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HALF-OF-THE-SITES REACTIVITY OF RAT SKELETAL MUSCLE D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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Summary

Apo-glyceraldehyde-3-phosphate dehydrogenase (D-Glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) from rat skeletal muscle is inactivated by stoichiometric amounts of 1-fluoro-2,4-dinitrobenzene (FDNB) in a time-dependent reaction. The maximal loss of activity attained in the presence of 1 and 2 equivalents of FDNB per mol of the tetrameric enzyme corresponds to 25% and 50% of the control, respectively. Further increase in FDNB concentration results in no additional loss of activity, and only two subunits per tetramer remain modified in the presence of an excess of the reagent. Full inactivation can however be achieved by addition of stoichiometric amounts of DTNB, which readily modifies the SH groups of the two active centers apparently inaccessible to FDNB.

This type of the half-of-the-sites reactivity is unusual and differs appreciably from the previously described effect of FDNB on glyceraldehyde-3-phosphate dehydrogenases from other sources. Namely, modification by FDNB of two subunits of the tetrameric molecule of the enzymes from rabbit skeletal muscle or from baker's yeast is known to inactivate the neighbouring subunits, whereas in the case of the rat muscle enzyme, the active centers remaining free are fully active and prevented from being modified by FDNB.

Apo-glyceraldehyde-3-phosphate dehydrogenase from rat skeletal muscle covalently bound to CNBr-activated Sepharose 4B, exhibits the half-of-the sites reactivity of the type characteristic for the soluble enzyme. The half-of-the-sites effect is retained in an immobilized dimeric form of the dehydrogenase prepared by ADP-induced dissociation of the matrix-bound tetrameric enzyme.

These results demonstrate that the non-equivalence among the active centers is conserved in the isolated dimeric species of glyceraldehyde-3-phosphate

Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; DNP, dinitrophenyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

dehydrogenase, and suggest that the tetrameric structure is not a prerequisite for the half-of-the-sites effect.

Introduction

Cooperative subunit interactions in a tetrameric molecule of glyceraldehyde-3-phosphate dehydrogenase have been the object of extensive studies. The enzyme was shown to exhibit the half-of-the-sites effect in the modification of cysteine residues of the active center. Only 2 of the 4 chemically identical subunits were capable of reacting with some (pseudo) substrates or inhibitors [1—4], and the reaction of a half of the enzyme active sites with a number of modifying agents resulted in complete inactivation [3—6].

This phenomenon reflects the asymmetric nature of the oligomeric protein molecule, organized as a dimer of dimers. Cooperative interactions, responsible for the half-of-the-sites reactivity, seem to be transmitted across one out of the three intersubunit contact regions revealed by X-ray crystallographic studies of lobster glyceraldehyde-3-phosphate dehydrogenase [7,8]. It remains obscure, however, if such cooperative interactions persist in the isolated dimer, under conditions precluding intra-dimeric subunit contacts.

In the present investigation we made an attempt to explore this problem. Advantage was taken of the fact that the apo-form of glyceraldehyde-3-phosphate dehydrogenase, isolated from rat skeletal muscle, is capable of reversibly dissociating into dimers under mild conditions [9]. The tetrameric enzyme, covalently bound to CNBr-activated Sepharose 4B, could be dissociated giving rise to the immobilized dimeric form of dehydrogenase, which proved to be enzymatically active [10]. It was decided therefore to elucidate if the non-equivalence of the active centers of subunits comprising the dimer is lost upon dissociation. For this purpose, the pattern of inactivation by FDNB, the well-known half-of-the-sites reagent for glyceraldehyde-3-phosphate dehydrogenase [4,6] has been investigated both with the soluble and immobilized forms of the enzyme.

Materials and Methods

Preparation and assay of glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase was isolated from rat skeletal muscle [11]. For preparation of the apoenzyme, a crystalline suspension of the dehydrogenase was dissolved in 10 mM sodium phosphate buffer containing 5 mM EDTA and 4 mM 2-mercaptoethanol (pH 6.7) and passed through a column of Sephadex G-50 equilibrated with the same buffer. The eluate was applied to a CM-cellulose column equilibrated with the above buffer. Only the apoenzyme is adsorbed on CM-cellulose under these conditions. It was eluted with 0.1 M sodium phosphate buffer/5 mM EDTA/4 mM 2-mercaptoethanol (pH 8.3).

