Biochimica et Biophysica Acta, 341 (1974) 248–255

BBA 67143

DIHYDROXYACETONE SULFATE AS SUBSTRATE ANALOGUE FOR α-GLYCEROL PHOSPHATE DEHYDROGENASE

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(Received September 10th, 1973)

SUMMARY

Dihydroxyacetone sulfate is a substrate for α-glycerolphosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD$^+$ 2-oxidoreductase, EC 1.1.1.8). The substitution of the sulfate for the phosphate does not affect the K_m of the enzyme for NADH. It affects, on the contrary, the K_m of the sugar ester, which is 45- to 100-fold larger, and the pH optimum, which is shifted from 8.0 to 6.0–6.5, with dihydroxyacetone sulfate as substrate. The similarity of the plot of the pK_m versus pH for dihydroxyacetone phosphate and dihydroxyacetone sulfate suggests that the same groups are involved in the binding of the two compounds to the enzyme. The kinetic data favor the hypothesis that the dianion of dihydroxyacetone phosphate is the active form of the substrate.

INTRODUCTION

α-Glycerolphosphate dehydrogenase (*sn*-glycerol-3-phosphate:NAD$^+$ 2-oxidoreductase, EC 1.1.1.8) is a soluble NAD$^+$-linked enzyme which catalyzes the interconversion of L-glycerol-3-phosphate and dihydroxyacetone 3-phosphate [1]. The enzyme has a molecular weight of 78 000 [2] and is composed of two subunits of 39 000 molecular weight [3, 4]. The dimeric structure of the enzyme is also confirmed by the analysis of the terminal amino acids. Two molecules of methionine are released by carboxypeptidase from 77 000 × g of enzyme; the amino terminals are, on the contrary, blocked [5]. Beside dihydroxyacetone phosphate and glycerol phosphate, the enzyme acts also on 1-fluoro-deoxy-DL-glycerol 3-phosphate [7]. It is also reported to be active on glyceraldehyde 3-phosphate [8]. We have not been able to confirm this.

We have now found that dihydroxyacetone sulfate is a substrate of α-glycerolphosphate dehydrogenase. The K_m of the new substrate is 45- to 100-fold larger that that of dihydroxyacetone phosphate, however, the pK_m versus pH curve shows the same pattern for both substrates. On the contrary, the pH optimum is shifted from 8.0 to 6.5, with dihydroxyacetone sulfate as substrate.

Abbreviation: DTNB: 5,5′-dithiobis-2-nitrobenzoic acid.

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MATERIALS AND METHODS

Chemicals

Cellulose phosphate (P11), nominal capacity 7.4 mequiv/g and DEAE-cellulose (DE11), nominal capacity 1.0 mequiv/g, were purchased from W. and R. Balston, Maidstone, Kent, U.K. Dihydroxyacetone sulfate was prepared as previously described. This product was at least 95% pure as judged by elemental and infrared analysis [9]. Dihydroxyacetone phosphate, dimethylketal dimonocyclohexylamine salt and dithioerythritol were from Sigma (U.S.A.). NADH was from Boehringer (West Germany). *p*-Hydroxymercuribenzoate was from the California Foundation, Los Angeles, U.S.A. 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) was from Aldrich Chemical Co., Milwaukee, U.S.A.

Preparation of enzyme

Column chromatography on phosphocellulose and DEAE-cellulose were performed at 20 °C; all the other operations were performed at 20 °C. α-Glycerophosphate dehydrogenase from rabbit muscle was prepared as follows. Rabbit muscle (675 g) was homogenized in a Waring Blendor with 1350 ml of 1 mM EDTA, pH 6.5. The homogenate was filtered through two layers of gauze, the residue was stirred with 350 ml of 1 mM EDTA, pH 6.5, filtered again and added to the first filtrate. The extract (1450 ml) was centrifuged for 10 min at 12 000 × *g*. To the supernatant were added with stirring 850 g (wet wt) of cellulose phosphate, that had been previously equilibrated with 5 mM EDTA, pH 6.0. pH was 6.0 in the course of the absorption and temperature was 20 °C. After this treatment more than 95% of the fructose 1,6-diphosphate aldolase activity was adsorbed. The mixture was filtered through a Buchner funnel, the resin was washed with 200 ml 5 mM EDTA, pH 6.0, and the filtrate (1450 ml) was treated again with 790 g (wet wt) of cellulose phosphate equilibrated as previously described. In this step α-glycerolphosphate dehydrogenase activity was completely adsorbed. The resin was then poured into a 8 cm × 29 cm column and washed with 1000 ml of 5 mM EDTA, pH 6.0. The resin was then eluted with 0.2 M NaCl in 5 mM EDTA, pH 6.0. The activity was recovered between 800 and 1200 ml. The eluate (400 ml) was treated with 116 g of $(NH_4)_2SO_4$, the protein precipitate was discarded, and the supernatant solution was treated again with 56 g of $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in the minimal volume of 1 mM EDTA, pH 6.0.

