Carbon Isotope Effects on the Fructose-1,6-bisphosphate Aldolase Reaction, Origin for Non-statistical $^{13}$C Distributions in Carbohydrates*

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The kinetic and equilibrium isotope effects on the fructose-1,6-bisphosphate aldolase reaction have been determined using the rabbit muscle enzyme. The natural $^{13}$C abundance for both atoms participating in the bond splitting were measured in position C-1 of dihydroxyacetone phosphate and glyceraldehyde 3-P after irreversible conversion to glycerol-3-P and 3-phosphoglycerate, respectively, and chemical degradation. The carbon isotope effects were determined comparing the $^{13}$C content of the corresponding positions after partial and complete turnover, and after complete equilibration of the reactants. The observed kinetic isotope effect on C-3 is discussed to originate from the formation of the enamine, which comes to equilibrium before the rate determining release of glyceraldehyde 3-P from the ternary complex. The equilibrium isotope effect is seen as the reason for an earlier-found relative $^{13}$C enrichment in position C-3 of carbohydrates from different compartments of cells. The kinetic isotope effect is suggested to cause $^{13}$C discriminations in the C-3 pool in context with the hexasen formation in competition with other dihydroxyacetone phosphate turnover reactions.

The relative enrichment of carbon-13 in the carbonyl group of amino acids, as observed by Abelson and Hoering (1) in 1961, was the first indication for the existence of non-statistical isotope distributions in biological compounds. Later results on acetic acid (2) and on acetoin (3) gave evidence that this observation was just one example of a common phenomenon. In order to find a general explanation for this observation Galimov (4) discussed that even in chemically unequilibrated systems, e.g. biological systems, a microscopic reversibility in enzymatic reactions is the origin for a thermodynamically ordered isotope distribution. The author’s calculations could in fact explain some of the isotopic patterns of natural compounds known at that time. However, the presumption of a general thermodynamic equilibrium in biological systems is probably not realistic. In our opinion kinetic isotope effects on enzymatic reactions should be considered as primary causes for isotope discriminations. This has been proven for the primary CO$_2$-fixing reactions (5–8). In secondary metabolism, the isotope effect on the pyruvate dehydrogenase reaction has been made responsible for the general depletion of $^{13}$C in metabolites of acetyl-CoA, such as fatty acids or isoprenoids (9–15).

More detailed interpretations were possible when the total isotopic patterns of primary and secondary metabolites became available. Especially, our corresponding investigations on glucose indicated an enrichment of $^{13}$C in positions C-3 and C-4, and a depletion of $^{13}$C in positions C-1 and C-6 (Fig. 1) of this important primary metabolite (16). We have also identified the corresponding pattern in some direct metabolites of glucose (15, 17). As one of the reasons for this glucose pattern, we have discussed isotope effects on several reactions of the pentose phosphate cycle, especially on the fructose-1,6-bisphosphate aldolase reaction. In order to verify this assumption we have now determined the kinetic and the equilibrium isotope effects on this reaction.

We used for our experiments the well characterized Class I rabbit muscle aldolase (18, 19), which belongs to the same class as the cytosolic plant enzyme (20, 21). Rabbit muscle aldolase is a homotetramer with a molecular mass of 160 kDa, each subunit occurring in an $\alpha/\beta$ barrel (22). The enzyme catalyzes the reversible aldol cleavage of 1-phospho-ketoses (mainly fructose 1,6-bisphosphate (FBP)) into dihydroxyacetone phosphate (DHAP) and aldehydes (mainly glyceraldehyde phosphate (glyceraldehyde 3-P)). In the condensation reaction it is specific for dihydroxyacetone phosphate with retention of pro-S-configuration of C-3 in the ketoses. More than 50 different aldehydes are condensed with DHAP (23, 24).

The currently understood enzyme mechanism consists of several distinct steps (25–27), as shown in Fig. 2. They are preceded by the opening of the predominant ring form of the substrate in solution; step I is building up of the enzyme substrate complex, $E$-FBP; step II a Schiff base ($E$-SB$_{amide}$) formation from this complex; in step III the -C-C-bond cleavage to the “ternary” complex, $E$-enamineglyceraldehyde 3-P, the enamine of DHAP and glyceraldehyde 3-P, takes place; step IV is the dissociation of glyceraldehyde 3-P from the ternary complex; step V is the DHAP-Schiff base formation, $E$-SB$_{DHAP}$; step VI is a proton transfer giving rise to the protonated Schiff base, $E$-DHAP; step VII is the dissociation of DHAP from its Michaelis complex, $E$-DHAP. It is still not clear whether the velocity of the total reaction rate is limited by the -C-C-bond cleavage (step VII) or by the release of the triose phosphates (step IV and VII).

