Rapid Publication

Identification, Cloning, and Heterologous Expression of a Mammalian Fructosamine-3-Kinase

Ghislain Delpierre, Mark H. Rider, François Collard, Vincent Stroobant, Florent Vanstapel, Helena Santos, and Emile Van Schaftingen

Fructosamines are thought to play an important role in the development of diabetic complications. Little is known about reactions that could metabolize these compounds in mammalian tissues, except for recent indications that they can be converted to fructosamine 3-phosphates. The purpose of the present work was to identify and characterize the enzyme responsible for this conversion. Erythrocyte extracts were found to catalyze the ATP-dependent phosphorylation of 1-deoxy-1-morpholinofructose (DMF), a synthetic fructosamine. The enzyme responsible for this conversion was purified ~2,500-fold by chromatography on Blue Sepharose, Q Sepharose, and Sephacryl S-200 and shown to copurify with a 35,000-M_r protein. Partial sequences of tryptic peptides were derived from the protein by nanoelectrospray-ionization mass spectrometry, which allowed for the identification of the corresponding human and mouse cDNAs. Both cDNAs encode proteins of 309 amino acids, showing 89% identity with each other and homologous to proteins of unknown function predicted from the sequences of several bacterial genomes. Both proteins were expressed in *Escherichia coli* and purified. They were shown to catalyze the phosphorylation of DMF, fructoselysine, fructoseglycine, and fructose in order of decreasing affinity. They also phosphorylated glycated lysozyme, though not unmodified lysozyme. Nuclear magnetic resonance analysis of phosphorylated DMF and phosphorylated fructoseglycine showed that the phosphate was bound to the third carbon of the 1-deoxyfructose moiety. The physiological function of fructosamine-3-kinase may be to initiate a process leading to the deglycation of fructoselysine and of glycated proteins. *Diabetes* 49:1627–1634, 2000

Fructosamines are the products of nonenzymatic reactions of glucose with primary amines followed by Amadori rearrangement. These reactions, known as glycation, typically modify the lysine and arginine side-chains and the NH_2-terminus of proteins (1–3), as well as low-molecular-weight compounds such as aminophospholipids (4).

Formation of fructosamines is a slow process, taking several weeks (at 37°C) to reach equilibrium in proportion to the glucose concentration. Hence, these compounds have aroused much interest in the field of diabetes. HbA_{1c}, a glycated form of hemoglobin A, and serum fructosamines are commonly assayed in patients to assess the treatment of diabetes, because they reflect an integrated value of blood glucose concentration over the preceding weeks (5–7). In addition, fructosamines can further react to form advanced glycation end products, which irreversibly alter the properties of proteins and may therefore play an important role in the development of long-term complications of diabetes (1–3).

Until recently, little was known about the reactions that could metabolize fructosamines in mammalian tissues. However, brief reports have recently appeared mentioning that tissue extracts contain a kinase converting fructoselysine to fructoselysine 3-phosphate (8,9). The latter appears to decompose into free lysine, inorganic phosphate, and 3-deoxyglucosone (9). Because this dicarbonyl compound is a potent glycating agent, Brown et al. (10) proposed that inhibitors of fructoselysine-3-kinase may help prevent diabetic complications. The enzyme catalyzing the phosphorylation of fructoselysine has been poorly characterized but appears to be the same as that which phosphorylates fructose on its third carbon (8), which is only partially characterized. Fructose 3-phosphate has been identified by nuclear magnetic resonance (NMR) spectroscopy, first in lenses from diabetic animals (11,12) and then in erythrocytes (13). Its formation can be induced by incubating erythrocytes with fructose, with an apparent K_M of ~30 mmol/l (14). We recently found that this formation of fructose 3-phosphate in intact erythrocytes is strongly inhibited by 1-deoxy-1-morpholinofructose (DMF), a synthetic fructosamine, and that the latter is converted to DMF 3-phosphate with an apparent K_M of ~100 µmol/l. These

From the Laboratory of Physiological Chemistry (G.D., F.C., E.V.S.), and the Hormone and Metabolism Unit (M.H.R), Université Catholique de Louvain; the Brussels branch of the Ludwig Institute for Cancer Research (V.S.), Christian de Duve Institute of Cellular Pathology, B-1200 Brussels; the Biomedical Nuclear Magnetic Resonance Unit (F.V.), Katholieke Universiteit Leuven, B-3000 Leuven, Belgium; and Instituto de Tecnologica Química e Biológica (H.S.), Universidade Nova de Lisboa, Apartado 127,2780-156 Oeiras, Portugal.

Address correspondence and reprint requests to Emile Van Schaftingen, MD, PhD, Laboratory of Physiological Chemistry, Université Catholique de Louvain, Avenue Hippocrate 75, B-1200, Brussels, Belgium. E-mail: vanschaftingen@bchm.ucl.ac.be.

Received for publication 26 June 2000 and accepted in revised form 25 July 2000. Posted on the World Wide Web at www.diabetes.org/diabetes on 5 September 2000.

BLAST, basic local alignment search tool; COSY, correlation spectroscopy; DMF, 1-deoxy-1-morpholinofructose; EST, expressed sequence tag; HMQC, heteronuclear multiple quantum correlation; HPLC, high-pressure liquid chromatography; IPTG, isopropyl-1-thio-β-D-galatopyranoside; MS, mass spectrometry; NMR, nuclear magnetic resonance; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

findings not only support the conclusion that the same enzyme phosphorylates fructose and fructosamines, but also provide a convenient substrate to characterize fructose/fructosamine-3-kinase (G.D., V.S., F.V., E.V.S., unpublished data).

The purpose of this work was to further characterize this enzyme. We are reporting the purification of fructosamine-3-kinase from human erythrocytes and the sequences of the cDNAs encoding the human and mouse enzymes. The recombinant enzymes have been expressed in *Escherichia coli* and their properties compared with those of the enzyme purified from erythrocytes.

RESEARCH DESIGN AND METHODS

Materials. AG 1-X8 200–400 mesh resin and Biogel P2 fine were purchased from BioRad. Blue Sepharose CL-6B, Q Sepharose fast flow, Sephacryl S-200 superfine, and radiochemicals were from Amersham Pharmacia Biotech. Ion-exchange papers, DE81, and P81 were from Whatman. All other reagents (analytical grade) were from Acros, Merck, or Sigma. Alkaline phosphatase, glycerol-3-phosphate dehydrogenase, and enzymes for molecular biology were from Roche Molecular Biochemicals.

Synthesis of fructosamines. Radiolabeled DMF was synthesized from [U-^{14}C]glucose and morpholine (15) (G.D., V.S., Fl.V., E.V.S., unpublished data). Fructoseglycine (16) and fructoselysine (17) were synthesized as indicated. For the synthesis of glycated lysozyme, a solution containing 60 mg/ml lysozyme, 1 mol/l glucose, and 25 mmol/l HEPES pH 7.1 was filtered on a 0.22-µm membrane and incubated at 37°C for 20 days. Glycated lysozyme was separated from glucose by gel filtration on a Biogel P2 equilibrated with water. Electrospray mass spectrometry (MS) analysis indicated a mean substitution level of 1.2 mol sugar per 1 mol lysozyme (range 0–3).

