Biochimica et Biophysica Acta, 377 (1975) 9—14

BBA 67394

KINETIC PROPERTIES OF AN ENZYME HIGHLY ENRICHED IN CARBON-13

R. T. EAKIN

Department of Chemistry, University of Texas at Austin, Austin, Texas (U.S.A.)

(Received July 3rd, 1974)

Summary

Preparations of *Candida utilis* 6-phosphogluconate dehydrogenase with extreme variation in carbon isotope composition have been obtained. Molecular activities, pH optima and substrate affinities of these enzyme preparations have been measured. It was observed that any effect of isotope composition on these physiologically important properties is minimal.

Introduction

Isotope effects in biological systems have long been of interest and have been extensively studied. Much of the early work in this field has been reviewed by Katz and Crespi [1]. Most studies have been on deuterium substitution which theoretically should give the largest perturbations but which is complicated by the extensive amount of proton—deuteron exchange possible among biomolecules and solvent water molecules. The ^{12}C—^{13}C isotope pair provides a system involved in all biological and biochemical phenomena, and in which constitutional isotope effects are predominant since isotope exchange is minimal. This carbon-isotope system has not yet been exploited significantly in terms of isotope effects, but biological discrimination between the ^{12}C and ^{13}C nuclei at natural abundance levels (98.9% ^{12}C and 1.1% ^{13}C) has been found in many systems and has been reviewed by Smith [2]. Knowledge of biological ^{13}C isotope effects at high levels of substitution is minimal but has recently been reviewed by Matwiyoff and Ott [3]. Reported effects are on the level of relative macromolecular content and descriptive cytology, but no information is yet available for isotopic variance in a single molecular species on a kinetic level. Therefore an enzyme labeled with 90 atom% ^{13}C was biosynthesized and purified for the study of ^{13}C kinetic isotope effects at the macromolecular level.

The enzyme 6-phospho-D-gluconate : $NADP^+$ oxidoreductase (decarboxylating) (EC 1.1.1.44) Type I from *Candida utilis* was chosen for this pur-

pose. It is easily isolated, very stable, and is biosynthesized in reasonable quantity when the yeast is grown on acetic acid as a sole carbon source. Two preparations of this particular enzyme, 90 atom% ^{13}C and 1.1 atom% ^{13}C (natural abundance), have been obtained. As will be seen in the results presented here, the magnitude of perturbation of kinetic parameters due to extreme variation of carbon-isotope composition is minimal and probably insignificant in a biological sense.

Materials and Methods

Culturing of yeast. A strain of *C. utilis* (CU-1) able to utilize acetate both as the only energy source and as the only carbon source (other than a trace level of biotin) was cultured on an inorganic medium with continuous feeding of acetic acid as previously described [4]. Two sources of acetic acid were used to prepare separate cultures containing different isotopic levels of carbon. Commercial acetic acid containing the natural abundance carbon isotope level (1.1 atom% ^{13}C and 98.9 atom% ^{12}C) randomly distributed in each of the two carbon atoms was obtained from the J.T. Baker Chemical Company. Acetic acid containing 90 atom% ^{13}C and 10 atom% ^{12}C randomly distributed in each of the two carbon atoms was obtained as a gift from Donald G. Ott and Vernon Kerr of the Los Alamos Scientific Laboratory of the University of California. Yeast cells in an active metabolic state were harvested in a Sharples centrifuge when the cultures were at high absorbances (>10 g/l cells). The harvested cells were washed with normal saline, frozen at −20° C and freeze-dried.

Purification of 6-phosphogluconate dehydrogenase. The enzyme was purified from both cultures using a procedure based on that of Rippa et al. [5]. When crystallizing the enzyme from low ionic strength buffer, solid ammonium sulfate was added in small increments to 40% of saturation while keeping the pH at 6.2 with 5% NH_4OH. Precipitated protein was removed by centrifugation at 20 000 × *g* for 10 min and crystallization was induced by addition of saturated $(NH_4)_2SO_4$ solution, pH 6.2, to a point of slight turbidity. Enzyme preparations were recrystallized after centrifuging crystals at 20 000 × *g* for 10 min and dissolving the pellet in a sodium phosphate buffer ($2 \cdot 10^{-2}$ M phosphate, 10^{-4} M EDTA, pH 6.2).