The solution (18 ml) was dialyzed 4 h against 1 mM EDTA, pH 6.0. The dialyzed solution was poured through a 2.2 cm × 13 cm (50 ml) DEAE column, equilibrated with 8 mM phosphate buffer, pH 7.0. The resin was washed with the same buffer (250 ml) until the absorbance at 280 nm was zero. The resin was then eluted with 20 mM phosphate buffer, pH 7.0. α-Glycerolphosphate dehydrogenase was recovered between 40 and 85 ml. The eluate (45 ml) was treated with 19.6 g of $(NH_4)_2SO_4$ and the precipitate was dissolved with 1 mM EDTA, pH 6.0 (2 ml).

Enzymatic activity was routinely assayed at 22 °C in the presence of 25 mM Tris–HCl buffer. 0.1 mM NADH and either 0.4 mM dihydroxyacetone phosphate or 3.6 mM dihydroxyacetone sulfate. The pH was 7.5 with the first of the two substrates and 6.5 with the second one.

A unit was defined as that amount of enzyme that catalyzes the reduction of 1 μmole of dihydroxyacetone phosphate per min.

Protein concentration was measured from the absorbance at 280 nm. The method was standardized against a known dry weight of dialyzed enzyme. A solution containing 1 mg/ml (light path 1 cm) yielded an absorbance value of 0.600 at 280 nm.

Disc gel electrophoresis was performed at 2 °C in 7% crosslinked polyacrylamide gel polymerized in 7 mm × 0.4 mm tubes. The current was 5 mA/tube. Gels were prepared and electrophoresis performed for 2 h in 0.2 M glycine and 0.025 M Tris–HCl buffer, pH 8.5. Enzymatic reaction kinetics were studied for the forward reaction. For each determination of K_m and V four substrate concentrations were used ranging either from 250 to 60 μM (dihydroxyacetone sulfate) or from 33 to 8 μM (dihydroxyacetone phosphate). NADH was 0.1 mM. Glycerolphosphate dehydrogenase (spec. act. 170–190 units/mg) was between 0.056 and 4.5 μg/ml depending on the pH and the substrate used. Either 25 mM Tris–HCl buffer (pH from 6.0 to 8.5) or 25 mM Tris–acetate buffer (pH 5.5–8.5) were employed. I was adjusted to 0.025 by the addition of NaCl. Temperature was 22 °C. Double-reciprocal plots ($1/v$ against $1/s$) were made by a least squares fit.

RESULTS

Dihydroxyacetone sulfate is a substrate for α-glycerolphosphate dehydrogenase

The preparation of crystalline α-glycerolphosphate dehydrogenase purchased from Boehringer catalyzes the NADH-dependent reduction of both dihydroxyacetone phosphate and sulfate. NADH was consumed in an equimolar amount to the dihydroxyacetone sulfate added. The product of the reaction was chromatographically indistinguishable from the glycerol sulfate obtained by reduction of dihydroxyacetone sulfate with $NaBH_4$. To investigate whether the activity on dihydroxyacetone sulfate was linked to a contaminant protein, α-glycerolphosphate dehydrogenase was prepared from rabbit muscle. The purification of the extracts, following either one of the two activities, yielded an enzyme preparation which presented a single protein band when submitted to disc gel electrophoresis at pH 8.5. The specific activity, with dihydroxyacetone phosphate as the substrate, was 204 international units, that is of the same order of magnitude as that obtained by Telegdi [10] which, apparently, records the highest specific activity so far reported. In the course of the purification procedure, the ratio between the activity on dihydroxyacetone phosphate and the activity on dihydroxyacetone sulfate changed from 1.35 to 1.79, even though the two activities coincided throughout all the steps (phosphocellulose and DEAE chromatography included) (Table I).