Measurement of the fructosamine-kinase activity. Except where otherwise indicated, fructosamine kinase was assayed by the conversion of [^{14}C]DMF to [^{14}C]DMF-phosphate. The assay mixture contained 25 mmol/l Tris pH 7.8, 1 mmol/l EGTA, 5 mmol/l ATP Mg, 100 µg/ml bovine serum albumin, 20,000 cpm [^{14}C]DMF, as well as nonradiolabeled DMF to reach a final concentration of 50 µmol/l. Samples (typically 100 µl of erythrocyte lysate in a final volume of 150 µl or 5–50 µl of purified fractions in a final volume of 100 µl) were incubated in this reaction medium at 30°C for 15–30 min to reach adequate conversions (at most 40%). In the case of intensely red samples (e.g., erythrocyte lysates), the reaction was stopped by the addition of three volumes of 10% (vol/vol) perchloric acid. After neutralization, 350 µl of the extract was diluted with 2.65 ml water and applied on an anion-exchange column (AG-1 X8 Cl$^-$ resin, ~1 ml wet gel in a Pasteur Pipette), which was washed with 3 ml water. The phosphorylated DMF was then eluted with 6 ml 150 mmol/l NaCl. The eluate was mixed with 15 ml OptimaGold (Packard) scintillation fluid and counted for radioactivity. In the case of clear samples, the reaction was stopped by spotting 35 µl of the medium on anion-exchanger papers (2 × 2 cm, DE81), which were washed three times in ice-cold water (at least 40 ml/paper) and then once with alcohol and once with diethyl ether. After drying, the papers were placed in vials with 10 ml scintillation fluid and counted for radioactivity. One unit of fructosamine kinase is the amount of enzyme catalyzing the conversion of 1 µmol DMF/min under the conditions defined above.

Measurement of the phosphorylation of other substrates. The phosphorylation of fructoselysine, fructoseglycine, and glycated lysozyme was measured at 30°C in the presence of 25 mmol/l Tris pH 7.8, 1 mmol/l EGTA, 100 µg/ml bovine serum albumin, 100 µmol/l ATP Mg, and 750,000 cpm [γ-^{32}P]ATP in a final volume of 100 µl. In the case of fructoselysine and fructoseglycine, the reaction was stopped by mixing 85 µl of the incubation medium with 400 µl of an ice-cold 2.5% (wt/vol) charcoal suspension in 25 mmol/l Mes and 5 mmol/l EDTA, pH 6.5. The mixture was vortexed for 30 s to allow ATP adsorption on charcoal. After a 15-min centrifugation at 16,000*g* and 4°C, the supernatant was counted for radioactivity. Radioactivity present in inorganic phosphate (18) was subtracted. In the case of glycated lysozyme, 35 µl of the reaction medium was spotted onto cation-exchange papers (2 × 2 cm, P81), which were washed three times with ice-cold 75 mmol/l H_3PO_4 (at least 40 ml/paper), then once with alcohol and once with diethyl ether, and counted for radioactivity.

Purification of the fructosamine kinase from human erythrocytes. Fructosamine kinase was purified from packed erythrocytes obtained from patients suffering from hemochromatosis. All purification steps were carried out at 4°C, and the preparation was stored at –70°C between steps. Protein concentration was monitored either by measuring A_{280} or by the Bradford assay (19) with bovine γ-globulin as a standard in samples containing ATP. Protein was concentrated by ultrafiltration in Amicon cells with membranes of 10 kDa molecular weight cutoff.

After separation from the plasma by centrifugation, packed red cells (~450 ml) were washed twice with Krebs-Henseleit bicarbonate buffer (20) and diluted in 2 l of a hypotonic lysis buffer containing 5 mmol/l HEPES, pH 7.5,1 mmol/l dithiothreitol, 1 µg/ml leupeptin, and 1 µg/ml antipain. The hemolysate was centrifuged for 1 h at 11,000*g* and the membrane pellet discarded. The supernatant was gently mixed for 30 min with ~120 ml Blue Sepharose equilibrated with the lysis buffer. The preparation was centrifuged at 3500*g* for 5 min, and the sedimented gel was packed into a column (2.6 cm internal diameter) on top of a layer of ~35 ml fresh Blue Sepharose, equilibrated in buffer A (25 mmol/l HEPES, pH 7.5, and 1 mmol/l dithiothreitol). The column was washed with ~250 ml buffer A and eluted with a linear gradient of NaCl (0–1 mol/l in 300 ml) in buffer A containing 1 µg/ml leupeptin and antipain. Fructosamine kinase was measured in the fractions, and those containing activity were pooled (~150 ml) and concentrated to ~15 ml. The concentrate obtained from two such preparations was diluted 10 times in 25 mmol/l Tris, pH 8.5, 1 mmol/l dithiothreitol (buffer B) containing 1 µg/ml leupeptin and 1 µg/ml antipain and applied on a Q Sepharose column (2.6 × 20 cm) equilibrated with buffer B. After washing with the same buffer, a linear gradient of NaCl (0–500 mmol/l in 250 ml buffer B containing protease inhibitors) was applied. Fractions (2.5 ml) were collected and those containing fructosamine-kinase activity were pooled and concentrated to a final volume of ~10 ml.

The preparation was diluted six times in buffer A, supplemented with protease inhibitors, and applied on a Blue Sepharose column (1.6 × 10 cm) equilibrated with buffer A. The column was washed with 300 ml buffer A and then 200 ml buffer A containing 200 mmol/l NaCl. Fructosamine kinase was then eluted with buffer A containing 200 mmol/l NaCl and 1 mmol/l ATP Mg. Fractions (2 ml) containing activity were pooled and concentrated to a volume of ~2 ml. This preparation was applied on a Sephacryl S-200 column (1.6 × 50 cm) and equilibrated with buffer A containing 100 mmol/l KCl. Protein was eluted with the same buffer in the presence of protease inhibitors. Fractions (1 ml) containing activity were pooled, concentrated to ~2 ml, and stored at –70°C before use. For determination of the molecular mass, the Sephacryl S-200 column was calibrated with pyruvate kinase (M_r 237,000), lactate dehydrogenase (M_r 144,000), and glycerol-3-phosphate dehydrogenase (M_r 68,000).

Peptide sequencing by nanoelectrospray-ionization MS. Bands corresponding to the 35,000-M_r protein (~10 µg) were cut from a 10% (wt/vol) polyacrylamide-SDS gel (21), concentrated in agarose in a Pasteur Pipette tip, and digested with trypsin (22,23). Peptides were separated by narrowbore high-pressure liquid chromatography (HPLC) in an acetonitrile gradient (22). Peaks eluting from the column were detected by UV absorption (A_{214}) and collected by hand. Two major peaks eluting at ~35 and 45 min were dried under vacuum and redissolved in 5 µl 60% methanol/1% (vol/vol) acetic acid. Two microliters of these solutions were analyzed by nanoelectrospray-ionization MS in an LCQ ion-trap mass spectrometer (Finnigan MAT) fitted with a nanoelectrospray probe (24). The source voltage was set at 0.8 kV and the scan time was 3.6 s. Spectra were taken in full MS and zoom-scan mode to determine parent ion masses and their charge state. For MS/MS, the collision energy was adjusted to the minimum needed for fragmentation.

