STUDIES ON THE MECHANISM BY WHICH INORGANIC ARSENATE FACILITATES THE ENZYMATIC REDUCTION OF DIHYDROXYACETONE BY α-GLYCEROPHOSPHATE DEHYDROGENASE

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1. Introduction

Glycerol 3-phosphate dehydrogenase (glycerol 3-phosphate:NAD oxidoreductase, EC 1.1.1.8) is a soluble NAD-linked enzyme which catalyzes the interconversion of dihydroxyacetone phosphate and L-α-glycerol 3-phosphate [1,2]. It has been reported that several enzymes whose natural substrates are the phosphorylated derivatives also act on the non-phosphorylated analogs if arsenate is present [3]. A kinetic study of the effect of arsenate on the activity of glucose phosphate isomerase (EC 5.3.1.9) when fructose is used as substrate [4] suggests that, subsequent to the binding of arsenate by the enzyme, fructose is bound, following which an interaction occurs between arsenate and the hydroxyl group of fructose, yielding an ester-like compound capable of substituting for the homologous phosphate ester fructose 6-phosphate.

Furthermore, studies of the effects of inorganic arsenate on the reactions catalyzed by purified enzymes in which inorganic phosphate is a reactant, have provided results suggesting that inorganic arsenate reacts to form an arsenylated intermediate that is unstable in aqueous systems [5]. This paper reports results obtained in a study of the effect of arsenate on the glycerol 3-phosphate dehydrogenase-mediated reduction of dihydroxyacetone. The evidence obtained suggests that, at least in this case, an ester-like compound is formed between DHA and arsenate without involvement of the enzyme active site. The resulting analog is acted upon by the enzyme in a manner similar to that for the phosphorylated substrate.

2. Materials and methods

L-α-Glycerolphosphate dehydrogenase from rabbit skeletal muscle (spec. act. 180 units/mg, pH 7.5, 25°C) and Tris–HCl were purchased from Sigma, St Louis. Dihydroxyacetone phosphate as the dimethylketal dicyclohexylammonium salt, NAD^+, NADH (disodium salt) and triethanolamine–HCl were obtained from Boehringer-Mannheim. Dihydroxyacetone and 3-hydroxy-2-butanone were from Eastman Kodak, Rochester, NY; hydroxy-2-propanone from Pfaltz-Bauer, Flushing, NY; sodium arsenate and 1,2-propanediol from Fisher Scientific, NJ; cyclohexanone was from Rhone-Poulenc, France. ^{74}As, as sodium arsenate, was obtained from the Radiochemical Centre, Amersham, England. All other reagents were the best available grade.

Enzyme activity was measured as described previously [6]. The stopped-flow experiments were carried out in a Durrum Spectrophotometer, model D-160, at 25°C. Proton nuclear magnetic resonance spectra were obtained with a Varian XL-100 NMR Spectrometer, using tetramethylsilane as internal standard. Labile protons of the samples were exchanged with deuterium through repeated solution-evaporation in D_2O (99.98%). Three-times recrystallized dihydroxyacetone was used throughout. The possibility of binding of arsenate to the enzyme was checked by gel filtration as described by Hummel

and Dreyer [7] using ^{73}As (as sodium arsenate). The method of Wilkinson [8] was used for calculation of the kinetic parameters. Fluorometric titration of the binding of NADH to glycerol 3-phosphate dehydrogenase was done in an Aminco-Bowman spectrofluorimeter. The data was subjected to a modified Scatchard analysis [9] in order to evaluate the number of binding sites and the dissociation constant.

3. Results and discussion

When dihydroxyacetone is used as a substrate for GPDH, no reaction can be detected unless arsenate is present in the reaction mixture. Furthermore, DHA probably does not bind to the enzyme in the absence of arsenate, as suggested by the fact that no inhibition of the reaction with the phosphorylated substrate (DHAP), is observed in the presence of the non-phosphorylated analog. Increasing arsenate concentration increases the rate of catalysis and, as shown in fig.1, saturation is obtained at about 120 mM arsenate.

At saturating concentrations of arsenate (270 mM), the plot of initial velocity versus DHA concentration displays a Michael-Menten pattern (fig.2). A similar pattern was obtained when NADH was the varied substrate. The calculated K_m app. for DHA is 64 mM, K_m app. for NADH 15 μM and K_m app. for arsenate was 27 mM. The value of 64 mM for DHA in the presence of arsenate is about 30-times higher than previously published values for the phosphorylated substrates [6,10,11]. The value of K_m app. for NADH is close to the values reported for the reaction with the phosphorylated substrate [6,10,11].