The original ratio was, however, restored by treating the enzyme for 3 h at 2 °C with 10 mM dithioerythritol (Table II).

Effect of the modification of cysteine and histidine residues on catalytic activity

Approximately 16 sulfhydryl groups per molecule of enzyme (molecular weight 77 000) were titrated with DTNB in the presence of 8 M urea following the procedure of Ellman [11].

In the absence of urea, titration of about 10 sulfhydryl groups with *p*-hydroxy-

TABLE I

PURIFICATION OF GLYCEROLPHOSPHATE DEHYDROGENASE FROM RABBIT MUSCLE

	Dihydroxyacetone phosphate as substrate (a)		Dihydroxyacetone sulfate as substrate (b)		a/b
	Total units	Specific activity (a)	Total units	Specific activity (b)	
Homogenate	14 500	0.38	10 500	0.28	1.35
Phosphocellulose	10 050	24.9	4 500	11.2	2.22
$(NH_4)_2SO_4$ and dialysis	9 000	39.3	4 000	17.7	2.2
DEAE-cellulose	4 100	204	2 260	114	1.79

TABLE II

EFFECT OF DITHIOERYTHRITOL ON THE RATE OF REDUCTION OF DIHYDROXYACETONE SULFATE

The incubation mixtures (0.1 ml) contained glycerolphosphate dehydrogenase, 1 mg, and 10 mM dithioerythritol where indicated. pH was 6.0. At the beginning of the experiment and after 3 h of incubation at 2 °C, samples were taken and dehydrogenase activity was assayed as described in Methods.

Assay system	Specific activity (units/mg)	
	Before treatment	After treatment
NADH + dihydroxyacetone phosphate	195	198
NADH + dihydroxyacetone sulfate	85	150

mercuribenzoate inactivated almost completely the dehydrogenase activity on both dihydroxyacetone phosphate and sulfate (Fig. 1).

Carbethoxylation of 6 histidyl residues per molecule of enzyme was found to lead to a 70% decrease of the activity on dihydroxyacetone sulfate, and to leave unchanged the activity on dihydroxyacetone phosphate (Fig. 2).

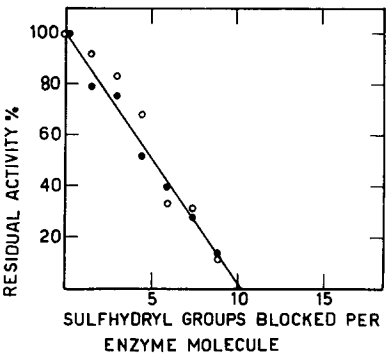
The partially blocked enzyme was stable for a few hours at 2 °C and pH 6.0.

Kinetic properties of the dehydrogenase reaction

The effect of NADH concentration on the rate of the reaction was studied, and it was found that the K_m for NADH was essentially the same with either dihydroxyacetone phosphate or sulfate at three different pH values (Table III).

The effect of the concentration of either dihydroxyacetone sulfate or phosphate on the rate of the reaction, at different pH, was also studied.

The experiments were performed at constant ionic strength. This was obtained by suitable addition of NaCl. However, essentially the same results were obtained in separate experiments where the addition of NaCl was omitted. In the experiments where Tris–acetate instead of Tris–HCl was used, a significant increase of the catalytic activity was noticed.



