Biochimica et Biophysica Acta, 397 (1975) 288—293

BBA 67558

STEREOSPECIFICITY OF HYDROGEN TRANSFER BY PHOSPHOGLYCERATE DEHYDROGENASE

ILGA WINICOV*

Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pa. 19174 (U.S.A.)

(Received January 31st, 1975)

Summary

Phosphoglycerate dehydrogenase (EC 1.1.1.95) has been shown to be A site specific in its hydrogen transfer capacity unlike other dehydrogenases which use phosphorylated substrates. The experiments have been carried out using a coupled assay system with yeast alcohol dehydrogenase. The specific activity measurements of the reaction products indicate the possible influence of an isotope effect on this system.

Introduction

All dehydrogenases, which have been studied to date, have shown stereospecificity for the hydrogen transfer to and from either the A or B site of NADH [1,2]. Glyceraldehyde-3-phosphate dehydrogenase and α-glycerol phosphate dehydrogenase have been shown to belong to the B class of enzymes [3,4]. In fact, all the hydrogenases tested to date, which utilize phosphorylated substrates, appear to be B specific [5]. It was of interest to determine whether phosphoglycerate dehydrogenase (EC 1.1.1.95) also belonged to the B class of dehydrogenases, or whether is showed A specificity like glycerate dehydrogenase [5]. For this purpose phosphoglycerate dehydrogenase was coupled in an assay system with yeast alcohol dehydrogenase, an enzyme with known A stereospecificity [6].

The method is similar to that which was developed by Shapiro and Dennis [7] for preparation of deutero L-(+)-lactic acid. Thus, if phosphoglycerate dehydrogenase belonged to the A class of enzymes, it would be able to quantitatively transfer ^3H from the A site of NADH (generated by ethanol oxidation with yeast alcohol dehydrogenase) to hydroxypyruvate phosphate yielding

* Present address: The Institute for Cancer Research, 7701 Burholme Avenue, Philadelphia, Pa. 19111, U.S.A.

[^{3}H] 3-phospho-glycerate. If, however, phosphoglycerate dehydrogenase belonged to the B class of enzymes, most of the ^{3}H counts should be recovered in NAD as can be seen from the control reaction utilizing α-glycerol phosphate dehydrogenase, a known B-specific enzyme.

Materials and Methods

Phosphoglycerate dehydrogenase was purified from *Escherichia coli* and crystallized as described previously [8]. Yeast alcohol dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and the oxidized form of β-diphosphopyridine nucleotide were purchased from Sigma Chemical Co. The α-glycerol phosphate dehydrogenase was obtained from C.F. Boehringer and Soehne, Mannheim. Hydroxypyruvate phosphate was purchased from Calbiochem, Inc. The [1-^{3}H] ethanol (methylene label), 27.6 Ci/mol, was obtained from New England Nuclear, Inc. and diluted about 1 : 50 with 95% ethanol and water, so that the calculated final specific activity of [1-^{3}H] ethanol was 0.6 Ci/mol in a 50% ethanol solution.

Assay systems

The coupled reaction for yeast alcohol dehydrogenase and phosphoglycerate dehydrogenase contained 25 μmol Tris · HCl, pH 7.5, 0.84 μmol NAD, 100 μg yeast alcohol dehydrogenase, 1.8 μmol hydroxypyruvate-*P* and 1.17 mmol [^{3}H] ethanol (0.7 mCi) in a 1 ml volume. An aliquot was withdrawn at zero time to determine the total amount of radioactivity in the system. After a 2-min preincubation at 35° C, 4.8 nmol of phosphoglycerate dehydrogenase were added to start the hydroxypyruvate-*P* reduction. The reaction was carried out in a closed system connected to a cold trap (water at 0° C), to recover any [^{3}H] acetaldehyde volatilized during the time of the reaction.

The reaction was terminated by addition of 1 ml 95% ethanol and immersion of the reaction vessel in a boiling water bath for 5 min to denature the two enzymes. The reaction mixture was then chilled to 0° C, the "cold trap" disconnected and the protein precipitate removed by centrifugation at 0° C. The isotope was further diluted in the supernatant fraction by addition of another 1 ml of 95% ethanol and the excess ethanol was vacuum distilled. After this step 99.99% of the initial counts were removed and the non-volatile products of the reaction were further characterized.