To prepare the apoenzyme solution used in the experiments with FDNB, the column of CM-cellulose with adsorbed protein was washed with 10 mM sodium phosphate/5 mM EDTA (pH 6.7) to remove 2-mercaptoethanol. The apoen-

zyme was then eluted with 0.1 M sodium phosphate buffer/5 mM EDTA (pH 8.3). The apoenzyme thus obtained had an $A_{280} : A_{260}$ ratio of 1.9—2.0. The protein concentration was estimated spectrophotometrically at 280 nm, using an extinction coefficient of 0.83 $cm^2 \cdot mg^{-1}$ for apoenzyme. The molecular weight of the dehydrogenase was taken to be 144 000 [12]. SH-groups titration, carried out by the method of Ellman [13], revealed the existence of 4 SH groups per tetramer that react "instantaneously" with DTNB.

Enzyme activity, assayed in 0.1 M glycine/NaOH (pH 9.2)/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 of protein.

Immobilization of glyceraldehyde-3-phosphate dehydrogenase

Immobilization of the apoenzyme was carried out by the method of Axén et al. [14] with small modifications. Sepharose 4B was thoroughly washed on a glass funnel with 0.1 M sodium phosphate (pH 11.0) and then with water to achieve the neutral pH. The gravity-packed gel was brought to twice its volume by addition of 1.0 M sodium phosphate solution (pH 11.3—11.5) at 8°C. To this suspension was added dropwise with constant mixing a 20% solution of freshly prepared CNBr in dioxane to achieve a concentration of 5 mg CNBr/ml packed gel. This procedure was completed within 8 min. The activated agarose beads were immediately washed with water to achieve neutral pH and then with 0.1 M sodium phosphate/4 mM 2-mercaptoethanol/5 mM EDTA (pH 8.0—8.5).

Apo-glyceraldehyde-3-phosphate dehydrogenase was coupled to CNBr-activated agarose in the above buffer, at a protein concentration of 1 mg/ml at 20°C in the course of 3—6 h under continuous gentle stirring. Free enzyme was removed by several cycles of washing on a glass funnel with the coupling buffer. A standard suspension was made consisting of 1 part Sepharose (packed volume) and 1 part standard buffer (0.1 M sodium phosphate/4 mM 2-mercaptoethanol/5 mM EDTA (pH 8.3). The protein content of the immobilized enzyme preparation was determined as described below; it usually corresponded to 120—140 μg protein/ml packed gel.

Preparation of immobilized dimers of glyceraldehyde-3-phosphate dehydrogenase

To achieve the dissociation of a matrix-bound tetrameric protein we used the experimental approach described in the previous publication [10]. A column of 2 × 0.7 cm was filled with a suspension of the immobilized apoenzyme (0.12 mg/ml packed gel) in 0.1 M sodium phosphate buffer (pH 8.3)/5 mM EDTA/4 mM 2-mercaptoethanol at 20°C. 50 mM ADP in the same buffer was slowly (in the course of 1.5—2.0 h) passed through the column. The column was then extensively washed with the above buffer to remove the nucleotide and the dissociated protein.

The nucleotide treatment resulted in the release of nearly 50% of the matrix-bound protein, which suggests dissociation of the immobilized tetrameric molecule into dimers to have taken place. The dimeric form of enzyme thus obtained was shown to be capable of reassociating with the native enzyme dimeric species existing in solution of the apoenzyme, which has been added to the suspension of Sepharose-bound dimers.

Determination of Sepharose-bound protein

Polyethylene glycol solution was used in the spectrophotometric measurements to diminish the light scattering of agarose beads. A 1-ml suspension of the matrix-bound protein was put into a 1-cm light path quartz cell, and 50% polyethylene glycol (M_r = 20 000) solution in water was added using a syringe to obtain a total volume of 3 ml. After careful gentle stirring to avoid the appearance of bubbles, the absorbance was measured at 280 nm with a SF-4 Model Spectrometer.

