DAEHAN HWAHAK HWOEJEE
(*Journal of the Korean Chemical Society*)
Vol. 24, No. 4, 1980
Printed in the Republic of Korea

Gluconobacter melanogenus 로부터의 폴리올 탈수소효소에 대한 반응속도론적 특성에 관한 연구

金康華 · 李賢宰
한국과학원 생물공학과
(1980. 3. 31 접수)

Kinetic Properties of the Dye-Coupled Cytoplasmic Polyol Dehydrogenase from *Gluconobacter melanogenus*

Kang-Wha Kim and **Hyun-Jae Lee**
Department of Biological Science and Engineering
Korea Advanced Institute of Science, Seoul 131, *Korea*
(Received March 31, 1980)

요 약. *G. melanogenus* 로부터 분리한 폴리올 탈수소 효소는 이미 알려진 바의 다른 폴리올 탈수소 효소와는 달리 조효소로서 2, 6-dichlorophenolindophenol (DPIP)와 같은 인위적 전자 수용체를 필수로 요구하고 있음으로 이 특수 효소의 반응메카니즘을 반응속도론적 연구를 통하여 규명코져 시도하였으며, 폴리올 산화반응에 대한 초기속도 측정실험과 효소반응 산물인 케토산에 의한 저해 실험을 통하여 이 반응은 Ping-Pong Bi-Bi형의 반응메카니즘으로 진행됨을 확인하였다. 따라서 두 기질 즉 포리올로서 ***D***-mannitol 및 전자수용체로 DPIP가 효소에 의하여 반응이 진행될 경우 ***D***-mannitol이 우선 효소와 작용하며 첫 반응산물로서 해당하는 케토산인 ***D***-fructose가 생성될 것으로 기대되며 이 반응이 전체 반응속도를 조절하는 과정일 것이라고 추측하였다.

ABSTRACT. A steady-state kinetic study on a dye-coupled cytoplasmic polyol dehydrogenase from *G. melanogenus* was carried by the initial velocity measurements in the direction of the polyol oxidation and the product inhibition by ***D***-fructose. For the initial rate experimrnts, ***D***-mannitol and ***D***-sorbitol were employed as the specific polyol substrates and 2, 6-dichlorophenolindophenol (DPIP) as the specific cofactor substrate for the enzyme. When the polyol and DPIP were examined by varying one of substrates and by fixing the second, the corresponding reciprocal plots showed the typical parallel pattern. This suggests that the enzyme from *G. melanogenus* proceeds by a Ping Pong Bi-Bi mechanism in which the polyol may account as the first reactant-in, and the ketose formed as the first product-out, respectively. The product inhibition patterns obtained by ***D***-fructose (one no-inhibition, one non-competitive, and two competitive) may also provide an additional conformatory evidence for the above mechanism. Based on the kinetic parameters obtained, it was also suggested that the rate-limiting step in the direction of polyol oxidation is associated with the release of the ketose from the Enzyme · Polyol complex.

INTRODUCTION

A variety of polyol dehydrogenases, each with distinct substrate and cofactor specificities, have been reported to catalyze the the specific oxidation of several acyclic polyols to the corresponding ketoses[1~5], but most of the enzymes so far studied were found to be linked to the nicotinamide nucleotides as the cofactor for their catalytic activities. Although there found some reports on the membrane-bound particulate enzyme as a cytochrome-linked ***D***-mannitol dehydrogenase (EC 1. 1. 2. 2)[6,7], virtually not much attention has yet been paid to the NAD (P)-independent or other cofactor-dependent cytoplasmic polyol dehydrogenase system. The enzyme we found recently from the cell-free extracts of *Gluconobacter melanogenus*, however, showed a very distinct cofactor requirement of 2, 6-dichlorophenolindophenol (DPIP) as an electron acceptor for the specific polyol oxidation[8]. Since this enzyme as a dye-coupled polyol dehydrogenase requires unique cofactor for its catalytic activity and shows a very limitted substrate specificity toward the polyols having ***D***-lyxo configuration such as ***D***-mannitol and ***D***-sorbitol, *etc.*, it prompts us to investigate further the nature of the enzyme and its specific mode of action toward the polyol oxidation.