The main purpose of this work is to elucidate the role of the enzyme reaction for the non-statistical $^{13}$C pattern of glucose, however, in the context of this investigation we also expect to identify the rate-limiting step in the mechanism by the carbon

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1 The abbreviations used are: FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GC-C-IRMS, gas chromatograph-combustion-isotope ratio mass spectrometer; kat, katal.
isotope effect on the reaction. Yet, our main interest deals with the influence of the aldolase reaction on the non-statistical isotope distribution in carbohydrate metabolism.

EXPERIMENTAL PROCEDURES

Chemicals—All ordinary chemicals were of analytical grade and purchased from local suppliers. Fructose 1,6-bisphosphate-trisodium salt, C_{12}H_{21}O_{11}P_{3}Na_{3}·8H_{2}O, was from Boehringer Mannheim GmbH, Mannheim, Germany.

Enzymes—Triose-phosphate isomerase (EC 5.3.1.1), glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), and phosphoglycerate kinase (EC 2.7.2.3), used for enzymatic assays of fructose 1,6-bisphosphate, 3-phosphoglycerate, and glyceral 3-phosphate, were from local suppliers (Sigma, München, and Boehringer Mannheim GmbH). Fructose-1,6-bisphosphate aldolase (EC 4.2.1.13) from rabbit muscle, specific activity 0.15 μkat/mg, was from local suppliers (Sigma, München, and Boehringer Mannheim GmbH). Triose-phosphate isomerase (EC 5.3.1.1) from rabbit muscle, specific activity 0.118 μkat/mg, was from Sigma (München, Germany). The activity of triose-phosphate isomerase in these enzymes, which is a common impurity of glycolytic enzymes, was below 0.1%.

Determination of the Kinetic Isotope Effect on the Aldolase Reaction and Isolation of the Reaction Products—The measurement of the kinetic isotope effect on the aldolase reaction was performed under pH- and temperature-controlled conditions (Dasim, Methrom, Herisau, CH), with the competitive method after O’Leary (29). 1 mmol of fructose 1,6-bisphosphate, 0.1 mmol of NAD+, 25 μmol of phosphoglcolate, and 2 mmol of NaH_{2}AsO_{4} were dissolved in 100 ml of water (high performance liquid chromatograph system (Sykam, Glicching, Germany)). Before addition of the medium, the column was equilibrated for 30 min with 5 mM KH_{2}PO_{4} (pH 2.8). The reaction products were eluted. The fractions containing the reaction products, identified by their UV-absorption (200-350 nm) and enzymatic assays, were pooled, lyophilized, and stored at 4°C. The column was regenerated with 25 ml of 500 mM KH_{2}PO_{4} (pH 2.8).

Determination of the Equilibrium Isotope Effect on the Aldolase Reaction and Isolation of the Reaction Products—270 mg of fructose 1,6-bisphosphate and 10 mg of 2-phosphoglycerolate (for inhibition of the triose-phosphate isomerase) were dissolved in 10 ml of 20 mM sodium arsenate solution (pH 7.6, 25°C). The solution was divided into 4 aliquots. In each, the reaction was started by the addition of 3,5 nkat of fructose-1,6-bisphosphate aldolase. The reaction was stopped after 8 h (2 aliquots), respectively, 24 h (2 aliquots), by heating (65°C, 10 min), and the denatured protein was eliminated by filtration (0.45 μm, Millipore type HA).

To the solution, 0.1 μmol of NADH, 0.1 μmol of NAD+, 283 nkat of glyceraldehyde-3-phosphate dehydrogenase solution, and 133 nkat of glyceraldehyde-3-phosphate dehydrogenase solution were added, and by 1 h incubation the triose phosphates were converted into glycerol 3-phosphate and 3-phosphoglycerate, respectively. After this conversion the protein was denatured by heating. The filtrate of the medium was concentrated to 1 ml, and the products were separated as described before, by anion-exchange chromatography. The pooled fractions (about 5 ml) were concentrated under reduced pressure to 500 μl and finally lyophilized directly in 700-μl autosampler vials, in which the chemical degradation was performed.

