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FEBS Letters 582 (2008) 3330–3334

Characterization of mammalian sedoheptulokinase and mechanism of formation of erythritol in sedoheptulokinase deficiency

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Received 9 July 2008; revised 6 August 2008; accepted 22 August 2008

Available online 5 September 2008

Edited by Judit Ovádi

Abstract **Our aim was to identify the product formed by sedoheptulokinase and to understand the mechanism of formation of erythritol in patients with sedoheptulokinase deficiency. Mouse recombinant sedoheptulokinase was found to be virtually specific for sedoheptulose and its reaction product was identified as sedoheptulose 7-phosphate. Assays of sedoheptulose in plant extracts disclosed that this sugar is present in carrots (≈7 μmol/g) and in several fruits. Sedoheptulose 1-phosphate is shown to be a substrate for aldolase B, which cleaves it to dihydroxyacetone-phosphate and erythrose. This suggests that, in patients deficient in sedoheptulose-7-kinase, sedoheptulose is phosphorylated by fructokinase to sedoheptulose 1-phosphate. Cleavage of the latter by aldolase B would lead to the formation of erythrose, which would then be reduced to erythritol.**
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Keywords: Sedoheptulose; Heptose; Cystinosis; Sugar kinase; Pentose-phosphate pathway; Volemitol

1. Introduction

Recent analysis of cystinosis patients with a 57 kbp deletion inactivating the gene encoding the lysosomal cystine transporter (CTNS), as well as a contiguous gene (CARKL, now renamed SHPK) encoding a putative carbohydrate kinase, led to the finding that these patients have abnormally high amounts of sedoheptulose and erythritol in urine [1]. This abnormal excretion is not found in cystinosis patients who are heterozygous for this deletion. Extracts of control fibroblasts were shown to display sedoheptulokinase activity and this activity was markedly reduced in fibroblasts obtained from patients with a homozygous deletion of the CARKL gene. These findings led to the conclusion that sedoheptulosuria and erythritoluria both result from a deficiency of a new enzyme, sedoheptulokinase, encoded by the CARKL gene. The product of the reaction catalyzed by sedoheptulokinase was proposed to be sedoheptulose 7-phosphate on the basis of its coelution with authentic sedoheptulose 7-phosphate in HPLC. However, the possibility that sedoheptulokinase phosphorylates sedoheptulose on its 1st carbon was not excluded. Earlier work has indeed indicated that liver contains a sedoheptulokinase that converts sedoheptulose to sedoheptulose 1-phosphate [2]. Furthermore, the mechanism leading to abnormal erythritol excretion in sedoheptulokinase deficiency is not elucidated. The purpose of this work was to identify the product of the reaction catalyzed by sedoheptulokinase and to elucidate the mechanism of formation of erythritol.

2. Materials and methods

2.1. Materials

2,7-Sedoheptulosan (except if otherwise indicated, all sugars and polyols mentioned in this work are of the D-series), transketolase, altrose, mannoheptulose, glyceraldehyde 3-phosphate, and auxiliary enzymes were from Sigma (St. Louis, Mo). Other chemicals were from Merck. 2,7-Sedoheptulosan (200 mM) was heated in the presence of 0.05% perchloric acid for 2 h at 100 °C. This resulted in the formation of ≈25 mM sedoheptulose, as checked with sedoheptulokinase. More prolonged incubations did not lead to a higher conversion of sedoheptulosan to sedoheptulose, most likely because the thermodynamic equilibrium of the hydrolysis of sedoheptulosan is in favor of the anhydride [3]. The identity of the compound as sedoheptulose was confirmed by the finding that it was converted by sedoheptulokinase to a substrate for transketolase (see Section 3). Ribulose-5-phosphate reductase was prepared as previously described [4]. Molecular biology enzymes were from Fermentas (St. Leon Rot, Germany).

