Biochimica et Biophysica Acta, 341 (1974) 85–92

BBA 67166

RABBIT MUSCLE L-GLYCEROL-3-PHOSPHATE DEHYDROGENASE

SUBSTRATE ACTIVITY OF 3,4-DIHYDROXYBUTYL 1-PHOSPHONATE AND 4-HYDROXY-3-OXOBUTYL 1-PHOSPHONATE

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(Received October 22nd, 1973)

SUMMARY

The phosphonic acid analogs of glycerol 3-phosphate, 2,3-dihydroxypropyl 1-phosphonate and 3,4-dihydroxybutyl 1-phosphonate, were examined as substrates for the rabbit muscle NAD-linked glycerol-3-phosphate dehydrogenase (L-glycerol-3-phosphate:NAD oxidoreductase, EC 1.1.1.8). The three-carbon analog was completely inert while the four-carbon analog was oxidized at approximately the same rate and had nearly the same K_m (240 μM for glycerol 3-phosphate compared to 190 μM for 3,4-dihydroxybutyl 1-phosphonate) as the natural substrate. The rate of reduction of dihydroxyacetone phosphate was approx. 25 times faster than that for its analog, 4-hydroxy-3-oxobutyl 1-phosphonate. This difference was not due to a difference in K_m values since the value for the natural substrate is 130 μM compared to 182 μM for the analog. The difference in rate does not appear to be due to a difference in the acidity of the phosphonate as compared to the natural substrate.

INTRODUCTION

We have previously described conditions under which 3,4-dihydroxybutyl 1-phosphonate, an analog of glycerol 3-phosphate, inhibits the growth of *Escherichia coli* [1]. More recent investigations revealed that the four-carbon phosphonate has a profound effect upon phospholipid metabolism [2]. These observations led us to explore whether the dihydroxybutyl 1-phosphonate could serve as a substrate or an inhibitor for several reactions catalyzed by enzymes isolated from *E. coli* and other sources. In the course of these studies our attention became focused on glycerol-3-phosphate dehydrogenase activities. The present report describes observations concerning the substrate specificity of the rabbit muscle NAD-linked glycerol-3-phosphate dehydrogenase (L-glycerol-3-phosphate:NAD oxidoreductase, EC 1.1.1.8).

Compounds containing the $-CH_2PO_3H_2$ group in place of the $-OPO_3H_2$ group are structurally similar but not identical. Structural differences involving bond lengths and angles have been reported among compounds of the P–X–P type, where X is either O, NH, or CH_2 [3]. Furthermore, the phosphonic acid analogs are weaker acids

than their phosphate counterparts. These differences are on the order of 2.5 pK_a units for each of the dissociation constants. From a biological point of view it is obvious that enzymes responsible for phosphoester cleavage would not be able to perform their catalytic function on a phosphonic acid analog. It is less clear as to whether an enzyme catalyzing a reaction at some site other than the phosphoester bond would be able to function with such an analog.

2,3-Dihydroxypropyl 1-phosphonate, an analog of glycerol 3-phosphate, has been tested as a substrate for the rabbit muscle dehydrogenase and was observed to be inert [4]. One possible conclusion from such an observation is that the phosphoester bond of glycerol 3-phosphate is required for activity. An alternative conclusion is that the steric dissimilarities between glycerol 3-phosphate and the three-carbon analog are too great and therefore preclude any activity. This paper presents evidence indicating that 3,4-dihydroxybutyl 1-phosphonate and 4-hydroxy-3-oxobutyl 1-phosphonate, an analog of dihydroxyacetone phosphate, serve as substrates for the rabbit muscle glycerol-3-phosphate dehydrogenase.

MATERIALS AND METHODS

Materials

NAD^+ (Grade 111), NADH (Grade 111), DL-glycerol 3-phosphate, disodium salt (grade X), di-monocyclohexylamine salt of dihydroxyacetone phosphate dimethylketal, glycine, triethanolamine·HCl, bovine serum albumin, and L-glycerol-3-phosphate:NAD oxidoreductase (EC 1.1.1.8) were purchased from the Sigma Chemical Co., St. Louis, Mo. Hydrazine sulfate was purchased from the Fisher Scientific Co., Pittsburgh, Pa. Dihydroxyacetone phosphate was generated using the Dowex-50 X4-200R provided by the manufacturer for this purpose and following the manufacturer's instructions.