The non-volatile ^{3}H counts, which indicate the extent of the reaction at a given time, were determined after evaporation of an aliquot of the supernatant fraction in a scintillation vial two times with methanol, followed by counting of the residue in Aquasol scintillation system (New England Nuclear). The counting efficiency was determined for each set of samples.

The coupled reaction for yeast alcohol dehydrogenase and glycerol phosphate dehydrogenase contained: 25 μmol Tris · HCl, pH 7.5, 0.84 μmol NAD, 100 μg yeast alcohol dehydrogenase, 2.2 μmol dehydroxyacetone-*P*, and 1.17 mmol [^{3}H] ethanol (0.7 mCi) in a 1 ml volume. The dihydroxyacetone-*P* reduction was started by addition of 10 μg α-glycerol phosphate dehydrogenase and the reaction mixture was treated as described for the yeast alcohol dehydrogenase-phosphoglycerate dehydrogenase coupled assay system.

Identification and quantitation of non-volatile reaction products

(A) *Reaction with phosphoglycerate dehydrogenase.* An aliquot of the non-voliate reaction products was streaked on a cellulose, Polygram Cell 300 (Brinkman Instr., distributors) thin-layer plate, and NAD, NADH, hydroxypyruvate-*P*, and 3-phosphoglycerate standards were spotted on each side of the streaked material. Thin-layer chromatography was carried out according to Bieleski [9] in *n*-propyl acetate/90% formic acid/water (55 : 25 : 15, by vol.) at room temperature, twice for 80 min. The chromatogram was air dried between runs.

The separated reaction products were identified as follows: NAD and NADH were identified by ultraviolet absorbance and fluorescence (Mineral light, short wave SL 2537), respectively; hydroxypyruvate-*P*, 3-phosphoglycerate and P_i were identified by Hanes-Isherwood reagent spray for phosphorus as modified by Axelrod and Bandurski [10].

The radioactivity profile of the chromatogram was determined by cutting a 1 cm wide strip from one end of the streaked sample and counting 1-cm^2 pieces of this strip in toluene scintillation system. Thus identified, the separated radioactive areas were scraped and the cellulose eluted in 3 ml water, followed by a 1 ml wash. Aliquots of the eluate were counted in Aquasol, assayed for inorganic and acid-labile phosphorus [11] and total phosphorus [12]. Enzymatic determination of 3-phospho[^{3}H]glycerate concentration was also carried out as described by Bücher [13]. The specific activity of 3-phospho[^{3}H]glycerate was calculated from phosphoglycerate concentrations determined both enzymatically and from acid-stable phosphorus determinations.

(B) *Reaction with α-glycerol phosphate dehydrogenase.* Recovery and quantitation of products in yeast alcohol dehydrogenase- and α-glycerol phosphate dehydrogenase-coupled reaction was carried out as described for the phosphoglycerate dehydrogenase reaction. Except, thin-layer chromatography was done using ammonium versene/ammonium acetate/95% ethanol (0.2 : 30 : 70; by vol.) for 100 min at 37°C. Specific activity for glycerol phosphate and NAD were calculated using values obtained from phosphorus determinations.

Results and Discussion

To determine the time for maximum accumulation of the reaction products, the time course for hydroxypyruvate-*P* reduction by phosphoglycerate dehydrogenase was followed in the coupled assay system with yeast alcohol dehydrogenase. The disappearance of acid-labile organic phosphate (hydroxypyruvate-*P*) was measured [11] as a function of incubation time at 35°C and is shown in Fig. 1. The coupled assay system of yeast alcohol dehydrogenase and phosphoglycerate dehydrogenase was incubated as described in Materials and Methods using unlabeled ethanol, and aliquots were withdrawn at the indicated times for phosphate determinations. All assays were done in duplicate and corrected for presence of inorganic phosphate. The reaction can be seen in Fig. 1 to proceed for at least 30 min, after which time an equilibrium situation probably becomes established. Therefore, 30 min incubation time was chosen for subsequent assays in which the reaction products were identified and quantitated.