2.2. Overexpression and purification of mouse sedoheptulokinase

The open-reading-frame of mouse SHPK (GenBank™ accession number NP_083307) was PCR-amplified using Phusion DNA polymerase and mouse liver cDNA as a template. A 5′ primer containing the initiator codon (CACT**CATATG**GCTTCGCGACCTGTCACTC) in a NdeI site (in bold) and a 3′-primer containing the putative stop codon (GAGC**CTCGAG**AACTAAGGCTCCTTCTGGCTA) flanked by an XhoI site (in bold) were used. The 1450-bp PCR-product was digested with NdeI and XhoI restriction enzymes, cloned in pBlueScript and checked by sequencing. A NdeI–XhoI fragment was removed from the pBlueScript plasmid and ligated in pET-15b expression vector [5]. This vector was used to transform *E. coli* BL21(DE3) pLysS. Protein expression and preparation of bacterial extracts were performed as described previously [6]. The polyHis-tagged protein was purified on HisTrap columns (Ni^{2+} form) [7]. It was eluted with 150 mM imidazole, as indicated by SDS–PAGE analysis, and desalted on PD-10 columns equilibrated with 25 mM HEPES, pH 7.4 and 25 mM KCl. With this procedure, 3.5 mg of pure sedoheptulokinase was obtained per litre of culture. Protein concentration was estimated by measuring A_{280} assuming an extinction coefficient of 1.18 $(mg/ml)^{-1}\ cm^{-1}$ for sedoheptulokinase.

2.3. Measurement of enzymatic activities

The enzymatic activities were assayed spectrophotometrically at 30 °C by monitoring the changes in A_{340}. Except if otherwise indicated, all assays were performed in 600 μl of *assay buffer* containing 25 mM HEPES, pH 7.4, 25 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol

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doi:10.1016/j.febslet.2008.08.024

and 0.1% bovine serum albumin. Sedoheptulokinase activity was determined in *assay buffer* containing 1 mM ATP-Mg, 0.15 mM NADH, 0.3 mM phosphoenolpyruvate, different concentrations of sedoheptulose, 10 μg/ml rabbit muscle pyruvate kinase, 5 μg/ml rabbit muscle lactate dehydrogenase, and 0.25 μg/ml sedoheptulokinase.

Aldolase was assayed through the production of dihydroxyacetonephosphate in a reaction mixture containing 25 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 0.15 mM NADH, rabbit muscle triose phosphate isomerase (3.5 U/ml) and glycerol-3-phosphate dehydrogenase (1 U/ml), the indicated concentrations of fructose 1,6-bisphosphate, fructose 1-phosphate or sedoheptulose 1-phosphate, and 0.01 mg/ml purified rat liver aldolase B.

2.4. Preparation of the phosphorylation product of sedoheptulokinase and of sedoheptulose 1-phosphate

Sedoheptulokinase (7 μg/ml) was incubated in *assay buffer* (5 ml final volume) containing 10 mM sedoheptulose and 10 mM ATP-Mg for 2 h at 37 °C. The incubation was arrested by heating at 80 °C for 10 min. After centrifugation, the supernatant was diluted 3-fold with water and applied onto a 15 ml AG1X8 column, which was eluted with a continuous 0–1 M NaCl gradient. The phosphate esters were measured through the formation of inorganic phosphate [8] after alkaline phosphatase treatment. A peak of monophosphate ester (≈20 μmol), corresponding to sedoheptulose-phosphate, was eluted from the column before the peaks of ADP and ATP. It was used to test whether the sedoheptulokinase product was a substrate for transketolase or aldolase B.

Sedoheptulose 1-phosphate was similarly prepared with partially purified liver fructokinase [9]. The phosphorylation product (≈12 μmol) was purified by anion exchange chromatography as described above. NaCl was removed by gel filtration on Sephadex G10 and the purified product was concentrated to ≈20 mM in a Speed-Vac.

2.5. Assays of sedoheptulose 7-phosphate and of sedoheptulose

Sedoheptulose 7-phosphate was assayed spectrophotometrically, at a wavelength of 340 nm, by following the formation of ribose 5-phosphate in *assay buffer* containing 0.1 mM CTP-Mg, 0.15 mM NADPH, 0.1 mM thiamine-pyrophosphate, 0.5 mM glyceraldehyde 3-phosphate, 0.05 U/ml yeast transketolase, 1.7 U/ml recombinant ribose-5-phosphate isomerase from spinach, and 0.3 U/ml recombinant *Haemophilus influenzae* ribulose-5-phosphate reductase [4].