The protein content of the samples was estimated according to a calibration curve, which was obtained as follows. Increasing concentrations of the protein solution were added to series of samples containing a fixed amount of the inactivated agarose suspension. The volume was made up to 3.0 ml with 50% polyethylene glycol and after stirring the absorbance was measured. The readings were corrected for the absorbance of agarose without protein. The amount of agarose present in a sample to be analyzed should correspond to that used to construct the calibration curve.

Activity determination of the immobilized enzyme

Matrix-bound activities were determined by adding an aliquot (0.025—0.1 ml) of a suspension containing the immobilized derivative to the following assay mixture: 0.1 M glycine/NaOH (pH 9.9)/15 mM EDTA/15 mM sodium arsenate/1.2 mM NAD^+ in a 1-cm light path quartz cell at 25°C. The final volume of the sample was 3.0 ml. The reaction was started by addition of glyceraldehyde-3-phosphate (final concentration 1.5 mM) and followed for 30 s. Under these conditions the assay was linear with the amount of the matrix-bound enzyme added. The specific activity of the matrix-bound tetrameric glyceraldehyde-3-phosphate dehydrogenase was approx. 50—80% of that of the pure enzyme in solution. Immobilized dimers of the enzyme were previously shown to have catalytic properties similar to those of the immobilized tetrameric form [10].

NAD^+ and EDTA were purchased from Reanal, 2-mercaptoethanol was a product of Austrowaren, Sepharose 4B and Sephadex G-50 were obtained from Pharmacia Fine Chemicals. Polyethylene glycol (M_r = 20 000) was obtained from Ferak and Schuchardt, DTNB from Aldrich Chem. Co. ADP was purchased from Merck A.G. (Darmstadt). D-glyceraldehyde-3-phosphate was prepared according to the method of Szewczuk et al. [16]. FDNB (prepared in this laboratory) was dissolved in 50% ethanol and kept in the dark at 4°C. To determine the exact concentration of the reagent, the aliquots of the solution (1—2 μl of the 1 mM solution) were mixed in a spectrophotometric cell with 3.0 ml of 4 mM 2-mercaptoethanol in 0.1 M sodium phosphate buffer (pH 8.35). The increase in absorbance at 340 nm was measured, and the concentration of FDNB was calculated using an extinction coefficient of the SH adduct of 11 400 $M^{-1} \cdot cm^{-1}$ [6].

Results

Fig. 1 shows the time-course of the inactivation of rat muscle glyceraldehyde-3-phosphate dehydrogenase in the presence of stoichiometric amounts of

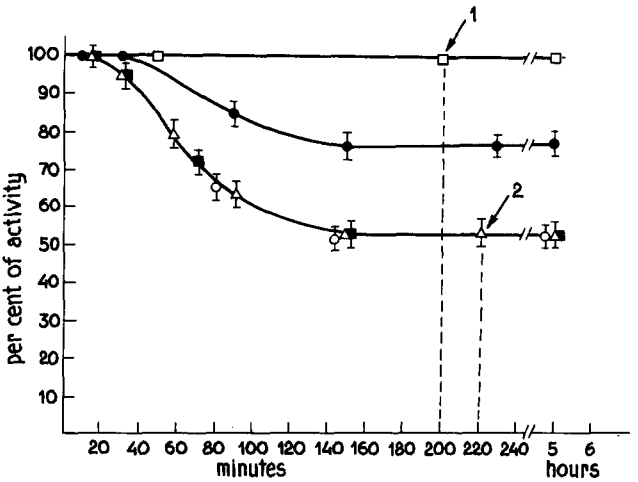

Fig. 1. Inactivation of apo-glyceraldehyde-3-phosphate dehydrogenase from rat skeletal muscle in the presence of FDNB. Apoenzyme ($4.0 \cdot 10^{-6}$ M) in 0.1 M sodium phosphate buffer (pH 8.3)/5 mM EDTA was incubated at 20°C in the dark with no additions (□) and in the presence of $4.0 \cdot 10^{-6}$ M (●), $8.0 \cdot 10^{-6}$ M (△), $12.0 \cdot 10^{-6}$ M (■) and $16.0 \cdot 10^{-6}$ M (○) FDNB. Inactivation was started by addition of 2—3 µl of the FDNB solution to 1.0 ml of the apoenzyme solution. At indicated time intervals aliquots were taken for activity determination. At intervals indicated by the arrows aliquots were withdrawn to examine the effect of DTNB, which was added in amounts corresponding to four (1) or three (2) equivalents per mol protein.