Amplification and sequencing of cDNAs. For the human sequence (accession number AJ404615), a 5′ primer containing the putative ATG codon (GGGAATTCCATATGGAGCAGCTGCTGCGCGCC) in an *Nde*I site (underlined) and a 3′ primer containing the putative stop codon (GGATCC TACTTGAGCAGCCTTCGCATG) flanked by a *BamH*I site were constructed based on human expressed sequence tag (EST) sequences (T09491 and AI291863). They were used to polymerase chain reaction (PCR)-amplify kidney cDNA (prepared with Moloney murine leukemia virus [M-MLV] transcriptase) with *Pwo* polymerase, in the presence of 2 mol/l DMSO. An ~950-bp product was obtained, which was subcloned in pBluescript. EST clones 171540 and 173578 were ordered from the U.K.–Human Genome Mapping Project Resource Centre and resequenced to determine the sequence of the 3′ region (from nucleotide 763) of the human cDNA.

The open reading frame encoding the mouse enzyme was similarly amplified using the same 5′ primer as for the human sequence, a 3′ primer with sequence CGCGGATCCCTACCTGAGCAGCTTCTCCATC and mouse brain cDNA or EST clone 580142. The ~950-bp fragment that was obtained was subcloned and sequenced as above. EST clones 580142 and 480259 were also completely sequenced. In addition, rapid amplification of cDNA ends (RACE) experiments were performed using 5′ RACE amplification kit (Gibco-BRL) and three reverse primers corresponding to nucleotides 241–261 (for cDNA synthesis), 131–151, and 91–111 (for the first and second PCR reactions) of the cDNA sequence. Final products with sizes of ~950 bp were subcloned in pBluescript and sequenced. Sequencing was performed by the dideoxy-method (25 cycles using T7 Thermosequenase and primers labeled with an infra-red dye [IRD 41]). Products were analyzed in an automated laser fluorescence DNA sequencer 4000L (LI-COR).

Expression and purification of recombinant fructosamine kinases. *Nde*I-*Bam*HI fragments were removed from the pBluescript plasmid containing the PCR-amplified open reading frames and ligated in pET3a (26). *E. coli* BL21(DE3)pLysS strain (26) transformed with these plasmids was grown in M9 (human enzyme) or LB (mouse enzyme) medium at 37°C until the A_{600} reached 0.5–0.6. The cultures were cooled in ice for 30 min and the inducer isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.4 mmol/l. The cultures were then incubated for 70 h at 13°C. At this time, the cells were collected by centrifugation and extracted as described previously (27), except that the extraction medium contained 20 mmol/l HEPES, pH 7.5, instead of 20 mmol/l phosphate, pH 7.4.

Purification was carried out starting from 3-liter cultures. The bacterial extracts (150 ml) were made 22% (wt/vol) in polyethyleneglycol 6000 and centrifuged for 20 min at 20,000*g*. The protein pellet was resupended in 350 ml buffer A containing 1 µg/ml each of leupeptin and antipain. A Blue Sepharose column (100 ml) was loaded with this preparation and extensively washed with buffer A until the A_{280} decreased to ~0.15. Fructosamine kinase was eluted with the same buffer supplemented with 0.5 mol/l NaCl, 1 mmol/l ATP Mg, and 1 µg/ml each of leupeptin and antipain. The active fractions were pooled and concentrated to ~2 ml. The preparation was further purified on a Sephacryl S-200 column as described above.

Preparation and NMR analysis of phosphorylated DMF and phosphorylated fructoseglycine. Fifteen micromoles of DMF or fructoseglycine were incubated for 20 h at 30°C in a volume of 1 ml containing 25 mmol/l HEPES, pH 7.1, 1 mmol/l EGTA, 1 mmol/l dithiothreitol, 20 mmol/l ATP Mg, and 7.5 mU of recombinant human fructosamine kinase. The reaction medium was mixed with 2 ml ice-cold 10% (vol/vol) $HClO_4$ and centrifuged for 15 min at 16,000*g* and 4°C. The supernatant was neutralized with $KHCO_3$ and, after elimination of the $KClO_4$ precipitate by centrifugation, it was diluted to 10 ml with water and applied on an anion-exchange column (AG 1-X8 Cl^- resin, 1.6 × 15 cm). The column was washed with 30 ml water, and a linear gradient of NaCl (0–500 mmol/l in a total volume of 250 ml) was applied to elute the phosphoric esters of DMF and fructoseglycine. Fractions (3 ml) were collected for the measurement of inorganic phosphate (28) before and after incubation with alkaline phosphatase. The fractions containing monophosphate esters, eluting just before (DMF-phosphate) or just after (fructoseglycine-phosphate) inorganic phosphate, were pooled and concentrated under vacuum to ~1 ml. The sample was desalted by gel filtration on a Biogel P2 fine column equilibrated with water (0.9 × 55 cm). Fractions containing phosphoric esters and <5 mmol/l NaCl (as estimated by conductimetry) were pooled and freeze-dried for NMR analysis. The residue was dissolved in deuterated water and the pH adjusted to 5.1 with ^2HCl. All NMR spectra were acquired on a Bruker DRX-500 spectrometer at 27°C using a 5-mm probe head for inversion detection. Standard Bruker pulse programs were used to obtain ^1H-^1H correlation spectroscopy (COSY) spectra and ^1H-^{31}P-spectra (heteronuclear multiple quantum correlation [HMQC]). A delay of 50 ms was used for evolution of $^3J_{PH}$ constants.

RESULTS

Identification and purification of fructosamine kinase. When incubated in an erythrocyte lysate in the presence of ATP, DMF (35 µmol/l) was phosphorylated at a rate of ~0.7 nmol · min^{-1} · g^{-1} protein. The apparent K_M of this conversion for DMF was ~10 µmol/l. Similar activities were observed in the supernatant after centrifugation of the lysate at 10,000*g* for 90 min. Fructosamine kinase was purified from such supernatants by a procedure involving the following: *1*) batch adsorption on Blue Sepharose, from which the enzyme was eluted in a salt gradient (not shown); *2*) adsorption on a Q Sepharose column and elution in a salt gradient (not shown); *3*) Blue Sepharose chromatography with stepwise elution with salt and ATP (Fig. 1*A*); and *4*) gel filtration on Sephacryl S-200 (Fig. 1*B*). The enzyme was purified ~2500-fold compared with the activity in the erythrocyte lysate with an overall yield of ~5% (Table 1).