Sedoheptulose was assayed in neutralized perchloric acid extracts of plant tissues as the ADP formed in the presence of sedoheptulokinase in the same assay mixture as for sedoheptulokinase (see above). A_{340} was measured 5 min after all assay constituents except sedoheptulokinase had been added. Sedoheptulokinase (2.5 μg/ml) was then added to initiate the reaction, which was allowed to proceed until A_{340} stabilized. The concentration of sedoheptulose was computed from the change in A_{340}.

Fruit and vegetables were homogenized in a Waring Blendor with 2 vol of water. The resulting extracts were centrifuged for 5 min at 1000 × g and a portion of the supernatant was mixed with perchloric acid (2% final concentration). Freshly pressed orange or grapefruit juice was mixed with perchloric acid (2% final concentration). The resulting perchloric acid extracts were centrifuged for 5 min at 15000 × *g*, neutralized with K_2CO_3 and re-centrifuged. The concentration of sedoheptulose was determined with purified sedoheptulokinase in the resulting supernatant.

2.6. Action of liver aldolase on sedoheptulose 1-phosphate

For the purification of liver aldolase, a rat liver extract (25 ml) prepared in 4 vol of 25 mM HEPES, pH 7.1, 50 mM KCl, 5 μg/ml leupeptin and 5 μg/ml antipain was centrifuged and the resulting supernatant was applied onto a 25 ml DEAE-Sepharose column. The flow-through fractions (25 ml) were pooled and applied onto a 20-ml SP-Sepharose column equilibrated in HEPES 25 mM, pH 7.1. The column was washed with the same buffer and aldolase was eluted with a NaCl gradient (0–500 mM) in 100 ml of the same buffer. Aldolase was purified about 40-fold in this way to a specific activity of 10 μmol/min/mg protein as determined with fructose 1,6-bisphosphate as a substrate.

For the characterization of the products formed from sedoheptulose 1-phosphate, the aldolase preparation (0.37 mg/ml) was incubated with 2 mM sedoheptulose 1-phosphate for 150 min in the presence of 3 mM HEPES, pH 7.1, and the reaction was stopped by heating for 5 min at 80 °C. Protein was eliminated by centrifugation and the resulting supernatant was analyzed by mass spectrometry. All mass spectral analyses were performed on a LCQ Deca XP ion-trap spectrometer equipped with an electrospray source (ThermoFinnigan, San Jose, CA). The sample dissolved in methanol was introduced directly into the source at a flow rate of 4 μl/min. The LCQ was operated in positive mode under manual control in the Tune Plus view with default parameters and active Automatic Gain Control. MS/MS analysis was done to confirm the structure of the precursor ions using low energy collision-induced dissociation with a relative collision energy of 25%.

3. Results

3.1. Characterization of recombinant mouse sedoheptulokinase

We prepared an expression vector allowing the production of mouse sedoheptulokinase as a N-terminal fusion protein with a polyHis tag. The protein was expressed in *Escherichia coli* at 16 °C for 19 h in the presence of the inducer isopropyl-thiogalactoside. Extracts were prepared and the protein was purified to homogeneity (not shown) by metal affinity chromatography.

We determined its kinase activity using a spectrophotometric assay in which ADP formation was followed. Fig. 1A shows that the enzyme was active with sedoheptulose as a substrate. The enzyme displayed a K_M of 190 ± 15 μM for sedoheptulose and a V_{max} of 128 ± 3 μmol/min/mg protein, corresponding to a k_{cat} of ≈115 s^{-1}. Sedoheptulokinase did not show any activity on 2,7-sedoheptulosan (tested at 50 mM), and was only weakly active on two structural analogs of sedoheptulose, namely altrose (analog of C2–C7) and mannoheptulose (C4 epimer) (Fig. 1B), for which we calculated catalytic efficiencies of 2.2 and 1.4 s^{-1} M^{-1}, respectively, as compared to 6.0 ×

