Tissue Distribution and Evolution of Fructosamine 3-Kinase and Fructosamine 3-Kinase-related Protein*

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Fructosamine 3-kinase (FN3K) and FN3K-related protein (FN3K-RP) catalyze the phosphorylation of the Amadori products ribulosamines, psicosamines, and, in the case of FN3K, fructosamines. BLAST searches in chordate genomes revealed two genes encoding proteins homologous to FN3K or FN3K-RP in various mammals and in chicken but only one gene, encoding a protein more similar to FN3K-RP than to FN3K, in fishes and the sea squirt *Ciona intestinalis*. This suggests that a gene duplication event occurred after the fish radiation and that the FN3K gene evolved more rapidly than the FN3K-RP gene. In agreement with this distribution, only one enzyme, phosphorylating ribulosamines and psicosamines but not fructosamines, was found in the tissues from a fish (*Clarias gariepinus*), whereas two enzymes with specificities similar to either FN3K or FN3K-RP were found in mouse, rat, and chicken tissues. FN3K is particularly active in brain, heart, kidney, and skeletal muscle. Its activity is also relatively elevated in erythrocytes from man, rat, and mouse but barely detectable in erythrocytes from chicken and pig, which correlates well with the low intracellular concentration of glucose in erythrocytes from these species. This is in keeping with the specific role of FN3K to repair protein damage caused by glucose. FN3K-RP was more evenly distributed in tissues, except for skeletal muscle where its activity was particularly low. This may be related to low activity of the pentose phosphate pathway in this tissue, as suggested by assays of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. This finding, together with the high affinity of FN3K-RP for ribulosamines, suggests that this enzyme may serve to repair damage caused by the powerful glycating agent, ribose 5-phosphate.

Fructosamine 3-kinase (FN3K) and fructosamine 3-kinase-related protein (FN3K-RP) are two recently described mammalian enzymes that phosphorylate ketoamines on the third carbon of their sugar moiety (1–4). Ketoamines are produced through a spontaneous reaction of aldoses or aldose derivatives with an amine, followed by an Amadori rearrangement (5, 6). Both FN3K and FN3K-RP phosphorylate ribulosamines and psicosamines, the glycation products of ribose and allose, but only the former catalyzes the phosphorylation of fructosamines, which are derived from glucose and are well known to be formed in animal tissues. Phosphorylation of ketoamines on their third carbon leads to their destabilization and their spontaneous detachment from proteins with a half-life of the order of 6–8 h (fructosamines and psicosamines) or 25 min (ribulosamines) (3, 7). FN3K and FN3K-RP are therefore responsible for protein deglycation, a new variety of protein repair. Evidence for the involvement of FN3K in the removal of fructosamines from hemoglobin and of FN3K-RP in the removal of ribulosamines and psicosamines has been obtained by incubating erythrocytes in the presence of elevated concentrations of glucose, ribose, or allose with or without competitive inhibitors of the two enzymes (7, 8). Of interest is the conclusion that fructosamines are removed from only some of the glycation sites of hemoglobin, apparently those that are most accessible to FN3K (9).

Many questions still remain about protein deglycation, most particularly about FN3K-RP. The origin of the substrates of this enzyme remains enigmatic, as there do not seem to be significant amounts of free ribose or allose in tissues, contrasting with the occurrence of concentrations of glucose in the millimolar range. The purpose of the present work was to study the species and tissue distributions of FN3K and FN3K-RP, as this could give some hint as to the function of these enzymes, most particularly FN3K-RP. As FN3K might prevent some damaging effects of glucose, it was also decided to test the effect of starvation and diabetes on the expression of this enzyme.