The dilithium salt of 3,4-dihydroxybutyl 1-phosphonic acid was prepared as previously described [5]. The monosodium salt of 4-hydroxy-3-oxobutyl 1-phosphonic acid was a generous gift of Dr S. Goldstein. The method of preparation of this compound will be reported in a subsequent communication. The dilithium salt of 2,3-dihydroxypropyl 1-phosphonic acid was prepared by the method of Rosenthal and Geyer [6]. All other chemicals used were of analytical or reagent grade. All solutions were prepared with glass distilled water.

Enzyme assay

The rabbit muscle glycerol-3-phosphate dehydrogenase was diluted as described by Black [7]. The dehydrogenase activity was determined spectrophotometrically by measuring the rate of formation or disappearance of NADH at 340 nm using either a Gilford Model 2400 spectrophotometer or a Gilford Model 240 fitted with a constant temperature cuvette holder and Honeywell recorder. Reaction rates were determined during the first 30 s for the oxidation of glycerol 3-phosphate and 3,4-dihydroxybutyl 1-phosphonate. The initial rates during this time period were linear in all cases. The assay procedure used in all kinetic experiments was that described by Black [7]. Although racemic mixtures of glycerol 3-phosphate and its phosphonic acid analogs were used throughout the present studies, the results are expressed in terms of the L-isomers. All assays were performed at 26 °C.

The reaction mixture for assaying the rate of oxidation of glycerol 3-phosphate and 3,4-dihydroxybutyl 1-phosphonate contained 33 μmoles triethanolamine buffer at pH 9.0, 2 μmoles EDTA, 2 μmoles β-mercaptoethanol, 1.0 mg bovine serum albumin, and the indicated concentration of glycerol 3-phosphate, 3,4-dihydroxybutyl 1-phosphonate, NAD^+, and enzyme per ml. The Li^+ concentration was kept constant at 10 mM for the reaction. The extent of oxidation of L-glycerol 3-phosphate and L-3,4-dihydroxybutyl 1-phosphonate was determined enzymatically according to the method of Hohorst [8].

The reaction mixture used for following the reduction of dihydroxyacetone phosphate and 4-hydroxy-3-oxobutyl 1-phosphonate contained 50 μmoles triethanolamine buffer (pH 7.5) and no Li^+ but was identical in all other respects to that used for measuring the oxidation of glycerol 3-phosphate. The pH of all components were preadjusted to 7.5 individually. Solutions of NAD^+ and NADH were prepared freshly before use. Calculations were made using $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ as the molar extinction coefficient for NADH at 340 nm.

RESULTS

Baer et al. [4] reported that 2,3-dihydroxypropyl 1-phosphonate cannot serve as either a substrate or an inhibitor for rabbit muscle glycerol-3-phosphate dehydrogenase. Experiments performed in the course of the present investigations completely support these conclusions (data not shown). However, preliminary experiments revealed that 3,4-dihydroxybutyl 1-phosphonate could serve as a substrate for the muscle dehydrogenase. The fact that the substrate activity is not due to a contamination present in the preparation of the four-carbon phosphonate was revealed by measuring the extent of oxidation of glycerol 3-phosphate and 3,4-dihydroxybutyl 1-phosphonate under assay conditions favoring the completion of the reaction. Fig. 1 indicates that greater than 95% of the L-form of each of the compounds was converted to the oxidized form.

A series of experiments was initiated to determine the true K_m values for glycerol 3-phosphate, 3,4-dihydroxybutyl 1-phosphonate, and NAD^+. A Lineweaver-

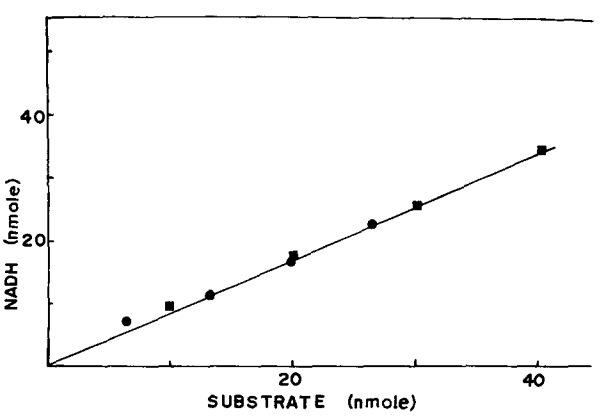

Fig. 1. Extent of oxidation of various concentrations of L-glycerol 3-phosphate (■—■) and L-3,4-dihydroxybutyl 1-phosphonate (●—●) as determined by the reduction of NAD^+ according to the method of Hohorst [8].

