FUNCTIONAL ARGININE RESIDUES OF D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM RAT SKELETAL MUSCLE

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1. Introduction

Extensive studies on the structure of D-glyceraldehyde-3-phosphate dehydrogenase have revealed the existence of the anion-binding sites in the active centers of the tetrameric enzyme molecule [1–4]. The probable involvement of arginine residues in the formation of such sites was demonstrated by chemical modification of yeast D-glyceraldehyde-3-phosphate dehydrogenase with butanedione [5]. Two arginine residues per subunit of the enzyme were found to be modified concomitant with the loss of the dehydrogenase activity. In the present paper we describe the effect of butanedione on the enzyme isolated from the mammalian muscle.

2. Materials and methods

D-Glyceraldehyde-3-phosphate dehydrogenase was isolated from rat skeletal muscle [6], the apoenzyme was prepared as previously described [7]. Butanedione was obtained from Sigma. The incubation with butanedione was carried out as indicated in the legend to fig.1. Sodium phosphate (0.01 M) was added to the reaction mixture to increase the stability of the apoenzyme. Higher concentrations of inorganic phosphate were found to exhibit a protective effect against inactivation. After incubation the enzymatic activity was measured under standard conditions by adding 5 μl of the incubation solution to the assay mixture (0.1 M glycine buffer, pH 8.7, 0.4 mM NAD^+, 5 mM sodium arsenate, 5 mM EDTA, 0.6 mM glyceraldehyde-3-phosphate, 20°C).

The amount of modified arginine residues was estimated on the basis of amino acid analysis of the protein after a 24-h hydrolysis, as described in our previous communication [3].

3. Results

We have previously reported a nearly complete inactivation of yeast D-glyceraldehyde-3-phosphate dehydrogenase in the course of incubation with

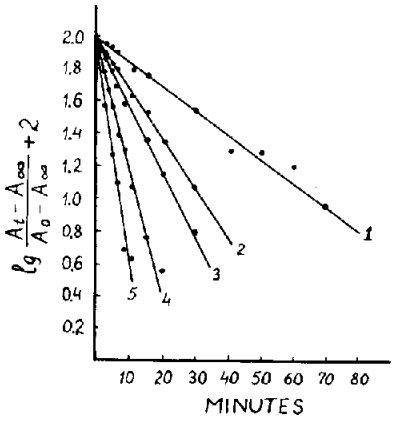

Fig.1. Time course of inactivation of apo-glyceraldehyde-3-phosphate dehydrogenase by butanedione. Apoenzyme (0.85 mg/ml) was incubated at 20°C in 0.01 M sodium phosphate buffer, pH 8.3, containing 0.14 M NaCl, 5 mM EDTA and 4 mM 2-mercaptoethanol, with butanedione at the following concentrations: 7.5 mM (1), 15 mM (2), 30 mM (3), 60 mM (4) and 120 mM (5). Samples were taken for assay at indicated intervals. A_t represents the enzyme activity at time t and A_∞ is the constant level of activity remaining when the reaction was completed; A_0 is the activity in the absence of butanedione.

butanedione. Modification of two arginine residues per subunit of the enzyme appeared to be responsible for inactivation [5]. Similar results were obtained in the present investigation carried out with the enzyme isolated from rat skeletal muscle. Amino acid analysis of the 90% inactivated enzyme revealed modification of two arginine residues per enzyme subunit to have occurred. All other amino acid residues were unaffected.

The time dependence of the inactivation of the dehydrogenase by butanedione was examined under various conditions, and it was found, that the inactivation process could be described as a pseudo-first order reaction (fig.1).

The inactivation of the enzyme by butanedione is reversible. This was demonstrated by first inactivating the enzyme to 14% of the initial activity and then gel filtering of the reaction mixture through Sephadex G-50 (20 × 1.0 cm) equilibrated with 0.1 M sodium phosphate, 5 mM EDTA, pH 8.3, at 20°C to remove excess reagent. The eluting protein was incubated for 10 min and then assayed for enzymatic activity. After this time the activity was increased to 50% of that of the native enzyme.

The presence of inorganic phosphate (0.1 M) in the reaction mixture in the course of incubation with butanedione largely protects the enzyme from inactivation, suggesting that the modified arginine residues constitute the anion binding sites of the protein. Several pyridine nucleotide-dependent dehydrogenases were recently reported to possess arginine residues essential as recognition sites for the negatively-charged oligophosphate moiety of the nucleotide [8–10]. It seemed of interest in this connection to elucidate if arginine residues modified under conditions of our experiments are important for the binding of enzyme to D-glyceraldehyde-3-phosphate dehydrogenase. The effect of NAD^+ on the enzyme inactivation by butanedione was then studied.