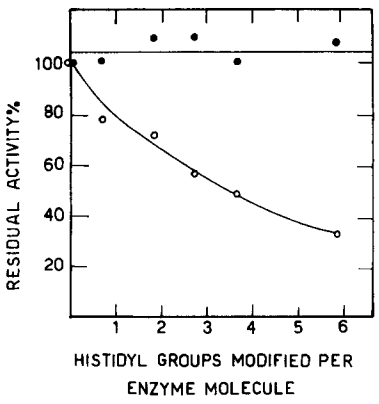


Fig. 1. Effect of the treatment with *p*-hydroxymercuribenzoate on the dehydrogenase activity. The incubation mixtures (1 ml) contained 6.8 nmoles of enzyme (specific activity 170 units/mg of protein) in 50 mM acetate buffer, pH 6.0. Temperature was 22 °C. At the beginning of the experiment and every 5 min thereafter, 0.005 ml of 2 mM *p*-hydroxymercuribenzoate was added for a total of 6 additions. The reaction with *p*-hydroxymercuribenzoate was determined by the increase in the absorbance at 250 nm. Identical additions of the reagent were made to the sample and to the blank. The readings were taken in quartz cuvettes with a light path of 1 cm. The number of sulfhydryl groups reacted was determined on the basis of a molecular weight of 77 000 [2]; glutathione was used as a standard. Dehydrogenase activity, as a function of sulfhydryl groups titration, was determined with 0.4 mM dihydroxyacetone phosphate (●—●), or 3.6 mM dihydroxyacetone sulfate (○—○), 0.1 mM NADH, and 25 mM Tris–HCl buffer, pH 7.0.

Fig. 2. Effect of carbethoxylation of histidyl residues on the dehydrogenase activity. The incubation mixtures (1 ml) contained 11.4 nmoles of enzyme (specific activity 170 units/mg of protein) in 50 mM acetate buffer, pH 6.0. At the beginning of the experiment and every 10 min thereafter, 0.001 ml of 0.1 M ethylpyrocarbonate in ethanol was added for a total of 6 additions. The reaction was determined by the increase in the absorbance at 242 nm (E_M, 3200) [19]. Identical additions of the reagent were made to the sample and to the blank. The readings were taken in quartz cuvettes with a light path of 1 cm. The number of histidyl groups reacted was determined on the basis of a molecular weight of 77 000 [2]. Dehydrogenase activity was determined as described in Fig. 1. at pH 7.0 with both dihydroxyacetone phosphate (●—●) and sulfate (○—○).

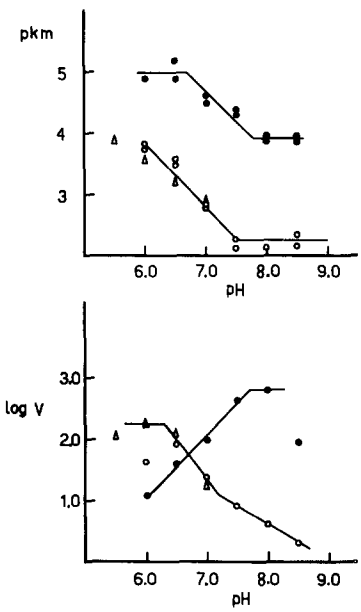
TABLE III

EFFECT OF THE pH ON THE K_m OF THE NADH OXIDATION REACTION IN THE PRESENCE OF EITHER DIHYDROXYACETONE PHOSPHATE OR DIHYDROXYACETONE SULFATE AS SUBSTRATES

The incubation mixtures (1 ml) contained glycerolphosphate dehydrogenase (specific activity 190 units/mg): from 9 to 0.045 μg (depending of the pH of the assay system); 25 mM Tris–HCl buffer at the pH indicated, from 50 to 3 μM NADH and either 0.5 mM dihydroxyacetone phosphate or 2.7 mM dihydroxyacetone sulfate. *I* was adjusted to 0.025 by the addition of NaCl. Temperature was 22 °C. The progress of the reaction was followed in a ACTA III, Beckman recording spectrophotometer.

Assay system	K_m for NADH (μM)		
	pH 6.5	pH 7.5	pH 8.0
NADH + dihydroxyacetone phosphate	12.5	5.1	3.5
NADH + dihydroxyacetone sulfate	10.0	4.0	3.4

The plot of pK_m against pH (Fig. 3) showed that two groups with pK values of 6.75 and 7.75 were relevant in the catalysis with dihydroxyacetone phosphate as substrate, while, with dihydroxyacetone sulfate as substrate, only a group with a pK of 7.5 was detected. According to Dixon [12], the two groups with pK values of 7.5 and 7.75, respectively, should belong to the enzyme–coenzyme–substrate complexes, while the groups with a pK of 6.75 could belong either to the enzyme–NADH complex or to dihydroxyacetone phosphate (pK_2 = 6.45) [13]. When the correction for the dissociation of the phosphate group of the substrate was included in the calculation of the pK_m, the results presented in Fig. 4 were obtained.