Burk plot [9] of the reciprocal of the initial velocity (v) versus the reciprocal of the molar concentration of NAD^+ (or glycerol 3-phosphate) at several different concentrations of glycerol 3-phosphate (or NAD^+) indicated, as expected [7], that the K_m for each substrate was dependent upon the concentration of the other substrate.

A similar series of experiments was performed for 3,4-dihydroxybutyl 1-phosphonate and NAD^+. The results were qualitatively similar to those obtained for the natural substrate. The true Michaelis constants for L-3,4-dihydroxybutyl 1-phosphonate and NAD^+ were obtained by treating the data obtained from the Lineweaver–Burk plots according to the method of Florini and Vestling [10] (Fig. 2A). The corresponding data for L-glycerol 3-phosphate and NAD^+ is presented in Fig. 2B. The

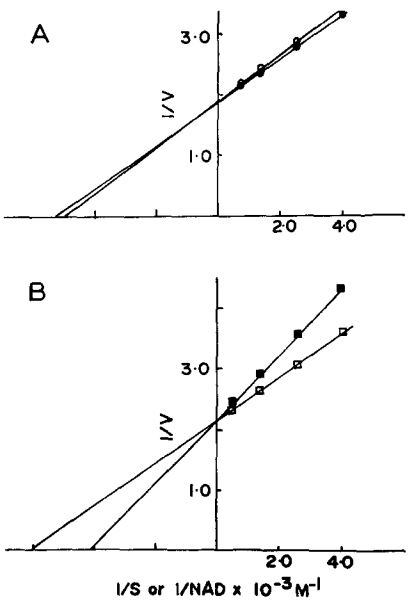

Fig. 2. Plot of the reciprocal of the maximal reaction velocity ($1/V$) versus the reciprocal of substrate concentration (1/S) or ($1/NAD^+$) for the oxidation of L-glycerol 3-phosphate and L-3,4-dihydroxybutyl 1-phosphonate. The reaction mixture and conditions for incubation were as described in the Materials and Methods section. Although the initial rates were actually measured during the first 30 s of reaction the rates are expressed as the change in absorbance over 5 min. The reaction was initiated by the addition of 250 ng of glycerol 3-phosphate dehydrogenase (95 units/mg of protein) per ml. The data is treated as described by Florini and Vestling [10]. A. ○—○, NAD^+; ●—●, L-3,4-dihydroxybutyl 1-phosphonate. B. □—□, NAD^+; ■—■, L-glycerol 3-phosphate.

true K_m values reported by Black [7] for the natural substrates as well as the values obtained in the present study are presented in Table I. It was not possible to calculate the true turnover numbers in terms of moles of enzyme because of questions concerning the presence of inactive proteins. However, the values for the ratios of V per g of enzyme for L-3,4-dihydroxybutyl 1-phosphonate and L-glycerol 3-phosphate are 0.066 and 0.059 moles/min per g, respectively.

It is clear that the rabbit muscle glycerol-3-phosphate dehydrogenase catalyzed the oxidation of glycerol 3-phosphate and 3,4-dihydroxybutyl 1-phospho-

TABLE I

	True K_m values observed (μM)	True K_m values reported by Black [7] (μM)
Glycerol 3-phosphate	240	260
NAD^+	160	160
3,4-Dihydroxybutyl 1-phosphonate	190	—
NAD^+	200	—
Dihydroxyacetone phosphate	130	80
NADH	2.9	6.3
4-Hydroxy-3-oxobutyl 1-phosphonate	182	—
NADH	2.2	—

nate at nearly the same rate. However, when the reaction was assayed in the reverse direction the reduction of dihydroxyacetone phosphate proceeded at approx. 25 times the rate of the reduction of 4-hydroxy-3-oxobutyl 1-phosphonate. However, the reduction of both the analog and the dihydroxyacetone phosphate proceeded to approximately the same extent (data not shown).