**EXPERIMENTAL PROCEDURES**

*Synthesis of Fructosamines—Radiolabeled 1-deoxy-1-morpholino-fructose (DMF) and 1-deoxy-1-morpholino-ribulose (DMR) were synthesized according to Refs. 1 and 4, respectively. Lysozyme-bound osamines utilized for the measurement of activities were prepared as described (4). Unlabeled, low molecular weight osamines were either obtained from Sigma (DMF) or synthesized as described (fructoselysine, Ref. 8) and (psicoselysine, DMR, Ref. 4).*

*Animal Handling—Male Wistar rats and NMRI mice were obtained from Charles River and fed on regular rodent laboratory pellets. Diabetes was induced in rats by a single injection of streptozotocin (75 mg/kg, diluted in 0.15 M NaCl). The fish (*C. gariepinus*), obtained from the Centre de Recherches et de Formation en Aquaculture, were bred at 28 °C.*

*RP, fructosamine 3-kinase-related protein; DMF, 1-deoxy-1-morpholino-fructose; DMR, 1-deoxy-1-morpholino-ribulose; DTT, dithiothreitol; contig, group of overlapping clones.*

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1 The abbreviations used are: FN3K, fructosamine 3-kinase; FN3K-RP, fructosamine 3-kinase-related protein; DMF, 1-deoxy-1-morpholino-fructose; DMR, 1-deoxy-1-morpholino-ribulose; DTT, dithiothreitol; contig, group of overlapping clones.
Partial Purification of FN3K and FN3K-RP from Animal Tissues—All purification steps were performed at 4 °C. Tissues were harvested on animals killed by decapitation (rat, chicken), vertebral dislocation (mouse), or stunning (fish). They were frozen between Wollenberger clamps cooled in liquid nitrogen and maintained at −70 °C until used. The frozen samples were homogenized in a Potter-Elvehjem device with 2 ml/g tissue of buffer A (20 mM Hepes, pH 7.1, 10 mM KCl, 2.5 mM MgCl₂, 1 mM dithiothreitol (DTT), and 1 μM/ml leupeptin/antipain). The homogenates were centrifuged for 15 min at 10,000 × g. Heparinized blood was centrifuged for 10 min at 2,000 × g, and the plasma and theuffy coat were discarded. The erythrocytes were washed twice with ice-cold 150 mM NaCl and kept at −70 °C until used. 1 volume of tissue extract or packed red blood cells was diluted with 2 volumes of buffer B (10 mM Hepes, pH 7.1, 1 mM DTT, 1 μM/ml leupeptin/antipain) and centrifuged for 15 min at 16,000 × g. The resulting supernatant (0.5 ml) was diluted in 1 ml of buffer B before application onto Blue-Sepharose columns (1 ml of wet gel for FN3K purification) or Blue Trisacryl columns (1 ml of wet gel for FN3K-RP purification) equilibrated with the same buffer. Blue-Sepharose columns were washed with 4 ml of buffer B, and retained proteins were eluted with buffer B supplemented with 500 mM NaCl and 1 mM ATP-Mg (5 fractions of 1 ml). In the case of the Blue Trisacryl columns, buffer B also contained 0.2 mM EDTA, and retained proteins were eluted with 500 mM NaCl (2 fractions of 1 ml) and then 1 N NaCl (5 fractions of 1 ml) without ATP-Mg.

Protein in the extracts was measured with the method of Bradford (10) with bovine γ-globulin as a standard, except for erythrocytes where the method of Drabkin and Austie (11) was used (Sigma).

Measurement of Enzymatic Activity—All assays were carried out at 30 °C. For the assay of FN3K, the phosphorylation of [γ-32P]ATP in lysozyme glycated with allase. The assay mixture contained 25 mM Tris, pH 7.8, 1 mM EGTA, 2 mM MgCl₂, 1 mM DTT, 2.1 mg/ml glycated hen egg lysozyme (∼220 μM psicosamines), 20 mM DMF (to inhibit FN3K), and 100 μM ATP-Mg and 800,000 cpm [γ-32P]ATP (Amersham Biosciences) in a final volume of 100 μl. Thirty μl of the reaction medium were spotted onto cation-exchanger papers (F81), which were washed three times with 50% methanol, 1 M H₃PO₄, dried, and then mixed with scintillation liquid (OptimaGold; Packard) for radioactivity measurement. The sum of the activities found in the fractions eluted with salt was used to compute FN3K activity.

