Biochimica et Biophysica Acta, 527 (1978) 319—326

BBA 68613

THE ROLE OF ARGININE RESIDUES IN THE FUNCTION OF D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

N.K. NAGRADOVA, R.A. ASRYANTS and N.V. BENKEVICH

A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow (U.S.S.R.)

(Received March 3rd, 1978)
(Revised manuscript received June 29th, 1978)

Summary

Inactivation of apo-glyceraldehyde-3-phosphate dehydrogenase from rat skeletal muscle in the presence of butanedione is the result of modification of one arginyl residue per subunit of the tetrameric enzyme molecule. The loss of activity follows pseudo-first-order kinetics. NAD^+ increases the apparent first-order rate constant of inactivation.

The effect of NAD^+ on the enzyme inactivation is cooperative (Hill coefficient = 2.3—3.2). Glyceraldehyde 3-phosphate protected the holoenzyme against inactivation, decreasing the rate constant of the reaction. At saturating concentrations of substrate the protection was complete. The Hill plot demonstrates that the effect is cooperative. This suggests that subunit interactions in the tetrameric holoenzyme molecule may affect the reactivity of the essential arginyl residues. In contrast, glyceraldehyde 3-phosphate had no effect on the rate of inactivation of the apoenzyme in the presence of butanedione. 100 mM inorganic phosphate protected both the apoenzyme and holoenzyme against inactivation. The involvement of the microenvironment of the arginyl residues in the functionally important conformational changes of the enzyme is discussed.

Introduction

Chemical modification studies with the use of reagents specific for arginyl residues (butanedione, phenylglyoxal) have shown that the enzymes which act upon anionic substrates contain arginyl residues at their active sites [1]. In previous publications we described inactivation of glyceraldehyde-3-phosphate dehydrogenase isolated from baker's yeast [2] and rat skeletal muscle [3] in the presence of butanedione. In both cases, the loss of two arginine residues per subunit of the tetrameric enzyme molecule was observed. Other amino acid residues were not affected by this treatment. No detectable conformational

changes were observed in the modified enzyme preparation. Investigation of the coenzyme-binding properties of the modified yeast enzyme [2], as well as analysis of the effect of NAD^+ on the inactivation of the rat muscle dehydrogenase [3] led us to conclude that arginyl residues affected by modification are located at the catalytic region of the active center, probably in the vicinity of the nicotinamide-binding site. This paper presents evidence that a single arginyl residue per subunit of the dehydrogenase is essential for the composition of the substrate-binding site in the catalytically active holo-conformation of the enzyme.

Materials and Methods

D-Glyceraldehyde-3-phosphate dehydrogenase was isolated from rat skeletal muscle as previously described [4]. For preparation of the apoenzyme a crystalline suspension of the dehydrogenase was dissolved, at 25 mg/ml in 10 mM sodium phosphate/5 mM EDTA/4 mM 2-mercaptoethanol (pH 6.7). 2—3 ml of this solution was passed through a column of Sephadex G-50 (1.8 X 20 cm) equilibrated with the same buffer. Fractions having an absorbance ratio ($A_{280\,nm} : A_{260\,nm}$) of about 1.4 were collected (8—10 ml, 7 mg/ml protein) and applied to a CM-cellulose column (2.5 X 6 cm) equilibrated with the above buffer. Only the apoenzyme was adsorbed on the CM-cellulose under these conditions. The column was washed with the above buffer and the apoenzyme eluted with 10 mM sodium phosphate/5 mM EDTA/4 mM 2-mercaptoethanol/150 mM NaCl (pH 8.3). The apoenzyme solution (1.2—1.5 mg/ml, $A_{280\,nm} : A_{260\,nm} = 1.9—2.0$) was used within 1—2 days.

