Lack of asymmetry in the active sites of tetrameric D-glyceraldehyde-3-phosphate dehydrogenase during alkylation in the crystalline state

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1. INTRODUCTION

D-Glyceraldehyde-3-phosphate dehydrogenase (GPDH; EC 1.2.1.12) catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate (GAP) through the formation of an acyl thiol enzyme. It is composed of 4 subunits of identical amino acid sequence (review [1]). The X-ray diffraction studies indicated that the subunit structure of crystalline GPDH from different sources [2–6], except that from *Bacillus stearothermophilus* [7], displays a pairwise asymmetry which may have functional consequences. In the case of lobster GPDH crystals it was also suggested that Cys-149 bearing the essential thiol group interacted with His-176 only in 2 of the 4 subunits [2]. This implies that the mercaptide–imidazolium ion-pair formation between Cys-149 and His-176, which enhances the nucleophilicity of the thiol group below its pK_a [8], is formed in 2 subunits only. Hence, it could be expected that the essential cysteine residues in the crystal may react at different rates in a simple alkylation. It has been shown that all 4 reactive sulphydryl groups of GPDH crystals could be alkylated with the negatively charged iodoacetate [9], but quantitative data on the reaction rates were not presented.

To test a possible difference in the reactivities of the essential –SH groups of the tetrameric GPDH in the crystalline state, we have chosen iodoacetamide, which is known to react equally with all four –SH groups in solution [8,10,11]. This is an important point since several alkylating NAD-analogues [12] and some negatively charged reactants, like β-(2-furyl)acryloyl phosphate [13] or *p*-hydroxymercuribenzoate [14] exhibit 'half-of-the-sites' reactivity even in solution and thus they are not suitable for studying this problem. Furthermore, with the neutral iodoacetamide, electrostatic effects which may complicate the reaction could also be eliminated. We have found that all 4 subunits of the crystalline enzyme react with iodoacetamide at identical rates and the rates of alkylation are similar in crystal and in solution.

2. MATERIALS AND METHODS

Four-times recrystallized pig muscle GPDH was prepared as in [15]. The M_r of the tetrameric enzyme was taken as 144 000 [16,17]. GPDH activity was assayed at 25°C in 0.1 M glycine buffer (pH 8.5) containing 0.1 M potassium chloride, 7×10^{-9} M active enzyme, 2×10^{-3} M NAD, 2×10^{-3} M GAP and 1×10^{-3} M Na_2HASO_4. Specific activity of the enzyme was 130 units/mg. The concentration of the –SH groups was measured with 5,5′-dithio-bis(2-nitrobenzoic acid) by using Ellman's procedure [18].

GAP was prepared from fructose 1,6-diphosphate (Reanal) according to [19]. All other chemicals were commercial preparations of reagent grade. Iodoacetamide and ammonium sulfate were recrystallized from carbon tetrachloride and from water, respectively. Concentration of the stock solution of iodoacetamide was determined spectrophotometrically by using the absorption coefficient $\varepsilon_{275} = 372$ $M^{-1} \cdot cm^{-1}$ [20].

Alkylations of crystalline GPDH were performed after centrifuging and resuspending the enzyme

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crystals in 0.1 M imidazole buffer containing 3.65 M ammonium sulfate at different pH-values. The amount of dissolved enzyme in the reaction mixture was < 1% of the total protein content, as measured in the supernatant after centrifugation. Solution studies were carried out in 0.1 M imidazole buffer in the presence and in the absence of 2.75 M ammonium sulfate.

Kinetics were followed both in crystal and in solution at 25°C under pseudo first-order conditions with an excess of iodoacetamide by reacting 0.8–3.2 × 10^{-5} M protein (tetramer) with 1.1–3.1 × 10^{-3} M iodoacetamide. Diffusion of iodoacetamide into the crystal was not a limiting factor. Aliquots withdrawn from the reaction mixtures were diluted 50-fold with 0.1 M KCl which practically stopped alkylation as indicated by the fact that the activity of diluted samples did not decrease with time. Residual activity was determined with GAP substrate in the assay mixture above. Alkylations in solution were also followed at 370 nm by measuring the disappearance of the broad absorption band characteristic of the enzyme–coenzyme complex [21] in a Cary 118 recording spectrophotometer equipped with a thermostatted cell compartment. Second-order rate constants were calculated by dividing the first-order rate constants by concentration of iodoacetamide.