In this communication, we report the results obtained from a series of steady-state kinetic experiments on the specific polyol oxidation by the dye-coupled cytoplasmic polyol dehydrogenase from *G. melanogenus*, in which the initial rate measurements were carried with ***D***-mannitol and ***D***-sorbitol as the polyol substrate and DPIP as the cofactor in the direction of the polyol oxidation. Based on the steady-state behavior of this enzyme catalyzed polyol oxidation, it can be noted that such a dye-coupled dehydrogenase may proceed by a Ping Pong mechanism wile the other types of NAD (P)-linked dehydrogenases such as ribitol dehydrogenase from *Acetobacter aerogenus*[9], ***D***-mannitol dehydrogenase from *Absidia glauca*[10] and ***D***-sorbitol dehydrogenase from sheep liver[11] are followed by a sequential binding order forming abortive ternary complexes or a rapid equibrium random mechanism.

EXPERIMENTAL MATERIALS AND METHODS

Materials. The enzyme as a dye coupled polyol dehydrogenase was prepared from the cell-free extracts of *G. melanogenus* (ATCC 15163) as previously decribed[8]. ***D***-Mannitol and 2, 6-dichlorophenolindophenol (DPIP) were purchased from Merk Chemicals, Co., and ***D***-fructose, ***D***-sorbitol, and other biochemicals from Sigma Chemical Co.

Enzyme Activity Assay. The activity of the enzyme was assayed by measuring the rate of the dye reduction accompanied with the polyol oxidation at 30°C. The standard reaction mixture contained 68 μmoles of ***D***-mannitol, 0. 12 μmoles of acetate buffer (pH 5. 0) and 0. 03 units of the enzyme preparation in a final volume of 2. 0 m*l*. The initial velocity measurements were made by monitoring the changes in the absorbance of the cofactor, DPIP, at 522nm (a_M= 8. 6$\times 10^3$ cm$^{-1}M^{-1}$) with a Beckman Acta CIII Recording Spectrophotometer. The progress curves of the rate measurement were linear with time throughout the whole ranges of experiments. For product inhibition studies, ***D***-fructose as a product of polyol oxidation was incorporated into the reaction mixture, and the initial velocities were measured as in the above by drawing the tangent line to that portion of the recorder trace extrapolated to the time of the reaction starts.

Graphical Analysis. All subsequent initial

velocity data were examined graphically by Lineweaver-Burk plots[12] using least-square anlysis to facilitate the choice of the appropriate rate equation, and all kinetic constants were estimated by pertinent replots of slope and intercept values of the appropriate reciprocal plots. Inhibition data were derived from fits of experimental values to the general Michaelis-Menten type rate equations involving competitive, non-competitive and uncompetitive inhibitors, respectively.

RESULTS

Initial Velocity Studies. The steady-state kinetic investigation on the enzymic polyol oxidation by the dye-coupled cytoplasmic polyol dehydrogenase from *G. melanogenus* were carried with ***D***-mannitol or ***D***-sorbitol and DPIP as the substrates. When ***D***-mannitol and DPIP were examined by holding one of the substrate at constant level and by varying the second, the parallel patterns of the double reciprocal plots were obtained as shown in *Fig.* 1. The same paralled plots were also obtained when ***D***-sorbitol and DPIP were examined as variable and changing-fixed substrates, and when ***D***-fructose and an oxidized form of DPIP were similarly manipulated as the substrates for the reverse reaction. Based on such parallel patterns of the double reciprocal plots, it was assumed that the enzyme reaction catalyzing the polyol oxidation may be involved in a doble displacement reaction type mechanism, *i.e.*, a Ping Pong Bi-Bi reaction mechanism described by Cleland[13]:

$$E + A \rightleftarrows EX \rightleftarrows E' + P$$
$$E' + B \rightleftarrows EY \rightleftarrows E + Q$$