In the last step of the purification, fructosamine kinase activity eluted after glycerol-3-phosphate dehydrogenase with a M_r of ~20,000. SDS-PAGE analysis indicated that several polypeptides were still present in the preparation at this stage (Fig. 1*C*). Inspection of the elution profiles of several

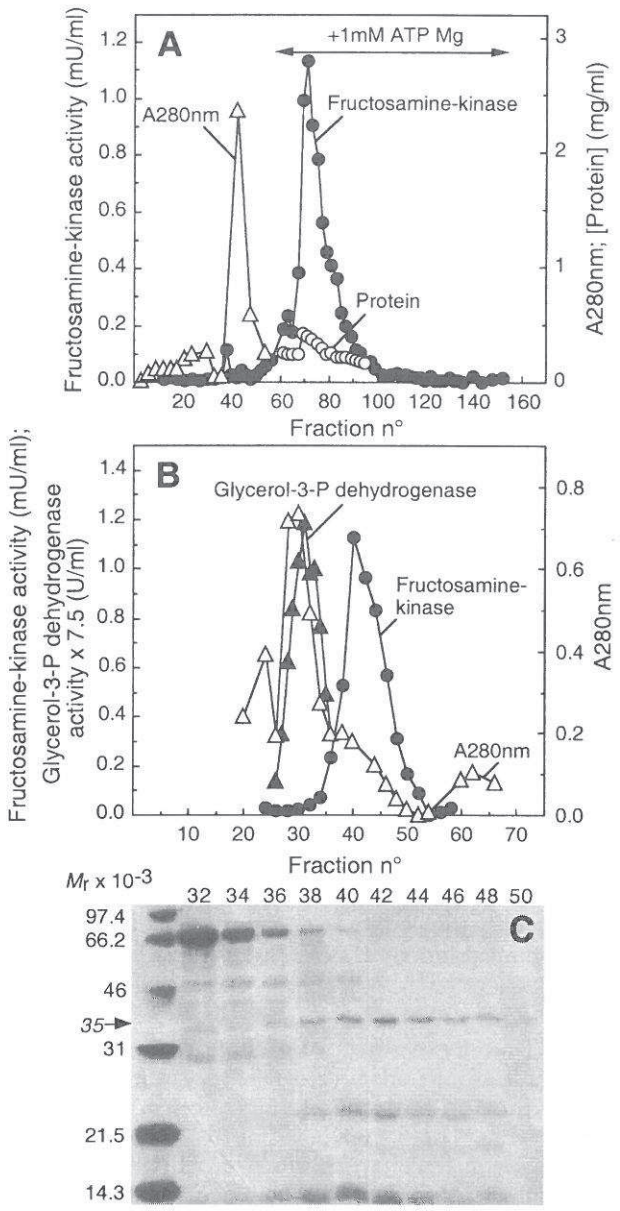

FIG. 1. Purification of human erythrocyte fructosamine kinase on Blue Sepharose and Sephacryl S-200 and analysis of the fractions of the last column by SDS-PAGE. *A*: A preparation containing 41 mU fructosamine kinase and purified by Blue Sepharose and Q Sepharose chromatography was applied on Blue Sepharose. The column was washed with buffer A (not shown), then with buffer A containing 200 mmol/l NaCl, and finally with buffer A containing NaCl and 1 mmol/l ATP Mg. *B*: The active fractions obtained from this column were concentrated, supplemented with 1.7 U rabbit muscle glycerol-3-phosphate dehydrogenase, used as a molecular weight standard, assayed as previously described (44), and chromatographed on a Sephacryl S-200 column. *C*: The indicated fractions of the latter were analyzed by SDS-PAGE and Coomassie Blue staining.

columns from three different purifications indicated that the polypeptide whose abundance in the fractions best correlated with the activity had a M_r of ~35,000. Bands corresponding to this protein were cut from the gel, concentrated in agarose, and digested with trypsin. The peptides were purified by narrowbore HPLC and two broad peaks eluting at 35 and 45 min were analyzed by nanoelectro-

TABLE 1
Purification table of human erythrocyte fructosamine kinase

Step	Volume (ml)	Protein (g)	Total activity (mU)	Specific activity (mU/g)	Purification fold	Yield (%)
Hemolysate	4,970	325	207.3	0.638	—	100
Blue Sepharose	25	0.244	49.2	202	129	23.7
Q Sepharose	7.8	0.090	46.6	519	813	22.5
Blue Sepharose	2.5	0.020	22.3	1,165	1,826	10.8
Sephacryl S-200	3	0.0062	10	1,626	2,549	4.8

spray-ionization MS. The peaks contained several peptide ions, which were fragmented for sequencing by tandem MS. The partial de novo sequences obtained did not allow identification of the protein in the protein sequence databases. Interestingly, one sequence, which was Ac-ME(K/Q)(L/I)(L/I)R (Fig. 2), indicated acetylation of the NH_2-terminal methionine residue, suggesting that the peptide corresponded to the NH_2-terminus of the protein.

Cloning of the cDNA encoding fructosamine kinase. Basic local alignment search tool (BLAST) (29) searches in EST databanks with a partial sequence of one of the peptides mentioned above [GEQMAD(L/I)H(L/I)Y] allowed for the identification of several ESTs from mouse and from human cDNA. Additional BLAST searches with these ESTs allowed the construction of contigs containing a complete open reading frame (ORF) in the case of the mouse cDNA and an incomplete one, missing an internal sequence, in the case of the human cDNA. With this information, we designed oligonucleotides corresponding to the putative ATG and stop codons, which were used to amplify the coding region starting from mouse brain and from human kidney cDNA. The amplified products were cloned in pBluescript and sequenced. We also sequenced several EST clones to complete the sequences at their 3′ and 5′ ends. In the case of the mouse enzyme, we also performed 5′-RACE starting from spleen and liver RNA to amplify the 5′ end. Four clones were eventually sequenced and found to start at nucleotide 1, 2 (2 x), and 14 of the mouse cDNA (Fig. 3*B*).

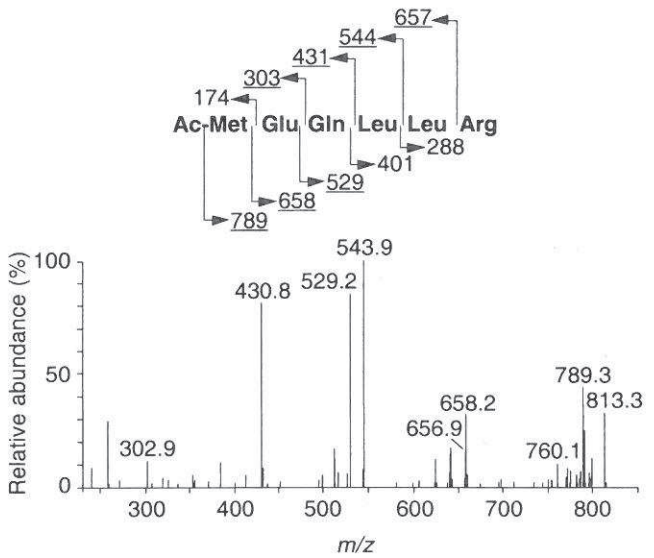

FIG. 2. MS/MS sequencing of a peptide from purified human erythrocyte fructosamine kinase. Bands of the 35,000-M_r polypeptide identified as fructosamine kinase were taken from a polyacrylamide gel, concentrated in agarose, and incubated with trypsin. Peptides were purified by HPLC and analyzed by nanospray-ionization tandem MS. The fragmentation pattern of an ion with charge/mass ratio (m/z) 831 is shown. The deduced sequence is shown above the spectrum with the m/z values of the expected fragments. Observed m/z values are underlined.