FDNB. Under these conditions only SH-groups of the active center are involved in the modification, since it has been shown that, when used in limiting amounts FDNB modifies selectively cysteine residues of glyceraldehyde-3-phosphate dehydrogenase [17]. In the presence of 1 and 2 mol of the reagent added per mol of enzyme, the activity loss is proportional to the number of the active sites modified, but no further inactivation occurs upon increasing the concentration of FDNB.

When the time-course of inactivation in the presence of an excess FDNB was outlined in the semi-log form, a monophasic linear plot was obtained. This indicates that the inactivation of two subunits affected by modification occurs with the same rate constant.

The reaction of FDNB with cysteine residues of the protein can be followed spectrophotometrically by measuring the increase of absorbance at 340 nm [6]. Fig. 2 shows that only 2 mol of the reagent are bound to the protein under conditions of our experiments. Half of the catalytic sites of the tetrameric enzyme molecule remain unmodified, despite the presence of an excess of FDNB. Prolonged (approx. 12 h) incubation of the enzyme under these conditions resulted in a certain increase in absorbance at 340 nm; it could not, however, be attributed to the modification of active sites, since the residual activity of the samples did not change and remained 50% of the control. It follows then that only 2 of the 4 active centers of the dehydrogenase molecule are accessible to FDNB. The effect is specific for FDNB, since the cysteine residues of the unaltered active centers are readily modified by the other SH-reagent, DTNB. As seen in Fig. 1, addition of stoichiometric amounts of the latter results in a

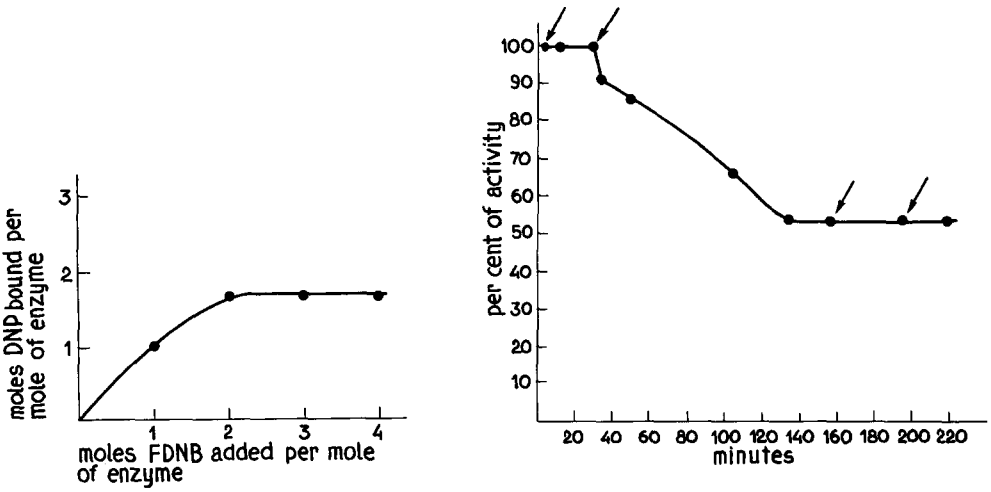

Fig. 2. Reaction of FDNB with apo-glyceraldehyde-3-phosphate dehydrogenase from rat skeletal muscle. Different amounts of FDNB were added to spectrophotometric cells, containing apoenzyme solution ($4.0 \cdot 10^{-6}$ M). The mixtures were incubated in the dark and the increase in absorption at 340 nm was measured. The constant value of absorbance was reached within 120 min. It was used to calculate the number of DNP groups bound per tetrameric enzyme (see Materials and Methods).