In this Ping Pong mechanism, a free modified-enzyme intermediate (E') was suggested,

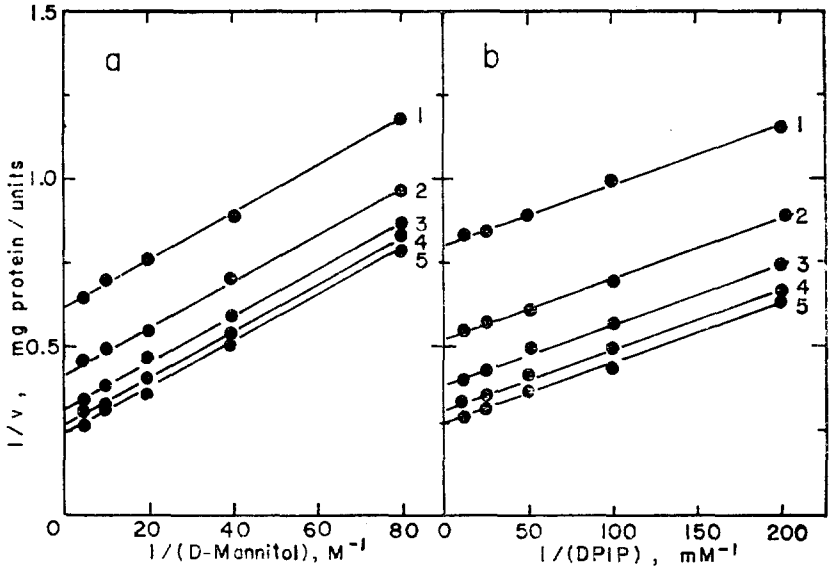


Fig. 1. Reciprocal plots of ***D***-mannitol oxidation by the polyol dehydrogenase from *G. melanogenus*. The initial velocities were measured under the standard assay condition. In (a), the reciprocal values of the initial valccities (1/v) were plotted against the reciprocal values of ***D***-mannitol as the variable substrate at the different fixed contrations of DPIP: (1) 5, (2) 10, (3) 25, (4) 50, and (5) 100 μM, respectively. In (b), 1/v versus 1/(DPIP) were plotted employing DPIP as the variable substrate and the polyol as the changing-fixed substrate. The ***D***-mannitol concentration were: (1) 12.5, (2) 25, (3) 50, (4) 100, and (5) 250 mM, respectively.

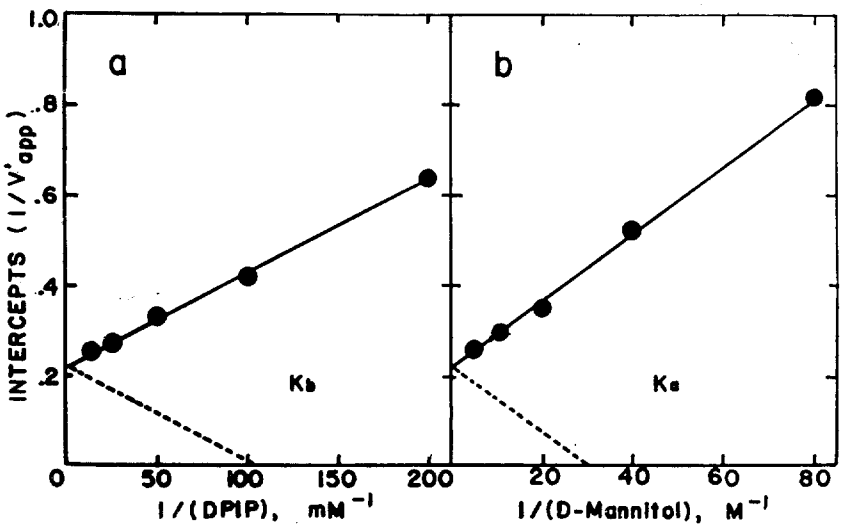


Fig. 2. Replots of the apparent maximum velocities with the changing-fixed substrates. The intercepts obtained from *Fig.* 1a and 1b were replotted against the reciprocal values of DPIP (a) and ***D***-mannitol (b) as the corresponding changing-fixed substrates, respectively.