The sequence of the human cDNA comprises ~1,400 bp (Fig. 3*A*) and that from mouse (Fig. 3*B*) comprises 1,010 bp. Both cDNAs have a short 5′ untranslated region of ~20 bp. In both cases, the predicted protein has 309 residues and a calculated molecular mass of 35 kDa. The human sequence contains the peptides analyzed by MS (underlined). Most particularly, the sequence of the peptide predicted to be at the NH_2-terminus corresponds to the first 6 residues of the human protein. This is in agreement with the fact that the first ATG of the human sequence is in a favorable Kozak consensus (30). Intriguingly, the corresponding ATG in the mouse sequence (accession number AJ404616) is preceded by a T at position –3, which is relatively infrequent (30). The 3′ noncoding sequence of the human cDNA is a poorly readable C-rich region of ~400 bases, which starts immediately after the stop codon. In contrast, the 3′ end of the mouse cDNA is quite short and is not particularly C rich. Both sequences contain typical polyadenylation signals.

Sequence comparisons. Alignment of the human and mouse fructosamine kinases showed that they share 89% sequence identity. BLAST searches in databanks indicated the presence of ORF's encoding homologous proteins in several genomes, including those of *Synechocystis* sp., *E.coli* (Fig. 4), and *Caenorhabditis elegans* (not shown) but not those of *Saccharomyces cerevisiae* and *Drosophila melanogaster*. Typically, these predicted proteins have a length of ~300 amino acids and share ~30–40% identity with human or mouse fructosamine kinase. A motif with the sequence HGDLWSGN (residues 214–221 of the human enzyme) appears to be particularly well conserved.

Expression of fructosamine kinase. The coding sequences of the human and mouse enzymes were inserted into a pET vector and used to express the corresponding proteins in *E. coli*. Extracts from cells incubated for 70 h at 13°C with the inducer IPTG displayed a fructosamine kinase activity of 0.17 (human construct) and 0.11 (mouse construct) μmol · min^{-1} · g^{-1} protein when tested with 50 μmol/l radiolabeled DMF, whereas no activity was observed when control extracts were tested. Both enzymes were purified by Blue Sepharose affinity chromatography and by gel-filtration on Sephacryl S-200 to specific activities of 12 and 16 μmol · min^{-1} · g^{-1} protein. As expected, the fructosamine kinase activity co-purified with a 35,000-M_r polypeptide, which represented the major band as judged by SDS-PAGE after the

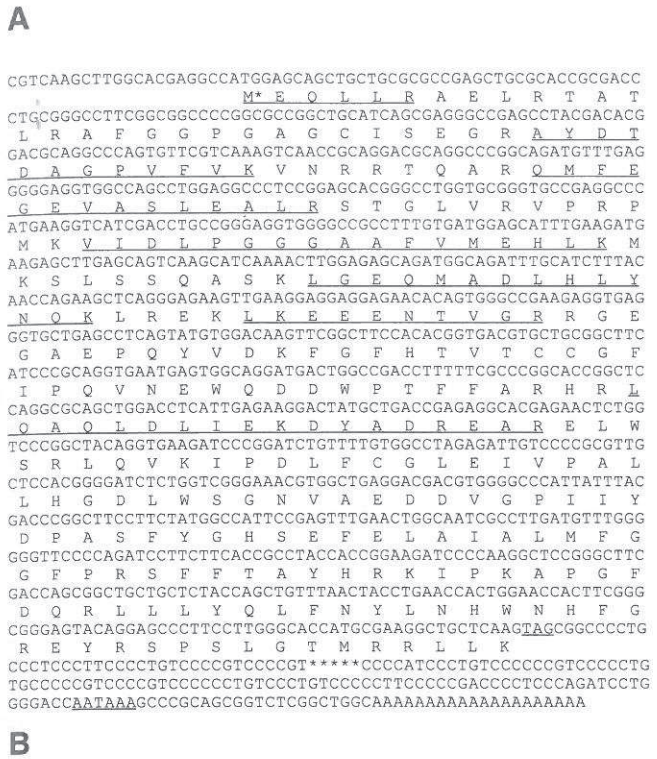

FIG. 3. Sequence of the human (*A*) and mouse (*B*) cDNAs and sequence of the predicted proteins. In the nucleotide sequences, the positions of the stop codon and of the polyadenylation signal are underlined. The underlined residues in the human amino acid sequence have been confirmed by MS/MS analysis of peptides derived from the enzyme purified from erythrocytes; ***, the presence of an ~340-bp poorly readable C-rich region at the 3′ untranslated end of the human cDNA; M*, an acetylated methionine.**

FIG. 4. Alignment of human and mouse fructosamine kinases with proteins predicted from the genomes of *Synechocystis* sp. and *E. coli*. The sequences of human (HS) and mouse (MM) fructosamine kinases are aligned with sequences from *Synechocystis* sp. (SY) and *E. coli* (EC). Residues of the last three sequences that are identical to those of human fructosamine kinase are indicated in bold.

second chromatography step (not shown). The yield of the purification was 54 and 39% for the human and mouse recombinant enzymes, respectively, corresponding to 225 and 165 mU (not shown).

Properties of the recombinant enzymes. The enzyme purified from erythrocytes and the recombinant human and mouse proteins phosphorylated not only DMF but also fructoselysine, fructoseglycine, and fructose (Table 2). DMF was the substrate for which the enzyme displayed the highest affinity. However, the low-specific radioactivity of [^{14}C]DMF made it difficult to estimate the K_M with precision. The second-best substrate was fructoselysine, for which the enzyme displayed ~10 times less affinity than for DMF, but with a 1.5- to 5-fold higher V_{max}. The K_M for fructoseglycine was ~2 orders of magnitude higher than for fructoselysine. It could not be determined reliably in the case of fructose but was estimated to be >100 mmol/l for the three enzymes. At the latter concentration, the rate of phosphorylation was 39 and 70 nmol · min^{-1} · mg^{-1} protein for the human and mouse recombinant enzymes and 21 nmol · min^{-1} · mg^{-1} protein for the human erythrocyte enzyme.

We also tested the ability of the fructosamine kinases to phosphorylate protein–bound fructosamines. As shown in Fig. 5 for the mouse recombinant enzyme, incorporation of ^{32}P from [γ-^{32}P]ATP was observed when the DMF kinase was incubated with glycated lysozyme but not with control lysozyme. Furthermore, the incorporation was inhibited by DMF. We calculated that ~0.1 µmol of phosphate had been incorporated per µmol of lysozyme after a 30-min incubation in the experiment shown in Fig. 5.

