A Biomimetic Biotechnological Process for Converting Starch to Fructose: Thermodynamic and Evolutionary Considerations in Applied Enzymology

Aydin Moradian and Steven A. Benner*

Contribution from the Laboratory for Organic Chemistry, Swiss Federal Institute of Technology, 8092 Zürich, Switzerland. *Received January 21, 1992*

Abstract: A process for preparing fructose from starch has been designed to have a thermodynamic profile similar to those found in natural metabolic pathways and implemented in a reactor containing five enzymes acting together. The process runs at equilibrium, with a final exergonic step pulling intermediates to fructose, the desired product. Therefore, the yields of fructose are high and not dominated by the glucose–fructose equilibrium constant that constrains the commercial process, which uses xylose isomerase to catalyze its final step. Three different strategies were used to find enzymes suitable for catalyzing the final irreversible step, the hydrolysis of fructose-6-phosphate: (a) recruiting an enzyme to operate backwards with respect to its physiological function; (b) recruiting an enzyme to accept a non-natural substrate through the use of a cosubstrate; and (c) developing an indirect route for converting fructose-6-phosphate to fructose. As presently implemented, the process converts starch and inorganic phosphate to glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, and then fructose and inorganic phosphate; the last is recycled. The net hydrolysis of fructose-6-phosphate to yield fructose is obtained via a transaldolase-catalyzed reaction between fructose-6-phosphate and glyceraldehyde to yield fructose and glyceraldehyde-3-phosphate, which is then hydrolyzed to regenerate glyceraldehyde and inorganic phosphate using a 3-phosphoglycerate phosphatase recruited to act on an unnatural substrate. This work illustrates general ideas that may prove useful in designing other multistep biocatalytic transformations, in particular, the focus on the energetics of the pathway and on the evolution of enzymes as a guide to selecting enzymes useful in biocatalytic processes.

Introduction

Of the many features that distinguish enzymic catalysts used by Nature from catalysts used in traditional chemical processes, two are particularly important to the design of natural biochemical pathways. First, most enzymes catalyze very specific transformations on their substrates. Second, except for those catalyzing regulated steps or the last step in a reaction sequence, enzymes important in metabolism generally catalyze freely reversible reactions.

Together, these features allow natural biochemical pathways to be more efficient than multistep synthetic sequences not involving enzymes. Because most of the steps in a natural biochemical pathway have equilibrium constants near unity, the starting materials and reagents need not be extremely reactive. Further, an enzyme-based reaction sequence can be extended indefinitely with essentially no extra energetic cost, allowing major rearrangement of the atoms in a starting material in one pot to yield a product with an entirely different structure. Because the final step in a biosynthetic pathway is exergonic, the yields are close to quantitative. The high specificity of natural enzymes allows such pathways to proceed without the chemical chaos that would result should the enzymes catalyze even small amounts of undesired side reactions.

Of course, cause and effect can be viewed conversely. The need to live in water effectively precludes highly reactive reagents in living systems. Without such reagents, multistep reaction sequences *must* run largely at equilibrium. Reaction sequences operating at equilibrium require in turn catalysts with extremely high specificity, as the conditions of a dynamic equilibrium give each catalyst several opportunities to make a "mistake" with each intermediate molecule. Under these conditions, even the most specific of conventional "chemical" reagents[1] would not be adequate. Not surprisingly, natural enzymes operating in pathways close to equilibrium have measured reaction specificities that are 5–10 orders of magnitude higher than even the best nonenzymatic catalysts.[2]

Many enzymes used in contemporary biotechnology are atypical in these respects, in that they have *broad* substrate specificity and catalyze *irreversible* reactions. This is because to date biotechnologists have largely worked to find single enzymes that catalyze isolated chemical reactions. Nevertheless, biotechnology can make the greatest impact on the economics of chemical production only if biocatalytic processes exploit typical enzymes and mimic natural metabolic pathways, especially if the biocatalysts used can be chosen from anywhere in Nature so that the process design is not limited to a particular package of enzymes that evolution has delivered in a particular organism.[3] In such processes, as with classical fermentation processes, the production costs would be largely independent of the length of the chemical reaction sequence. Avoiding a strong correlation between the cost of a product and the number of steps required for its synthesis should, in turn, allow the biotechnological engineer to focus on the selection of inexpensive starting materials and products with the optimal properties, rather than those that can be synthesized in the smallest number of steps. Should this vision be realized, the impact of biocatalysts on the manufacture and use of chemicals could be profound.

Some progress has been made in designing fermentations that work inside living cells.[4] Less work has been devoted to developing multienzyme reactions in vitro. Multienzyme reactions are used routinely in coupled enzyme assays and, occasionally, in the synthesis of research chemicals (e.g., 2-(*R*)- and 2-(*S*)-2-[^{2}H,^{3}H]acetate).[5] However, several difficulties complicate efforts to develop multistep processes with isolated enzymes designed to work in vitro. First, incompatibilities between enzymes that must work together in a single reactor are often encountered, especially for enzymes drawn from different biological sources. Second, it is not clear how easily enzymes can be found in Nature to effect unusual transformations, those that are not found in a standard biological pathway. Thus it is important to learn how generally

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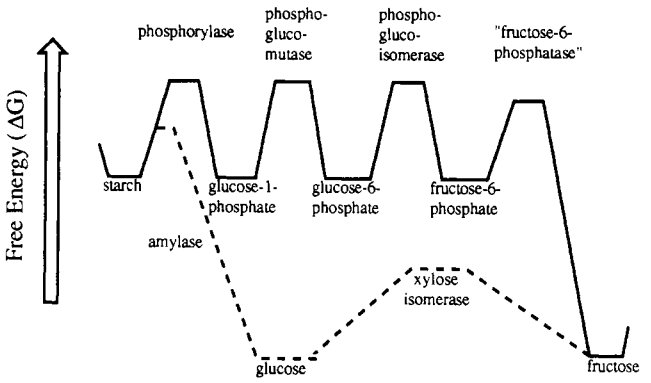

Figure 1. Energetics of the biotechnological process used commercially at present for producing fructose from starch (dotted line) and the process developed here, which is designed to have a free energy profile resembling that of a natural biochemical pathway (solid line).

biological catalysts can be recruited to perform a role different from the role that they have evolved to perform.

It is unlikely that general solutions exist for problems constructing multistep enzymatic pathways. Rather, progress will come only by tackling individual synthetic problems and resolving the difficulties that arise while implementing specific processes that prepare specific target molecules. Through accumulated experience, the design and implementation of multistep processes will, one hopes, become easier.