To prepare the holoenzyme, the CM-cellulose column was further washed, to remove inorganic phosphate with 10 mM NaCl/5 mM EDTA/4 mM 2-mercaptoethanol (pH 6.7). The apoenzyme was then eluted with 150 mM NaCl/5 mM EDTA/4 mM 2-mercaptoethanol (pH 7.6), and NAD^+ was added to the eluate at a final concentration of 0.15 mM. Protein concentration was estimated from absorbance at 280 nm using an extinction coefficient of 0.83 $cm^2 \cdot mg^{-1}$ for the apoenzyme. The enzyme activity, assayed in 0.1 M glycine/NaOH (pH 8.7)/5 mM EDTA/5 mM sodium arsenate/0.5 mM glyceraldehyde 3-phosphate/0.4 mM NAD^+ at 25°C corresponded to 150 μmol NADH/min per mg protein.

D-Glyceraldehyde 3-phosphate was prepared as the calcium salt from D-fructose 6-phosphate by the method of Szewczuk et al. [5]. Solutions of the sodium salt were prepared by removing Ca^{2+} using Dowex-50 (H^+-form) and adjusting the solution to pH 6.5 with NaOH. NAD^+ was obtained from Reanal. The concentrations of NAD^+ and D-glyceraldehyde 3-phosphate were determined as described by Ferdinand [6]. 2,3-Butanedione was purchased from Sigma and distilled before use.

Inactivation studies. The solution of apoenzyme or holoenzyme was diluted 2-fold to 0.7—0.8 mg/ml protein and pH 8.27 with 0.2 M veronal buffer/5 mM EDTA/4 mM 2-mercaptoethanol (pH 8.7). Incubation with butanedione was carried out at 20°C in samples containing 0.5 ml of the above enzyme solution and varying concentrations of the modifying agent (0.006—0.03 ml 10% butanedione in water). The spontaneous inactivation of dehydrogenase was negli-

gible under these experimental conditions. The inactivation reaction was initiated by addition of butanedione to the enzyme solution. Other conditions are specified in the legends to figures. The time-course of inactivation was followed by removing aliquots of the incubation mixture at specific intervals and assaying for dehydrogenase activity. The assay system contained 0.1 M glycine/NaOH (pH 8.7)/5 mM EDTA/5 mM sodium arsenate/0.2 mM glyceraldehyde 3-phosphate/0.2 mM NAD^+. The measurements were carried out at 20°C in a SF-4 spectrophotometer.

Inactivation was first order in the enzyme sites. The rate of inactivation was obtained from a plot of log enzyme activity versus time of incubation with butanedione. The effect of ligands on the rate of inactivation was analyzed as described by Byers and Koshland [7]. The Hill plot for the NAD^+ influence on the reaction was delineated according to Eqn. 1

$$\log(k_s - k_0) + \log[(1/(k_{obs} - k_0)) - (1/(k_s - k_0))] = n \cdot \log[NAD^+] + \log K_s \quad (1)$$

where k_0 and k_s are the pseudo-first-order rate constants of inactivation observed in the absence of NAD^+ and in the presence of its saturating concentrations, respectively, k_{obs} is the observed rate constant, K_s is the dissociation constant of NAD^+ from the enzyme and n is the Hill coefficient.

Protection by glyceraldehyde 3-phosphate against inactivation was analyzed according to Eqn. 2

$$\log k_0 + \log\left[\left(\frac{1}{k_i}\right) - \left(\frac{1}{k_0}\right)\right] = n \cdot \log[GAP] + \log\left(\frac{1}{K_s}\right) \quad (2)$$

where k_0 and k_i are the pseudo-first-order rate constants of inactivation observed in the absence and in the presence of glyceraldehyde 3-phosphate, respectively; K_s is the dissociation constant of glyceraldehyde 3-phosphate from the enzyme, n is the Hill coefficient and GAP stands for glyceraldehyde 3-phosphate.

Reactivation studies. Enzyme inactivated to approx. 15% of the initial activity was passed through a Sephadex G-50 column (1.0 X 25 cm) equilibrated with 10 mM sodium phosphate/5 mM EDTA/70 mM NaCl/4 mM 2-mercaptoethanol (pH 8.2) to remove excess butanedione, and assayed after 60—90 min incubation at 20°C. Another method of reactivation was to add to the inactivation mixture a solution of arginine (final concentration 20 mM).

