are phosphorylated. However, C2 seems unlikely as furanosyl-phosphates, such as fructose 2-phosphate [27] and fructose 2,6-bisphosphate [28], are extremely labile in acid even in the cold; a 2-phosphate derivative would have therefore been hydrolysed during the extraction with perchloric acid. Furthermore, the NMR spectra show several anomeric forms of the fructosamine-phosphate derivative, which indicates that the carbohydrate moiety is free to anomerize and therefore that the hydroxy group on C2 of the 1-deoxyfructose moiety is free. Finally, no proton coupling is expected in the case of a 2-phosphate derivative, as C2 bears no proton. These data therefore argue for a DMF 3-phosphate structure.

That the same enzyme is involved in the formation of both fructose 3-phosphate and DMF 3-phosphate is indicated by the strong inhibition exerted by DMF on fructose 3-phosphate formation, with an apparent K_i identical to the K_m for the phosphorylation of DMF. Since this enzyme phosphorylates DMF with at least 300-fold higher affinity than fructose, we conclude that its physiological function is, most likely, to phosphorylate fructosamines, as suggested by Szwegold et al. [7].

Based on these observations, we recently purified and cloned the cDNA of an enzyme that phosphorylates not only DMF, but also 1-deoxy-1-*N*- ϵ -lysine-fructose, 1-deoxy-1-*N*-glycine-fructose and glycosylated lysozyme, and, with a very low affinity ($K_m > 100 \text{ mM}$), fructose [29]. Our hypothesis is that fructosamine 3-kinase catalyses the first step of an as yet unknown, intracellular 'deglycation' process. Further work is needed to define the role of fructosamine 3-kinase. The identification of a good substrate

for this enzyme will certainly be useful to further characterize this enzyme and its physiological function.

We thank Professor H. G. Hers for helpful comments during the preparation of this manuscript. This work was supported by the Actions de Recherche Concertées, the Belgian Federal Service for Scientific, Technical and Cultural Affairs, the Fonds National de la Recherche Scientifique and the Juvenile Diabetes Foundation International. G.D. is aspirant of the Belgian FNRS.

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Received 25 July 2000/13 September 2000; accepted 10 October 2000