The goal of the work reported here was to develop a multienzyme process for the conversion of starch to fructose. The goal was a process that runs at equilibrium (except for the final step), provides high yields of fructose not dominated by the glucose–fructose equilibrium found in commercial processes dependent on xylose isomerase,[6] and does not involve the isolation of any intermediates. The goal was chosen for two reasons. First, fructose is one of the most important products presently manufactured with use of biotechnology.[6] The two-step commercial process uses amylase to hydrolyze starch to glucose as a first step and xylose isomerase to convert glucose to fructose in the second. Noteworthy is the fact that the thermodynamic design of the commercial process is *opposite* to the thermodynamic design of a natural biochemical pathway (Figure 1). The first step is *exergonic* in water; the hydrolysis of starch goes largely to completion. The second step is *isoenergetic*; the ratio of fructose to glucose at equilibrium is approximately 45:55. Of course, a natural biological pathway would have the opposite thermodynamic profile, with an exergonic step *last* so that the yield of product would be close to quantitative.

The suboptimal thermodynamics of the commercial process create economic problems. For acidic soft drinks,[6] high fructose corn syrup (HFCS) is desired that has the same sweetness as sucrose on a weight basis. This requires a higher level of fructose (55%) than is produced directly by the commercial process, which yields a syrup containing typically 42% fructose, 51% glucose, and 7% oligosaccharides remaining from incomplete breakdown of the starch.[7,8] Many ingenious strategies have been designed to obtain this higher percentage of fructose in the product stream, including running the process at very high temperatures, where the glucose–fructose equilibrium constant is shifted toward fructose,[9] and running the isomerization reaction in ethanol, where the equilibrium is also shifted.[10] Commercially, however, a chromatographic enrichment of fructose is used, a process that is expensive and time-consuming.[6-8]

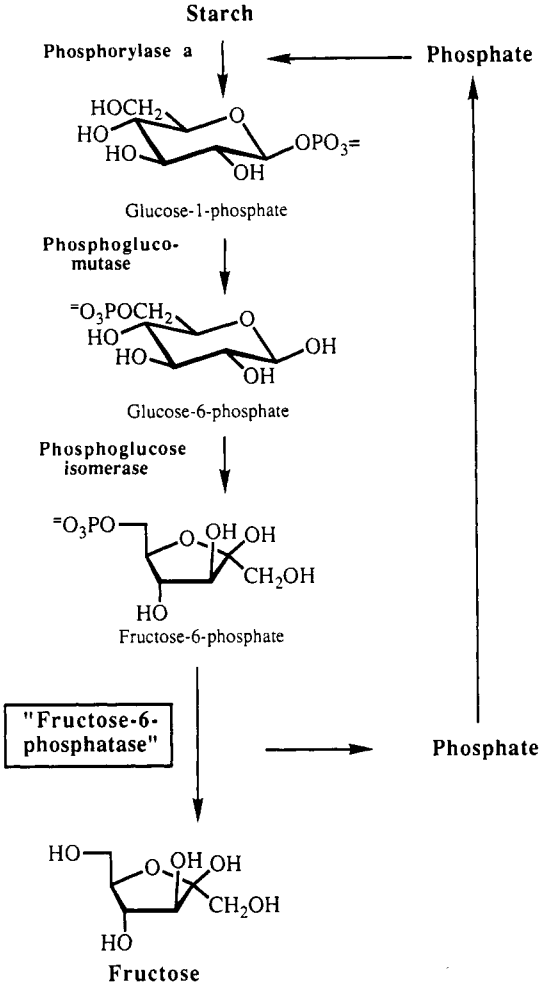

Figure 2. Outline of the multistep process for converting starch into fructose in very high yield. The energy present in the glycosidic bond in starch is stored as a high-energy phosphate bond (see Figure 1) until the oxidation state of the carbon chain is appropriately adjusted. The key to the process then is a selective enzymatic process (a "fructose-6-phosphatase") that converts the correct phosphorylated intermediate to the desired product (fructose) in an exergonic step that pulls all of the intermediates to products.

The process developed here has a thermodynamic profile similar to those of natural biosynthetic pathways. The thermodynamics of the process are outlined in Figure 1, and a general outline of chemistry is given in Figure 2. In this process, the energy in the glycosidic bond in starch is stored as a phosphate ester while the oxidation state of the carbon skeleton is adjusted. The energy is then released in a final exergonic step by hydrolysis of fructose-6-phosphate to yield fructose. The first steps are part of the well-known glycolytic pathway from starch. However, a search of natural sources did not yield an enzyme that catalyzes physiologically the final exergonic step, the selective hydrolysis of fructose-6-phosphate. Therefore, enzymic catalysts for this step were sought by (a) recruiting an enzyme to operate in the process in a direction opposite to its physiological direction, (b) recruiting an enzyme to accept a non-natural substrate through the use of cosubstrates, and (c) developing an indirect route for obtaining the net hydrolysis of fructose-6-phosphate. The first two approaches were successful to a degree; the last was sufficiently successful to sustain a small-scale continuous process. Each approach has some precedent in the literature (vide infra). However, we believe that the combination presented here illustrates

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many general aspects of the problems associated with the design of a process involving multiple enzymes operating in vitro. Especially interesting is how the course of the research was influenced by views of how evolutionary forces influence the behavior of enzymes.

Experimental Section

Sources of Materials. The following enzymes were obtained from Boehringer-Mannheim and used without further purification: glycerol kinase (2.7.1.30, *Candida mycoderma*, 85 U/mg), glucose-6-phosphate dehydrogenase (1.1.1.49, *Leuconostoc mesenteroides*, 550 U/mg), glucose oxidase (1.1.3.4, *Aspergillus niger*, 40 U/mg), peroxidase (1.11.1.7, horse radish, 250 U/mg), phosphoglucose isomerase (5.3.1.9, *Saccharomyces cerevisiae*, 350 U/mg), pyruvate kinase (2.7.1.40, rabbit muscle, 200 U/mg), and lactate dehydrogenase (1.1.1.27, rabbit muscle, 550 U/mg). The following enzymes were obtained from Sigma and used without further purification: ATPase (3.6.1.3, rabbit kidney, 1 U/mg), 5′-nucleotidase (3.1.3.5, *Crotalus adamantus*, 500 U/mg), phosphorylase a (2.4.1.1, rabbit muscle, 25 U/mg), phosphoglucomutase (5.4.2.2, rabbit muscle, 300 U/mg), fructose dehydrogenase (1.1.99.11, *Gluconobacter industrius*, 20 U/mg), transaldolase (2.2.1.2, *Candida utilis*, 20 U/mg), sorbitol dehydrogenase (1.1.1.14, sheep liver, 40 U/mg), lysozyme (3.2.1.17, egg white, 50,000 U/mg), DNase I (3.1.21.1, bovine pancreas, 2000 U/mg), and phosphofructokinase (2.7.1.11, rabbit muscle, 200 U/mg).

The following enzymes were purified for this work (vide infra): 3-phosphoglycerate phosphatase (3.1.3.38, from spinach), fructose kinase (2.7.1.4, from *Zymomonas mobilis*), and 5′-nucleotidase (3.1.3.5, from *Escherichia coli*). *Zymomonas mobilis* ZM4 (ATCC 31821) was obtained from the American Type Culture Collection. The *E. coli* was a standard laboratory strain, and fresh spinach was from the local grocer (Globus).