FN3K-RP was assayed in the fractions of the Blue Trisacryl column through the incorporation of 32P from [γ-32P]ATP in lysozyme glycated with allase. The assay mixture contained 25 mM Tris, pH 7.1, 1 mM DTT, 10 mM KCl, 2.5 mM MgCl₂, 250 μM NADP, and 250 μM 6-phosphogluconate. In both cases, the activity was calculated from the variation of the activities found in the fractions eluted with salt from the Blue Trisacryl columns used to compute the FN3K-RP activity.

The phosphorylation of protein-bound fructosamines and ribulosamines was measured in a similar way in some experiments. The concentration of substrates was 250 μM protein-bound fructosamines and 450 μM protein-bound ribulosamines.

Glucose-6-phosphate dehydrogenase activity was determined spectrophotometrically in a reaction mixture containing 25 mM Hepes, pH 7.1, 1 mM DTT, 10 mM KCl, 2.5 mM MgCl₂, 250 μM NADP, and 250 μM glucose-6-phosphate. 6-Phosphogluconate dehydrogenase activity was determined in a reaction mixture containing 50 mM Tris, pH 7.8, 1 mM DTT, 25 mM KCl, 1 mM MgCl₂, 250 μM NADP, and 250 μM 6-phosphogluconate. In both cases, the activity was calculated from the variation of A₅₄₀ observed after the addition of the appropriate volume of tissue homogenate.

RNA Extraction and Quantitative PCR—Total RNA was isolated from 0.5 g of frozen tissues from rats, using the Ambion RNA-WIZ kit. cDNA was synthesized by RT-PCR using oligo(dT) and Superscript II reverse transcriptase according to the manufacturer’s instructions. Gene-specific primers were designed to generate PCR products of 75–350 bp from the genomic DNA sequence (available at genome.jgi-psf.org/ciona and www.ncbi.nlm.nih.gov/geneview?gene=GenBank.html), taking into account the consensus sequences for splicing sites. All sequences were aligned using Clustal X (12). For the creation of a neighbor-joining tree, the “tree” option of the Clustal X program was used after exclusion of positions with gaps and with correction for multiple substitutions. For bootstrap analysis, 1000 samplings were carried out. For the creation of a maximum likelihood tree, the alignment was first transformed to the Phylib format after removal of all positions with gaps. This alignment was fed into the TreePuzzle program (13) using standard settings and the JTT matrix (14) as evolutionary model with analysis of 1000 quartet puzzling steps.

RESULTS

Genomic Environment of the FN3K and FN3K-RP Sequences—BLAST searches in vertebrate genomes with the human and mouse FN3K and FN3K-RP sequences indicate that the human, mouse, and rat genomes contain two genes corresponding to FN3K-RP and FN3K (Fig. 1). These genes are in tandem repeat in the telomeric region of chromosomes 17q (man), 11q (mouse), and 10q (rat). The neighboring genes encode a putative GTPase of the Ras family (RAB40) on the centromeric side and tubulin-specific chaperone D on the telomeric side. A similar genomic arrangement is found in the chicken genome with, successively, the genes encoding FN3K-RP (as indicated by the fact that it showed more identity with mammalian FN3K-RPs, 78%, than with FN3Ks, 67%), FN3K, and tubulin-specific chaperone D. The sequence of thecontig encoding these three genes (gi:44766232) is interrupted upstream of the FN3K-RP gene, preventing us from knowing the identity of the gene located 5’ to FN3K-RP.