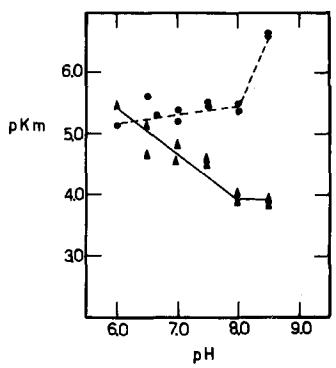


Fig. 3. pK_m values (upper part of the figure) and log V values (lower part of the figure) for dihydroxyacetone phosphate and sulfate as a function of pH. Dihydroxyacetone phosphate (●—●) Dihydroxyacetone sulfate (○—○, Tris–HCl buffer); (△—△, Tris–acetate buffer).

Fig. 4. pK_m values for the monoanion (●—●) and for the dianion (▲—▲) of dihydroxyacetone phosphate, calcultaed from the data of Fig. 3, on the assumption that either one of the two forms is the active substrate.

The pH optimum was 8.0 with dihydroxyacetone phosphate as the substrate, and 6.0 (Tris–acetate buffer) or 6.5 (Tris–HCl buffer) with dihydroxyacetone sulfate as the substrate. Differences in the pH optimum of the enzyme depending on the buffer system used were already reported [14].

Plot of V versus pH showed also that the rate of the reduction of dihydroxyacetone phosphate was increased by the deprotonation of groups with a pK of 7.75, while the rate of the reduction of dihydroxyacetone sulfate was dependent of the deprotonation of groups with pK 6.0–6.5 and 7.5 respectively (Fig. 3).

DISCUSSION

Dihydroxyacetone sulfate is a good substrate for glycerolphosphate dehydrogenase.

Substitution of dihydroxyacetone sulfate for dihydroxyacetone phosphate does not affect the K_m for NADH. This is as expected from the compulsory binding of the coenzyme and substrate to the enzyme [15, 16].

The plot of pK_m versus pH for the two substrates, differs essentially in the presence of a bend at pH 6.75 with dihydroxyacetone phosphate ($pK_2 = 6.45$). This bend is absent with dihydroxyacetone sulfate. It is likely, therefore, that the bend is due to the conversion of dihydroxyacetone phosphate from the monoanionic to the dianionic form and not to the change of the ionic state of an histidine residue [17]. Assuming that the dianion of dihydroxyacetone phosphate is the active substrate, and correcting the pK_m curve accordingly, the dependence, on pH, of the pK_m for the two substrates become almost identical. The ratio between the K_m for dihydroxyacetone sulfate and phosphate oscillates between 45 and 100 between pH 6.0 and 8.5.

According to Reynolds et al. [18], keto-dihydroxyacetone phosphate is the primary reactive species in the reaction catalyzed by glycerolphosphate dehydrogenase. The intensity of the absorbance peak at 263 nm, characteristic of the carbonyl group, is not very different in dihydroxyacetone sulfate ($\varepsilon_{263\,nm} = 10.2$) and in dihydroxyacetone phosphate ($\varepsilon_{263\,nm} = 26.0$). The extent of the hydration of the carbonyl group cannot be, therefore, the major reason for the difference in the K_m. Also the steric hindrance of the two compounds should be similar. The difference in the charge between the dianionic form of the phosphate and the sulfate esters could be, on the contrary, an important factor.

The large difference in the pH optimum with the two substrates is also of interest. For technical reasons, we have left partially unexplored the ascending limb of the activity versus pH curve with dihydroxyacetone sulfate as substrate, and the descending limb with dihydroxyacetone phosphate as substrate. It is clear, however, that in the presence of the latter, the rate of the reaction increase with the deprotonation of group(s) with a pK of 7.75, while, in the presence of the sulfate ester, the rate of the reaction decreases with the deprotonation of group(s) with a pK of 6.0–6.5. This last effect is partially counteracted by the deprotonation of group(s) with pK between 7.3 and 7.5.

ACKNOWLEDGMENT

This work was supported by the grant CT2.00768.04 of the Italian Consiglio Nazionale delle Richerche and by the NATO Grant 751.

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