The following materials were obtained from Boehringer-Mannheim and used without further purification: ADP, ATP, biotin, fructose-6-phosphate (F6P), glucose-1-phosphate (G1P), glucose-6-phosphate (G6P), D-glyceraldehyde, NAD^+, and NADH. The following materials were obtained from Sigma and used without further purification: bicichonic acid, bovine serum albumen, calcium pantothenate, copper (II) sulfate, fructose, Nonidet P-40, Tris–HCl, Tris base, and Triton X-100. Potato starch (catalog no. 85645, electrophoresis grade[11]) was obtained from Fluka.

DEAE-Trisacryl was obtained from IBF Biotechnics of Villeneuve-la-Garenner, France. Sepharose products were obtained from Pharmacia. Procion Brown 5-HR and Yellow MX-GR were gifts from Imperial Chemical Industries, Switzerland. All other chemicals were obtained from Fluka AG. UV spectra were obtained using a Shimadzu Model UV 160 or 240 spectrophotometer. Ultrafiltration units and membranes were from Amicon.

Assay of Substrates, Intermediates, and Products. Except where otherwise noted, all assays were done in a "reaction buffer" (20 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.0) at 37 °C. Inorganic phosphate was determined by the method of Saheki et al.[12] Fructose was determined by the method of Ameyama and Adachi,[13] or by incubating aliquots (100 μL) with sorbitol dehydrogenase (7.5 U) and NADH (1 mM) in reaction buffer and following spectroscopically the loss of NADH. Potato starch was analyzed by conversion of the available hexose to 6-phosphogluconate with phosphorylase *a* (3 U), phosphoglucomutase (8 U), glucose-6-phosphate dehydrogenase (8 U), and NAD^+ (1 mM) in reaction buffer containing sodium phosphate (10 mM). Free glucose in the starch sample was determined by the oxidation of glucose (glucose oxidase, hydrogen peroxide forming, 25 U) coupled to the decomposition of NADH by peroxidase (25 U) in reaction buffer containing NADH (10 mM).

To measure the concentrations of intermediates in the process, aliquots (1 mL) were quenched (85 °C, 5 min, or 200 μL of 12% trichloroacetic acid). Glucose-6-phosphate was determined by using glucose-6-phosphate dehydrogenase (10 U) and NAD^+ (0.97 mM) in a total volume of 1 mL. After the reaction was completed, phosphoglucomutase (10 U) was added and glucose-1-phosphate determined. Fructose-6-phosphate was assayed by the method of Kahana et al.[14] Alternatively, quenched aliquots (100 μL) containing fructose-6-phosphate were added at 37 °C to buffer (0.9 mL) containing NAD^+ (1 mM), phosphoglucose isomerase (20 U), and glucose-6-phosphate dehydrogenase (16 U). The formation of NADH was followed at 340 nm.

All commercially available enzymes were assayed in "reaction buffer" (20 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.0) at 37 °C using Lineweaver-Burk plots to obtain values for Michaelis constants, K_M, and V_{max}. Phosphorylase *a* was assayed by using the method of Lee,[15] phosphoglucomutase by the method of Joshi and Handler,[16] and phosphoglucose isomerase by the method of Kahana and Lowry.[14] 5′-Nucleotidase activity on fructose-6-phosphate and ribose-5-phosphate was determined by incubating solutions of the substrates (100 μL, 100 mM) in buffer (750 μL, 20 mM Tris–HCl, 10 mM $MgCl_2$, pH 9) with 5′-nucleotidase (5 U) at 37 °C and assaying aliquots (200 μL) withdrawn at intervals for inorganic phosphate. The effect of additives (organic solvents, metal ions) on the 5′-nucleotidase activity was determined in this medium.

Transaldolase was assayed by incubating varying amounts of fructose-6-phosphate (0–100 mM) in buffer (0.967 mL) containing glyceraldehyde (10 mM) and NADH (0.2 mM). Sorbitol dehydrogenase (20 μL, 500 U/mL) was then added, the mixture pre-equilibrated (37 °C, 5 min), and the reaction initiated by adding a solution of transaldolase (13 μL, 77 U/mL). The loss of NADH was followed at 366 nm. The kinetics of the enzyme with respect to D-glyceraldehyde were studied similarly, but with 100 mM fructose-6-phosphate and 0–200 mM D-glyceraldehyde.

Alternatively, the kinetics of transaldolase were studied in the absence of sorbitol dehydrogenase. The reaction mixtures were quenched by the addition of trichloroacetic acid (200 μL, 12% solution) and centrifuged, and the supernatant was analyzed for fructose.

Extraction of Fructose Kinase. Fructose kinase was purified from *Zymomonas mobilis* ZM4 by a procedure adapted from Scopes et al.[17] In our hands, the chromatographic step involving binding to the immobilized dye Yellow MX-GR was not successful; the fructose kinase, once bound, failed to elute with ATP. Therefore the procedure was modified. *Zymomonas mobilis* was grown in medium containing, per liter, glucose (14 g), fructose (6 g), yeast extract (10 g), KH_2PO_4 (2 g, pH 6), $Fe(NH_4)_2(SO_4)_2$ (20 mg), calcium pantothenate (2 mg), and biotin (1 mg) at 30 °C with shaking. Cells (50 g) were lysed enzymatically at 30 °C in lysis buffer (1 L, 150 U of lysozyme, 3 mg of DNase I, 0.78 g of 2-mercaptoethanol, 0.4 g of $MgCl_2$, KH_2PO_4, 1 mL of Nonidet P-40), the cell debris removed by centrifugation (13000 g, 15 min, 4 °C), and the pH adjusted to 6 (1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes) in 5 M acetic acid).

A dye column was prepared by drying Sepharose CL-4B (200 mL) under vacuum, swelling the resulting powder in distilled water (280 mL), and gently stirring the solution with Procion Brown H-5R (120 mg in 80 mL of water). A solution of NaCl (40 mL, 4 M) was then added, followed by a solution of NaOH (4 mL, 10 M). The mixture was gently stirred (60 °C, 16 h), the supernatant decanted, and the beads washed (water, then 1 M NaCl in 25% aqueous ethanol, then water). The material was equilibrated in buffer (1 M Mes, pH 6, 10 mM KOH, 10 mM NaCl, 2 mM $MgCl_2$) and poured into a column (2.2 × 12 cm). The extract was passed through the column and the eluant collected and concentrated by ultrafiltration (PM-10 membrane). The concentrate was then applied to a DEAE-Trisacryl column equilibrated with Tris–HCl buffer (20 mM, pH 8). The protein was eluted with a gradient of NaCl (0–0.25 M). Fructose kinase eluted at 0.2 M NaCl. The active fractions were pooled and concentrated (ultrafiltration, PM-10 membrane). Traces of glucokinase activity were precipitated with ammonium sulfate (1.9 M).

