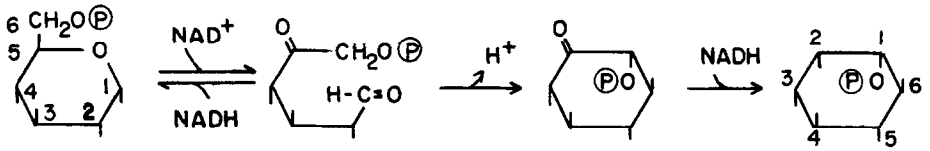
THE STEREOSPECIFICITY OF D-GLUCOSE-6-PHOSPHATE:1L-MYO-INOSITOL-1-PHOSPHATE CYCLOALDOLASE ON THE HYDROGEN ATOMS AT C-6.

Si Myung Byun, Robert Jenness, William P. Ridley, and Samuel Kirkwood, Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55101.

Received August 20,1973

SUMMARY: D-Glucose-6-phosphate:1L-myo-Inositol-1-phosphate cycloaldolase from rat testis or mammary gland removed stereospecifically the pro-S hydrogen atom at C-6 from D-glucose-6-phosphate. The pro-R hydrogen at C-6 remained in the product, 1L-myo-Inositol-1-phosphate and evidence is given that it is the hydrogen at C-1 of 1L-myo-Inositol-1-phosphate. The possible mechanism of cyclization is discussed.

D-Glucose-6-phosphate:1L-myo-Inositol-1-phosphate cycloaldolase (hereafter called cycloaldolase, E. C. number not assigned yet) catalyzes the cyclization of D-glucose-6-phosphate (Glc-6-P) to form 1L-myo-Inositol-1-phosphate (In-1-P) without carbon chain rearrangement (1) (2) (3) (4). The possible mechanism was proposed by Loewus and Kelly (1), in which internal aldolcondensation proceeds via an oxidation-reduction process as follows:



D-Glc-6-P 5-keto-Glc-6-P myo-Inosose-2-1-P 1L-myo-In-1-P

This proposal finds supports from other investigators working with the enzyme from plants (5), animals (6) (7) (8), Neurospora (9) and yeasts (10). That 5-keto-Glc-6-P actually is an intermediate was established recently by Barnett et al. (8) who prepared it chemically and showed that cycloaldolase from rat testis catalyzes its reduction by [4-^{3}H]-NADH to [5-^{3}H]-Glc-6-P. This finding has been confirmed by us by another approach (11).

One of the two hydrogens at C-6 of Glc-6-P is removed in the cyclization to myo-Inositol-1-phosphate (10) but hitherto it has not

been established whether this step is stereospecific. If the enzyme has the ability to differentiate between chemically like groups on the basis of their absolute geometrical differences, it could abstract stereospecifically only one of the two chemically like paired hydrogen atoms at C-6 of Glc-6-P (C_1 point group) during the cyclization. The synthesis of [pro-R-6-^{3}H]-Glucose by two of the authors (12) enabled us to examine this question. We wish to report data indicating that cycloaldolase catalyzes the stereospecific removal of the pro-S hydrogen at C-6 of Glc-6-P.

MATERIALS AND METHODS

Enzymes and Chemicals: Cycloaldolases were prepared from rat testis and mammary gland with the method of Barnett and Corina (13) except that 1 mM dithiothreitol was used instead of glutathione. After the fractionation with 30~40% saturated ammonium sulfate the enzyme was further purified on a Sepharose-6B column (2.5 x 100 cm) in 50 mM tris-acetate buffer pH 7.4 containing 1 mM dithiothreitol. Studies of the properties of the enzyme from both testis and mammary gland will be presented elsewhere. The enzyme preparations used in this work contained 4.0 mg of protein/ml for testis (69.3 fold purification after Sepharose-6B column chromatography) and 6.5 mg of protein/ml for mammary gland (77.9 fold purification). Inositol oxygenase (3.4 units/mg protein) was prepared from adult male rat kidney by the method of Charalampous (14). Bacterial alkaline phosphatase (44 U/mg) was obtained from Worthington Biochemical Corporation. Yeast hexokinase (specific activity 300 IU/mg), ATP·2Na·5H_2O, Glc-6-P·2Na·2H_2O, and D-GlcUA and NAD^+ were purchased from Cal Biochem. [6-^{3}H]-glucose (7.95 Ci/mmole) was a product of Amersham/Searle. [pro-R-6-^{3}H]-glucose (total count 7.8 x 10^4 DPM, 0.5 μmoles) was prepared enzymatically by two of the authors (12). It has been shown that at least 79% of the tritium was located in the pro-R position. Glc-6-P was prepared from glucose by the action of hexokinase and ATP and

purified by Dowex-1-X8 (Cl^- form) column chromatography (15). The yield was 65-73%.

Radioactivity Determinations: Chromatograms containing ^3H were scanned in a Packard Radiochromatogram Scanner Model 7201 and solutions were counted in a Beckman LS-235 Liquid Scintillation Counter using the scintillation solution of Bray (16). The system would accomodate 0.03 ml of water solution.