Table 1. Kinetic parameters for the dye-coupled cytoplasmic polyol dehydrogenase from *G. melanogenus.*

Substrates	Michaeles constants (M)*		Maximum velocity (V_1) μmoles/min/mg protein
	K_a	K_b	
D-Mannitol/DPIP	3.4×10^{-2}	10.7×10^{-6}	4.6
D-Mannitol/Ferricyanide	3.4×10^{-2}	1.3×10^{-3}	4.6
D-Sorbitol/DPIP	2.9×10^{-1}	9.8×10^{-6}	2.6

* K_a and K_b represent Michaelis constants for the polyol and electron acceptor substarates, respectively.

and thus the reciprocal form of the initial rate equation derived by King and Altman method[14] gives rise to the following:

$$\frac{V_1}{v}=1+\frac{K_a}{(A)}+\frac{K_b}{(B)} \qquad (1)$$

where V_1 is the maximum velocity for the forward direction of the polyol oxidation, and K_a and K_b are the Michaelis constants for the polyol and the electron acceptor, respectively. Based on equation 1, the true Michaelis constants for the substrates can be estimated by the secondary plot methed in the manner of Florini and Vastling[15] as shown in *Fig.* 2 in which the values of the intercepts obtained from *Fig.* 1, *i.e.*, the reciprocal values of the apparent maximum velocities, $1/V_1$, were replotted against the reciprocal values of the variable substrate to give the reciprocal values of K_a and K_b at the intercepts on the abscissae. The resulting kinetic parameters were listed in *Table* 1. As seen in *Table* 1, the enzyme has quite different affinities toward ***D***-mannitol and DPIP with K_m values of 34 mM and 9.8 mM, respectivly, and the maximum velocity for the forward direction with ***D***-mannitol was obtained 4.6 μmoles per min per mg protein under the standard assay condition at pH 5.0 while the

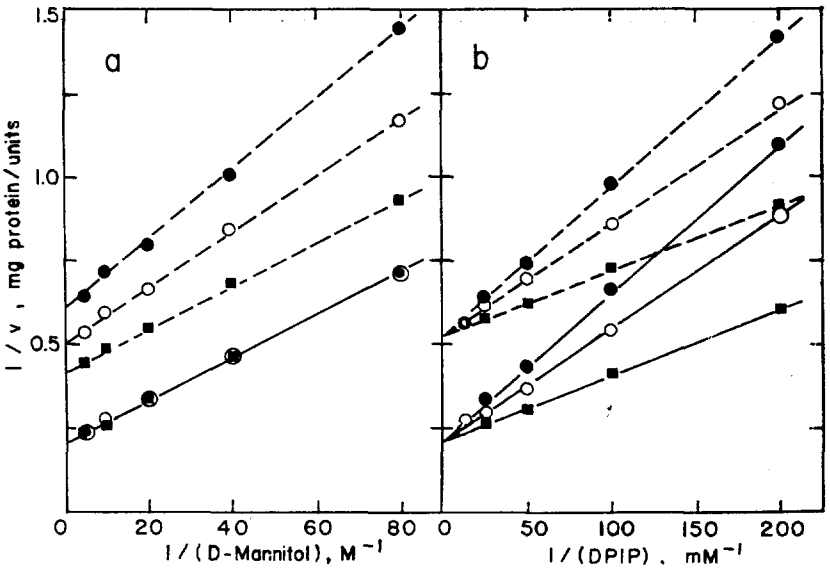


Fig. 3. Reciprocal plots of product inhibition by ***D***-fructose. The initial velocities were measured in the presence of ***D***-fructose at the concentions: (■) zero, (○) 0.5, and (●) 1.5 *M*, respectively. The reciprocal values of the initial velocities were plotted against the reciprocal values of ***D***-mannitol (a) and DPIP (b) as the variable sustrates: In (a), two fixed concentrations of DPIP, 200 μM (solid line) and 10 μM (dotted line), and in (b), two fixed concentrations of ***D***-mannitol, 250 m*M* (solid line) and 25 m*M* (dotted line), were employed as the changing-fixed substrates, respectively.