Degradation of Reaction Products for Positional Isotope Analysis—For the periodate degradation of glycerol 3-phosphate into formaldehyde and phosphoglycolaldehyde (30), the compound (isolation and determination of total amount see above) was dissolved in 500 mM KH_{2}PO_{4} buffer (pH 5.8) to a final concentration of at least 100 μM, and a 120% molar excess sodium metaperiodate was added. The reaction was complete after 5 min at room temperature. In the case of millimole samples, this was controlled by the determination of the formaldehyde formed (31), while for nanomole samples the turnover was determined simultaneously to the isotope ratio measurement in the GC-C-IRMS system. For the determination of the isotope ratio of the phosphoglycolaldehyde formed, the solution was evaporated to dryness; after addition of water, the evaporation was repeated twice. The residue was directly submitted to wet combustion by adding 1 ml of 50% H_{2}SO_{4} and a two times molar excess of KMnO_{4}. The reaction was performed in an evacuated vessel at room temperature, and was complete after 3 min. The CO_{2} produced was measured volumetrically and then condensed into a vial for isotope ratio determination.

3-Phosphoglycerate was oxidized by Ce(IV)(NH_{4})_{4}(SO_{4})_{4} to CO_{2} and phosphoglycolate (32). In a 10-ml vacutainer 3-phosphoglycerate (10-1000 nmol), isolated as described, was dissolved in 1 ml of 50 mM KH_{2}PO_{4} and 2-2000 μl of reaction mixture (12.7 g of Ce(IV)(NH_{4})_{4}(SO_{4})_{4} and 15 ml of concentrated H_{2}SO_{4}, diluted in 85 ml of water) were added. The vacutainer was evacuated and the mixture incubated for 1 h at 100°C. With millimole samples the yield was controlled volumetrically, and the CO_{2} was transferred to the isotope ratio mass spectrometer for measurement. In the case of nanomole samples, after reaction the vacutainer was filled to a final gas volume of 1 ml with 10% H_{2}SO_{4} and an aliquot of the gaseous phase was transferred by means of a gas tight syringe to the GC-C-IRMS system, where turnover and isotope ratio were determined.

Determination of Isotope Ratios and Isotope Effects—Gas samples in the millimole range and solid substances were analyzed in a MM 903 isotope ratio mass spectrometer (VG Isogas, Middelwich, Cheshire, Great Britain). Prior, solid samples were converted into CO_{2} in an elemental analyzer Roboprep CN (Europea Scientific, Crewe, Great Britain). For the periodate degradation of glycerol 3-phosphate into formaldehyde and phosphoglycolaldehyde (30), the compound (isolation and determination of total amount see above) was dissolved in 500 mM KH_{2}PO_{4} buffer (pH 5.8) to a final concentration of at least 100 μM, and a 120% molar excess sodium metaperiodate was added. The reaction was complete after 5 min at room temperature. In the case of millimole samples, this was controlled by the determination of the formaldehyde formed (31), while for nanomole samples the turnover was determined simultaneously to the isotope ratio measurement in the GC-C-IRMS system. For the determination of the isotope ratio of the phosphoglycolaldehyde formed, the solution was evaporated to dryness; after addition of water, the evaporation was repeated twice. The residue was directly submitted to wet combustion by adding 1 ml of 50% H_{2}SO_{4} and a two times molar excess of KMnO_{4}. The reaction was performed in an evacuated vessel at room temperature, and was complete after 3 min. The CO_{2} produced was measured volumetrically and then condensed into a vial for isotope ratio determination.

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Ce(IV)-fission of 3-phosphoglycerate was controlled gas volumes. 3-phosphoglycerate and glycerol 3-phosphate were isotope effects could also be implied in the degradation of the isomers were separated as described under "Experimental Procedures." The evaluation of the separation and degradation procedure for isotope effects was tested with reference mixtures corresponding to that of the original compound (Table I). For position C-1 in both products they did not differ by more than 0.5 %.

Hence an isotope effect on the separation and degradation procedures would be negligible.

In addition, an isotope balance of the whole reaction was established by comparison of the $\delta^{13}C$ value of fructose 1,6-bisphosphate to that of the sum of all fragments isolated (Table II). The difference in the $\delta^{13}C$ value of 1.3 % is small enough to exclude an isotope effect in the whole reaction, even though the errors on each position should not be neglected. We guess that the reason for these relatively high standard deviations of the positional $\delta^{13}C$ value is the application of an on-line method, permitting the use of the small amount of only 50–100 nmol of substrate for the determination of the isotope effects. On the other hand the advantage of this method was the possibility to supply small incubation volumes for the experiments (1–5 ml), to use small analytical columns for the separation, and hence to have the possibility of making more independent experiments with a limited amount of enzyme.