Only one copy of a FN3K/FN3K-RP homolog was found in the Fugu rubripes and zebra fish genomes; in both cases, the encoded protein showed more identity with FN3K-RP (69.9 and 69.3% with the human and mouse sequences, respectively) than with FN3K (60.0 and 60.8% sequence identity). In both cases, it was flanked by the tubulin-specific chaperone D gene on the 3’ side, as in the mammalian genomes. However, the 5’ flanking genes were different in the two fish genomes, encoding an ADP-riboseylation factor in F. rubripes and a reverse transcriptase in zebra fish. It should be added that there is no trace of FN3K-like sequences between the FN3K-RP and tubulin-specific chaperone D genes in the fish genomes.

Sequence Comparisons and Evolutionary Tree—Fig. 2 shows an alignment of FN3K and FN3K-RPs from several vertebrates as well as from the urochordate C. intestinalis (a sea squirt). The sequences of chicken FN3K and FN3K-RP and that of C. intestinalis were reconstructed from genomic sequences and EST (earthen) sequences, taking into account the known consensus for splice sites. From such an alignment, in which the rat sequences were also included (not shown), an evolutionary tree (Fig. 3) could be reconstructed that suggests that the duplication of an ancestral FN3K-RP gene leading to mammalian and bird FN3Ks and FN3K-RPs may have occurred after the fish speciation. That the branches leading to FN3K (most particularly to the mammalian enzymes, see “Discussion”) are longer than those of FN3K-RPs suggests that the rate of evolution was more rapid in the first case than in the latter case. The alignment suggests also that the following residues may be FN3K-specific: Phe-17, Ala-30, Gln-47, Arg-69, Tyr-113, Asn-126, Pro-165, His-171, Asp-183, Val-198, Asn-284, Pro-299, Leu-308. Intriguingly, the degree of identity of the last 60 residues of protein-bound fructosamines and ribulosamines was 25–35% sequence identity with FN3K/FN3K-RP are also
This procedure resulted in an FN3K rose on which FN3K is retained and from which it can be eluted. This was done by fractionating tissue extracts on Blue-Sepharose and substrates that compete for the added ATP. It was therefore necessary to partially purify FN3K before its assay. These proteins are at present unknown. They have therefore been excluded from the present analysis, which mainly focuses on the evolution of FN3K and FN3K-RP in vertebrates.

**Development of Assays of FN3K and FN3K-RP**—FN3K can be assayed in partially purified preparations through the phosphorylation of $[^{14}C]$DMF to $[^{14}C]$DMF-3-phosphate (2). Because the enzyme has a very low activity, this assay cannot be applied to unfractionated tissue extracts, due to the presence of enzymes and substrates that compete for the added ATP. It was therefore necessary to partially purify FN3K before its assay. This was done by fractionating tissue extracts on Blue-Sepharose on which FN3K is retained and from which it can be eluted with 0.5 M NaCl in the presence of 1 mM ATP-Mg (not shown). This procedure resulted in an -10-fold purification of the enzyme. It was checked that the assay of the partially purified enzyme through the phosphorylation of DMF was proportional with time and the concentration of enzyme. It was also verified, for at least one tissue of each of the investigated species, that DMF inhibited the phosphorylation of DMF to $[^{14}C]$DMF-3-phosphate (2). Because a potential source of ribulosamines, the most likely physiological substrate of FN3K-RP (4), is ribose 5-phosphate, an intermediate of the pentose phosphate pathway. We therefore determined the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, the two rate-limiting enzymes of this pathway, to check if there were any correlation of the expression of the two enzymes with FN3K-RP. As shown in Fig. 6, both enzymes were present in all tissues but their activities were particularly low in skeletal muscle, where they represented only a small fraction of the mean activities found in other tissues. These low activities in skeletal muscle therefore parallel the presence of a spacer that links the dye to the matrix. Under our conditions (most particularly in the presence of EDTA, which improves the retention), more than 90% of FN3K-RP was retained on the column and could be eluted with NaCl without ATP-Mg, whatever the tissue or the species from which the extract was made (not shown).