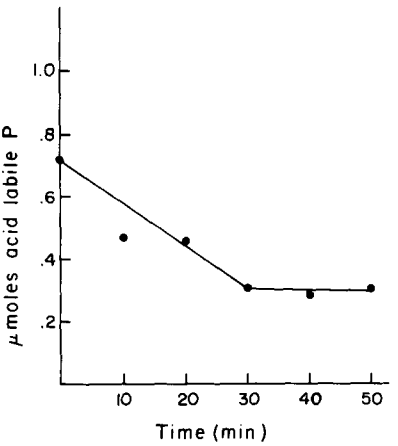

Fig. 1. Hydroxypyruvate-*P* reduction by phosphoglycerate dehydrogenase in an assay coupled to yeast alcohol dehydrogenase. The disappearance of acid-labile organic phosphate (hydroxypyruvate-*P*) was measured as a function of incubation time at 35°C as described in Materials and Methods. All assays were carried out in duplicate and corrected for presence of inorganic phosphate.

To determine the stereospecificity of hydrogen transfer by phosphoglycerate dehydrogenase, the reduction of hydroxypyruvate-*P* by this enzyme was coupled to [^{3}H]ethanol oxidation by yeast alcohol dehydrogenase. The course of ^{3}H transfer from [1-^{3}H]ethanol to 3-phosphoglycerate was followed not only by separation and identification of the reaction products after a 30 min incubation of the coupled reaction, but also by determination of the specific activity of each of the products.

Coupled reactions of yeast alcohol dehydrogenase and phosphoglycerate dehydrohenase or α-glycerol phosphate dehydrogenase (a B-specific enzyme) were incubated for 30 min as described in Materials and Methods. The products of the reaction were separated by thin-layer chromatography, eluted and the specific activity determined as dpm per μmol acid-stable phosphate. The results of duplicate assays on two reactions are shown in Table I.

Qualitatively the coupled reactions would predict that since yeast alcohol dehydrogenase is A-specific in its hydrogen transfer capacity, another A-specific dehydrogenase would transfer all of the tritium to the reduced substrate with no counts remaining in the oxidized form of the coenzyme (NAD), while a B-specific dehydrogenase would not be able to transfer ^{3}H from the A position of NADH, and would therefore, subsequently generate ^{3}H-containing NAD. Qualitatively this appears to be the case in the experiments reported here as shown in Table I. The B-specific enzyme, α-glycerol phosphate dehydrogenase, yields highly labeled NAD as its reaction product, while phosphoglycerate dehydrogenase gives no detectable counts in NAD. In addition, the isolated 3-phosphoglycerate contains a high level of ^{3}H counts. Since only A site specific transfer would result in all of the isotope being channeled into phosphoglycerate, with no counts recoverable in NAD, this finding leads to the conclusion that phosphoglycerate dehydrogenase is A site specific in its hydrogen transfer.

Quantitatively, however, the data required some additional comments.

TABLE I

STEREOSPECIFIC ^3H TRANSFER FROM [1-^3H]ETHANOL IN A COUPLED ASSAY

Coupled assays of yeast alcohol dehydrogenase were carried out with phosphoglycerate dehydrogenase or α-glycerol phosphate dehydrogenase as described in Materials and Methods. The products of the reaction were separated by thin-layer chromatography, eluted and the specific activity of each compound determined as dpm per mol organic phosphate; or in case of NAD, as dpm per 2 mol of organic phosphate.

Coupled assay	Specific activity (dpm/μmol)			dpm in substrate (%)
	Theoretical*	Substrate**	NAD	
Phosphoglycerate dehydrogenase and yeast alcohol dehydrogenase				
Experiment 1	561000	295000	0	71
Experiment 2	503000	249000	0	50
α-Glycerol phosphate dehydrogenase and yeast alcohol dehydrogenase	500000	177000	312000	35

* Calculated as half specific activity of [1-^3H] ethanol.
** 3-Phosphoglycerate or α-glycerol phosphate.

The finding of any ^3H counts in α-glycerol phosphate, although initially unexpected, can be explained if more than one turnover of the enzyme has occurred. Although the initial reduction of NAD by yeast alcohol dehydrogenase would still produce [^3H]NADH with ^3H in the A site, subsequent transfer of H from the B site of [^3H]NADH would give [^3H]NAD, which in the next cycle of reduction would now have ^3H in both the A and B positions, and therefore, serve as ^3H donor for either the A or B site-specific dehydrogenase. The finding that NAD has twice the specific activity found in α-glycerol phosphate (Table I), is consistent with this interpretation.