Results

Previously we have shown that inactivation of apo-glyceraldehyde-3-phosphate dehydrogenase from rat skeletal muscle in the presence of butanedione obeys pseudo-first-order kinetics and is accompanied by modification of two arginyl residues per subunit of the tetrameric enzyme molecule [3]. The pseudo-first-order rate constant of inactivation was measured as a function of butanedione concentration (Fig. 1). As seen in Fig. 1A, a plot of the observed rate constant versus concentration of the modifying agent is not linear, but rather exhibits saturation kinetics. This pattern of inactivation is consistent

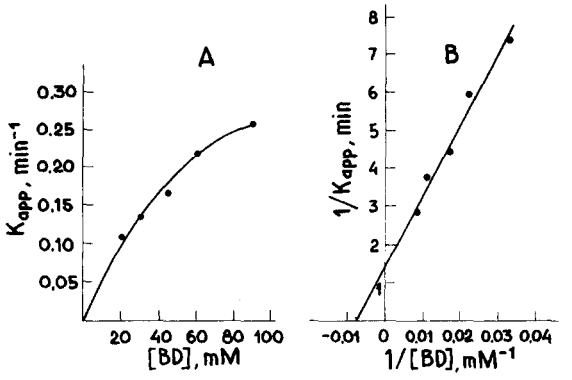

Fig. 1. Effect of the concentration of butanedione on the pseudo-first-order rate constant of the apoenzyme inactivation. k_{app}, the observed pseudo-first-order rate contant; BD, butanedione. (A) Plot of k_{app} versus butanedione concentration. (B) The double reciprocal plot of the data.

with the reversible binding of the reagent to the enzyme to form an enzyme-butanedione complex prior to covalent modification to yield the inactive enzyme. The latter is supposed to be capable of dissociating, since a slow reactivation was observed after removal of the excess of butanedione by gel filtration or by addition of free arginine to the reaction mixture. In both cases the activity rose from 7—10% to 50—60% of the original value.

NAD^+ present in the incubation mixture enhances the rate constant of enzyme inactivation (Fig. 2).

Fig. 3 represents determination of the reaction order with respect to butane-

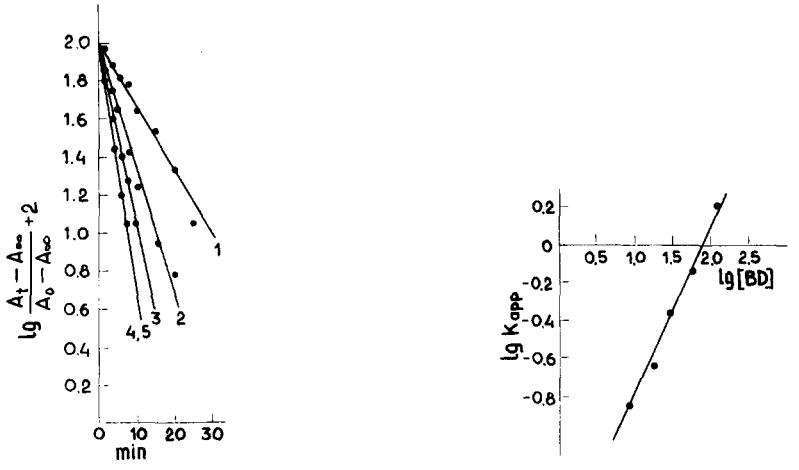

Fig. 2. Effect of NAD^+ on the pseudo-first-order rate constant of the apoenzyme inactivation in the presence of butanedione. 5.57 μM apoenzyme was incubated in the presence of 18 mM butanedione. NAD^+ was present at the following concentrations: 1, none; 2, 5.6 μM; 3, 11.2 μM; 4, 22.8 μM; 5, 150 μM. A_t represents the enzyme activity at time t and A_∞ is the constant level of activity remaining when the reaction was complete; A_0 is the activity in the absence of butanedione.

Fig. 3. Order of inactivation with respect to the concentration of butanedione. Holoenzyme was present in a concentration of 6.0 μM.

dione. As shown in the figure, the points fit a straight line with a slope of 0.9. This indicates that inactivation is the result of the reaction of one arginyl residue per active site of the dehydrogenase.