**Tissue Distribution of FN3K, FN3K-RP, and Dehydrogenases of the Pentose Phosphate Pathway**—As shown in Fig. 4, the tissues or cells displaying the highest FN3K activity in mice and rats were red blood cells, brain, heart, kidney, and muscle. Lower activities were observed in liver, and no significant activity was detected in lung, spleen, testis, and thymus. A similar profile was observed in chicken tissues, except that the activity was negligible in erythrocytes. The finding that FN3K activity was low in erythrocytes from birds, where the glucose concentration is low (15), led us to study this activity in the erythrocytes from pig, a mammalian species in which the intraerythrocytic concentration of glucose is low (16). As shown in Fig. 5, FN3K was found to be also quite low in pig erythrocytes, whereas FN3K-RP activity was comparable with that observed in other mammals.

The distribution of FN3K-RP in rat and mouse tissues was found to be more even than that of FN3K, except for skeletal muscle, which displayed less than 20% of the mean activity of other rat or mouse tissues. A very low FN3K-RP activity was also found in skeletal muscle, smooth muscle, and red cells in chicken.

A potential source of ribulosamines, the most likely physiological substrate of FN3K-RP (4), is ribose 5-phosphate, an intermediate of the pentose phosphate pathway. We therefore determined the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, the two rate-limiting enzymes of this pathway, to check if there were any correlation of the expression of the two enzymes with FN3K-RP. As shown in Fig. 6, both enzymes were present in all tissues but their activities were particularly low in skeletal muscle, where they represented only a small fraction of the mean activities found in other tissues. These low activities in skeletal muscle therefore parallel

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**Fig. 1.** **Organization of the FN3K and FN3K-RP genes and surrounding genes in different genomes.** The following abbreviations are used: TBCD, tubulin-specific chaperone D; NDEL1, similar to Lis1-interacting protein Nudel, endooligopeptidase A; RAB40B, Ras-related protein Rab-40B; Rev. T., reverse transcriptase-like protein; ADP ribosyl, similar to ADP ribosylation factor-like 1.

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**Tissue Distribution and Evolution of FN3K and FN3K-RP**

- **A.** Homo sapiens X chromosome 17q25.3
- **B.** M. musculus X chromosome 11q
- **C.** Zebrafish X chromosome 3q
- **D.** Fugu rubripes

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**Legend:**

- Tissue Distribution and Evolution of FN3K and FN3K-RP
- Development of Assays of FN3K and FN3K-RP
- Tissue Distribution of FN3K, FN3K-RP, and Dehydrogenases of the Pentose Phosphate Pathway

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**References:**

1. Caenorhabditis elegans
2. Drosophila melanogaster
3. Saccharomyces cerevisiae
4. Allose
5. Ribulosamines
6. DMF
7. Psicosamines
8. Ribulose 5-phosphate
9. Glucose-6-phosphate dehydrogenase
10. ADP ribosylation factor-like 1
Characterization of the Osamine Kinase Present in Fish Tissues—As the BLAST searches indicated that a FN3K/FN3K-RP homolog was present in fish genomes, we fractionated different tissue extracts from the catfish *C. gariepinus* on Blue-Sepharose and checked for the presence of a ketoamine kinase. Fractions eluted with salt contained an enzyme that phosphorylated protein-bound psicosamines but not protein-bound fructosamines. The highest activities were observed in brain, testis, and liver (0.29, 0.36, and 0.15 milliunits/g protein, respectively), whereas other tissues contained activities at the detection limit (not shown). As shown in Fig. 7 for an enzymatic preparation derived from liver, the activity of the fish enzyme was not inhibited by DMF (up to 10 mM) or fructoselysine, but rather by micromolar concentrations of DMR and ribuloselysine. Furthermore, the enzyme phosphorylated [14C]ribulose-lysine but not radiolabeled DMF, indicating that its properties are similar to those of FN3K-RP (not shown).