Fig. 3. The effect of incubation of apo-glyceraldehyde-3-phosphate dehydrogenase with 1 equivalent of FDNB per mol enzyme on the time course of inactivation in the presence of 2 equivalents of FDNB. Apoenzyme from rat skeletal muscle ($3.7 \cdot 10^{-6}$ M) in the buffer indicated for Fig. 1, was incubated at 20°C in the dark. At time intervals indicated by the arrows, the solution of FDNB was added, each time corresponding to 1 equivalent per mol of the enzyme tetramer.

rapid loss of the residual activity of the enzyme. These results indicate that the rat muscle glyceraldehyde-3-phosphate dehydrogenase exhibits half-of-the-sites reactivity towards FDNB.

As seen in Fig. 1, the inactivation of the apoenzyme in the presence of FDNB begins after an initial lag-period, in the course of which incubation with 1 or 2 mol equivalents of the modifying agent causes no loss in activity. If however, a 30-min incubation in the presence of 1 FDNB equivalent per mol protein was followed by addition of a new portion of the reagent, sufficient to modify the second active center, the inactivation started immediately (Fig. 3). The time-course of inactivation in this case closely resembles the time-course of inactivation beginning after a 20-min lag-period in the presence of 2 mol equivalents of FDNB per mol enzyme (Fig. 1).

Taking into consideration that inactivation of the dehydrogenase caused by FDNB is the result of the covalent modification of the functional SH-groups, it seems reasonable to assume that the lag-period, which precedes the beginning of the inactivation, responds to the first phase of the process, a non-covalent complex formation between FDNB and the enzyme active site. This non-covalent interaction may bring about some changes in the microenvironment of the active center cysteine residues, facilitating their modification.

It seemed of interest to reveal if detectable conformational changes are really involved in the process of enzyme inactivation by FDNB. An attempt was made to compare the reactivity of sulfhydryl groups of the apoenzyme samples,

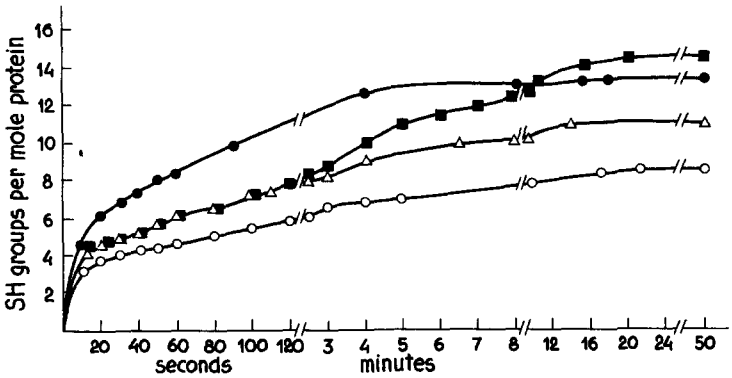

Fig. 4. Number of sulfhydryl groups of apo-glyceraldehyde-3-phosphate dehydrogenase from rat skeletal muscle reacting with DTNB vs. time. Apoenzyme was incubated under conditions indicated in Fig. 1 with no additions (●) and in the presence of 0.8 (■), 1.6 (△) and 2.0 (○) equivalents FDNB per mol protein. After 150 min incubation the enzymatic activity of the samples corresponded to 100, 80, 65 and 54% of the initial value, respectively. 2-mercaptoethanol was then added to each of the samples to achieve a final concentration of 4 mM, and incubation continued for another 60 min. This was followed by removal of 2-mercaptoethanol by gel filtration through a Sephadex G-50 column equilibrated with the buffer indicated in Fig. 1. The reaction of DTNB with sulfhydryl groups of the dehydrogenase was carried out in the same buffer, with a 40-fold excess of DTNB with respect to the enzyme concentration ($1.0 \cdot 10^{-6}$ M).

which differed in the degree of modification by FDNB. Fig. 4 illustrates the results of SH-groups titration with DTNB. The native apoenzyme contains 4 SH groups per tetramer, which react "instantaneously". The second phase of the reaction which is over within 2 min, includes modification of the another 4 SH-groups, which are supposed to belong to cysteine residues 153, being the second in reactivity after Cys-149 [18,19].