In the previous publication we have reported that the effect of NAD^+ on the inactivation of the apoenzyme is cooperative [3]. A more detailed investigation of this phenomenon was performed. Fig. 4 illustrates the results of this study plotted as described by Byers and Koshland [7]. The Hill coefficient obtained in different experiments was 2.3—3.2, suggesting a high degree of cooperativity. The acceleration of enzyme inactivation by butanedione in the presence of NAD^+ is most likely the result of a coenzyme-induced conformational change increasing the reactivity of essential arginyl residues. We may conclude that the cooperative process of transition from apo- to holo-conformation of the enzyme is associated with some alterations in the microenvironment of the essential arginyl residues.

To examine the possibility that these residues participate in the binding of substrate, the effect of glyceraldehyde 3-phosphate on the holoenzyme inactivation has been studied. As seen in Fig. 5A, substrate markedly decreases the rate constant of inactivation. The plot shown in Fig. 5B was constructed, where k_0 and k_i are the measured rate constants of holoenzyme inactivation in the absence and in presence of substrate, respectively [8,9]. Extrapolation of the data to glyceraldehyde 3-phosphate concentration = ∞ gave the value of $(k_0 - k_i) = 0.357\ min^{-1}$, whereas the experimentally determined value of k_0 was $0.361\ min^{-1}$. This means that at saturating concentrations of substrate the rate constant of inactivation approaches zero.

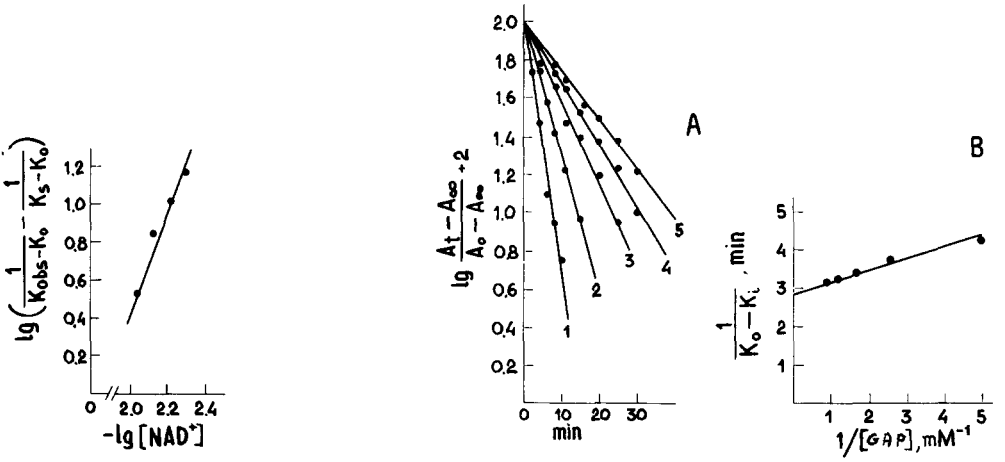

Fig. 4. Hill plot for the effect of NAD^+ on the apoenzyme inactivation by butanedione. 4.5 μM apoenzyme was incubated in the presence of 18 mM butanedione. The results are treated according to Byers and Koshland [7].

Fig. 5. Protection by glyceraldehyde 3-phosphate against inactivation of holoenzyme by butanedione. (A) Inactivation of 4.5 μM holoenzyme in the presence of 18 mM butanedione. The glyceraldehyde 3-phosphate (GAP) concentrations were 1, 0; 2, 3, 4 and 5, 0.2, 0.4, 0.6 and 0.9 mM, respectively. (B) Variation of the inactivation rate constants with glyceraldehyde 3-phosphate concentration. 5.7 μM holoenzyme was incubated under conditions of A. k_0 and k_i are the rate constants obtained in the absence and presence of glyceraldehyde 3-phosphate, respectively.