Table 2. Product inhibition patterns and inhibition constants.

Inhibitor	Substrate[a]		Inhibition[b] pattern	Inhibition constant (*M*)[c]
	D-Mannitol	DPIP		
D-Fructose	Var	Sat	N. I.	—
D-Fructose	Var	Unsat	NC	1.9
D-Fructose	Sat	Var	C	0.9
D-Fructose	Unsat	Var	C	1.4

[a]Var, sat and unsat. designate the variable and the fixed substrates at saturated and unsaturated conditions, respectively. [b]Inhibition patterns of N. I., NC and C designate, respectively, noinhibition, noncompetitive and competitive inhibtion. [c]Inhibition constants (K_i) were measured by fitting the experimental values to the general equations involving a specific inhibiter.

reverse reaction with ***D***-fructose showed a negligible rate under the same condition.

Product Inhibition Studies. Product inhibition by ***D***-fructose was studied with ***D***-mannitol and DPIP as the substrates. In a set of the double reciprocal plots as shown in *Fig.* 3, ***D***-fructose gave non-competitive inhibition against ***D***-mannitol as the variable substrate under the condition of a fixed unsaturated concentration of DPIP while no inhibition was observed when DPIP was saturated (*Fig.* 3a). When DPIP was used as a variable substrate (*Fig.* 3b), the competitive inhibition pattern by ***D***-fructose was obtained for both cases employing saturated and unsaturated concentrations of ***D***-mannitol as the fixed substrate.

The replots of slopes and intercepts obtained from *Fig.* 3 against the variable concentrations of ***D***-fructose showed linear lines, and provide K_i values for ***D***-fructose depending on the types of the product inhibition. A summary of product inhibition patterns and the corresponding inhibition constants were presented in *Table* 2. These along with the other initial rate experiments suggest strongly that the mode of polyol oxidation by this dye-coupled cytoplasmic polyol dehydrogenase from *G. melanogenus* is different from the other NAD(P)-linked polyol dehydrogenases.

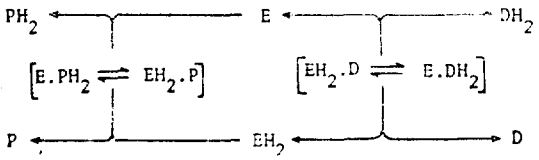
DISCUSSION

The results of the initial velocity experiments along with the array of product inhibition patterns indicate that the polyol oxidation by a dye-coupled cytoplasmic polyol dehydrogenase from *G. melanogenus* may proceed by a Ping Pong mechanism involving a modified-enzyme intermediate. However, since the general rate equation for the bi-substrate reaction involving ternary complex intermediates will also give the same parallel patterns of the double reciprocal plots while tne $K_{ia}K_b$ term is very small compare to the $(A)\cdot(B)$ term in equation 2, it is desirable to demonstrate an additional experiment for the elimination of such a possible deviation term.

$$v=\frac{V_1(A)(B)}{(A)(B)+K_a(B)+K_b(A)+K_{ia}K_b} \qquad (2)$$

In equation 2 where K_{ia} is a dissociation constant for the EA complex, such a deviation term can be increased by performing the initial rate measurements in the presence of a competitive inhibitol at a fixed high concentration level, and this would have the effect on the size of the deviation term of $K_{ia}K_b/(A)(B)$ which, in turn, results in a non-pallel pattern of the reciprocal plots if the reaction proceeds by the mechanism involving ternary complex intermedates. In fact, form such an experiment measuring initial velocities in the presence of 0.5 *M* ***D***-gluconic acid as a competitive inhibitor (K_i=0.15 *M*)[8], we could demonstrate the same parallel patterns of the double reciprocal plots having a different slop compared to that of *Fig.* 1. Therefore, this result can be accounted as an additional confirmatory evidence for a Ping Pong mechanism involving a double displacement type reaction.