One possible explanation for the difference in activity might be a difference in K_m values. For this reason the true K_m values were obtained for 4-hydroxy-3-oxobutyl

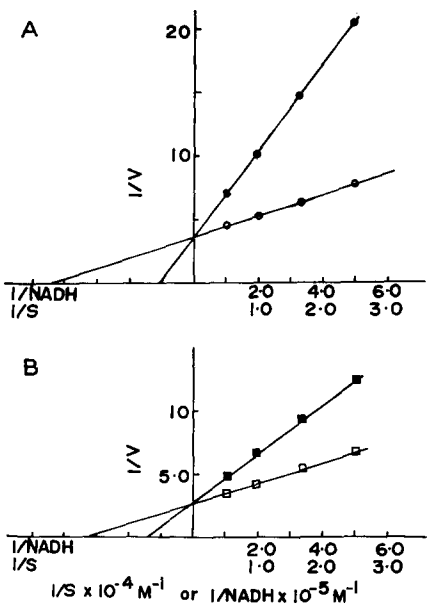

Fig. 3. Plot of the reciprocal of the maximal reaction velocity ($1/V$) versus the reciprocal of substrate concentration (1/S) or (1/NADH) for the reduction of dihydroxyacetone phosphate and 4-hydroxy-3-oxobutyl 1-phosphonate. The reaction mixture and conditions for incubation were as described in the Materials and Methods section. The initial rates are expressed as the change in absorbance over 10 min. The reaction was initiated by the addition of 9.4 ng of glycerol-3-phosphate dehydrogenase (134 units/mg of protein) per ml in the case of dihydroxyacetone phosphate and 188 ng of enzyme per ml of the same specific activity in the case of 4-hydroxy-3-oxobutyl 1-phosphonate. The data is treated as described by Florini and Vestling [10]. A. ○—○, NADH; ●—●, 4-hydroxy-3-oxobutyl 1-phosphonate. B. □—□, NADH; ■—■, dihydroxyacetone phosphate.

1-phosphonate, dihydroxyacetone phosphate, and NADH by the same general procedure described above for the components of the reverse reaction. The data for obtaining the true K_m for 4-hydroxy-3-oxobutyl 1-phosphonate and NADH is presented in Fig. 3A and that for obtaining the true K_m for dihydroxyacetone phosphate and NADH is depicted in Fig. 3B. The true K_m values for the substrates examined along with the values for the natural substrates reported by Black [7] are summarized in Table I. The ratios of V per g of enzyme for 4-hydroxy-3-oxobutyl 1-phosphonate and dihydroxyacetone phosphate are 0.024 and 0.61 moles/min per g of enzyme, respectively.

It is evident that the K_m values for the natural substrates and their corresponding analogs are quite similar (Table I). There is a major difference in pK_a values for phosphates and phosphonates. This difference could be responsible for the observation that the rate of oxidation of glycerol 3-phosphate and 3,4-dihydroxybutyl 1-phosphonate at pH 9.0 are similar while the rate of reduction of dihydroxyacetone phosphate was approx. 25-fold greater than that for 4-hydroxy-3-oxobutyl 1-phosphonate at pH 7.5. A higher pH optimum was expected for the phosphonic acid analogs because they are weaker acids than the natural substrates. When the affect of pH on rate was determined (Fig. 4), contrary to expectation, 4-hydroxy-3-oxobutyl 1-phosphonate had a lower pH optimum than the natural substrate.