An estimate of the dissociation constant of the protecting ligand from the enzyme can be obtained using the plot of Fig. 5B. The value found in our experiments (0.12 mM) is not the true dissociation constant of the enzyme-substrate complex, due to the rapid transformation of this complex into acyl-enzyme. This value agrees sufficiently well with the apparent Michaelis constant for glyceraldehyde 3-phosphate, which is 0.15 mM [10].

The complete protection of the holoenzyme from inactivation provided by glyceraldehyde 3-phosphate, and the character of the concentration dependence of this protective effect strongly suggest that the arginyl residue, modified under conditions of our experiments, constitutes a part of the substrate binding site in the active center of the holoenzyme.

Further analysis revealed a substantial difference to exist between the effect of glyceraldehyde 3-phosphate on the inactivation of holoenzyme and apoenzyme. As shown in Fig. 6, in the latter case the protection against inactivation is negligible. In the presence of the maximal concentration of the substrate tested (0.23 mM), the rate constant of inactivation decreased from 0.103 to 0.082 min^{-1} (according to the data in Fig. 6B). This suggests that the arginyl residue, affected by modification, is not involved in the binding of substrate to apoenzyme. We come to the conclusion that structure of the substrate-binding site should be different in the apoenzyme and holoenzyme.

Inorganic phosphate also affords protection against inactivation by butanedione, but the effect is observed only at rather high concentrations of this ligand (Fig. 7). Both the apoenzyme and holoenzyme are protected, in contrast to the results obtained with glyceraldehyde 3-phosphate.

When the data on substrate protection of the holoenzyme were analyzed according to Eqn. 2, a cooperativity was revealed (Fig. 8). It could be detected only at low substrate concentrations which are not sufficient to saturate more than two active sites of the tetrameric enzyme. The Hill coefficient found in

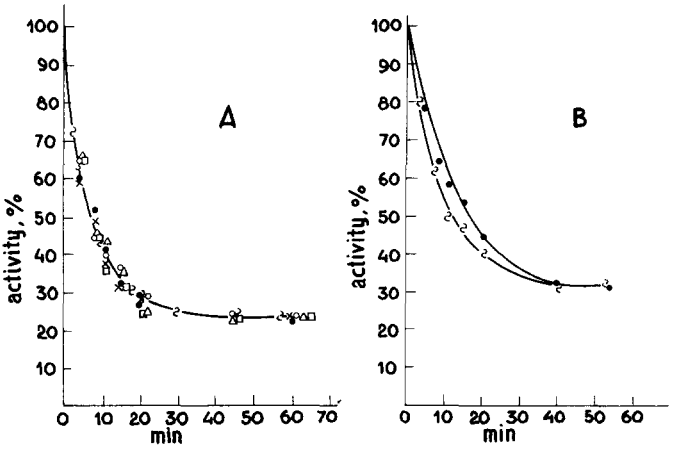

Fig. 6. Effect of glyceraldehyde 3-phosphate on the inactivation of apoenzyme in the presence of butanedione. 6 μM apoenzyme was incubated in the presence of 30 mM (A) or 18 mM (B) butanedione. ∞, no other additions; □, 0.1 mM; △, 0.13 mM; ○, 0.16 mM; X, 0.2 mM; ●, 0.234 mM glyceraldehyde 3-phosphate.

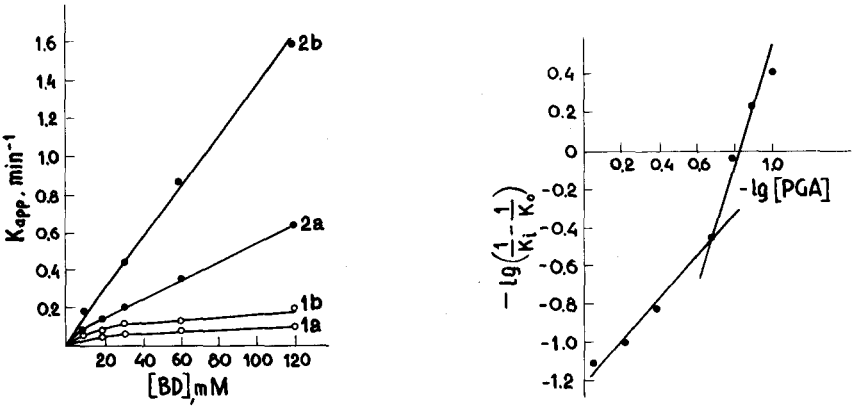

Fig. 7. Effect of inorganic phosphate on the inactivation of apoenzyme (1) and holoenzyme (2) in the presence of butanedione. Enzyme concentration, 4.3 μM. a, 0.1 M sodium phosphate; b, 0.01 M sodium phosphate.