TABLE 2
Kinetic properties of human erythrocyte, human recombinant and mouse recombinant fructosamine kinases

	Human erythrocyte fructosamine kinase		Human recombinant fructosamine kinase		Mouse recombinant fructosamine kinase	
	K_M	V_{max} (mU/mg)	K_M	V_{max} (mU/mg)	K_M	V_{max} (mU/mg)
DMF	~1 µmol/l	1.6	~1 µmol/l	12	~1 µmol/l	16
Fructoselysine	13.2 µmol/l	8.8	7.2 µmol/l	18	7.4 µmol/l	34
Fructoseglycine	1.0 mmol/l	34	2.2 mmol/l	102	1.0 mmol/l	134

Data are the means of duplicate determinations.

When tested with DMF, the human recombinant enzyme displayed a K_M for ATP of 18 µmol/l. With 1 mmol/l GTP, CTP, or UTP, the activity was equal to 106, 27, and 76%, respectively, of the activity observed with 1 mmol/l ATP.

NMR analysis of purified DMF-phosphate and fructoseglycine-phosphate. DMF and fructoseglycine were phosphorylated with fructosamine kinase, purified, and analyzed by NMR. Three major doublet resonances were detected in the phosphomonoester region of the proton-coupled ^{31}P-NMR spectrum of the two compounds, in addition to the resonance caused by inorganic phosphate (not shown). By analogy with fructose 3-phosphate (12), they were assigned to the β-fructopyranose, the β-fructofuranose, and the α-fructofuranose forms, in order of decreasing abundance and values of chemical shift. In the case of DMF-phosphate, the relative abundances were 100:43:24, and the chemical shifts at pH 5.1 were 5.6, 3.8, and 3.2 ppm with respect to the α-phosphate of ATP, respectively. ^{1}H-NMR spectra, with and without phosphorus broadband decoupling, were acquired to identify which proton resonances coupled to phosphorus. Differences were only detected in three multiplets resonating between 4.1 and 4.5 ppm (Fig. 6). These couplings were confirmed in two-dimensional proton-phosphorus correlation spectra HMQC (not shown). To determine which proton in the fructose molecule was coupled to phosphorus, proton-proton correlation spectra (COSY) were acquired. The assignment to protons bound to the third carbon of the deoxyfructose moiety followed from the observation that the corresponding resonances correlated with only one other in COSY spectra.

DISCUSSION

Purification and cloning of a fructosamine-3-kinase. Until now, the enzyme catalyzing the phosphorylation of fructose or fructosamines on their third carbon had not been purified extensively. Using DMF as a substrate, we have purified a fructosamine kinase close to homogeneity and have cloned the corresponding human and mouse cDNAs. The proteins encoded by these cDNAs were expressed in *E. coli* and shown to have kinetic properties similar (although not identical) to those of the enzyme purified from erythrocytes. The small differences in the kinetic properties are possibly due to differences in post-translational modification (e.g., acetylation of the NH_2-terminus). The native and recombinant forms of this enzyme catalyze the phosphorylation of DMF, fructoselysine, fructoseglycine, and fructose in order of decreasing affinity. The position of the phosphorylated carbon was investigated by NMR analysis in the case of DMF and

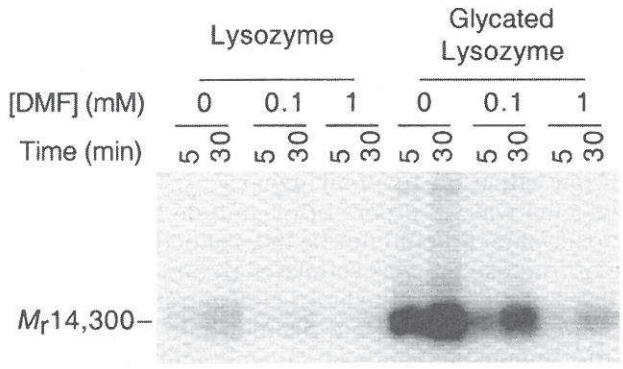

FIG. 5. Phosphorylation of unglycated and glycated lysozyme by fructosamine kinase and its inhibition by DMF. Thirty micrograms of glycated or unglycated lysozyme were incubated at 30°C in the presence of 25 mmol/l Tris, pH 7.8, 1 mmol/l EGTA, 100 µmol/l ATP Mg, 1,000,000 cpm of [γ-^{32}P]ATP, the indicated concentrations of DMF, and 30 µU of mouse recombinant fructosamine kinase in a final volume of 50 µl. At the indicated times, the reaction was stopped by adding 17 µl sample buffer (0.125 M Tris pH 7.8, 8% SDS, 40% sucrose, 0.03% Bromophenol Blue, 40 mmol/l dithiothreitol) for SDS-PAGE. The samples were loaded on a polyacrylamide gel, which was allowed to migrate at 4°C, dried under vacuum, and exposed to a PhosphorImager screen (Molecular Dynamics) for the detection of the radioactivity.

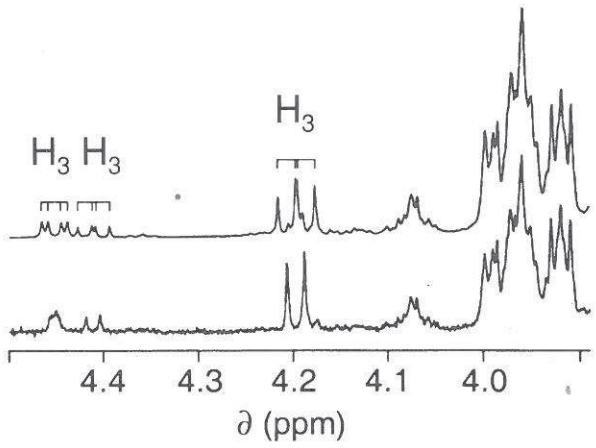

FIG. 6. Proton NMR spectra of phosphorylated DMF with (bottom trace) or without (top trace) phosphorus decoupling. Spectra were taken in a Bruker DRX-500 at a probe head temperature of 27°C and 128 scans (top spectrum) or 1 scan (lower spectrum). The pH value of the solution was 5.1. The collapse of the three multiplets on phosphorus decoupling enabled the identification of H_3 resonances in the three major forms of phosphorylated DMF: β-fructopyranose, the β-fructofuranose, and the α-fructofuranose forms. Chemical shift values are referenced to the water signal at 4.70 ppm.

fructoseglycine and found to be C3. We assume that it must be the same in the case of fructoselysine and fructose. Because this enzyme has >10,000-fold more affinity for DMF and fructoselysine than for fructose, it has to be considered as a fructosamine-3-kinase.

The enzyme also catalyzed the phosphorylation of glycated lysozyme but not of unglycated lysozyme, suggesting that the fructosamine moieties are the acceptors of the phosphoryl groups. The low phosphorylation stoichiometry of this reaction (~0.1 mol/l) may be caused by a problem of accessibility of the glycated residues or due to the fact that only certain kinds of glycated residues are phosphorylated. Glycated lysozyme is known to contain fructoselysine and fructosearginine residues (31).

