Biochimica et Biophysica Acta, 526 (1978) 1—12

BBA 68538

KINETICS OF INACTIVATION AND MOLECULAR ASYMMETRY OF NAD-SPECIFIC GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE OF *PISUM SATIVUM*

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(Received February 3rd, 1978)

Summary

Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) has been purified to homogeneity from pea seeds. The purified enzyme gave a single protein band on polyacrylamide gel electrophoresis (with and without sodium dodecyl sulfate; subunit molecular weight 38 000). It is free from bound nucleotides. The kinetics of heat inactivation of the crude enzyme extract as well as the purified enzyme are biphasic, in that exactly half of the activity is destroyed more rapidly than the residual half. The data are consistent with the rate equation:

$$A = \frac{A_0}{2} \cdot e^{-k_1 t} + \frac{A_0}{2} \cdot e^{-k_2 t}$$

where A_0 and A are activities at times zero and t, respectively, and k_1 and k_2 are first-order rate constants for the fast and slow phases, respectively. Addition of NAD^+ slows down thermal inactivation, without altering the overall kinetic pattern. The activity lost due to the fast component (k_1) of the reaction is regained on cooling ('annealing'), whereas the slow reaction (k_2) is not reversed, suggesting the following scheme:

$$\text{Active enzyme} \underset{\text{Cool}}{\overset{\text{Heat}(k_1)}{\rightleftharpoons}} \text{Half active enzyme} \xrightarrow{\text{Heat}(k_2)} \text{Inactive enzyme}$$

This is confirmed by plotting the activity after 'annealing' against initial period of heating. A single first-order rate constant (k_2) is observed.

The enzyme possesses about one reactive SH group per subunit which can be titrated with 5,5'-dithio-bis-(2-nitrobenzoic acid). Blocking of these groups inactivates the enzyme. Inactivation with 20 μM *N*-ethylmaleimide and 30 μM

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iodoacetate (at pH 8.6 and 33°C) follows simple first-order kinetics (rate constants 0.099 and 0.139 min^{-1}, respectively), suggesting that all SH groups react equally readily with these reagents. Reaction of the enzyme with 0.6 μM *p*-chloromercuri benzoate, however, shows biphasic kinetics similar to thermal inactivation. The reaction of *p*-chloromercuri benzoate with partially heat-inactivated enzyme (residual activity 37.5%) follows simple first-order kinetics.

The molecular asymmetry demonstrated by these results must arise from the unique quaternary structure of the enzyme molecule, which is apparently made up of chemically identical subunits (pseudo-isologous association).

Introduction

NAD-specific glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) has been purified from a variety of sources [1] and is a tetrameric protein made up of chemically identical subunits. The subunits are not functionally identical and are probably arranged pairwise, i.e., the enzyme molecule behaves as a dimer of dimers. This is evident from half-site reactivity shown by various glyceraldehyde-3-phosphate dehydrogenases [2—5], shown by X-ray crystallography on lobster enzyme [6] and kinetics and thermodynamics of hybridization of yeast and rabbit muscle enzymes [7].

Very little is known about plant glyceraldehyde-3-phosphate dehydrogenase. Duggleby and Dennis [8] purified this enzyme from pea seeds and have studied some of its properties and steady-state kinetics [9,10]. In our laboratory this enzyme has been purified by a somewhat different procedure, which has some advantages.

A casual observation suggested to us that inactivation kinetics might be used as a probe for molecular dissymmetry of some enzymes. Thus, freshly crystallised glyceraldehyde-3-phosphate dehydrogenase loses its activity gradually on storage till it has about half of the initial activity. Thereafter, it is stable for long periods [11]. We now describe some thermal and chemical inactivation studies with pea glyceraldehyde-3-phosphate dehydrogenase. Our results strongly suggest the presence of two sets of active sites in a tetrameric molecule, which apparently is composed of identical monomers.

Experimental

Materials

DL-Glyceraldehyde-3-phosphoric acid diethyl acetal barium salt, NAD^+, phenylmethylsulphonyl fluoride and 5,5′-dithio-bis(2-nitrobenzoic acid) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., *p*-Chloromercuri benzoic acid was from B.D.H., Poole, U.K., *N*-ethylmaleimide from Fluka, A.G., Switzerland and iodoacetic acid from L-Light Co., U.K. Buffer substances and other chemicals were of analytical reagent grade. All solutions were prepared in double-distilled water from an all glass assembly. Unless otherwise stated, the experiments were carried out in 10 mM potassium phosphate and 2 mM EDTA (pH 7.2) (extraction buffer).