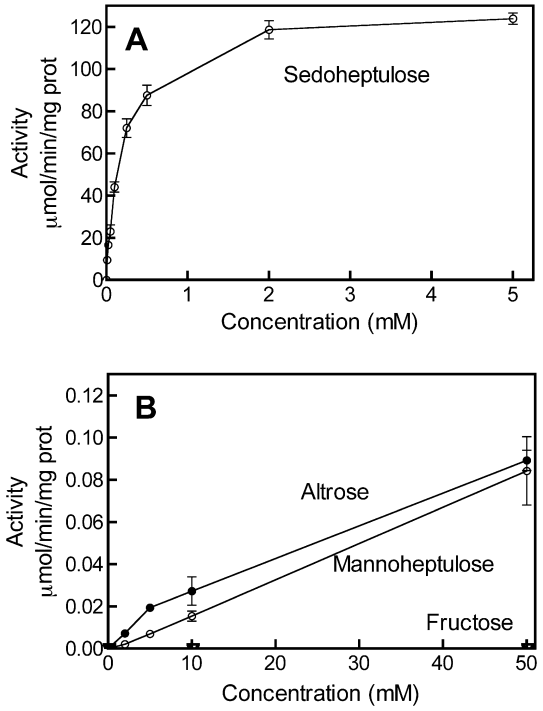

Fig. 1. Kinetic properties of sedoheptulokinase. The activity of purified mouse sedoheptulokinase was tested in the presence of the indicated concentrations of sedoheptulose (panel A), altrose, mannoheptulose and fructose (panel B).

10^5 s^{-1} M^{-1} for sedoheptulose. Sedoheptulokinase was inactive on fructose, glucose, galactose (tested at up to 50 mM), erythritol and D-erythrose (tested at up to 10 mM).

Sedoheptulose is most likely phosphorylated by sedoheptulokinase either on its 7th or on its 1st carbon. To determine which of these possibilities is true, we prepared the phosphorylation product, purified it by chromatography on anion-exchanger and titrated it through the release of inorganic phosphate by alkaline phosphatase. We then checked whether the phosphorylation product reacted with transketolase, using glyceraldehyde 3-phosphate as a co-substrate, and a combination of ribose-5-phosphate isomerase and ribulose-5-phosphate reductase to monitor the formation of ribose 5-phosphate. With this assay, we found that the sedoheptulose-phosphate produced by sedoheptulokinase was quantitatively converted to ribose 5-phosphate. We checked that no decrease in A_{340} was observed if one of the constituents of the assay (transketolase, thiamine-pyrophosphate, auxiliary enzymes, glyceraldehyde 3-phosphate) was omitted. These results indicated therefore that sedoheptulokinase phosphorylates its substrate on its 7th carbon.

3.2. Action of aldolase B on sedoheptulose 1-phosphate

Absence of sedoheptulokinase leads to the accumulation of sedoheptulose and erythritol [1]. The accumulation of the heptulose is the direct consequence of the lack of activity of sedoheptulokinase. Since this enzyme does not act on erythrose or erythritol (see above), the accumulation of erythritol must be the consequence of sedoheptulose accumulation. Previous studies have shown that this heptulose is a substrate for fructokinase (ketohexokinase), which phosphorylates it with a catalytic efficiency amounting to 6% of that observed with fructose [10]. We confirmed that sedoheptulose is a substrate for purified fructokinase (results not shown) and we prepared sedoheptulose 1-phosphate with this enzyme. Mass spectrometry analysis of the purified product indicated the presence of a positively charged ion of *m*/*z* 335, as expected for the (M−H+2Na) form of a heptose-monophosphate (not shown).

We checked if sedoheptulose 1-phosphate was a substrate for aldolase B by measuring the formation of dihydroxyacetone-phosphate. As shown in Fig. 2, liver aldolase displayed almost as much activity on sedoheptulose 1-phosphate as on fructose 1-phosphate. K_M values of 0.6 and 0.25 mM were calculated for sedoheptulose 1-phosphate and fructose 1-phosphate, respectively. V_{max} values were 0.4 and 0.5 μmol/min/mg protein, respectively.

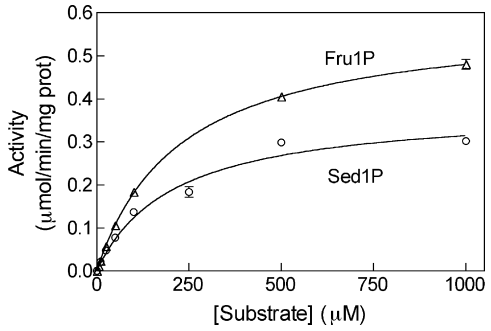

Fig. 2. Liver aldolase acts on sedoheptulose 1-phosphate. The activity of partially purified rat liver aldolase B was assayed through the formation of dihydroxyacetone-phosphate in the presence of the indicated concentrations of fructose 1-phosphate or sedoheptulose 1-phosphate. The results were fitted to the Michaelis–Menten equation.