Determination of concentration of protein and enzyme. Protein concentrations were estimated on a mass basis using the absorbance at 280 nm as described by Rippa et al. [5], taking into account the mass differences due to isotope compositions. Molecular or molar concentrations of enzyme were determined directly from absorbance at 280 nm using a molar absorptivity $\epsilon_M = 1.283 \cdot 10^8$ cm^2/mole derived from the mass extinction and molecular weight given by Rippa et al. [6] for natural abundance carbon preparations.

Determination of 6-phosphogluconate dehydrogenase activity. Activity of the enzyme was measured using the assay system described by Rippa et al. [5]. Reaction volume was 1 ml and enzyme was added in a volume of 10 µl. The reaction velocities were measured at ambient temperature on a Cary 14 recording spectrophotometer at 340 nm. This procedure monitors the rate of accumulation of NADPH and this rate has been used for determination of molecular

activity. Initial molecular activity was thus determined as moles NADPH/s/mole enzyme using a molar extinction coefficient of $6.22 \cdot 10^6$ cm^2/mole for NADPH.

Results

Isolation of 6-phosphogluconate dehydrogenase. Enzyme preparations were obtained from 150 g of dried *Candida* grown on 1.1 atom% [^{13}C]acetic acid and from 225 g of dried *Candida* grown on 90 atom% [^{13}C]acetic acid. These preparations were crystallized 8 times to a constant initial molecular activity of (75 ± 4) moles NADPH/s/mole enzyme. The two preparations were not distinguishable on the basis of their initial molecular activities. The final yield was 4.6 mg of 1.1 atom% ^{13}C-labeled enzyme and 7.2 mg of 90 atom% ^{13}C-labeled enzyme. The ultraviolet spectra of the two preparations were identical (Fig. 1) showing a maximum at 278 nm with a shoulder at 290 nm reflecting the large tryptophan content [5].

pH optima. The pH optima of the two enzyme preparations were obtained by measuring initial molecular activities at pH intervals of 0.2. This procedure showed a pH optimum of 8.0 for both preparations (Fig. 2). The initial molecular activities of both preparations dropped off more rapidly at higher pH values than at lower pH values. The experimental data indicate that the 90 atom% ^{13}C-labeled enzyme preparation is slightly more active at higher pH values than the 1.1 atom% ^{13}C-labeled preparation, but the magnitude of the difference is too small to ascribe it to an isotope effect with certainty.

Substrate binding. The affinities of 1.1 atom% [^{13}C]$NADP^+$ and 1.1 atom% 6-phospho[^{13}C]gluconate for each enzyme preparation were deter-

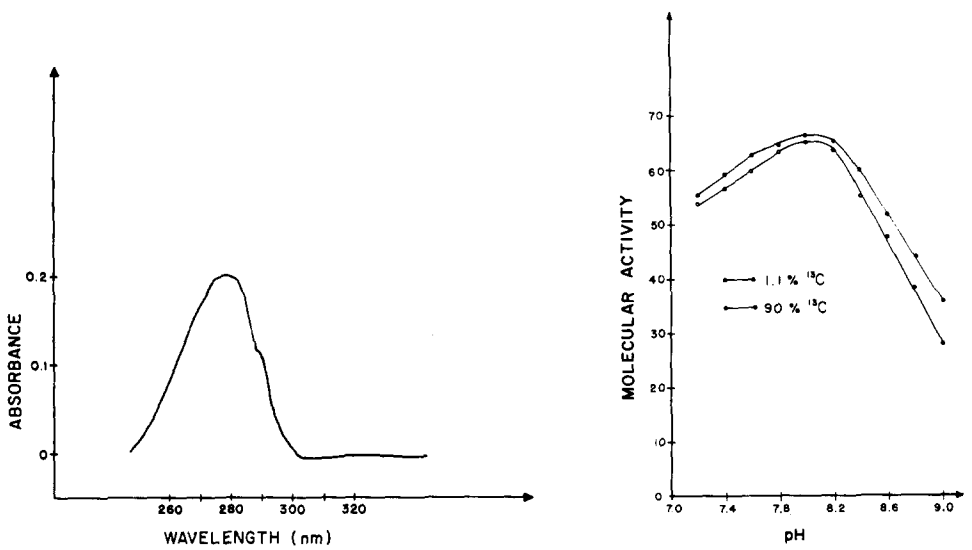

Fig. 1. Ultraviolet absorption spectrum of 6-phosphogluconate dehydrogenase. This spectrum is obtained from both the 1.1 atom% ^{13}C and 90 atom% ^{13}C preparations.