The time-course of the reaction of SH-groups with DTNB is markedly altered with the apoenzyme modified with 0.8 mol equivalents of FDNB. The number of "instantaneously" reacting SH groups is not diminished, in spite of the fact, that cysteine residues 149 are partly blocked by dinitrophenylation. Moreover, some additional SH-groups become highly reactive. The second phase of the reaction is greatly accelerated.

Quite different is the pattern of SH-group titration in the case of the apoenzyme having 2 active sites modified by FDNB. The second phase of the reaction is slower compared with the native apoenzyme; the difference is much more pronounced when the results are compared with those obtained with the enzyme having 0.8 SH-groups per mol dinitrophenylated. The diminished reactivity of SH-groups is also reflected in the incomplete accessibility to DTNB of the slowly-reacting "masked" cysteine residues. The maximum amount of sulfhydryl groups, which could be determined after 50-min incubation, corresponded to 15 in the case of the native apoenzyme, whereas only 8.5 SH-groups were detected in the enzyme with two active sites modified by FDNB. It appears therefore, that in addition to 2 cysteine residues which are arylated, 4.5 more SH-groups became inaccessible to DTNB.

These results suggest the existence of some conformational differences between the apoenzyme samples having been dinitrophenylated at one and two active sites, respectively. While in the former case a "loosening" of the struc-

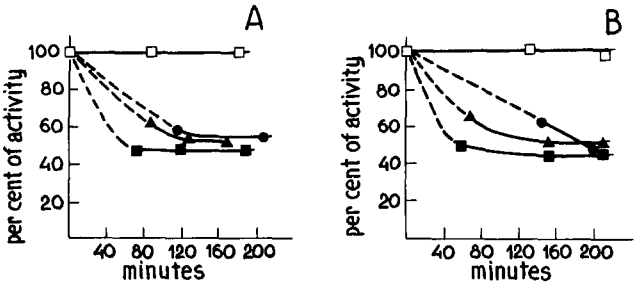

Fig. 5. Inactivation of the tetrameric (A) and dimeric (B) forms of rat muscle glyceraldehyde-3-phosphate dehydrogenase immobilized on Sepharose 4B, in the presence of FDNB. Suspension of Sepharose-bound apoenzyme (tetrameric or dimeric species) was washed on a glass funnel with 0.1 M sodium phosphate buffer (pH 8.3)/10 mM EDTA to remove 2-mercaptoethanol. A suspension (2.0 ml) of the immobilized tetrameric (A) or dimeric (B) forms of the apoenzyme (140 and 70 μg of protein, respectively) in the buffer indicated above, was incubated at 25°C in the dark under constant gentle stirring with no additions (□) and in the presence of 10 (●), 20 (▲) and 30 (■) equivalents of FDNB per mol protein. At indicated time intervals aliquots were taken for activity determination.

ture may be supposed to account for the enhanced reactivity of SH-groups, the opposite effect (a sort of "tightening") probably takes place upon modification of the second active site of the tetramer.

The "dimer of dimers" behaviour of glyceraldehyde-3-phosphate dehydrogenase, reflected in the half-of-the-sites phenomenon, suggests that the pairs of subunits comprising the "functional dimers" may be relatively independent from each other and capable of exhibiting cooperativity in the absence of interdimeric interactions. It was interesting in this connection to investigate the effect of the half-of-the-sites reagent on the isolated dimer of the dehydrogenase. An experimental approach to the study of the dissociated forms of enzymes was developed by Chan [20]. It is based on the immobilization of the oligomer onto a matrix with subsequent treatment by a dissociating agent to remove the non-covalently bound subunits. This approach was used in our previous study to prepare immobilized dimeric species of apo-glyceraldehyde-3-phosphate dehydrogenase from rat skeletal muscle [10]. The same technique was employed in the present investigation. Catalytically active dimers of the enzyme, covalently bound to Sepharose 4B, were obtained (see Materials and Methods) and were investigated under conditions preventing their reassociation into the tetramer.