Since sedoheptulose 1-phosphate is cleaved by aldolase with production of dihydroxyacetone-phosphate, the other product must be erythrose. Analysis by elecrospray mass spectrometry in positive ion mode of the reaction mixture obtained when 2 mM sedoheptulose 1-phosphate was incubated with liver aldolase indicated the presence of dihydroxyacetone-phosphate (*m*/*z* 215, corresponding to the M−H+2Na form), as well as of a product with *m*/*z* 143, corresponding to the (M+Na) form of a tetrose (not shown). Tandem mass spectrometry analysis of this ion disclosed that its main fragments had *m*/*z* values of 125 (loss of water), 113 (loss of C4) and 83 (loss of C3–C4), in full agreement with the fragmentation spectrum of commercial erythrose (not shown). Mass spectrometry indicated also that neither dihydroxyacetone-phosphate, nor erythrose were formed if aldolase had been omitted from the reaction mixture. These findings confirmed therefore that erythrose is the other product of sedoheptulose 1-phosphate cleavage by aldolase B.

We also checked that the product of phosphorylation of sedoheptulose by sedoheptulokinase was not split by aldolase B. This confirmed that this enzyme does not phosphorylate sedoheptulose on its first carbon.

3.3. Presence of sedoheptulose in vegetables and fruits

Sedoheptulokinase was used to assay sedoheptulose in neutralized perchloric acid extracts of a number of vegetables and fruits (Table 1). The heptose was found in several fruits at concentrations of the order of 1 μmol/g. This amount was variable, particularly in apples, possibly depending on the cultivar tested or on the degree of maturity, but this was not further explored. The heptose was most abundant in carrots, which contained about 6.7 μmol/g sedoheptulose. The other vegetables that we tested did not contain detectable amounts of this heptose.

4. Discussion

4.1. Identity of the product of sedoheptulokinase

The goal of the present work was to determine more precisely the function of the protein encoded by the CARKL/

Table 1
Concentration of sedoheptulose in fruits and vegetables

Species	Content (μmol/ml or μmol/g)
Apple	1.30, 0.97, 0.44, 0.20, <0.20 (3×)
Apricot	1.00, 0.83, 0.75
Banana	<0.20 (2×)
Carrots	5.5, 4.3, 10.9
Grapefruit (juice)	1.06, 0.73, <0.20
Lettuce	<0.20
Orange (juice)	2.31, 2.37, 0.87
Potato	<0.20 (2×)
Spinach leaves	<0.20
Tomato	1.18, 1.18, 0.73

The concentration was determined with purified sedoheptulokinase on neutralized perchloric acid extracts prepared from freshly pressed juice or from tissue extracts. Because of the wide variability from sample to sample in some cases, individual values are shown. (2×) and (3×) indicate that a value below the detection limit (0.2 μmol/ml or g) has been observed in 2 or 3 different specimens.

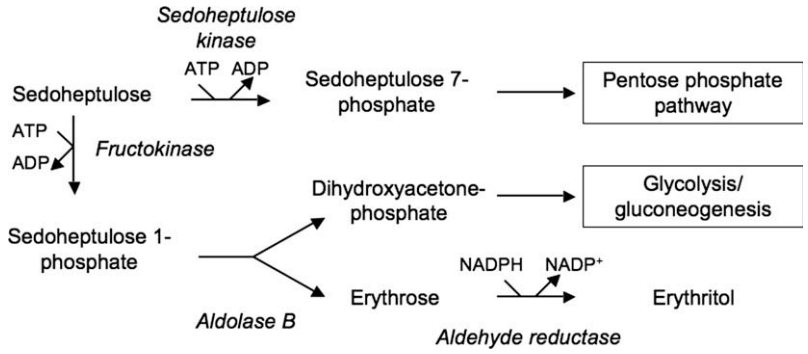

Fig. 3. Metabolism of sedoheptulose and its alteration in sedoheptulokinase deficient patients. Sedoheptulokinase normally converts sedoheptulose to sedoheptulose 7-phosphate, which is metabolized by enzymes of the pentose phosphate pathway. Based on the properties of fructokinase and aldolase B, we propose that, in the absence of sedoheptulokinase, sedoheptulose would be phosphorylated by fructokinase to sedoheptulose 1-phosphate, which would then be cleaved by aldolase B to dihydroxyacetone-phosphate and erythrose. The latter would be reduced to erythritol by aldehyde reductase. Both sedoheptulose and erythritol are excreted in urine.