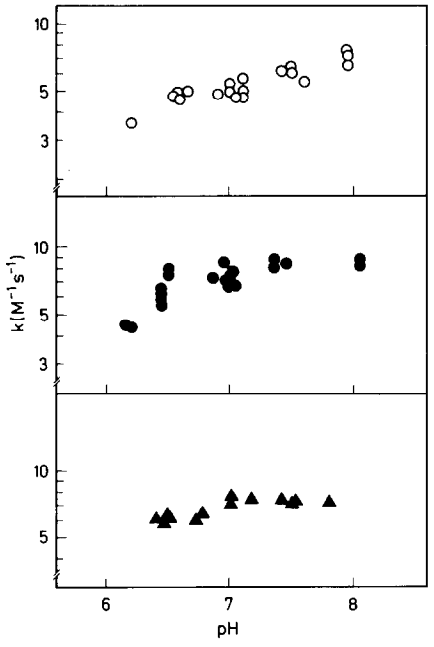

Fig.1. The pH-dependence of alkylation of D-glyceraldehyde-3-phosphate dehydrogenase by iodoacetamide in solution in the absence (○) and in the presence of 2.75 M ammonium sulfate (●), and in crystals (▲). Reaction conditions are in section 2.

3. RESULTS AND DISCUSSION

The pH-dependence of alkylation rate of GPDH with iodoacetamide displays a double sigmoid curve [8]. This profile shows a relatively pH-independent region around pH 7, where a mercaptide–imidazolium ion-pair exist [8]. The comparison of alkylations of crystalline and dissolved enzymes could be in this pH-independent range. We have measured the alkylation of the enzyme at several pH values around pH 7, both in solution with and without ammonium sulfate, and in the crystalline state. The pH-dependencies of rate constants are similar in all 3 cases (fig.1). The values of the second-order rate constants in table 1 show that the reactivity of the essential –SH group of GPDH does not differ in the dissolved and crystalline forms. This is consistent with results on acylation of GPDH by a substrate analogue, β-(2-furyl)acryloyl phosphate where the enzyme exhibits half-site reactivity in both physical states [13]. Whereas acylation with β-(2-furyl)acryloyl phosphate is strongly inhibited by the high concentration of ammonium sulfate, alkylation with the neutral iodoacetamide is much less affected.

Iodoacetamide inactivates GPDH crystals in a monophasic reaction down to < 10% residual

Table 1

Second-order rate constants of alkylation of D-glyceraldehyde-3-phosphate dehydrogenase with iodoacetamide at 25°C

Physical state of GPDH	k ($M^{-1} \cdot s^{-1}$)	
	pH 7	pH 7.5
Dissolved		
without $(NH_4)_2SO_4$	4.8	6.0
with 2.75 M $(NH_4)_2SO_4$	7.5	8.5
Crystalline		
with 3.65 M $(NH_4)_2SO_4$	7.5	7.3

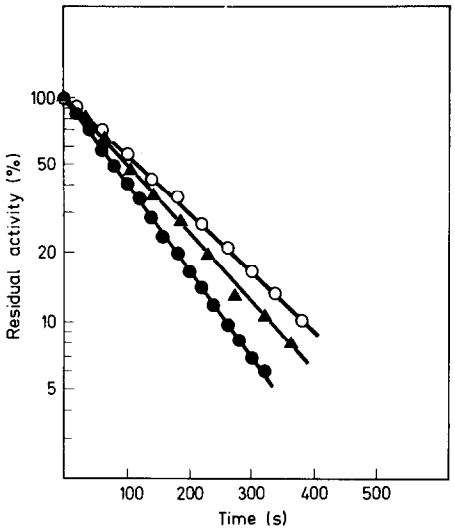

Fig.2. Kinetics of alkylation of D-glyceraldehyde-3-phosphate dehydrogenase by iodoacetamide at pH 7.5 in solution in the absence (○) and in the presence of 2.75 M ammonium sulfate (●), and in crystals (▲). Reaction conditions are in section 2.

activity (fig.2). Determination of the –SH group content of the enzyme with 5,5′-dithio-bis(2-nitrobenzoic acid) also indicated the disappearance of 4 –SH groups/tetramer during alkylation. It should be noted that a monophasic reaction could also occur if two distinct conformations of the Cys-149–His-176 diad were in a rapid equilibrium. This is, however, not probable in the light of X-ray diffraction studies, which indicate the presence of two relatively stable forms [2].

We show that the chemical behaviour of GPDH in the crystal is similar to that observed in solution where the essential –SH groups of all 4 subunits exist in an equally activated mercaptide–imidazolium ion-pair form [8]. These results are not consistent with a pairwise asymmetry suggested previously for the 2 groups at the active sites of GPDH.

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