Aliquots (200 μL) containing fructose kinase were assayed in an imidazole–phosphate buffer (20 mM imidazole, 20 mM potassium phosphate, 5 mM $MgCl_2$, 10 mM fructose, 1 mM ATP, 1 mM NAD^+, pH 6.8, total volume 800 μL) by converting fructose-6-phosphate to glu-

Table I. Extraction of Fructose Kinase

purification step	activity (units), μmol/min	protein, mg	specific activity, U/mg	purification factor
crude extract	63000 (100%)	174	362	1
Brown column	7436 (12%)	14	537	1.5
DEAE–Trisacryl column	6108 (10%)	4.6	1330	3.7
ammonium sulfate	1880 (3%)	0.22	8180	22.5

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Table II. Extraction of 3-Phosphoglycerate Phosphatase

purification step	activity (Units), μmol/min	protein, mg	specific activity, U/mg	purification step
crude extract	108 (100%)	7650	0.014	1
ammonium sulfate	93 (86%)	108	0.85	60
Sephadex G-200	5.6 (5%)	0.8	7.0	500

cose-6-phosphate (phosphoglucose isomerase, 2 U) and then to 6-phosphogluconate (glucose-6-phosphate dehydrogenase, 2 U) in a coupled assay, where the formation of NADH was monitored at 340 nm. Protein content was measured by the method of Smith et al.[18] Table I shows the results obtained with the protocol, which yielded a 22-fold purification.

To couple fructose kinase and ATPase to synthesize fructose, ATPase (0.4 U) and fructose kinase (10 U) were incubated at 37 °C in reaction buffer (1 mL) containing ADP (1 mM) and fructose-6-phosphate (10 mM). Aliquots (100 μL) were withdrawn at intervals (15–60 min), quenched (85 °C for 5 min, or 0.5 mL of 12% trichloroacetic acid), and assayed for fructose. Fructose kinase (100 U in 30 mL of H_2O at 0 °C) was also entrapped in polyacrylamide gels[19] prepared from acrylamide (2.7 g), *N,N'*-methylenebisacrylamide (0.3 g), and TEMED (150 μL) initiated with ammonium peroxysulfate (150 μL, 0.4 g/mL). Alternatively, the kinase was trapped between two ultrafiltration membranes (molecular weight cutoff 10000), where it could be stored (4 °C) for at least 4 weeks without loss of activity.

Extraction and Purification of 3-Phosphoglycerate Phosphatase. To assay 3-phosphoglycerate phosphatase, 3-phosphoglycerate (6 mM) was incubated in buffer (20 mM Tris–maleate, 1 mM EDTA buffer, pH 6.3, 0.4 mL) for 10 min at 37 °C with an aliquot (1 mL) of the sample to be analyzed. The reaction was terminated by the addition of 12% trichloroacetic acid (0.5 mL). The mixture was centrifuged (Eppendorf) to remove the denatured protein, and aliquots (200 μL) were then assayed for inorganic phosphate.

3-Phosphoglycerate phosphatase was extracted by using a procedure modified from Randall and Tolbert.[20] Spinach (1 kg) was deribbed, soaked (30 min, room temperature) in buffer (20 mM Tris–maleate, 1 mM EDTA, 2% polyvinylpyrrolidone, pH 6.3), and homogenized (3–5 min) in a Waring blender. The homogenate was centrifuged (5000 g, 4 °C), the precipitate discarded, the supernatant brought to pH 4.5 (3 N HCl), and the resulting precipitate removed by centrifugation and discarded. After centrifugation, the pH of the supernatant was immediately returned to 6.3 (3 N NaOH).

Acetone (chilled to 0 °C, 35% of the volume extract) was then added dropwise to the supernatant (5 mL/min) and the mixture was equilibrated (4 °C for 1 h) and centrifuged (18000 × g). The precipitate was discarded, another portion of acetone (20% by volume of original extract) was added to the solution, and the mixture was equilibrated (4 °C for 1 h) and centrifuged (18000 × g). The precipitate was dissolved in buffer (as above, except lacking polyvinylpyrrolidone, 20% of original extract by volume).

The mixture was then fractionated with $(NH_4)_2SO_4$, first to 330 g/L (precipitate recovered by centrifugation at 18000 × g and discarded) and then to 550 g/L. The precipitate was recovered by centrifugation (18000 × g) and resuspended in Tris–maleate buffer (4 mL). The solution was then applied directly to a Sephadex G-200 sizing column (2.5 × 35 cm) that had been pre-equilibrated with the Tris–maleate buffer, and the column was eluted with Tris–maleate buffer. Fractions (3.5 mL) were collected and tested for activity on glycerate-3-phosphatase, glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate. The activity fractions were pooled and concentrated by ultrafiltration (XM-50 membrane, cutoff 10000). The progress of the purification is shown in Table II. The final, active solution could be stored at 4 °C for at least 2 months.

Protein contents of aliquots (100 μL) were determined[21] by dilution with a solution (2 mL) of copper(II) sulfate (2%) in bicichonic acid according to the "Total Protein Analysis" kit from Sigma. Internal standards with bovine serum albumin were used in every assay.

Analysis of Thermostability. The thermostability of each of the enzymes was measured by following the loss of their catalytic activities at 37 and 50 °C in reaction buffer.

The Process. The process was built in three stages, first on a small scale, then as a batch process, and last as a semicontinuous process. First, phosphorylase *a* (150 U), phosphoglucomutase (120 U), and phosphoglucose isomerase (120 U) were combined in reaction buffer (1 mL, 20 mM Tris–HCl, 10 mM $MgCl_2$, pH 7.0) comtaining inorganic phosphate (5 mM, Na salt) and starch (20 mg/mL). After incubation (10 min) at 37 °C, the reaction medium was ultrafiltered (YM10 membrane, cutoff 10000) to separate the enzymes from the small molecule products, and the concentrations of glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate were determined.

In the batch process, all enzymes were combined in an Eppendorf tube in reaction buffer (1 mL) containing starch (24 mg/mL, 150 mM glucose equivalent), phosphate (10 mM), and D-glyceraldehyde (0.25 M). Ten units each of phosphorylase *a*, phosphoglucomutase, phosphoglucose isomerase, and transaldolase were added. 3-Phosphoglycerate phosphatase (1.3 U, measured with natural substrate) was then added, and the suspension was vigorously mixed prior to incubation at 37 °C for 2 h (periodic agitation). After the incubation, the reation was terminated by the addition of 12% trichloroacetic acid (200 μL), and the precipitated enzymes were removed by centrifugation (Eppendorf). Aliquots of the clear supernatant were then analyzed for glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose, and glucose.

The semicontinuous process was run in an Amicon ultrafiltration cell (10 mL) fitted with a YM10 membrane (cutoff 10000) at 37 °C with stirring (200 rpm) with use of reaction buffer (3 mL) containing phosphorylase *a* (30 U), phosphoglucomutase (30 U), phosphoglucose isomerase (30 U), transaldolase (10 U), 3-phosphoglycerate phosphatase (2 U), D-glyceraldehyde (0.25 M), and starch (3 mg/mL, 18.5 mM glucose equivalent). The reactor was emptied through the membrane by applying nitrogen gas pressure. Fresh reaction buffer containing starch, phosphate, and glyceraldehyde as above was then added to the reactor. This procedure was repeated every 3–6 h for 170 h. The output was analyzed for the reaction intermediates and fructose.