Fig. 5 shows the effect of FDNB on the activity of the immobilized enzyme. Sufficiently large amounts of the reagent were used in these studies to overcome the diffusion limitations and the consequences of a possible adsorption of FDNB on the matrix. As seen in the figure, the immobilized apo-glyceraldehyde-3-phosphate dehydrogenase retains 50% of its initial activity after incubation with a considerable excess of FDNB, which proves that the half-of-the-sites behaviour of the tetramer is not altered upon covalent attachment to the matrix. The results also demonstrate that the non-equivalence of the active sites is preserved in the dimeric enzyme species. We may conclude therefore that the tetrameric structure of glyceraldehyde-3-phosphate dehydrogenase is not a prerequisite for exhibiting the half-of-the-sites phenomenon.

Discussion

The time-course of inactivation of rat muscle glyceraldehyde-3-phosphate dehydrogenase in the presence of stoichiometric amounts of FDNB is characterized by an initial lag period, when no loss of activity is observed. The inactivation which starts thereafter follows the first-order kinetics. If the enzyme has been preincubated in the presence of 1 mol equivalent of FDNB and then the second mol equivalent of the reagent is added, the inactivation starts immediately.

To explain these observations we suppose that the modification of the functional cysteine residue of the dehydrogenase is preceded by a non-covalent complex formation between FDNB and the active site of the enzyme. Dinitrophenylation of Cys-149 takes place in this complex as a first order intramolecular transformation. A similar mechanism was propsed by Batke et al. [21], who studied the kinetics of reaction of Cys-149 of pig muscle glyceraldehyde-3-phosphate dehydrogenase with *p*-hydroxymercuribenzoate. The evidence was presented by these authors that the process involves two steps. The noncovalent complex formation, which follows second-order kinetics, is succeeded by the first-order reaction of mercaptide bond formation. On analogy with the interaction of the dehydrogenase with *p*-hydroxymercuribenzoate, it seems likely that the binding of FDNB is facilitated by hydrophobic interactions between the benzene ring of the reagent and an apolar area of the active center.

The results of Fig. 3 may be interpreted if we assume that the non-covalent binding of FDNB to one of the subunits of the enzyme makes a neighbouring subunit more accessible to the modifying agent. Fig. 6 schematically illustrates the possible steps of the process of enzyme inactivation by the half-of-the-sites reagent. The subunits of the unmodified apoenzyme (I) interact with each other through three different types of contacts. Let us suppose that the half-of-the-sites effect is realized due to the interaction of subunits within the dimers 1—3 and 2—4, respectively.

The binding of the first FDNB equivalent to subunit 1 may make the structure of the tetramer change and come to state II at the end of the lag-period. The conformational state of two subunits is altered due to the noncovalent binding of FDNB with only one of them. The next step is transformation to state III, caused by covalent modification of one of the active centers with the proportional loss of avtivity. This conformational state of the protein was

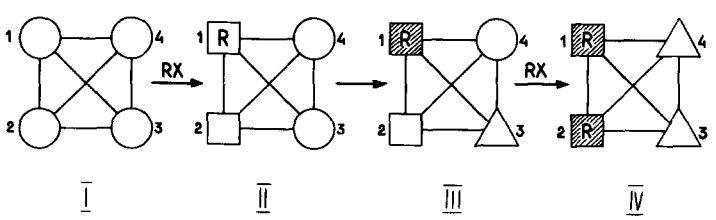

Fig. 6. Schematic representation of the tetrameric molecule of glyceraldehyde-3-phosphate dehydrogenase and of possible structural changes in the course of FDNB-induced inactivation. Open and shaded symbols, active and inactive subunits, respectively, RX, FDNB.

shown to be characterized by increased reactivity of SH-groups, possibly due to the "unmasking" of the cysteine residues which follow Cys-149 in accessibility. It is tempting to suppose that this "loosening" of the structure may facilitate FDNB binding and is possibly initiated in the course of the lag-period.