Methods

Enzyme assay. The substrate solution was prepared and standardised from the barium salt of its diethyl acetal as described by Ferdinand [12]. The glyceraldehyde-3-phosphate dehydrogenase activity was measured spectrophotometrically by monitoring the rate of formation of NADH in the reaction of 0.625 mM D-glyceraldehyde-3-phosphate, 0.625 mM NAD^+ and 43.75 mM arsenate in 40 mM pyrophosphate buffer (pH 8.6) at 30°C [13]. The increase in absorbance with time was measured at 366 nm in an Eppendorf Photometer. The initial rate of reaction was obtained graphically. ϵ_{NADH} at 366 nm is $3.11 \cdot 10^3\ M^{-1} \cdot cm^{-1}$. An enzyme unit was defined as that amount which brought about the reduction of 1 μmol NAD^+ to NADH in 1 min under the test condition. Protein was estimated by the method of Lowry et al. [14]. The specific activity is given in terms of enzyme units/mg protein.

Purification of enzyme. Glyceraldehyde-3-phosphate dehydrogenase was purified from pea seeds (*Pisum sativum)* by the following steps, modified from the procedure of Duggleby and Dennis [8].

(1) Crude extract: 120 g pea seeds were soaked overnight in the extraction buffer and crushed next day and suspended in the same buffer containing a protease inhibitor (phenylmethylsulphonyl fluoride, 16 mg/1000 ml extract), filtered through a muslin cloth and centrifuged to get a clear extract.

(2) First $(NH_4)_2SO_4$ fractionation: The pH of the extract was adjusted to 7.0 and it was gradually brought to 62% saturation of $(NH_4)_2SO_4$ keeping the pH constant at 7.0. The precipitate was rejected and the supernatant brought to 87% saturatuon of $(NH_4)_2SO_4$. After 1 h stirring, the precipitate was collected by centrifugation and suspended in the extraction buffer to 30 mg protein/ml.

(3) Heat treatment: The above solution was heated at 60°C for 2 min with constant shaking and chilled immediately. The precipitated proteins were removed by centrifugation and clear solution collected. Better and more reproducible results were obtained if this step was carried out with smaller lots (5 ml) instead of taking the entire solution of the previous step.

(4) Second $(NH_4)_2SO_4$ fractionation: The above solution was diluted to about 15 mg protein/ml, the pH adjusted to 7.8 and then brought to 65% saturation of $(NH_4)_2SO_4$ at this pH. The precipitate was discarded and the supernatant brought to 80% saturation of $(NH_4)_2SO_4$. The precipitated protein was collected by centrifugation and dissolved in 10 ml extraction buffer.

(5) Ethanol fractionation: To the above solution, pre-cooled ethanol (−15°C) was added at −15°C with vigorous shaking (70 ml ethanol/100 ml solution). The resulting suspension was centrifuged and the precipitate discarded. To the supernatant, more ethanol was added (60 ml ethanol/100 ml supernatant). The resulting precipitate was collected and suspended in 7 ml buffer. The suspension was centrifuged to obtain a clear solution.

(6) DEAE-cellulose chromatography: The above solution was dialyzed overnight against three changes of the extraction buffer and the dialysed enzyme solution was fed to a DEAE-cellulose column (2.5 × 30 cm) pre-equilibrated with the extraction buffer. The enzyme was adsorbed. It was eluted with extraction buffer containing 25 mM KCl.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed at pH 8.3 as described by Reisfeld et al. [15]. Sodium dodecyl sulfate (SDS) gel elec-

trophoresis was performed by the method of Weber and Osborn [16].

Heat inactivation. Kinetics of heat inactivation were studied with crude extract as well as with the purified enzyme. In the case of purified enzyme, the solution was diluted in extraction buffer to a protein concentration of 300—400 μg/ml. The crude extract or the diluted solution of purified enzyme was maintained at the desired temperature. At different time intervals, a small aliquot was withdrawn, chilled and 0.01 ml added directly to the test solution which was kept ready for this purpose. The increase in absorbance with time was noted as usual.

For reactivation (or 'annealing') experiments, enzyme solution was heated for the desired period of time at specified temperature after which the solution was immediately chilled. The activity of cooled enzyme was determined at different times till it became constant.