Results

The key step in this process is the final hydrolysis of fructose-6-phosphate in an exergonic reaction that pulls to products the intermediates from the part of the sequence running at equilibrium. Much effort was devoted to identify enzymes that catalyze this transformation efficiently.

Search for a Specific Fructose-6-phosphatase in Nature. As fructose is found in many fruits, several were screened for fructose-6-phosphatase activity, including banana, kiwi fruit, grape, and a variety of melons. In all cases, the level of phosphatase found was low. The most promising results were obtained with extracts from banana peel.[22] However, this activity was found not to be specific for fructose-6-phosphate. In general, it appears that free fructose is synthesized in nature by the NAD^+-dependent oxidation of sorbitol rather than by the hydrolysis of fructose-6-phosphate, at least in higher organisms. We considered using this reaction for the production of fructose. However, the redox reaction also operates near equilibrium under physiological conditions, and the recycling of nicotinamide cofactors remains a challenging technological problem.[23]

Reversing the Fructose Kinase Reaction. Fructose kinase is highly specific for fructose-6-phosphate and should catalyze the conversion of fructose-6-phosphate and ADP to fructose and ATP with high specificity as well. However, this reverse reaction is not physiological. It is endergonic by ca. 3 kcal/mol and must be "pulled" by coupling to an ATPase to yield the net hydrolysis of fructose-6-phosphate.

Synthesis of fructose from starch in the multistep process (Figure 2) was indeed observed when the "fructose-6-phosphatase" activity was obtained by a combination of fructose kinase from *Zymomonas mobilis* and ATPase from rabbit kidney. None was observed in the absence of ATPase. The rate of synthesis was slow, however, ca. 1.2 nmol min^{-1} mL^{-1} with 10 units of fructose kinase. This rather slow rate was not entirely unexpected. The

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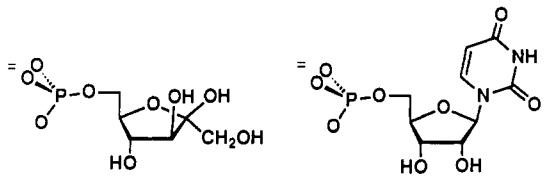


Fructose-6-phosphate Nucleoside 5'-phosphate

Figure 3. Structural similarity of fructose-6-phosphate and a nucleoside 5'-phosphate.

equilibrium constant for the reaction is ca. 340:1 in favor of the ADP–fructose-6-phosphate pair, and the kinase is inhibited by product when run in reverse. Thus, while the fructose kinase–ATPase couple provides a route to fructose via the pathway outlined in Figure 2, the slow rate of the reaction led us to search for alternative ways of hydrolyzing fructose-6-phosphate.

Recruitment of 5'-Nucleotidase. Extracellular 5'-nucleotidases apparently involved in digestion are available from a variety of sources, including enterobacteria[24] and snake venom (*Bothrops atrox* and *Crotalus adamantus*).[25] Fructose-6-phosphate and nucleoside 5-phosphates have structural similarities (Figure 3), suggesting that 5'-nucleotidases might catalyze the hydrolysis of fructose-6-phosphate. Further, extracellular enzymes, especially those that act physiologically in digestion, are generally less specific than intracellular enzymes, presumably because enzymes with low specificity are less likely to have a negative impact on the survival of an organism when outside the cell.[26] Finally, extracellular enzymes are often quite stable, and therefore suitable for use in a reactor.

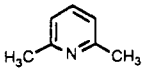
The 5'-nucleotidase from *E. coli*[27,28] had a half-life of only 3 days in reaction buffer at room temperature. Bovine serum albumen (0.1 M) stabilized the enzyme, although not sufficiently to make the enzyme suitable for use in a multienzyme reactor. The 5'-nucleotidase from *Crotalus adamantus*, which accepts the ribonucleoside monophosphates AMP (100%) > CMP (89%) > GMP (42%) > UMP (36%), was more robust. Further, while a β-hydroxyl group on the 3'-position of the ribose is an essential recognition feature of the enzyme, a β-hydroxyl group on the 2'-position of the substrate is not essential for activity,[26] suggesting that fructose-6-phosphate in its cyclic form (Figure 3) might be a substrate.

5'-Nucleotidase from *Crotalus adamantus* at 37 °C did indeed accept both fructose-6-phosphate and ribose-5-phosphate as substrates, although poorly; the specific activities were 0.05% and 0.04% respectively in relation to the enzyme's natural activity on 5'-AMP (data not shown). Replacing magnesium by cobalt enhances the rate of the reaction under standard assay conditions by 33%. Therefore, cobalt was used in all subsequent work with this enzyme.

Efforts were made to increase still further the rate with which 5'-nucleotidase hydrolyzes fructose-6-phosphate. The major principal structural difference between a nucleotide and fructose-6-phosphate is, of course, the presence of a heterocyclic base on the former. Binding of the base presumably induces a conformational change necessary for catalytic activity. Consistent with this is the fact that the conformationally constrained substrate analog (*R*)-8,5'-cycloadenosine-5'-phosphate is 200 fold less reactive than 5'-AMP (Figure 3). Presumably the nucleotidyl base in this analog cannot adopt the conformation necessary for it to occupy this pocket and induce the necessary conformational change.[29]

Filling a binding pocket with a cosubstrate often induces catalytic activity in enzymes. For example, horse liver alcohol dehydrogenase (E.C. 1.1.1.1) has low activity with both nicotinamide mononucleotide (NMN^+) and nicotinamide ribose as substrate. In the presence of the cosubstrate AMP, however, the enzyme's activity is greatly enhanced, presumably because NMN^+ and AMP together provide all of the substrate–enzyme interactions necessary for catalytic activity.[30]

Table III. Effect of Cosubstrates on the Catalytic Activity of 5'-Nucleotidase with Fructose-6-phosphate as Substrate

compd	structure	relative rate, %
none		100
pyridine		195
imidazole		138
2,6-lutidine		100
tetrahydrofuran		100
1,4-dioxane		100
methanol	H_3COH	100
ethanol	H_3CCH_2OH	100

The activity of nucleotidase with fructose-6-phosphate as substrate was increased by adding a heterocyclic compound as a cosubstrate (Table III). These results can be most easily explained by assuming that the cosubstrate binds to a specific site on the enzyme, rather than invoking a general "solvent effect". For example, both pyridine and imidazole enhanced the activity of 5'-nucleotidase on fructose-6-phosphate. However, 2,6-dimethylpyridine did not, nor did a variety of organic cosolvents that lack an aromatic ring (Table III).