SHPK gene. The protein was produced in *E. coli* and purified to homogeneity. We showed that it phosphorylates sedoheptulose on its seventh carbon and not on the first one. This conclusion is based on the finding that the phosphorylation product is a substrate for transketolase, but not for aldolase B. It agrees also with the finding that altrose, an analog of C2–C7 of sedoheptulose, is slowly but detectably phosphorylated by this enzyme, but that this is not the case for fructose, an analog of C1–C6. The catalytic efficiency of sedoheptulokinase was more than five orders of magnitude higher when sedoheptulose was used as a substrate than with any other sugar that we tested. This enzyme is therefore very specific and suitable to assay sedoheptulose.

The conclusion on the identity of the product formed by sedoheptulokinase differs from that of a previous work [2] showing that a sedoheptulokinase partially purified from liver phosphorylates sedoheptulose on its 1st carbon. This partially purified enzyme also phosphorylated fructose and L-sorbose, and we may therefore conclude that it corresponded to fructokinase. It is likely that the 'true' sedoheptulokinase was lost in the purification process used by Iwai and coworkers [2].

4.2. Mechanism of accumulation of erythritol

Sedoheptulokinase does not phosphorylate erythrose or erythritol. Therefore the accumulation of erythritol in patients with a deficiency in this enzyme must be indirect. The most likely explanation (Fig. 3) is that when sedoheptulose is not phosphorylated by sedoheptulokinase to sedoheptulose 7-phosphate, it accumulates to concentrations at which it would become a significant substrate for fructokinase [10]. The resulting sedoheptulose 1-phosphate would be converted by aldolase B to erythrose and dihydroxyacetone-phosphate. The only known fate for erythrose is to be reduced to erythritol by aldehyde reductase, a NADPH-dependent enzyme that uses D-erythrose as one of its best carbohydrate substrates [11].

It is reasonable to assume that most erythritol found in urine of patients with sedoheptulokinase deficiency is derived from sedoheptulose. From the median excretion of erythritol and sedoheptulose in these patients (0.32 mol/mol creatinine), we calculate, for a child weighing 30 kg, a sedoheptulose load of ≈1.4 mmol/day (assuming a creatinine excretion of 16.3 mg/kg/day). One hundred grams of carrots represent about 40% of that load and 100 ml of orange or grapefruit juice, about 10%. This suggests that food is the principal source for this sugar, but does not exclude the contribution of endogenous sources, e.g. the hydrolysis of cellular sedoheptulose 7-phosphate by cytosolic phosphatases.

4.3. Role of sedoheptulose in carrots

Our results indicate that sedoheptulose is usually a minor sugar in plants. Its concentration in carrots (≈0.14 g % fresh weight), the richest source that we identified, is much lower than those of fructose, glucose and sucrose (0.92, 1.22 and 2.83 g% fresh weight, respectively, according to Rodriguez-Sevilla et al. [12]). The role of this heptulose is presently unknown. It is tempting to relate its presence in carrots with that of (−)-daucic acid, a seven-carbon sugar dicarboxylic acid, which was initially identified in this vegetable [13]. However, the D-*lyxo* configuration of (−)-daucic acid argues against this possibility, as do also tracer studies with potential precursors of the related compound, chelidonic acid [14]. Sedoheptulose is a precursor for the polyol volemitol in polyanthus [15]. It would be interesting to know if carrots also contain this heptitol.

Acknowledgements: We thank Delphine Lebbe for help in the preparation of the manuscript. This work was supported by the Interuniversity Attraction Pole Programme – Belgian Science Policy (Network P6/05), the Fonds National de la Recherche Scientifique and the Actions de Recherche Concertées of the French Community of Belgium and by a generous grant of ASCO industries. T.K. was supported successively by the FNRS and the Marguerite-Marie Delacroix foundation

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