Effect of Diabetes—As FN3K repairs damage induced by glucose, it was of interest to test the effect of starvation and diabetes on the level of expression of the enzyme. Rats were starved for 2 days or rendered diabetic with streptozotocin and analyzed 5 days later. No detectable change in the activity of FN3K was observed in liver, heart, kidney, and brain (not shown). We also determined the level of FN3K and FN3K-RP mRNA by quantitative PCR. No difference was observed between the two experimental conditions and the control for liver, lung, heart, skeletal muscle, testis, and thymus (not shown).

DISCUSSION

Evolution of the FN3K Gene—We show in the present work that two distinct but related genes encoding orthologs of FN3K and FN3K-RP are present in the mammalian and chicken genomes, whereas there is only one FN3K/FN3K-RP homolog in the genomes of two fishes and of the urochordate *C. intestinalis*.

**Fig. 2.** Alignment of FN3K and FN3K-RP sequences. The following sequences are shown: human FN3K (FHs, gi:13959371) and FN3K-RP (RHs, gi:47606765); mouse FN3K (FMm, gi:13959370) and FN3K-RP (RMm, gi:47605894); chicken FN3K and FN3K-RP (FGg and RGg, respectively, contig accession number gi:44766232); FN3K-RP from *Danio rerio* (RDr, from emb AL935316.7), *Fugu rubripes* (RFr, SINFRUP-0000008981), and *Ciona intestinalis* (RCi, from Scaffold_198; 67907-69392). Putative FN3K-specific residues are indicated above the alignment. Underlined residues at the end of the chicken FN3K sequence are identical to those found in the chicken FN3K-RP. The conserved HGD motif found also in aminoglycoside kinases is indicated by asterisks.
Accordingly, two different enzymes were found to be present in rat, mouse, and chicken tissues with similar substrate specificities as human FN3K and FN3K-RP, respectively. The properties of the enzyme found in the tissues of a fish (C. gariepinus) were similar to those of FN3K-RP, consistent with the higher degree of similarity of the fish sequences with mammalian FN3K-RP than with FN3K. We speculate that the enzyme of C. intestinalis has also ribulosamine and psicosamine kinase activity, but no fructosamine kinase activity.

These experimental findings together with our phylogenetic analysis suggest that the FN3K gene may have arisen by an event of gene duplication of the ancestral FN3K-RP gene, fol-
The activities of FN3K and FN3K-RP have been determined after partial purification of the enzymes using DMF (FN3K) or lysozyme-bound psicosamines (FN3K-RP) as substrates. They are related to the amount of hemoglobin in the extract. Mean of 2 values.

followed by an increased mutation rate of the gene copy that has resulted in the appearance of the present-day FN3K gene. The absence of FN3K from fishes suggests that this event may have taken place just after the time of separation of the fishes from the main line of animal evolution. The precise time of the event cannot be deduced from the tree, because the branching order in this part of the tree does not receive high bootstrap support. Therefore a gene duplication before the fish-animal separation followed by the subsequent loss of the FN3K gene from fish cannot be excluded, but this explanation is not favored because it would be a less parsimonious one. The incorporation of an additional number of mutations (see Fig. 2) resulting in longer branch lengths for the members of the newly arisen FN3K clade would be the explanation for FN3K's wider substrate specificity and subsequent new function (see below). Although the branch leading to chicken FN3K is not as long as those leading to its mammalian orthologs, this may be the result of a gene conversion event in which part of the FN3K sequence has been replaced by the FN3K-RP sequence, as indicated by the high degree of identity of the last 60 amino acid residues of chicken FN3K with those of the corresponding FN3K-RP.

The evolution of the FN3K/FN3K-RP genes appears, therefore, to conform with the hypothesis of Ohno (17) that after duplication of a gene one copy preserves the original function, whereas the other copy would be free to diverge. This behavior is, however, far from being the rule, as most paralogs evolve at similar rates (18). A recent study on the evolution of the S. cerevisiae genome (19) indicates that it underwent a global duplication event followed by the loss of most of the duplicated genes, with only about 457 gene pairs persisting in the present day genome. Among these, accelerated evolution (defined as a >1.5-fold higher rate of amino acid substitutions) of one copy compared with the other was observed in 115 pairs, i.e. ~25%.