It should be noted that the specific activity of 3-phosphoglycerate, recovered as a product of the coupled reaction (Table I), is appreciably lower than expected from the calculated specific activity of ethanol. Since only 4.8 nmol of phosphoglycerate dehydrogenase is present in the assay system and about 400 μmol of phosphoglycerate is formed, the lowered specific activity cannot be due to a few enzyme turnovers during the heat denaturation step in excess cold ethanol. However, the decrease in specific activity can probably be explained by an isotope effect of the system, since as described in Materials and Methods, [1-^3H]ethanol was diluted 1 : 50 with unlabeled ethanol for use in the experiments, giving a preponderance of unlabeled ethanol molecules in the transfer mixture. A faster turnover rate with unlabeled material in any of the coupled reaction steps could easily have produced the observed lower specific activity of phosphoglycerate. Since the measured product was derived from a coupled reaction, the data cannot identify the step at which the isotope effect is manifested. However, an isotope effect has been observed with phosphoglycerate dehydrogenase using steady-state reactions and deuterated phosphoglycerate [14], and may therefore suggest that such an isotope effect also manifests itself in the reduction step by phosphoglycerate dehydrogenase in this system. This interpretation is further strengthened by a calculation of the expected ^3H isotope effect using the equation described by Melander [15] and

the known isotope effect observed with ^{2}H and steady-state phosphoglycerate dehydrogenase reaction at pH 7.5 [14]. This calculation gives a predicted ^{3}H isotope effect of 1.96 for phosphoglycerate dehydrogenase which is in good agreement with the observed isotope effect of 1.80 calculated from the data in Table I.

The finding that phosphoglycerate dehydrogenase is an A site specific dehydrogenase (like glycerate dehydrogenase [5]) and is unlike in stereospecificity to the other dehydrogenases which utilize phosphorylated substrates [2], shows that the phosphate group on a substrate does not generally influence the active site interactions required in stereospecificity of hydrogen transfer. Instead, conformation of the NAD molecule at the site of binding may expose the A or B sites of the nicotinamide ring to the substrate as suggested by X-ray crystallographic studies on D-glyceraldehyde-3-phosphate dehydrogenase [16].

Acknowledgments

I thank Dr Lewis I. Pizer, in whose laboratory this work was carried out, for his continued help and encouragement throughout the course of these experiments, and to Dr Judith Klinman for her critical review of the manuscript.

This work was supported by U.S. Public Health Service grant AI-03957 to Dr L.I. Pizer.

References

1 Kaplan, N.O. (1960) The Enzymes, Vol. 3, p. 115, Academic Press, New York
2 Rossman, M.G., Moras, D. and Olsen, K.W. (1974) Nature 250, 194—199
3 Loewus, F.A., Levy, H.R. and Vennesland, B. (1956) J. Biol. Chem. 223, 589—597
4 Levy, H.R. and Vennesland, B. (1957) J. Biol. Chem. 228, 85—96
5 Bentley, R. (1970) Molecular Asymmetry in Biology, Vol. 3, p. 6, Academic Press, New York
6 Fisher, H.F., Conn, E.E., Vennesland, B. and Westheimer, F.H. (1953) J. Biol. Chem. 202, 687—697
7 Shapiro, S.S. and Dennis, D. (1965) Biochemistry 4, 2283—2288
8 Winicov, I. and Pizer, L.I. (1974) J. Biol. Chem. 249, 1348—1355
9 Bieleski, R.L. (1965) Anal. Biochem. 12, 230—234
10 Axelrod, B. and Bandurski, R.S. (1951) J. Biol. Chem. 193, 405—410
11 Shibuyo, I., Honda, H. and Maruo, B. (1967) Agric. Biol. Chem. 31. 111—114
12 Bartlett, G.R. (1958) J. Biol. Chem. 234, 466—468
13 Bücher, T. (1947) Biochim. Biophys. Acta 1, 292—314
14 Winicov, I. (1975) J. Biol. Chem. 250, 1640—1647
15 Melander, L. (1960) Isotope Effects on Reaction Rates, p. 24, The Ronald Press, New York
16 Buehner, M., Ford, G.C., Moras, D., Olsen, K.W. and Rossmann, M.G. (1974) J. Mol. Biol. 90, 25—49