Further, 5'-nucleotidase does not accept the other phosphorylated intermediates in the process, and cosolvent did not decrease this specificity. Thus, 5'-nucleotidase could perform as a selective fructose-6-phosphatase in the process outlined in Figure 2. However, even in the presence of a heterocyclic cosubstrate and cobalt, nucleotidase hydrolyzes fructose-6-phosphate still only slowly. Further, the additives are problematic in a process ostensibly intended to yield a foodstuff. Therefore, alternative approaches were sought.

An Indirect Route: Use of Transaldolase for Effecting the Hydrolysis of Fructose-6-phosphate. The net hydrolysis of fructose-6-phosphate might also be achieved by the reaction of fructose-6-phosphate with glyceraldehyde to yield glyceraldehyde-3-phosphate and fructose in a reaction catalyzed by transaldolase, followed by hydrolysis of glyceraldehyde-3-phosphate. Transaldolase catalyzes a nearly isoenergetic reaction, and has found application in many laboratories.[31] Following the work of Bonsignore,[32] fructose could be obtained from fructose-6-phosphate and glyceraldehyde using transaldolases from both *Candida utilis* and *Saccharomyces cerevisiae*. As the specific activity of the transaldolase from *C. utilis* was found to be ca. 70% higher in this reaction than transaldolase from *S. cerevisiae*, it was used in all subsequent work.

A specific glyceraldehyde-3-phosphatase was then sought in Nature. Again, no enzyme was identified that acts physiologically in this capacity. However, a 3-phosphoglycerate phosphatase from plant sources was reported to accept glyceraldehyde-3-phosphatase as a substrate.[20] Enzymes from land plants are components of

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Table IV. Relative Activity of Purified 3-Phosphoglycerate Phosphatase on Different Substrates

substrate	relative activity, %
3-phosphoglycerate	100
glyceraldehyde-3-phosphate	44
glucose-6-phosphate	0
fructose-1-phosphate	0
glucose-1-phosphate	0

Table V. Thermostabilities of the Enzymes Used in the Process

enzyme	source	half-life, h 37 °C	50 °C
phosphorylase *a*	rabbit muscle	>100	>50
phosphoglucomutase	rabbit muscle	>100	8
phosphoglucose isomerase	*S. cerevisiae*	>100	33
transaldolase	*C. utilis*	>100	28
3-phosphoglycerate phosphatase	spinach	>100	30

bioreactors, as they seem to be (as a rule) more thermally stable than enzymes from mammals, not surprising given the greater temperature fluctuations experienced by such plants in their natural environment. Further, 3-phosphoglycerate phosphatase isolated from spinach (Table II) accepted glyceraldehyde-3-phosphate at a rate of ca. 41% that of its natural substrate.[20]

For transaldolase and 3-phosphoglycerate phosphatase to yield fructose from fructose-6-phosphate via the coupled reaction in the process, they must not catalyze transformations on other intermediates. Transaldolase is known not to accept glucose-6-phosphate as a substrate, not surprising considering that glucose-6-phosphate is present physiologically and its participation in a transaldolation reaction is not desired metabolically. However, 3-phosphoglycerate phosphatase was reported to hydrolyze glucose-6-phosphate (at a rate 7% that of phosphoglycerate itself) and fructose-6-phosphate (6%) as substrates.[20] This report was confirmed with crude preparations of this enzyme. The hydrolysis of fructose-6-phosphate is, of course, desired, and would cause no difficulties in the process as envisioned (Figure 2). However, hydrolysis of glucose-6-phosphate is undesirable, as it would destroy a high-energy intermediate before the oxidation state of the carbon skeleton had been appropriately adjusted in the reaction sequence running at equilibrium.

Because of its cellular location,[20] evolutionary arguments suggested that 3-phosphoglycerate phosphatase should have a higher substrate specificity than that reported. Therefore, the enzyme was carefully purified. Elution profiles of a Sephadex G-50 column, where each fraction was assayed for catalytic activity against glycerate-3-phosphate, glucose-6-phosphate, and fructose-6-phosphate, showed that a highly purified preparation of the enzyme had no catalytic activities against the other phosphorylated intermediates in the process (Table IV), contrary to earlier reports. This suggests that the activity observed with partially purified protein against other intermediates in the process results from contaminating proteins and that a purified preparation

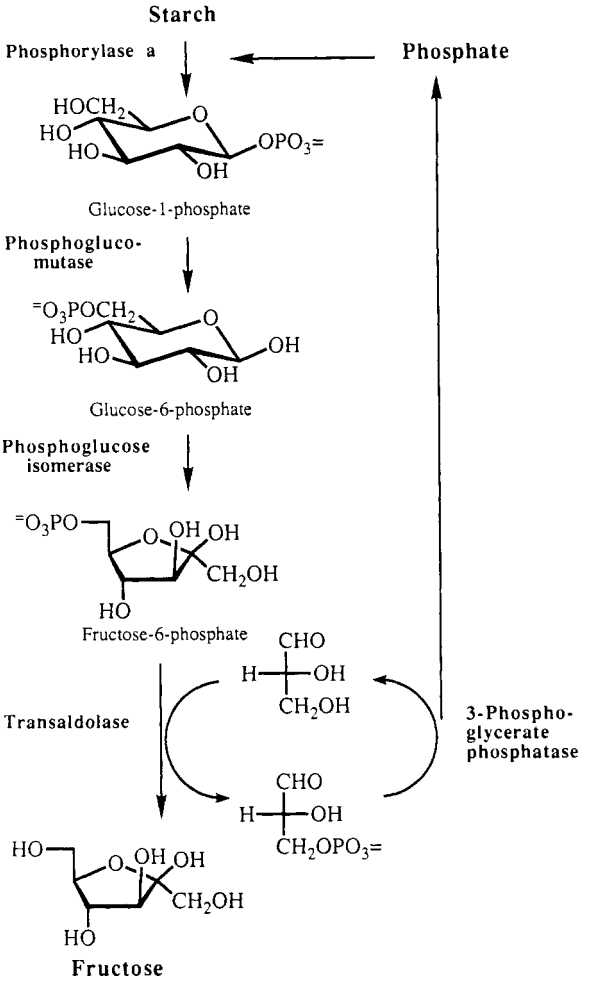

Figure 4. Semicontinuous process for converting starch to fructose in high yield.

could be used in a multistep process (Figure 2).

Constructing the Process. As transaldolase and 3-phosphoglycerate phosphatase effected the net hydrolysis of fructose-6-phosphate without effecting the dephosphorylation of other intermediates, a process containing all enzymes (Figure 4) was constructed. All enzymes in the process were found to be quite stable to thermal inactivation under the conditions used in the reactor (Table V). No loss of activity was detected at both 25 and 37 °C. Only at 50 °C could thermal inactivation be measured, with phosphoglucomutase being the least stable enzyme. Notably, the 3-phosphoglycerate phosphatase from spinach was rather robust, with a half-life of 30 h at 50 °C.