This isotope balance and the $\delta$ value assignment to the fragments also reveals the specific isotope distribution in the substrate fructose 1,6-bisphosphate used. In contrast to natural glucose (16) the positions C-3 and C-4 are depleted in $^{13}C$. As the origin of the compound is not known, a discussion of the reasons is not possible. In the present context it has to be pointed out that the measurement of the isotope effect is always based on a relative shift in a given position, and absolute values of the C-atom in question are not important.

**Kinetic Isotope Effect on the Fructose-1,6-bisphosphate Aldolase Reaction**—The kinetic isotope effect on the fructose-1,6-bisphosphate aldolase reaction was determined according to the competitive method (29). In the present case the $\delta^{13}C$ value of the products in positions corresponding to positions C-3 and C-4 of fructose 1,6-bisphosphate after partial and complete turnover were determined after,

$$V_{\text{max}}/K_m = \log(1-\delta) / \log(1 - P_{R^*}/P_R) \quad (\text{Eq. 1})$$

The calculated kinetic isotope effect on position C-3 of fructose 1,6-bisphosphate was $13(V_{\text{max}}/K_m) = 1.016 \pm 0.007$, on position C-4 $\delta^{13C}(V_{\text{max}}/K_m) = 0.997 \pm 0.009$ (Table III). The range of these values is realistic and their difference is obvious.

**Equilibrium Isotope Effect on the Fructose-1,6-bisphosphate Aldolase Reaction**—The thermodynamic equilibrium of the fructose-1,6-bisphosphate aldolase reaction should be expressed as,

$$\text{FBPcyclic} \Leftrightarrow$$

DHAP(50% gem diol) + glyceraldehyde 3-P(98% diol) \quad (\text{Eq. 2})

The existence of the two different forms of DHAP has not been taken into account in the determinations of the isotope effects.

\[ \delta^{13}C = \frac{R_{\text{comp}} - R_{\text{PDB}}}{R_{\text{PDB}}} \times 1000[\%]_{\text{PDB}}, \]

where PDB is the international carbon isotope standard and $R_{\text{PDB}} = 0.0112372$.\[2\]

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**RESULTS**

**Assay Conditions for the Measurement of the Isotope Effects on the Fructose-1,6-bisphosphate Aldolase Reaction**—The usual enzyme assay (28) for the determination of the enzyme activity of the fructose-1,6-bisphosphate aldolase is based on the isomerization of the trioses formed by triose-phosphate isomerase and the reduction of dihydroxyacetonphosphate using an excess of the subsidiary enzymes.

For the determination of the kinetic isotope effects it was important, not only to convert the products as fast as possible, but also to do this by independent reactions, in order to obtain two distinct compounds as carriers of atoms 3 and 4 of the substrate. Therefore the triose-phosphate isomerase, if present at all, was blocked by 2-phosphoglycolate (34), and the glyceraldehydephosphate formed was oxidized by glyceraldehyde phosphate dehydrogenase to 3-phosphoglycerate, while dihydroxyacetone phosphate was independently reduced to glycerol 3-phosphate (Fig. 3). The coupled reactions had the advantage of recycling NAD$^+$, and thus the coenzyme concentration could be kept constant. In addition, the irreversibility of the reaction was attained by incubation in 20 mM arsenate (35).

**Control for Isotope Effects on the Developed Method Itself**—The evaluation of the separation and degradation procedure for isotope effects was tested with reference mixtures corresponding to the reaction medium but without enzymes. These mixtures were separated as described under “Experimental Procedures,” and the products were degraded as indicated. As an isotope effect could also be implied in the degradation of the products, 3-phosphoglycerate and glycerol 3-phosphate were degraded in millimole quantities under the conditions applied for the analysis of the incubation products. The turnover of the Ce(IV)-fission of 3-phosphoglycerate was controlled gas volumetrically on the CO$_2$ formed, in the case of the glycerol 3-phosphate degradation (NaIO$_4$) by colorimetric determination of the formaldehyde formed (32). The $\delta^{13}C$ values of CO$_2$, formaldehyde, and phosphoglycolaldehyde were measured as described under “Experimental Procedures.” For both reaction products, the $\delta^{13}C$ values as calculated from that of the fragments were compared to that of the original compound (Table I). For position C-1 in both products they did not differ by more than 0.5 %.