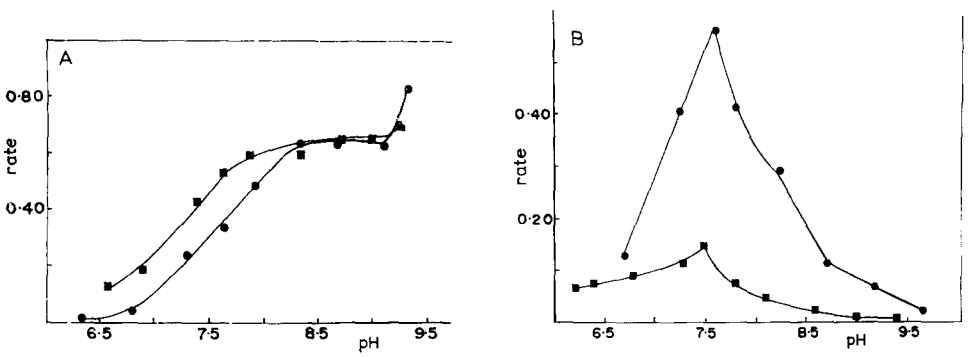

Fig. 4. A. Effect of pH on the initial rate of oxidation of L-glycerol 3-phosphate (●—●) and 3,4-dihydroxybutyl 1-phosphonate (■—■). The reaction mixture consisted of NAD^+, 25 mM; L-glycerol 3-phosphate or L-3,4-dihydroxybutyl 1-phosphonate, 5 mM; triethanolamine buffer, 50 mM; EDTA, 1 mM; mercaptoethanol, 1 mM; and 1 mg of bovine serum albumin per ml. The reactions were initiated by the addition of 150 ng of glycerol-3-phosphate dehydrogenase (134 units/mg) per ml. B. Effect of pH on the initial rate of reduction of dihydroxyacetone phosphate (●—●) and 4-hydroxy-3-oxobutyl 1-phosphonate (■—■). The reaction mixture consisted of NADH, 60 μM; dihydroxyacetone phosphate or 4-hydroxy-3-oxobutyl 1-phosphonate, 2 mM; triethanolamine buffer, 50 mM; EDTA, 1.0 mM; mercaptoethanol, 1.0 mM and 1 mg bovine serum albumin per ml. The reduction of dihydroxyacetone phosphate was initiated by the addition of 30 ng of L-glycerol-3-phosphate dehydrogenase (134 units/mg) per ml and the reduction of 4-hydroxy-3-oxobutyl 1-phosphonate by the addition of 150 ng of enzyme. The initial rates are expressed as the change in absorbance per 5 min. The incubation conditions were as described in Materials and Methods.

DISCUSSION

The phosphonic acid analogs of natural organic phosphates may have some potential as metabolic regulators and chemotherapeutic agents. It was previously

demonstrated that 3,4-dihydroxybutyl 1-phosphonate can inhibit the growth of *E. coli* [1] and has a profound effect upon phospholipid metabolism [2]. In vitro studies have been performed to evaluate the ability of several enzyme activities derived from *E. coli* to distinguish between the natural substrate and the phosphonic acid analogs. Glycerol-3-phosphate:CDP-diglyceride phosphatidyl transferase assayed by the procedure of Chang and Kennedy [11] can catalyze the incorporation of 3,4-dihydroxybutyl 1-phosphonate into a chloroform soluble material and the anabolic glycerol-3-phosphate: NAD(P) oxidoreductase assayed by the procedure of Kito and Pizer [12] can catalyze the reduction of 4-hydroxy-3-oxobutyl 1-phosphonate (Unpublished observations of this laboratory). However, 3,4-dihydroxybutyl 1-phosphonate is not a substrate and is not an inhibitor for the L-glycerol 3-phosphate acyltransferase described by Ray et al. [13] or the membrane-bound catabolic glycerol-3-phosphate dehydrogenase described by Weiner and Heppel [14] (unpublished observations of this laboratory). It is not clear at this time why the phosphonic acid analogs serve as substrates for certain enzymes and not for others. The steric and pK_a factors are undoubtedly important considerations.

The rabbit muscle dehydrogenase is the only enzyme studied to date that does not appear to distinguish between glycerol 3-phosphate and 3,4-dihydroxybutyl 1-phosphonate in the sense that both the K_m values and the rates of oxidation for each substrate are quite similar. However, this enzyme does distinguish between dihydroxyacetone phosphate and 4-hydroxy-3-oxobutyl 1-phosphonate. While the K_m values for these compounds are similar, there is a 25-fold greater rate of reduction of the natural substrate. The pH profiles (Fig. 4) do not appear to be consistent with the hypothesis that the differences in the rate of reduction are due to differences in the charge on the substrates. Studies with the muscle dehydrogenase indicate the importance of steric factors since 2,3-dihydroxypropyl-1-phosphonate is neither substrate or inhibitor for this enzyme. There may be many additional factors involved in explaining the differences in the substrate activity of the phosphonic acid analogs and their natural counterparts. Such explanations must await further experimentation. It seems clear that the phosphonic acid analogs have potential as metabolic inhibitors. It is therefore important to learn more about their interactions with enzymes.

ACKNOWLEDGMENT

This work was supported by Grant GB 33716 from the National Science Foundation and a grant from the City University of New York Faculty Research Award Program.

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