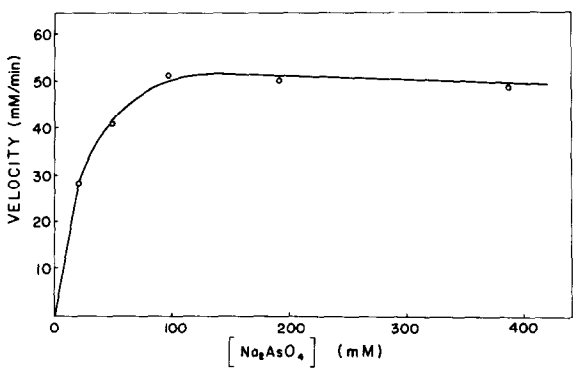

Fig.1. Effect of arsenate on the enzymatic reduction of DHA catalyzed by GPDH. DHA concentration was 270 mM, NADH was 40 μM, Tris–HCl 50 mM, pH 7.5, 25°C.

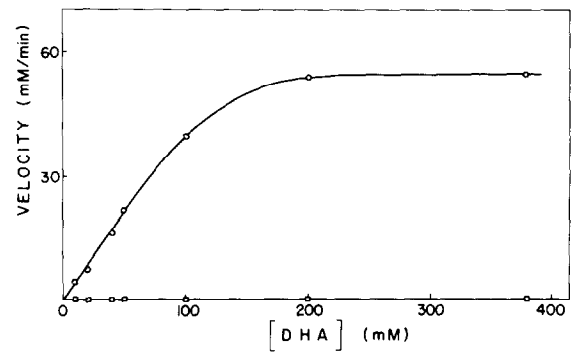

Fig.2. Velocity versus substrate curve for the enzymatic reduction of DHA by GPDH. NADH concentration was 40 μM, Tris–HCl 50 mM, pH 7.5, 25°C. (□——□) Without arsenate; (○——○) 270 mM arsenate.

The dependence of the initial rate of reaction on pH, for the enzymatic reduction of DHA in the presence of arsenate is shown in fig.3. The optimum pH app. of 7.3 is very close to the pH optimum (7.5) for the enzymatic reduction of DHAP [12].

These results suggest that when arsenate is present, the enzymatic reduction of DHA follows a kinetic

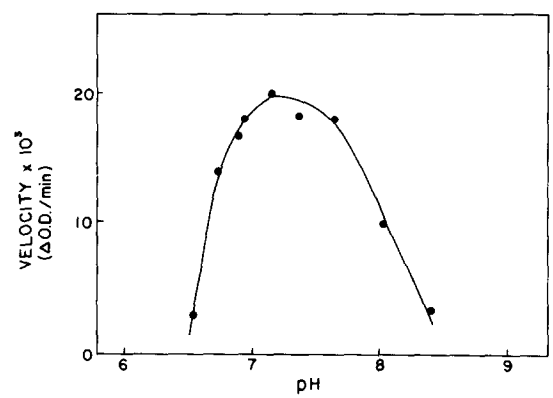

Fig.3. pH-Rate profile for GPDH-catalyzed reduction of dihydroxyacetone in the presence of 200 mM arsenate and 50 μM NADH. GPDH concentration was 5 μg/ml, 25°C; pH-Range 6–7, Triethanolamine–HCl; pH-range 7.2–8.5, Tris–HCl.