Physical properties and sequence comparisons. The relative molecular mass of the protein estimated by gel filtration (M_r 20,000) compared with the value obtained by SDS-PAGE (M_r 35,000) suggests that the purified protein is a monomer, perhaps with some degree of asymmetry accounting for the difference between the two relative molecular masses. Sequencing of peptides derived from the enzyme purified from erythrocytes demonstrated that the initiator methionine is NH_2-acetylated. Acetylation of the NH_2-terminus is a rather frequent post-translational modification, which occurs mainly on alanine, serine, and methionine residues. Acetylation of methionine residues preferentially occurs if the second residue is an aspartate or a glutamate (32). This reaction is catalyzed by NH_2-terminal acetyl-transferase 3 in *S. cerevisiae* (33). The human and mouse proteins both contain a glutamate in the second position, which suggests that the mouse enzyme would also be NH_2-acetylated.

BLAST searches did not reveal homology with proteins of known function but allowed us to identify ORFs of ~30% identity to mammalian fructosamine-3-kinase in several bacterial genomes. These presumably also encode fructosamine-3-kinases. Alignment of these sequences indicated the presence of a conserved HGDLWSGN motif. Interestingly, a conserved HGDxxxxN motif is found in aminoglycoside-kinases (34), which also catalyze the phosphorylation of a secondary alcohol of aminated compounds, suggesting that these enzymes may be distantly related to fructosamine-3-kinase.

Physiological role. Fructosamine-3-kinase phosphorylates fructose with low affinity (K_M >100 mmol/l); therefore, it is likely that this enzyme is responsible for the formation of fructose 3-phosphate in intact erythrocytes and in lenses. Accordingly, this reaction can be strongly inhibited in erythrocytes by a synthetic fructosamine, DMF (G.D., V.S., F.V., E.V.S., unpublished data). In animals, fructose is essentially metabolized in the liver and kidneys through fructose 1-phosphate (35), and the enzyme that catalyzes this reaction (ketohexokinase) has a V_{max} ~1,000-fold higher (expressed on a per weight basis) and a K_M ~100-fold lower than the enzyme catalyzing the phosphorylation of fructose on C3 in erythrocytes. Moreover, because there is no known enzyme able to metabolize fructose 3-phosphate, the conversion of fructose to fructose 3-phosphate probably has little physiological relevance. However, as a result of a side-reaction catalyzed by fructosamine-3-kinase on fructose, fructose 3-phosphate may accumulate in lenses when the concentration of free fructose pathologically increases in this tissue, as in diabetes (36). Fructose 3-phosphate can then decompose to inorganic phosphate and 3-deoxyglucosone, and the latter may participate in glycation reactions or be reduced to 3-deoxyfructose (36).

The enzyme we have characterized is most likely to be responsible for the conversion of DMF to DMF 3-phosphate in intact erythrocytes (G.D., V.S., F.V., E.V.S., unpublished data). The higher overall K_M value (100 µmol/l) observed for this conversion in intact cells compared with the K_M of the purified enzyme (1 µmol/l) is partly due to the fact that the transport of DMF is rate limiting, so that its intracellular concentration is lower than its extracellular concentration (G.D., E.V.S., unpublished data). In addition, competitive inhibitors of fructosamine-3-kinase may be present in intact erythrocytes, as indicated by the fact that the K_M value of fructosamine kinase in crude extracts was ~10-fold higher than the value obtained with the purified enzyme. Further work is needed to identify these inhibitors, which may correspond to endogenous fructosamines.

Although DMF is the best-known substrate of fructosamine-3-kinase, it is clearly not a physiological substrate. In contrast, fructoselysine, the second-best substrate, is a compound that could be derived from the digestion of glycated proteins or from the glycation of free lysine. The enzyme that we have characterized is most likely the same or closely related to the fructoselysine-3-kinase mentioned by Kappler et al. (9). According to these authors, fructoselysine 3-phosphate spontaneously decomposes to 3-deoxyglucosone, inorganic phosphate, and free lysine, thus leading to the recovery of this amino acid. Fructosamine-3-kinase would then be the first mammalian enzyme reported to degrade fructosamines. Two kinds of enzymes able to degrade low–molecular weight fructosamines, called amadoriases, have been identified in microorganisms. These are as follows: *1*) oxidases that convert fructosamines to free amines and glucosone with concomitant production of H_2O_2 (37–39) and *2*) an oxidase that cleaves ε-fructosyl-aminocaproate to free fructosamine and adipic acid (40,41). Only the first type allows the recovery of the initial animo-compound.

The most important physiological role of fructosamine-3-kinase may be its capacity to phosphorylate protein-bound fructosamines. We propose that such a reaction initiates a pathway leading to regeneration of lysine residues, hence preventing further reaction to advanced glycation end products. This pathway may be crucial in tissues or cells types with inexistent or low protein turnover. It is indeed intriguing that fructosamine-3-kinase is present in erythrocytes, which are unable to synthesize proteins. We may also infer from the presence of fructose-3-kinase in lenses (42) that this tissue also contains fructosamine-3-kinase. Again, this tissue is characterized by a very low protein turnover (43). If our hypothesis is correct, a congenital defect in fructosamine-3-kinase may lead to pathological effects such as cataract. In addition, inhibitors of fructosamine-3-kinase may have a detrimental effect, rather than the postulated beneficial effect (10) because of their inhibition of the formation of 3-deoxyglucosone.

In conclusion, the purification and cloning of a fructosamine-3-kinase is an important step in the identification of a new metabolic pathway, which may have far-reaching implications in the understanding of the development of diabetic complications.

ACKNOWLEDGMENTS

This work was supported by the Actions de Recherche Concertées; the Belgian State Program on Interuniversity Poles of

Attraction; the Federal Office for Scientific, Technical, and Cultural Affairs; the Fonds National de la Recherche Scientifique; and the Juvenile Diabetes Foundation International. G.D. is an aspirant of the Belgian Fonds National de la Recherche Scientifique, and F.C. is a fellow of the Fonds pour l'Encouragement à la Recherche dans l'Industrie et dans l'Agriculture.

We thank E. Sonveaux and J.M. Tilquin for guidance in chemical syntheses, H.G. Hers for helpful comments during the preparation of this manuscript, G. Berghenouse for her help in the biochemical work, and G. Noël for her help in the cloning and sequencing of the cDNAs.