A preview of the kinetics and thermodynamics of the process was obtained from the kinetic parameters of the various enzymes

Table VI. The K_M, K_{eq}, and ΔG Values for Steps in the Process[a]

enzyme	substrate	K_M, M	K_{eq}[b]	ΔG, kcal/mol	ref
phosphorylase *a* 2.4.1.1	starch	0.75 mg/ml	0.7	0.21	37
	phosphate	$5.50\ 10^{-4}$			
phosphoglucomutase 2.7.5.1	glucose-1P	$2.84\ 10^{-4}$	17.2	−1.69	38
phosphoglucose isomerase 5.3.1.9	glucose-6P	$8.70\ 10^{-4}$	0.30	0.72	14
transaldolase 2.2.1.2	fructose-6P	$1.97\ 10^{-4}$	3.8	−0.79	32
	glyceraldehyde	$9.35\ 10^{-4}$			
3-phosphoglycerate phosphatase 3.1.3.38	glyceraldehyde-3P		206 M[c]	−3.20	39

[a] All kinetic values were measured in 20 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.0, at 37 °C. For phosphorylase *a*, K_M refers to concentration that yields half maximal velocity. [b] Equilibrium constants are written in the direction of the reaction as it appears in the process and are unitless except for the hydrolysis of glyceraldehyde-3-phosphate. [c] Calculated from the equilibrium constant for the reactions catalyzed by triose phosphate isomerase (23), glycerol-3-phosphate dehydrogenase (1.0×10^{-12} M), glycerol phosphatase (39 M), and the equilibrium constant for the redox couple between propane-1,2-diol and 2-hydroxypropanal.[39]

Table VII. Concentrations of Reactants, Intermediates, and Products in the Batch Process

intermediate	concn,[a] mM
starch (initial)	133 ± 7.98[b]
starch converted	87.5 ± 10.07[b]
glucose	4 ± 0.25
glucose-1-phosphate	1.5 ± 0.14
glucose-6-phosphate	18 ± 1.30
fructose-6-phosphate	4 ± 0.52
fructose	60 ± 7.86

[a] Standard deviations from multiple independent runs; after a 2-h incubation. [b] Glucose equivalent.

determined under the conditions of the process (Table VI). Further, the concentration of 3-phosphoglycerate phosphatase was chosen in the final process (vide infra) such that the rate-limiting step was the dephosphorylation of glyceraldehyde-3-phosphate. Under these conditions, the concentration of intermediates up to and including glyceraldehyde-3-phosphate will be close to their equilibrium values.

(a) Combining the First Three Enzymes. A process that converts starch to fructose using only phosphorylase *a*, phosphoglucomutase, phosphoglucose isomerase, and a nonspecific phosphatase[33] would yield fructose and glucose in a ratio determined by the equilibrium concentrations of these species and the relative k_{cat}/K_M values of the phosphatase for the various substrates. Experiments showed that this approach would not yield product streams with the desired enrichment of fructose. At equilibrium, glucose-6-phosphate and glucose-1-phosphate constitute 76% of the hexose mixture, with fructose-6-phosphate only 24% of the mixture. A nonspecific dephosphorylation of the mixture at equilibrium would therefore yield a 24:76 mixture of fructose to glucose. Further, as glucose-6-phosphate and glucose-1-phosphate are the first intermediates in the sequence, running the sequence under nonequilibrium conditions could not increase the fraction of fructose present in the product stream.

(b) The Complete Process. A batch process containing all enzymes (Figure 4) in reaction buffer (1 mL) containing 1.3 units of 3-phosphoglycerate phosphatase and 10 units of the other enzymes yielded a final fructose concentration of 60 mM (Table VII) starting with a starch concentration of 24 mg/mL. Coloring of the solution was observed only after incubation for 24 h at 37 °C. Consequently, batches were incubated for 6 h, during which time neither coloring nor precipitation was observed. The concentrations of intermediates were close to values expected from the relevant equilibrium constants (Table VII). Thus, the process operated largely at equilibrium with the final step both exergonic and rate limiting.

A semicontinuous process was then tested at 37 °C in a stirred reactor fashioned from an ultrafiltration cell (10 mL) using an Amicon YM10 membrane (molecular weight cutoff of 10 000). The product stream was extracted through the membrane at intervals of 3–6 h and replaced with fresh medium containing more starch. Over a period of 170 h, no coloring was observed, and over 80% of the enzymic activities were retained. The yield of fructose and the small amounts of the process intermediates (glucose-6-phosphate, glucose-1-phosphate, and fructose-6-phosphate) are shown in Table VIII. Under these conditions, the reactor produced ca. 1 g of fructose per hour per liter of reactor volume.

The concentrations of the small amounts of phosphorylated intermediates present in the product stream were again near equilibrium values. Indeed, this simply indicates that the rate-limiting step in the process is the hydrolysis of glyceraldehyde-3-phosphate. Given the relative cost of the individual enzymes (in particular, of 3-phosphoglycerate kinase), the ratio of enzymes in the process was close to economically optimal. Should 3-phosphoglycerate phosphatase become cheaper (for example, by cloning), the optimal relative concentrations of the enzymes in the process would change, the product stream would contain lower concentrations of intermediates, and the process itself would yield a higher net flux of fructose.

Table VIII. Contents of the Product Stream from the Semicontinuous Process

compd	concn,[a] mM
starch (initial)	18.5 ± 1.1[b]
starch converted	11.5 ± 1.5
glucose	0.89 ± 0.05
glucose-1-phosphate	0.04 ± 0.01
glucose-6-phosphate	0.07 ± 0.01
fructose-6-phosphate	0.05 ± 0.01
fructose	11.4 ± 1.5

[a] The values reported represent the mean values of the analyses done at 3–8-h intervals during a 170-h run. [b] Glucose equivalent.

Discussion

The process described here illustrates several principles important in the design of biomimetic biotechnological processes. First, the process has a thermodynamic profile similar to those found in natural biochemical pathways. Second, alternative approaches were explored and compared to overcome an obstacle presented by Nature: the unavailability of an enzyme catalyzing a key reaction (the direct hydrolysis of fructose-6-phosphate). Third, an understanding of the physiological roles played by various enzymes and how the behavior of enzymes adapted to fill these roles is influenced by evolutionary process assisted in the search for enzymes to be recruited.

No effort was made to optimize the process past a bench top scale (ca. 10 mL reactor volume). The need for substantial amounts of ATPase (variant 1), a heterocyclic cofactor (variant 2), and glyceraldehyde as a cofactor (variant 3) will almost certainly preclude commercialization of the process as it is presently implemented. Nevertheless, the process can be evaluated using commercial criteria, in particular, the ratio of fructose and glucose in the product stream and the fraction of total hexose in the starch converted to fructose. By both standards, the process is very good.

The fructose:glucose ratio in the product stream is 93:7, far better than the process presently used commercially. Even including phosphorylated intermediates present in the product stream, products that might be dephosphorylated by a nonspecific phosphatase, the fructose:glucose ratio is high (92:8). Further, the glucose in the product stream does not arise from starch, but rather comes from free glucose contaminating the starch used in this work. Solubilized potato starch is partially hydrolyzed with acid,[11] and contains ca. 7% free glucose. Thus, to within the experimental error, fructose is the exclusive product of hexose present in starch.