In operating a Ping Pong Bi-Bi reaction by a dye-coupled polyol dehydrogenase from *G. melanogenus*, if the polyol substrate PH_2 acts as a leading substrate, the natural free enzyme E should be existed in an oxidized form to react with the polyol substrate, and the reaction of the free enzyme and the polyol may yield a binary transition state complex of $E\cdot PH_2$ which is, inturn, converted rapidly into the complex of $EH_2\cdot P$ from which the first oxidation product P departs and a modified-enzyme EH_2 as a reduced form is formed as shown in the following mechanism:



The reduced form of the free enzyme then reacts with the dye as the second substrate D for the reoxidation of the enzyme to the native state. However, in the above reaction mechanism, the DPIP like dye can also be acted as a leading substrate depending on the nature of the enzyme available since the kinetic-rate equation for such a Ping Pong mechanism shows the symmetric nature with respect to both of the polyol and the dye.

In conclusion, the picture emerged from this

kinetic study indicates that the enzymic oxidation of the ***D***-lyxo polyols by a dye-coupled cytoplasmic polyol dehydrogenase from *G. melanogenus* proceeds by a Ping Pong Bi-Bi mechanism with the polyol and the ketose as the first substrate-in and first product-out, respectively. Since the values of the Michaelis constants for the polyol and DPIP are quite different, and the rate-limiting step in the direction of the polyol oxidation seems to be associated with the release of the ketose from the E'P (or EH_2. P) complex, it appears that the natural free enzyme may exist predominantly in the oxidized form. Although the details in the nature of the enzyme and its significance in the metabolic pathways such as the aldose-ketose interconversion are still unknown, the enzyme from *G. melanogenus* seems to be uniquely susceptible to metabolic control by reacting initially with the polyol to produce the rate limiting product of the ketose.

REFERENCES

1. G. H. Martinez, H. A. Barker, and B. L. Horecker, *J. Biol. Chem.*, **238**, 1598 (1963).
2. J. M. Edmundowicz and J. C. Wriston, Jr., *J. Biol. Chem.*, **238**, 3539 (1963).
3. K. Sasajima and M. Isono, *Agr. Biol. Chem.*, **32**, 161 (1968).
4. S. D. Varma and J. H. Kinoshita, *Biochim. Biophys Acta*, **338**, 632 (1974).
5. S. T-H Ueng, P. Hartonowicz, C. Lewandoski, J. Keller, M. Holick and E. T. McGuinness, *Biochrm istry*, **15**, 1743 (1976).
6. K. Kersters, W. A. Wood and J. De Ley, *J. Biol. Chem.*, **240**, 965 (1965).
7. S. Birken and M. A. Pisano, *J. Bact.*, **125**, 225 (1975).
8. K. W. Kim and H. J. Lee, *Korean J. Biochem.*, **13**, 77 (1980).
9. H. J. Fromm and D. R. Nelson, *J. Biol. Chem.*, **237**, 215 (1962).
10. S. T-H Ueng and E. T. McGuinness, *Biochem istry*, **16**, 107 (1977).
11. U. Christensen, E. Tüchsen and B. Andersen, *Acta Chem. Scad. Ser. B*, **29**, 81 (1975).
12. H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.*, **56**, 658 (1934).
13. W. W. Cleland, *Biochim. Biophys Acta*, **67**, 1041 (1963).
14. E. L. King and C. Altman, *J. Phys. Chem.*, **60**, 1375 (1956).
15. J. R. Florini and C. S. Vestling, *Biochim. Biophys. Acta*, **25**, 575 (1975).