In many cases, this accelerated evolution was accompanied by the emergence of a new function, as is indeed the case for FN3K compared with FN3K-RP.

FN3K-specific Residues—Enzymes, most particularly dehydrogenases but also phosphotransferases (e.g. galactokinase (20) and α- and β-phosphoglucomutases, (21)) usually show a high degree of stereospecificity. It is therefore intriguing that the same enzyme (FN3K) can catalyze the phosphorylation of a hydroxyl group either in the D orientation (psicosamines, ribulosamines) or in the L orientation (fructosamines), whereas the related enzyme FN3K-RP appears to be specific for the D orientation. What appears to be critical for substrates of FN3K is (a) the presence of an amino group on carbon 1, as indicated by the fact that the Km is more than 1000-fold higher for fructose than for DMF and fructoselysine (2), and (b) a keto group or an hemiacetal group on carbon 2, as suggested by the fact that reduction of fructoselysine with borohydride decreases the catalytic efficiency by at least 20-fold.2 It is therefore likely that, with all types of ketoamine substrates, carbon 1–3 of the osamine moiety assumes the same position in the catalytic site of FN3K. As this is most likely also the case for the oxygen that has to be phosphorylated on C3, the accommodation of compounds with an L- or D configuration in C3 is most likely achieved by allowing two different types of orientation for C4-C6 (or C4-C5). Comparison of the mammalian and bird

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2 F. Collard and E. Van Schaftingen, unpublished results.
FN3K sequences with other vertebrate sequences allows one to pinpoint a number of residues that are conserved among FN3Ks, but not in FN3K-RPs. FN3K and FN3K-RPs are distantly related to aminoglycoside kinases, sharing about 15% sequence identity. Three-dimensional structure prediction using 3D-PSSM (22) (available at www.sbg.bio.ic.ac.uk) and the structure of Enterococcus faecalis aminoglycoside kinase (1J7U; Ref. 23) indicated that several of the potential FN3K-specific residues cluster in helices or loops that are near the putative catalytic pocket. These residues are Gln-47 (replaced by Glu in FN3K-RP), His-171 (replaced by Gln or Asn in FN3K-RPs), Asp-183 (replaced by other polar residues in FN3K-RPs), and Asn-284 (replaced by His in FN3K-RP). They align with Tyr-50, Asp-137, Asp-167, and Tyr-262, respectively, in the aminoglycoside kinase sequence. We hypothesize that the substitution of these residues for other amino acids with different size or charge is responsible for the change in specificity, most particularly because they are polar residues that are likely to form hydrogen bonds with hydroxyl groups of ketoamines.

**Tissue and Species Distribution—**The tissular distribution of the enzyme correlates well with the amount of mRNA for both FN3K and FN3K-RP (4) with the exception of spleen, where the activity of FN3K appears to be low compared with the mRNA level, and muscle, where the reciprocal observation was made. If FN3K and FN3K-RP are involved in protein repair mechanisms, they are expected to be most active in tissues such as erythrocytes, brain, and skeletal muscle where proteins are poorly or not renewed and which contain the “offending” agent. As for fructosamine 3-kinase, which has the unique property of phosphorylating fructosamines, the important offending agent is glucose. Remarkably, the activity of FN3K is elevated in erythrocytes from rat, mouse, and man, where the intracellular concentration of glucose is close to that of the plasma, but it is low in erythrocytes from chicken and pig, where the glucose concentration is low or near zero (15, 16). This correlation indicates that FN3K is dispensable in erythrocytes if glucose is absent. Thus there was no longer any selective pressure to maintain the expression of this enzyme in erythrocyte precursors during evolution in species in which glucose transport across the erythrocyte membrane became negligible.