Incubation with the Cycloaldolase: Radioactive Glc-6-P in 50 mM tris-acetate buffer pH 7.4 (30 mM, 0.2 ml), NAD^+ in 50 mM tris-acetate buffer pH 7.4 (30 mM, 0.2 ml), tris-acetate buffer pH 7.4 containing magnesium acetate (5 mM) and ammonium acetate (20 mM) (50 mM, 0.25 ml), H_2O (0.1 ml) and enzyme preparation in 50 mM tris-acetate buffer pH 7.4 (0.25 ml) were incubated in a 37°C water bath for 4 hours and then heated for 2 min. in a boiling water and cooled to room temperature. Alkaline phosphatase (0.2 ml of 1 mg protein/ml) was added and the solution incubated for another hour at 37°C. Incubation was terminated by heating in boiling water. Water (medium) was collected quantitatively (1.2 ml) using a microfreeze dryer and its radioactivity determined. The residue in the freeze-dry tube was dissolved in distilled water and deionized by passage through a mixed bed ion exchange column (1 x 8 cm) consisting of IR-120 (H^+) and IR-45 (OH^-) and evaporated to 0.5 ml under an infrared lamp. Aliquots of 25 to 50 µl were spotted on Eastman Cellulose 6065 Chromatogram sheet (30 x 30 cm) and developed for 3.5 hrs. in nBuoH-pyridine water (6:4:3 v/v). Inositol, which traveled identically with authentic myo-Inositol, was scraped from the chromatogram into counting vial and its radioactivity determined.

Incubation of Radioactive Inositol with Inositol Oxygenase: After incubation of [6-^3H]-Glc-6-P with testis cycloaldolase as described, the mixture was streaked on Whatman #3MM filter paper. The chromatogram was developed for 24 hrs. in nBuoH-pyridine-water (6:4:3 v/v) and

Table 1: Stereochemistry of Cyclization of [pro-6-^{3}H]-Glc-6-P by Cyclo-aldolase.

Enzyme	DPM in water [%]*	DPM in Inositol [%]*	Recovery DPM [%]**
Testis	13	86	97.1
control	1.0	0	101.7
Mammary gland	12	88	107
control	1.1	0	101.5

*Expressed as % of DPM's converted to myo-Inositol

**Expressed as the total counts recovered in the form of unreacted glucose, inositol and water.

1 ml of incubation mixture contained 0.2 ml of 30 mM [pro-R-6-^{3}H]-Glc-6-P, 0.2 ml of 30 mM NAD^+, 0.25 ml of 50 mM tris-acetate buffer pH 7.4 containing Mg^{++} and NH_4^+, 0.25 ml of enzyme preparation and 0.1 ml of H_2O and was incubated at 37°C for 4 hours. After terminating the reaction, 0.2 ml of alkaline phosphatase (1 mg protein/ml) was added and incubated for another hour. The medium was collected by lyophilization using a microfreeze dryer and DPM determined. Inositol was separated from residue by cellulose plate chromatography, deionized by passage through a mixed bed, concentrated, and the DPM determined.

scanned. Radioactive inositol was eluted with water and concentrated to dryness. This radioactive inositol was incubated with inositol oxygenase from rat kidney. Water was collected quantitatively (1 ml) by the method described earlier and its radioactivity determined. The residue was again streaked on Whatman #3MM paper. The chromatogram was scanned for radioactivity of GlcUA after development in nBuoH-acetic acid-water (120:30:50 v/v) for 36 hrs. by the descending method.

RESULTS AND DISCUSSION

Incubation of [6-^{3}H]-Glc-6-P with Cycloaldolase: Commercially available [6-^{3}H]-Glc-6-P (9 x 10^6 DPM) was incubated with testis enzyme preparation. The radioactivity recovered from inositol (3.0 x 10^4 DPM) was twice that

in the medium (1.4×10^4 DPM). The same result was observed by Chen and Charalampous (10). Since commercial [6-^{3}H]-Glc-6-P is an equimolecular mixture of [pro-R-6-^{3}H]-Glc-6-P and [pro-S-6-^{3}H]-Glc-6-P, the cycloaldolase releases the hydrogen faster than tritium in the medium due to a kinetic isotope effect.