Fig. 2. pH dependence of the initial molecular activities (moles NADPH/s/mole enzyme) of 6-phosphogluconate dehydrogenase preparations. Assay systems were buffered with 0.05 M Tris—HCl.

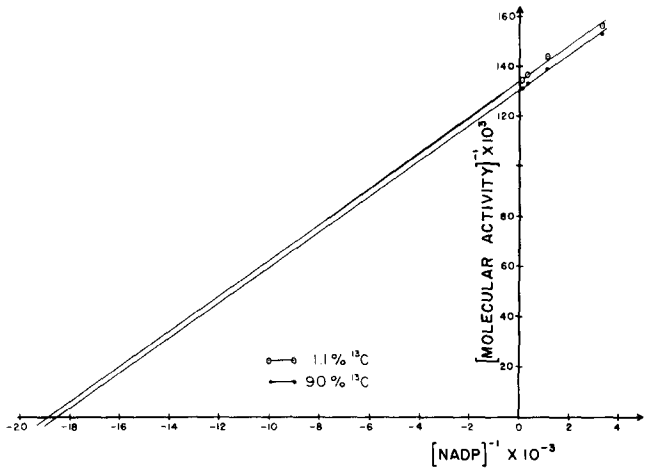

Fig. 3. Binding of $NADP^+$ to 6-phosphogluconate dehydrogenase preparations. The reciprocals of initial molecular activities (moles NADPH/s/mole enzyme) are presented as functions of reciprocal molar $NADP^+$ concentrations.

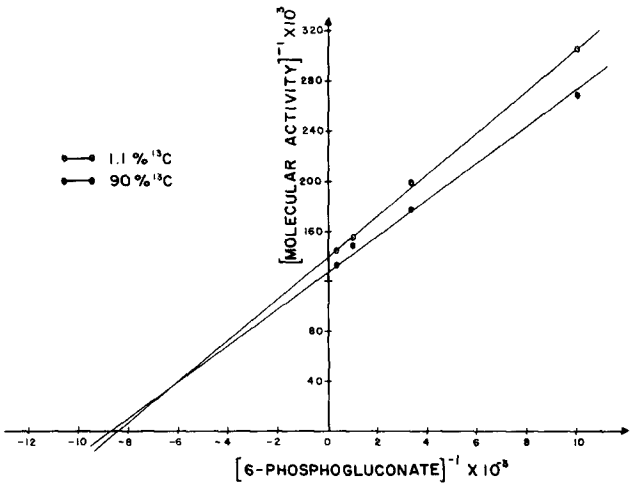

Fig. 4. Binding of 6-phosphogluconate to 6-phosphogluconate dehydrogenase preparations. The reciprocals of initial molecular activities (moles NADPH/s/mole enzyme) are presented as functions of reciprocal molar 6-phosphogluconate concentrations.

TABLE I

MAXIMUM INITIAL VELOCITIES AND SUBSTRATE K_m VALUES FOR 6-PHOSPHOGLUCONATE DEHYDROGENASE PREPARATIONS

Reaction velocities are expressed as moles NADPH/s/mole enzyme.

Substrate	Kinetic parameter	Enzyme preparation	
		1.1 atom% ^{13}C	90 atom% ^{13}C
$NADP^+$	Apparent K_m	54 μM	53 μM
$NADP^+$	V_{max}	75	77
6-Phosphogluconate	Apparent K_m	116 μM	119 μM
6-Phosphogluconate	V_{max}	72	78

mined by measuring initial molecular activities at different substrate concentrations. When varying the initial $NADP^+$ concentration an initial $NADP^+$ concentration of $3 \cdot 10^{-4}$ M was used. The $NADP^+$ affinity (Fig. 3) and the 6-phosphogluconate affinity (Fig. 4) for the two enzyme preparations were very similar. Table I lists the apparent K_m of both substrates and V for the two enzyme preparations as obtained from Fig. 3 and Fig. 4.