The marked changes in SH-group reactivity, which occur as a result of covalent modification of the second subunit, point to an opposite effect: the "tighthening" of the protein structure; the total amount of SH-groups determined by DTNB titration becomes lowered as compared with the native enzyme. Conformational state IV is characterized by the inaccessibility of two free active centers to dinitrophenylation. They are catalytically active and capable of reacting with DTNB.

The results obtained in this series of experiments are consistent with the hypothesis of the ligand-induced negatively co-operative conformational changes as the explanation of the half-of-the-sites reactivity [3—6]. Non-equivalence of the active sites of the enzyme molecule is likely to be induced by binding of FDNB to the active SH-groups. Upon arylation of two subunits of the tetramer the unoccupied active centers become inaccessible to this reagent. It should be noted, however, that the existence of some inherent asymmetry of the enzyme as the molecular basis of a "dimer of dimers" behaviour of this protein [2,22] seems quite probable. In our opinion, the combination of both factors, a pre-existing asymmetry on the one hand, and a ligand-induced effect, on the other, should be considered to explain the phenomenon of half-of-the-sites reactivity.

It is noteworthy that the half-of-the-sites behaviour of the rat muscle glyceraldehyde-3-phosphate dehydrogenase described above markedly differs from the effects previously observed with the homologous enzymes isolated from baker's yeast or from rabbit muscle. Each of these two dehydrogenases was shown to be completely inactivated upon dinitrophenylation of two subunits of the tetrameric enzyme molecule. Moreover, the reactivity of Cys-149 of the subunits which remained free, was considerably lowered [4—6], in contrast to the results obtained with the rat muscle enzyme.

Another difference between the rat and rabbit enzymes is that only 2 subunits of the former could be modified by FDNB whereas in the case of rabbit dehydrogenase, all 4 active sites were accessible to this reagent [5,17]. It appears therefore that the manifestations of the asymmetry between subunits forming the "functional dimers" within the tetrameric molecule of the dehydrogenase are determined by the peculiarity of the protein structure. The unusual behaviour of the rat muscle dehydrogenase seems to be an example to substantiate the prediction of Stallcup et al. [23], that an evolutionary amino acid replacement in a homologous protein can alter the cooperativity pattern.

No definite information is available at this time concerning the nature of structural differences underlying the dissimilar behaviour of the rat muscle enzyme, on the one hand, and the rabbit muscle and yeast enzymes, on the other. We should like to mention, however, that apart from the differences in the amino acid composition (and in particular, histidine content), the rat muscle dehydrogenase has an Asn residue at the 6th position instead of Asp found in the yeast and rabbit muscle enzymes (manuscript in preparation). According to the X-ray crystallography data, this residue is implicated in the

formation of the active center of the dehydrogenase [7]. Preliminary indications of the structural differences in the environment of Cys-149 existing in glyceraldehyde-3-phosphate dehydrogenases from various sources [24] are of interest in connection with the problem of elucidation of the structural basis of differences in cooperative behaviour of homologous proteins.

Rat skeletal muscle dehydrogenase proved to be an appropriate object to investigate the problem of subunit interactions in the isolated dimeric enzyme species. The results obtained in the study of FDNB effect on the activity of immobilized tetrameric and dimeric forms of the apoenzyme are consistent with the concept of the relative independence of dimers within the tetrameric dehydrogenase molecule. Immobilized dimers obtained by ADP-induced dissociation of the apoenzyme, retain both the catalytic activity and half-of-the sites behaviour.

In accordance with the X-ray crystallographic data, it has been suggested that the interaction, that leads to half-of-the-sites reactivity, occurs across the R-R intersubunit domain [6]. It seems reasonable to assume that the same type of subunit contacts exists in the dimers obtained under conditions of our experiments. The results of the present investigation demonstrate therefore that the tetrameric structure of glyceraldehyde-3-phosphate dehydrogenase is not a prerequisite for the half-of-the-sites reactivity of this protein. Cytidine triphosphate synthetase was the first polydimeric enzyme to be proven capable of exhibiting the half-of-the-sites effect in the isolated dimeric species [25], and glyceraldehyde-3-phosphate dehydrogenase appears to be another example of this sort of behaviour.

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