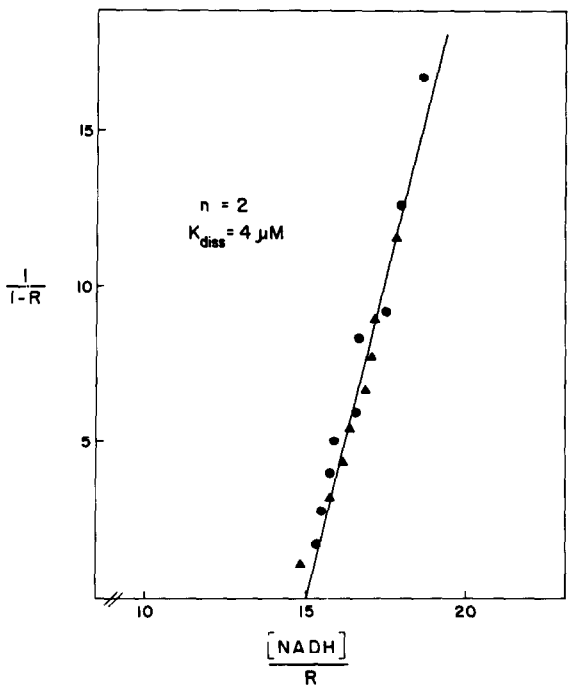

Fig.4. Modified Scatchard plot for the enhancement of fluorescence as NADH is bound to GPDH. Enzyme concentration was 7.7 μM, 25°C. (●——●) No arsenate present; (▲——▲) in the presence of 190 mM arsenate. Tris–HCl 50 mM, pH 7.5.

pattern similar to that for the reduction of DHAP. The principal difference is that the substrate inhibition observed with the phosphorylated substrate [13] is not present when the non-phosphorylated analog is used. Other substrate analogs tested such as hydroxy-2-propanone, 3-hydroxy-2-butanone and 1,2-propanediol showed no reactivity even in the presence of arsenate.

Gel filtration experiments designed to study the possibility of a direct effect of arsenate on the enzyme showed that, even at concentrations of arsenate beyond saturating ones (up to 0.5 M), there is no detectable binding of the anion to the protein. This is also true in the presence of DHA. These results and the fact that neither the number of sites nor the dissociation constant for NADH are altered by arsenate (fig.4) suggested that arsenate may be acting on the substrate, DHA, directly, or through a ternary complex (As–*E*–DHA), but not on the enzyme.

These two alternatives were clearly differentiated in the stopped-flow experiments. When arsenate is placed in the same syringe with the enzyme and NADH, the reaction exhibits a lag phase before becoming zero-order (fig.5a). However, when arsenate and dihydroxyacetone are mixed together in the same syringe, this lag phase is not observed; the plot of product formation versus time is linear from the beginning (fig.5b). This result strongly

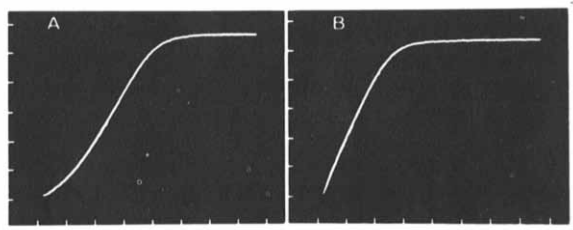

Fig.5. Progress curves for GPDH-catalyzed reduction of dihydroxyacetone in the presence of arsenate. [DHA] 250 mM, [NADH] 36 μM, [arsenate] 250 M, GPDH 1 μM. Tris–HCl 50 mM, pH 7.5. Ordinate, 0.5 V/div; Abscissa, 0.1 seg/div. (A) Arsenate and DHA in separate syringes. (B) Arsenate and DHA in the same syringe.

Table 1
Stopped-flow kinetics experiments

Syringe 1	Syringe 2	Curve observed
DHA, GPDH, NADH	Arsenate	With lag
GPDH, DHA	Arsenate, NADH	With lag
GPDH, Arsenate	DHA, NADH	With lag
GPDH	DHA, Arsenate, NADH	Zero order
GPDH, NADH, Arsenate	DHA, Arsenate	Zero order

After mixing the solutions from syringes 1 and 2, the light absorption was measured, indicating the appearance of NAD. Graphs are in figs.4 and 5

suggests that an adduct between DHA and arsenate must be formed in order to generate a suitable enzyme substrate. Other combinations were also tested, and the results, summarized in table 1, support this conclusion.

Formation of the As–DHA adduct seems to be a spontaneous reaction by which an ester-like product is probably formed.

Nuclear magnetic resonance studies of the interaction of arsenate and DHA (both compounds used at 10% concentration by weight) show that the NMR spectrum of DHA changes significantly upon addition of arsenate. The main change observed is a shift of the signal from the α-protons of DHA by 0.1 ppm toward higher frequency. When phosphate is used instead of arsenate, no detectable change in the NMR spectrum is observed. The lack of effect of phosphate is in agreement with the fact that this anion does not promote any detectable activity when used with the nonphosphorylated analog of the substrate.

The experimental results described strongly suggest that the enzymatic reduction of dihydroxyacetone, catalyzed by L-α-glycerol 3-phosphate dehydrogenase in the presence of arsenate, is mediated by the spontaneous formation of a DHA–arsenate complex, which is then recognized by the enzyme as a substrate.

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