Discussion

A fundamental assumption in the analysis of enzyme kinetics is that the enzyme can be considered as a single species with respect to chemical properties related to catalysis. However, even with natural abundance ^{13}C, random internal isotope distributions within an enzyme will make each enzyme molecule isotopically distinguishable. This is usually ignored in the analysis of enzyme kinetics, but here isotope effects are of interest so that internal isotope distribution cannot be dismissed a priori. Nevertheless, most carbon atom positions within the molecule would not be as sensitive to isotope variation as certain critical positions in the reaction coordinate. Thus only a few specific carbon atom positions within an enzyme, particularly ones associated with vibrational modes involved in the making or breaking of bonds during the catalytic process, would be of primary significance in any observable isotope effect. The results presented here for *Candida* 6-phosphogluconate dehydrogenase indicate very little if any effect of carbon isotope composition on the catalytic properties of this particular enzyme. If enzyme carbon atoms are in the reaction coordinate, the variations in vibrational modes generated from differences in isotope mass have only a minimal effect on the enzyme activity.

These results contrast with the significant deuterium isotope effects observed by Rokop et al. [7] in fully deuterated alkaline phosphatase. However, deuterium substitution for hydrogen will perturb the reduced mass of vibrational systems to a much greater extent than ^{13}C substitution for ^{12}C, deuterium is more likely to be a component of the reaction coordinate because of hydrogen bonding, and deuterium exchange can introduce isotope substitution into critical substrate positions of the reaction coordinate. It is interesting, though, that where experimental data show a slight difference between 1.1 atom% ^{13}C-labeled enzyme and 90 atom% ^{13}C-labeled enzyme preparations (e.g., molecular activity at high pH), the possible isotope effects are opposite from the isotope effects seen in deuterium substitution. Perhaps this is related to the antagonistic effects of ^{2}H- and ^{13}C-labeled substitutions which have been observed on a cellular level [8].

Whole organisms have been grown on highly enriched sources of ^{13}C without any appreciable effect ascribable to carbon isotope composition [3,9]. This present study extends the observation of minimal biological effects to a macromolecular level and thus enhances the potential of the ^{13}C isotope as a tracer and as an internal spin label in the study of biological systems where minimal perturbation from the natural state is desired.

Acknowledgements

The assistance of Dr C.T. Gregg with the culturing of yeast cells is greatly appreciated. Discussions with and manuscript criticisms from Dr N.A. Matwiyoff and Dr L.O. Morgan have been most helpful. Dr R.P. Wagner kindly donated the use of instrumentation in his laboratory. This work was performed in part at the University of California Los Alamos Scientific Laboratory under the auspices of the United States Atomic Energy Commission. Financial support was provided by the Robert A. Welch Foundation.

References

1 Katz, J.J. and Crespi, H.L. (1970) Isotope effects in biological systems, in Isotope Effects in Chemical Reactions (Collins, C.M. and Bowman, N.S. eds) pp. 286—363, Van Nostrand Reinhold Company, New York, N. Y.
2 Smith, B.N. (1972) Bioscience 22, 226—231
3 Matwiyoff, N.A. and Ott, D.G. (1973) Science 181, 1125—1133
4 Eakin, R.T., Morgan, L.O., Gregg, C.T. and Matwiyoff, N.A. (1972) FEBS Lett. 28, 259—264
5 Rippa, M., Signorini, M. and Picco, C. (1971) Ital. J. Biochem. 19, 361—369
6 Rippa, M., Signorini, M. and Pontremoli, S. (1967) Eur. J. Biochem. 1, 170—178
7 Rokop, S., Gadja, L., Parmerter, S., Crespi, H.L. and Katz, J.J. (1969) Biochim. Biophys. Acta 191, 707—715
8 Flaumenhaft, E., Uphaus, R.A. and Katz, J.J. (1970) Biochim. Biophys. Acta 215, 421—429
9 Gregg, C.T., Hutson, J.Y., Prine, J.R., Ott, D.G. and Furchner, J.E. (1973) Life Sci. 13, 775—782