As shown in fig.2, NAD^+ confers no protection on the dehydrogenase against modification with butanedione: on the contrary, it accelerates the inactivation. This result suggests that arginine residues affected by modification are not involved in coenzyme binding to the rat muscle dehydrogenase. The enhanced rate of inactivation observed in the presence of NAD^+ may be a consequence of the increased accessibility

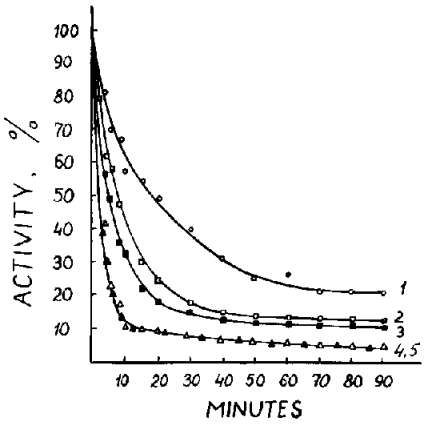

Fig.2. Effect of NAD^+ on the inactivation of apo-glyceraldehyde-3-phosphate dehydrogenase by butanedione. Apoenzyme (5.9 μM) was incubated with 15 mM butanedione under conditions of fig.1 with no additions (1) and in the presence of 5.9 μM (2), 11.8 μM (3), 29.6 μM (4) and 0.15 mM (5) NAD^+.

of arginine residues for modification due to structural changes upon coenzyme binding. It seems likely that the conformational alteration induced by the coenzyme affect the microenvironment of the anion-binding sites, and the chemical reactivity of the essential amino acid residues serves as a sensitive index of the changes.

Figure 2 illustrates the effect of NAD^+ successively bound to the four subunits of the enzyme. The coenzyme binding characteristics of the rat muscle apoenzyme are very similar to those of the rabbit muscle dehydrogenase [7]. Under conditions employed, curves 2 and 3 of fig.2 refer to the samples, containing one and two firmly bound NAD^+ molecules per tetrameric enzyme, respectively, whereas curve 4 corresponds to the enzyme having the third active center nearly 90% saturated with NAD^+. The excess of NAD^+ added to the sample 5, should ensure the complete saturation of four active centers.

The results shown in fig.2 suggest some cooperativity to exist in the NAD^+ effect on subunits constituting the firmly NAD^+-binding and the loosely NAD-binding pairs, respectively. It is seen, that the effect of the second bound NAD^+ molecule is less than that of the first, and the effect of the fourth molecule of the coenzyme is negligible as compared to the influence of NAD^+ bound to the third subunit.

4. Discussion

Together with the results obtained with the yeast enzyme [5], the present paper demonstrates that two arginine residues per subunit are essential for the activity of D-glyceraddehyde-3-phosphate dehydrogenase. It is to be established if these readily modified arginine residues occupy similar positions in the sequences of yeast and rat muscle dehydrogenases.

The arginine residues which are modified with butanedione in the rat muscle enzyme probably do not act as a positively charged recognition sites for the pyrophosphate moiety of the enzyme.

They are supposed to constitute the anion-binding sites for inorganic phosphate and perhaps for substrate. NAD^+ confers no protection against butanedione inactivation, on the contrary, it enhances the inactivation, suggesting an alteration in reactivity of the essential arginine residues upon coenzyme binding. This is not the case with yeast D-glyceraldehyde-3-phosphate dehydrogenase. The presence of NAD^+ was demonstrated to protect this enzyme from inactivation in the presence of butanedione, but the protection was partial even with the large excess of the coenzyme (unpublished results). The modified protein retained the capability of binding NAD^+, although with the lowered affinity [5]. A possible explanation of this effect is only a partial involvement of modified arginine residues in coenzyme binding.

The different pattern of the effect of coenzyme on the butanedione inactivation of the yeast and rat muscle D-glyceraldehyde-3-phosphate dehydrogenase points to some differences in the function of the modified residues in these homologous proteins. There are other reports in the literature indicating that the specific roles of active site arginine residues modified by butanedione may vary in isofunctional enzymes from various sources (horse liver and yeast alcohol dehydrogenase, [9], bovine liver and *Neurospora* glutamate dehydrogenases, [11]. The data suggests some peculiarities in the structural organization of certain active site regions of the homologous proteins.

The dissimilarity between yeast and skeletal muscle D-glyceraldehyde-3-phosphate dehydrogenases is revealed in the opposite effect of the coenzyme on the rate of butanedione inactivation. These data conform to a series of observations on different NAD^+ effects on the yeast and muscle species of the furyl-acryloyl-enzyme [12,13]. It seems likely that the conformational changes brought about by the binding of NAD^+ are highly specific and differ in the yeast and skeletal muscle enzymes. The results obtained in the present work suggest the structure of anion-binding sites of the rat muscle dehydrogenase to be involved in the rearrangement of the active center by the coenzyme.

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