It may seem quite intriguing that starvation and, most particularly, diabetes does not change the level of expression of FN3K in the investigated rat tissues. This is consistent with the finding that FN3K activity is not different between controls and patients with type I diabetes.3 One has to remember that what is most likely rate-limiting for the deglycation reaction is not the phosphorylation of fructosamines by FN3K but the spontaneous reaction that follows and takes place with a half-life of about 8 h. Calculations indicate that the phosphorylation capacity of FN3K in erythrocytes represents more than twice the glycation rate occurring at 200 mM glucose. Thus, an increase in the expression of FN3K in diabetes represents more than twice the glycation rate occurring at 200 mM glucose. Thus, an increase in the expression of FN3K in diabetes, where the glucose concentration is low or near zero (15, 16), indicates that FN3K is dispensable in erythrocytes if glucose is absent. Thus there was no longer any selective pressure to maintain the expression of this enzyme in erythrocyte precursors during evolution in species in which glucose transport across the erythrocyte membrane became negligible.

The role of FN3K-RP is puzzling as the nature of its physiological substrate is not known. Previous investigations have shown that it does phosphorylate ribulosamines with an 40-fold higher affinity than psicosamines. The fish enzyme appears to have similar kinetic properties, indicating that the function of the FN3K/FN3K-RP ancestor was most likely to phosphorylate ribulosamines. As there does not seem to be free ribose in mammalian tissues (most of which contain ribokinase, an enzyme displaying a $K_m$ of about 30 $\mu M$ for ribose value for the recombinant human enzyme),4 the source of ribulosamines must be different. One potential source is ribose 5-phosphate, derived from the pentose phosphate pathway and known to be an extremely potent glycating agent (24). Assay of the latter in tissues is difficult for several reasons. One of these is that phosphoribosyl-pyrophosphate spontaneously hydrolizes in acid (25) so that assays of ribose 5-phosphate in perchloric acid extracts measure the sum of ribose-5-phosphate and phosphoribosyl-pyrophosphate. Another reason is that anoxia leads in a few seconds to the degradation of adenine nucleotides and their conversion to inosine, ribose 1-phosphate, and ribose-5-phosphate. Thus, it is extremely difficult to sample tissues while completely avoiding an artificial increase in the formation of ribose-5-phosphate. Because the most important source of ribose 5-phosphate under normal (non-anoxic) conditions is most likely the pentose phosphate pathway, we have assayed its two rate-limiting enzymes. Remarkably, in both cases the lowest activity was observed in skeletal muscle, the tissue in which FN3K-RP is the least active. Although this is not absolute proof that this is the tissue where the concentration of free ribose 5-phosphate is the lowest, it is at least a strong indication for this and, therefore, indirect evidence for FN3K-RP playing a role in the deglycation of ribulosamine 5-phosphate. Because ribulosamine 5-phosphates are not substrates for FN3K-RP, we postulate that an as yet unknown phosphatase removes the phosphate bound to carbon 5, allowing FN3K-RP to phosphorylate the third carbon.

Another potential source of ribulosamines is free ADP-ribose, a potent glycating agent that reacts with amines to form ADP-ribulosamines (26). It would be most interesting to correlate the tissue concentration of this compound with the distribution of FN3K-RP. However, the assay of ADP-ribose in tissues is made difficult by the lability of NAD1 and NADH in acid or alkaline extracts (27). Furthermore, the diversity of its sources (hydrolysis of NAD, poly(ADP-ribose), cyclic ADP-ribose and other mono-ADP-ribose derivatives) makes it difficult to draw meaningful correlations between FN3K-RP and ADP-ribose-producing enzymes. The low concentration of ADP-ribose (~0.5 $\mu M$) reported to be present in erythrocytes (28) suggests, however, that in this cell type, known to have quite an active pentose phosphate pathway, ribose 5-phosphate is more likely to be a precursor for ribulosamines than ADP-ribose.

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Tissue Distribution and Evolution of Fructosamine 3-Kinase and Fructosamine 3-Kinase-related Protein

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