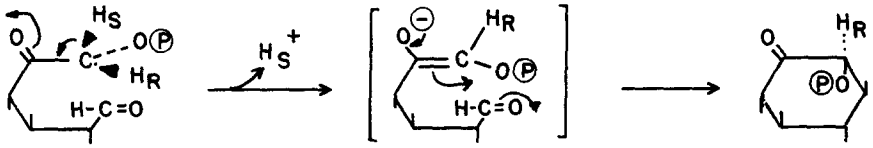
Incubation of Radioactive Inositol with Inositol Oxygenase to Locate ^{3}H in myo-Inositol: Labeled inositol was isolated by paper chromatography after incubation with the testis cycloaldolase in the presence of [6-^{3}H]-Glc-6-P and NAD^+. An amount containing 1.26×10^5 DPM (0.1 mmoles) was subjected to inositol oxygenase in the presence of O_2 and 9.0% of the tritium was recovered in the medium (less than 1% for control system). No detectable radioactivity was found in GlcUA. Since inositol oxygenase cleaves inositol between C-1 and C-6 (14), tritium in the medium could originate either from C-1 or C-6 (or both). We were not able to distinguish between these possibilities with the data at hand. An attempt should be made by subjecting [^{3}H]-Inositol formed from (1-^{3}H)-Glc-6-P by cycloaldolase, to degradation by inositol oxygenase.

Radioactive [^{3}H]-Inositol was also subjected to inositol dehydrogenase from Aerobacter aerogenes (11) (17) and no radioactivity was observed in the myo-Inosose-2 formed. Inositol dehydrogenase catalyzes the removal of hydrogen from C-2 of myo-Inositol to form myo-Inosose-2. Therefore, the data at hand indicate that the [^{3}H]-Inositol formed by cycloaldolase from [6-^{3}H]-Glc-6-P has tritium located at C-1 position even though the possibility of location at C-6 has not been excluded completely.

Incubation of [pro-R-6-^{3}H]-Glc-6-P with Cycloaldolase: [pro-R-6-^{3}H]-Glc-6-P (1.63×10^4 DPM, 6 μmoles) was incubated with testis and mammary gland cycloaldolase preparations respectively, followed by alkaline phosphatase and the amounts of ^{3}H in the medium, in myo-Inositol and in glucose (unreacted) were determined. The results are

shown in Table 1. For both testis and mammary gland preparations, 86-88% of the radioactivity calculated on the basis of Glc-6-P conversion to myo-Inositol appeared in myo-Inositol (23.8~27.4% of total ^3H) and 12-13% in the aqueous medium (3.3~4.4% of total ^3H). If cycloaldolase stereospecifically removes the pro-S hydrogen during the cyclization one would expect to observe 21% of the radioactivity in the medium and 79% in the inositol since the [pro-R-6-^3H]-Glc-6-P used in this study contained at least 79% of the ^3H in the pro-R position. The fact that 12-13% of the radioactivity appears in the medium is evidence of the previously observed kinetic isotope effect. On the other hand, if cycloaldolase acts randomly on the two hydrogens a greater percentage of the radioactivity would have been released into the medium. The data support the conclusion that it is the pro-S hydrogen at C-6 of Glc-6-P which is stereospecifically released in the cyclization process.

At first glance the results are somewhat surprising since one would expect that release of the pro-R hydrogen at C-6 of Glc-6-P would project the hydroxyl group at C-6 downward to favor formation of In-1-P. No carbanion is involved at C-6 and therefore no radioactivity from [^3H]-H_2O can be incorporated (13). The result, showing that the pro-S hydrogen is actually removed, indicates that the sp^3 configuration of C-6 may become the sp^2 in the transition state as follows:



The neighboring carbonyl group at C-5, which is produced in the first oxidation step by NAD^+, appears to accelerate the activation and dissociation of pro-S hydrogen at C-6.

REFERENCES

1. F. A. Loewus and S. Kelly, Biochem. Biophys. Res. Comm., 7, 204 (1962).
2. Y. Imai, J. of Biochem. (Tokyo), 53, 50 (1963).
3. F. Eisenberg, Jr., A. H. Bolden, and F. A. Loewus, Biochem. Biophys. Res. Comm. 14, 419 (1964).

4. I. W. Chen and F. C. Charalampous, Biochem. Biophys. Res. Comm., 17, 521 (1964).
5. M. M. Loewus and F. A. Loewus, Plant Physiol., 51, 263 (1973).
6. W. R. Sherman, M. A. Stewart, and M. Zinbo, J. Biol. Chem., 244, 5703 (1969).
7. F. Eisenberg, Jr., J. Biol. Chem., 242, 1375 (1967).
8. J. E. G. Barnett, A. Rasheed and D. L. Corina, Biochem. J., 131, 21 (1973).
9. M. Mogyoros, A. Brunner and E. Pina, Biochim. Biophys. Acta, 289, 420 (1972).
10. I. W. Chen, and F. C. Charalampous, Biochim. Biophys. Acta, 136, 568 (1967).
11. S. M. Byun and R. Jenness, unpublished data.
12. W. P. Ridley and S. Kirkwood, Biochem. Biophys. Res. Comm.,
13. J. E. G. Barnett and D. L. Corina, Biochem. J., 108, 125 (1968).
14. F. C. Charalampous, J. Biol. Chem., 235, 1286 (1960).
15. G. R. Bartlett, J. Biol. Chem., 234, 459 (1959).
16. G. A. Bray, Anal. Biochem., 1, 279 (1960).
17. J. Larner, Methods in Enzymology, 5, 326 (1962).