REFERENCES

1. Brownlee M, Vlassara H, Cerami A: Nonenzymatic glycosylation and the pathogenesis of diabetic complications. *Ann Intern Med* 101:527–537, 1984
2. Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40:405–412, 1991
3. Brownlee M: Glycation and diabetic complications. *Diabetes* 43:836–841, 1994
4. Ravandi A, Kuksis A, Marai L, Myher JJ: Preparation and characterization of glucosylated aminoglycerophospholipids. *Lipids* 30:885–891, 1995
5. Goldstein DE, Little RR, Wiedmayer HM, England JD, McKenzie EM: Glycated hemoglobin: methodologies and clinical applications. *Clin Chem* 32:B64–B70, 1986
6. Johnson RN, Metcalf PA, Baker JR: Fructosamine: a new approach to the estimation of serum glycosylprotein: an index of diabetic control. *Clin Chem Acta* 127:87–95, 1982
7. Armbruster DA: Fructosamine: structure, analysis and clinical usefulness. *Clin Chem* 33:2153–2163, 1987
8. Szwergold BS, Taylor K, Lal S, Su B, Kappler F, Brown TR: Identification of a novel protein kinase activity specific for Amadori adducts on glycated proteins (Abstract). *Diabetes* 46 (Suppl. 1):108A, 1997
9. Kappler F, Su B, Nguyen V, Taylor K, Wang J, Brown TR: Enzymatic production of 3-deoxyglucosone (Abstract). *Diabetes* 49 (Suppl. 1):A423, 2000
10. Brown TR, Kappler F, Szwergold BS, Lal S, Su B: Compounds and methods for therapeutic intervention in preventing diabetic complications and procedures for assessing a diabetic's risk of developing complications and determining the efficacy of therapeutic intervention. U.S. patent 6,004,958, 1999
11. Szwergold BS, Kappler F, Brown TR, Pfeffer P, Osman SF: Identification of D-sorbitol 3-phosphate in the normal and diabetic mammalian lens. *J Biol Chem* 264:9278–9282, 1989
12. Szwergold BS, Kappler F, Brown TR: Identification of fructose 3-phosphate in the lens of diabetic rats. *Science* 247:451–454, 1990
13. Petersen A, Szwergold BS, Kappler F, Weingarten M, Brown TR: Identification of sorbitol 3-phosphate and fructose 3-phosphate in normal and diabetic human erythrocytes. *J Biol Chem* 265:17424–17427, 1990
14. Petersen A, Kappler F, Szwergold BS, Brown TR: Fructose metabolism in the human erythrocyte: phosphorylation to fructose 3-phosphate. *Biochem J* 284:363–366, 1992
15. Hodge JE, Rist CE: The Amadori rearrangement under new conditions and its significance for non-enzymatic browning reactions. *J Am Chem Soc* 75: 316–322, 1953
16. Mossine VV, Glinsky GV, Feather MS: The preparation and characterization of some Amadori compounds (1-amino-1-deoxy-D-fructose derivatives) derived from a series of aliphatic ω-amino acids. *Carbohydr Res* 262:257–270, 1994
17. Thornalley PJ, Langborg A, Minhas HS: Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. *Biochem J* 344:109–116, 1999
18. McClard RW: Synthesis and purification of [1-^{32}P]fructose-1,6-bisphosphate with high specific radioactivity. *Anal Biochem* 96:500–503, 1979
19. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254, 1976
20. Krebs HA, Henseleit K: Untersuchungen über die Harnstoffbildung im Tierkörper. *Hoppe-Seyler's Z Physiol Chem* 210:33–60, 1932
21. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
22. Rider MH, Puype M, Van Damme J, Gevaert K, De Boeck S, D'Alayer J, Rasmussen HH, Celis JE, Vandekerckhove J: An agarose-based gel-concentration system for microsequence and mass spectrometric characterization of proteins previously purified in polyacrylamide gels starting at low picomole levels. *Eur J Biochem* 230:258–265, 1995
23. Gevaert K, Verschelde JL, Puype M, Van Damme J, Goethals M, De Boeck S, Vandekerckhove J: Structural analysis and identification of gel-purified proteins, available in the femtomole range, using a novel computer program for peptide sequence assignment, by matrix-assisted laser desorption ionization-reflection time-of-flight mass spectrometry. *Electrophoresis* 17:918–924, 1996
24. Wilm M, Mann M: Analytical properties of the nanoelectrospray ion source. *Anal Chem* 68:1–8, 1996
25. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 74:5463–5467, 1977
26. Studier FW, Moffatt BA: Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* 189:113–130, 1986
27. Veiga-da-Cunha M, Detheux M, Watelet N, Van Schaftingen E: Cloning and expression of a *Xenopus* liver cDNA encoding a fructose-phosphate-insensitive regulatory protein of glucokinase. *Eur J Biochem* 225:43–51, 1994
28. Fiske CH, Subbarow Y: The colorimetric determination of phosphorus. *J Biol Chem* 66:375–400, 1925
29. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic local alignment search tool. *J Mol Biol* 215:403–410, 1990
30. Kozak M: An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res* 15:8125–8133, 1987
31. Tagami U, Akashi S, Mizukoshi T, Suzuki E, Hirayama K: Structural studies of the Maillard reaction products of a protein using ion trap mass spectrometry. *J Mass Spectrom* 35:131–138, 2000
32. Persson B, Flinta C, von Heijne G, Jörnvall H: Structures of N-terminally acetylated proteins. *Eur J Biochem* 152:523–527, 1985
33. Polevoda B, Norbeck J, Takakura H, Blomberg A, Sherman F: Identification and specificities of N-terminal acetyltransferases from *Saccharomyces cerevisiae*. *EMBO J* 18:6155–6168, 1999
34. Perlin MH, Brown SA, Dholakia JN: Developing a snapshot of the ATP binding domain(s) of aminoglycoside phosphotransferases. *Front Biosci* 4:63–71, 1999
35. Van den Berghe G: Inborn errors of fructose metabolism. *Annu Rev Nutr* 14:41–58, 1994
36. Lal S, Szwergold BS, Taylor AH, Randall WC, Kappler F, Wells-Knecht K, Baynes JW, Brown TR: Metabolism of fructose 3-phosphate in the diabetic rat lens. *Arch Biochem Biophys* 318:191–199, 1995
37. Horiuchi T, Kurokawa T, Saito N: Purification and properties of fructosylamine oxidase from *Corynebacterium* sp.2-4-1. *Agric Biol Chem* 53:103–110, 1989
38. Horiuchi T, Kurokawa T: Purification and properties of fructosylamine oxidase from *Aspergillus* sp. 1005. *Agric Biol Chem* 55:333–338, 1991
39. Takahashi M, Pischetsrieder M, Monnier VM: Molecular cloning and expression of amadoriase isoenzyme (fructosyl amine:oxygen oxidoreductase, EC 1.5.3.) from *Aspergillus fumigatus*. *J Biol Chem* 272:12505–12507, 1997
40. Gerhardinger C, Marion MS, Rovner A, Glomb M, Monnier VM: Novel degradation pathway of glycated amino acids into free fructosamine by a *Pseudomonas* sp. soil strain extract. *J Biol Chem* 270:218–224, 1995
41. Saxena AK, Saxena P, Monnier VM: Purification and characterization of a membrane-bound deglycating enzyme (1-deoxyfructosyl alkyl aminoacid oxidase, EC 1.5.3) from a Pseudomonas sp. soil strain. *J Biol Chem* 271:32803–32809, 1996
42. Lal S, Szwergold BS, Kappler F, Brown TR: Detection of fructose-3-phosphokinase activity in intact mammalian lenses by ^{31}P NMR spectroscopy. *J Biol Chem* 268:7763–7767, 1993
43. Delcour J, Papaconstantinou J: Biochemistry of bovine lens proteins. IV. Synthesis and aggregation of α-crystallin subunits in differentiating lens cells. *J Biol Chem* 247:3289–3295, 1972
44. Bergmeyer HU, Grassl M, Walter HE: *Methods of Enzymatic Analysis*. Vol. 2, 3rd ed. Weinheim, Germany, VCH, 1974, p. 215–216