**Table I**

| Substance | $\delta^{13}C$ values [%]$_{\text{PDB}}$ in position | 1 | 2 + 3 | 1 - 3 calculated | 1 - 3 measured |
|-----------|--------------------------------------------------|-----|------|-----------------|---------------|
| Glycerol 3-P |                                             |    |      |                 |               |
| Incubated | 29.7 ± 0.8                                    | -27.7 ± 0.6 | -28.4 | -28.3 ± 0.2     |               |
| Isolated  | 29.3 ± 0.9                                    |               |       | ND*             |               |
| G3P |                                             |    |      |                 |               |
| Incubated | 20.6 ± 0.2                                    | -25.4 ± 0.3  | -23.8 | -23.8 ± 0.1     |               |
| Isolated  | 21.1 ± 1.3                                    |               |       | ND*             |               |

* ND, not determined.
The equilibrium is, in the concentration range used, more than 99.9% toward FBP (28). Taking this into account, the equilibrium isotope effect on the reaction was determined by incubation of fructose 1,6-bisphosphate of known isotopic pattern with aldolase for a given time in the absence of any other enzyme, and by subsequent pattern analysis of the products in equilibrium with that of the triose phosphates (P₃) using the isotope ratio after complete turnover, KIE = kinetic isotope effect.

The calculation of the isotope distribution and hence the equilibrium isotope effect was done by comparing the isotope ratios of the positions C-3 and C-4 of fructose 1,6-bisphosphate (S₀) and the glyceraldehyde 3-P and dihydroxyacetone phosphate (P₃) using the isotope ratio after complete turnover, KIE = kinetic isotope effect.

\[ \delta^{13}C_{\text{in}} = \frac{5 \times 10^{-3} \times \delta^{13}C_{\text{iso}} - 6.364 \times 10^{-4} \times \delta^{13}C_{\text{PE}}}{4.363 \times 10^{-3}} \]  

(Eq. 3)

The equilibrium isotope effect calculated for position C-3 was \( K_{12}^{13} = 1.004 \) and for position C-4 \( K_{12}^{13} = 1.005 \). The identical results for the two independent equilibration times indicate that the equilibration was already attained after 8 h.

**DISCUSSION**

At the beginning, it has to be pointed out that presently in the reaction sequence in question (Fig. 2), the final release of DHAP (step VII) is discussed to be the rate-limiting step of the whole sequence (37, 38), however, on the other side in the performed \( V_{\text{max}}/K_{\text{m},FBP} \) experiments, only the reactions before the first irreversible step, here the release of glyceraldehyde 3-P, can contribute to the observed isotope effects. Intriguingly we found on C-3 a kinetic isotope effect significantly different from unity, while the one on C-4 was practically unity. This is not compatible with the -C-C- bond fission itself as a reason for the isotope effect, but with the formation of the \( sp^2 \) configuration between C-2 and C-3 leading to the enamine of DHAP. The measured value 1.016 is reasonable for such a conversion of a secondary alcohol to an \( sp^2 \) terminal carbon, accompanied by the simultaneous fission of the adjacent C-C bond (39).

![Image](image_url)
(1.33 and 1.37, respectively, Ref. 40) proves the existence of an equilibrium between intermediate sp² structures before the release of glyceraldehyde-3-P, which is hence definitely the slowest partial step in the isotope effects in respect to the observed ¹³C enrichments in glucose. As a matter of fact we have found that this compound from various sources is depleted in ¹³C relative to glucose from the same origin (Fig. 4). The depletion is exclusively limited to position C-1, and a “normalization” of the pattern in positions C-2 and C-3 as reference shows that it can attain, relative to the expected pattern from DHAP, 25 % (e.g. glyceral in wine, less intense in glyceral from cattle fat). As glyceral is a minor product in the sources investigated, this depletion compensates the enrichments of a few % in the main products. A quantitative calculation of a metabolic and isotopic balance, as has been possible in the system CO₂/CH₄ for a rumen simulation (42) will, in a complicated system like a plant, only be possible after the determination and isotopic analysis of all main compounds. This would in principle permit the measurement of metabolic fluxes in plants and cell compartments and will be investigated in the future.

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