Fig. 8. Hill plot for glyceraldehyde 3-phosphate protection of the holoenzyme against inactivation by butanedione. Conditions as in Fig. 5. The results are treated as described by Byers and Koshland [7].

various experiments was 1.37—3.0. This suggests that the binding of substrate to a part of the active centers of the holo-tetramer induces changes in the microenvironment of the essential arginyl residues of the neighbouring subunits.

Discussion

Rat skeletal muscle glyceraldehyde-3-phosphate dehydrogenase like the homologous enzymes from other species, contains 10 arginyl residues per subunit [11]. Only two of them are modified under mild conditions employed in the present study, and a single arginyl has been demonstrated to be essential for activity. The X-ray crystallographic data obtained with the holo-glyceraldehyde-3-phosphate dehydrogenases from lobster muscle [12,13] and from *Bacillus stearothermophilus* [14] indicate that the active center contains two arginyl residues, Arg-10, involved in the binding of the pyrophosphate group of the coenzyme molecule, and Arg-231, located in the catalytic domain, at or near the substrate-binding site.

The results of our study on the chemical modification of the dehydrogenases isolated from yeast [2] or from rat skeletal muscle have shown that the arginyl residue, which is modified by butanedione under mild conditions, is not involved in the binding of coenzyme. The data point to the participation of this residue in the formation of the substrate-binding site in the active center of the holoenzyme. Considering the high degree of structural similarity between the homologous glyceraldehyde-3-phosphate dehydrogenases, it seems probable that the modified essential arginyl residue is Arg-231.

Chemical modification of the enzyme was used to detect the alterations in the microenvironment of this residue in the course of conformational changes induced by the binding of specific ligands. Our studies have demonstrated that

the cooperative transition from the apo- to holo-conformation of the enzyme is accompanied by the change of the reactivity of the essential arginyl residue. This could be the result of a particular change in the localization of this residue involved in the organization of the substrate-binding site. The conformation of this region of the active center of the apoenzyme appears to differ from that of the holoenzyme. The results of the protective experiments suggest that the arginyl residue does not participate in the binding of glyceraldehyde 3-phosphate to the apoenzyme. The "incorrect" orientation of the substrate in this case may account for the fact that the apoenzyme-glyceraldehyde 3-phosphate complex was found in kinetic studies to be a dead-end complex [15]. It is now a well established fact that the substrate binding to the dehydrogenase is ordered, with NAD^+ being the first substrate [16—19].

The reactivity of the arginine residue was found to be an indicator of the conformational changes induced by the covalent modification of the essential cysteine residue with substrate. The addition of glyceraldehyde 3-phosphate to the holoenzyme results in the formation of the phosphoglyceroyl-thioester in the active sites saturated with the substrate. The experimental evidence suggests that this process is accompanied by a decrease of the reactivity of the essential arginyl residues in the adjacent subunits. The effect is only observed under the conditions of partial saturation of the active centers with glyceraldehyde 3-phosphate. Since the molar concentration of the enzyme in muscle fluid exceeds by far that of glyceraldehyde 3-phosphate (0.4 and 0.08 mM, respectively [20]), the full saturation of the active sites is probably never attained under physiological conditions.

This consideration together with the fact that the evidence of conformational changes induced by acylation of the enzyme active sites was obtained with the true substrate, suggest that subunit interactions may be involved in the catalytic mechanism of the rat skeletal muscle glyceraldehyde 3-phosphate dehydrogenase functioning in vivo.

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