However, only 62% of the total hexose present in the starch appears as fructose in the product stream. This results from the fact that the starch used in this process, in addition to containing the standard α-[1,4]-glucosidic linkages degraded by phosphorylase *a*, also contains branching α-[1,6]-glucosidic linkages that are not degraded by the phosphorylase.[34] Similarly incomplete conversion is also observed in the commercial process for the same reason. Potato starch has typically 3–4% branching. Adding 4-α-D-glucanotransferase, which transfers the maltotriosyl residue on a limit dextrin to a 1,4-α-position in another chain making the hexose units available for conversion by phosphorylase *a* to glucose-1-phosphate,[35] pullulanase,[36] or isoamylase[6a] would obviously permit recovery of still more of the hexose units in starch. Nevertheless, it is worth noting that even if the unavailable hexose in the starch samples used here were simply converted to a mixture of glucose and fructose by the conventional process, the net

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fructose:glucose ratio would be 75:25, better than the ratio used commercially.

Finally, some minor alterations could further improve the productivity of the process. First, the cycle time (3–6 h) of the semicontinuous process using transaldolase was longer than necessary for optimal productivity. Second, higher amounts of phosphoglycerate phosphatase (the rate-limiting enzyme) would improve productivity. Finally, concentrations of glyceraldehyde used were much greater than the K_M of the transaldolase. As the transaldolase-catalyzed step is not rate limiting, the amount of glyceraldehyde could be reduced substantially without greatly influencing the rate of production of fructose.

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Registry No. ATPase, 9000-83-3; DNase I, 9003-98-9; G6P, 56-73-5; G1P, 59-56-3; F6P, 643-13-0; THF, 109-99-9; MeOH, 67-56-1; EtOH, 64-17-5; fructose, 57-48-7; starch, 9005-25-8; glucose-6-phosphate dehydrogenase, 9001-40-5; glucose oxidase, 9001-37-0; peroxidase, 9003-99-0; phosphoglucose isomerase, 9001-41-6; pyruvate kinase, 9001-59-6; lactate dehydrogenase, 9001-60-9; phosphorylase *a*, 9032-10-4; phosphoglucomutase, 9001-81-4; fructose dehydrogenase, 37250-85-4; transaldolase, 9014-46-4; sorbitol dehydrogenase, 9028-21-1; lysozyme, 9001-63-2; phosphofructokinase, 9001-80-3; 3-phosphoglycerate phosphatase, 62213-13-2; fructose kinase, 9030-51-7; 5′-nucleotidase, 9027-73-0; D-glyceraldehyde, 453-17-8; glucose, 50-99-7; glycerol kinase, 9030-66-4; fructose 6-phosphatase, 76774-41-9; pyridine, 110-86-1; 1*H*-imidazole, 288-32-4; 2,6-lutidine, 108-48-5; 1,4-dioxane, 123-91-1; 3-phosphoglycerate, 820-11-1; glyceraldehyde 3-phosphate, 142-10-9.

Calculated and Experimental Absolute Stereochemistry of the Styrene and β-Methylstyrene Epoxides Formed by Cytochrome $P450_{cam}$

Julia A. Fruetel,[†] Jack R. Collins,*[,‡] Debra L. Camper,[‡] Gilda H. Loew,*[,‡] and Paul R. Ortiz de Montellano*[,†]

Contribution from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, California 94143-0446, and Molecular Research Institute, 845 Page Mill Road, Palo Alto, California 94304. *Received January 31, 1992*

Abstract: Cytochrome $P450_{cam}$ oxidizes styrene to styrene oxide and a trace of phenylacetaldehyde; *cis*-β-methylstyrene to *cis*-β-methylstyrene oxide, *cis*-3-phenyl-2-propen-1-ol, and a trace of 1-phenyl-2-propanone; and *trans*-β-methylstyrene to *trans*-β-methylstyrene oxide, *trans*-3-phenyl-2-propen-1-ol, and a trace of 1-phenyl-2-propanone. Aromatic ring hydroxylation is also observed as a very minor process with each of the three substrates. Benzaldehyde is formed as a major product in all the reactions, but its formation is due to oxidation of the olefins by the H_2O_2 produced by uncoupled turnover of the enzyme. Stoichiometry studies indicate that oxidation of the styrenes is highly uncoupled relative to the oxidation of camphor. Analysis of the absolute stereochemistry of the three epoxides by chiral gas–liquid chromatography shows that the stereoisomers are formed in the following ratios: styrene oxide, *S*:*R* 83:17; *cis*-β-methylstyrene oxide, 1*S*,2*R*:1*R*,2*S* 89:11; *trans*-β-methylstyrene oxide, 1*S*,2*S*:1*R*,2*R* 75:25. Calculation of the minimum energy conformations of the three olefins, docking of the preferred conformations in the active site of cytochrome $P450_{cam}$ with use of AMBER to calculate the minimum energy orientations, and molecular dynamic simulations independently predict the following epoxide stereoisomer ratios: styrene oxide, *S*:*R* 65:35; *cis*-β-methylstyrene oxide, 1*S*,2*R*:1*R*,2*S* 84:16; *trans*-β-methylstyrene oxide, 1*S*,2*S*:1*R*,2*R* 75:25. The excellent agreement between theory and experiment supports the validity of the computational methodology and provides insight into the factors that control the stereoselectivity and outcome of cytochrome P450-catalyzed oxidations.

Cytochrome P450 enzymes play critical roles in the biosynthesis and metabolism of the steroids, fatty acids, eicosanoids, and other lipophilic endobiotics.[1-3] They also catalyze the oxidation of drugs and xenobiotics to more polar, more readily excreted, and sometimes more toxic metabolites.[1,4,5] Despite their biosynthetic and catabolic importance, little information is available on the active sites of these enzymes except for cytochrome $P450_{cam}$, a cytosolic enzyme from *Pseudomonas putida* that catalyzes the 5-hydroxylation of camphor.[6] The crystal structure of this enzyme, which has been solved in the presence and absence of camphor to a resolution of 1.6 and 2.2 Å, respectively,[7,8] currently serves as the template for all efforts to model the active sites of other cytochrome P450 enzymes. Analysis of the substrate-bound crystal structure suggests that hydrogen bonding of the camphor oxygen to Tyr 96 and interaction of its methyl groups with Val 295 and Val 247 are primarily responsible for orienting the substrate in the active site and promoting its regio- and stereospecific hydroxylation.[7-9] Experimental support for the primacy of these interactions is provided by the fact that site-specific replacement of Tyr 96 by a phenylalanine or either of the two valines by an isoleucine decreases the regio- and stereospecificity of camphor hydroxylation.[10,11] Conversely, replacement of the camphor oxygen by a sulfur, yielding a much poorer hydrogen bond acceptor with a great mobility in the active site, also results

* Authors to whom correspondence should be addressed.
[†] University of California, San Francisco.
[‡] Molecular Research Institute.

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