Inactivation with SH reagents. The enzyme (4 μg/ml) was incubated with 0.625 mM NAD^+ and the SH reagent (20 μM *N*-ethylmaleimide, 30 μM iodoacetate or 0.6 μM *p*-chloromercuri benzoate) in assay buffer (pH 8.6) at 33° C. At different intervals, 0.75 ml aliquots were withdrawn directly into the cuvette and reaction started by adding 0.05 ml 10 mM substrate solution. The rate of increase of absorbance was measured at 366 nm.

Results

Purification and characterization of enzyme

A summary of the results of the purification procedure is given in Table I. After the final step, the enzyme is purified about 200-fold with an overall recovery of 32%. The specific activity of the final preparation varies between 110 and 120 units/mg protein. This corresponds to the maximum specific activity described by Duggleby and Dennis [8] (viz. about 200 under their test conditions). Our purification procedure is different from that of the previous authors in the following four respects: (i) The preparation of acetone powder has been done away with; (ii) The enzyme is extracted in the presence of a pro-

TABLE I

SUMMARY OF PURIFICATION OF PEA GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (FROM 120 g OF PEA SEEDS)

Step	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification	Recovery (%)
Crude extract	400	5600	9960	0.56	—	—
First $(NH_4)_2SO_4$ fractionation	35	4305	1165	3.70	6.5	76.8
Heat treatment	35	4025	1036	3.90	6.9	71.8
Second $(NH_4)_2SO_4$ fractionation	10	3150	302	10.40	18.6	56.2
Ethanol fractionation	7	2716	35.7	76.00	135.7	48.5
DEAE-cellulose chromatography	90	1800	16.2	111.00	198.7	32.1

tease inhibitor (phenylmethylsulphonyl fluoride). This results in greater stability and better recovery of the enzyme in early purification steps; (iii) Heat treatment is performed after first $(NH_4)_2SO_4$ step, when the volume is more easily managed; (iv) DEAE-cellulose chromatography has been introduced as the final step. In some batches where alcohol fractionation is more effective, there is practically no purification in the chromatographic step. In other cases it brings about some purification, so that the final specific activity is always in the range of 110—120 units/mg protein. In all cases, however, the DEAE-cellulose chromatography leads to a complete removal of bound nucleotides, whereas the enzyme preparation of Duggleby and Dennis [8] is reported to contain 2 NAD^+ bound/mol of enzyme. The A_{280nm}/A_{260nm} ratio increases from about 0.9 to 1.5 as a result of this step. After DEAE-cellulose chromatography, any further treatment of the enzyme with animal charcoal or CM-cellulose did not bring about any change in the A_{280nm}/A_{260nm} ratio, suggesting that the enzyme at this stage did not have any bound nucleotides.

At this stage, the enzyme preparation gives a single band on polyacrylamide gel electrophoresis (with and without SDS). Thus, the enzyme is made up of more or less identical subunits. Molecular weight of the subunit as determined from SDS gel electrophoresis is 38 000, in good agreement with the earlier reported value of 36 640 [8].

Duggleby and Dennis [8] found the sedimentation coefficient of their enzyme to be equal to that of the rabbit muscle enzyme, suggesting more or less equal values for the molecular weights of the two enzymes (viz. 140 000—150 000). Therefore, pea glyceraldehyde-3-phosphate dehydrogenase must also be a tetramer made up of identical monomers (subunit molecular weight 36 000—38 000).

The purified enzyme solution shows a typical protein spectrum with maximum absorption at 278 nm and the A_{280nm}/A_{260nm} ratio is equal to 1.48. At a concentration of 1 mg protein/ml, the enzyme gives an absorbance of 1.04 at 280 nm.

Thermal inactivation

The time-dependent inactivation of pea glyceraldehyde-3-phosphate dehydrogenase has been studied with crude preparation (at 55°C) as well as pure enzyme (at 50 and 55°C). The results are shown in Sigs. 1, 2 and 3. In all cases, biphasic kinetics are observed and each phase accounts for destruction of exactly half of the initial activity. This is more clearly seen from the semilog plots, shown as insets of Figs. 1, 2 and 3. The overall kinetic behaviour is consistent with rate equation (Eqn. 1).

$$A = \frac{A_0}{2} \cdot e^{-k_1 t} + \frac{A_0}{2} \cdot e^{-k_2 t} \quad \ldots \qquad (1)$$

where A_0 and A are activities at times 0 and t, respectively, and k_1 and k_2 are first-order rate constants for the fast and slow phases of the reaction, respectively.

The values of k_1 and k_2, under various conditions, are shown in Table II. The value of k_1 at 55°C for purified enzyme could not be determined, as the half life of the fast phase was very small. This phase was completed in less than

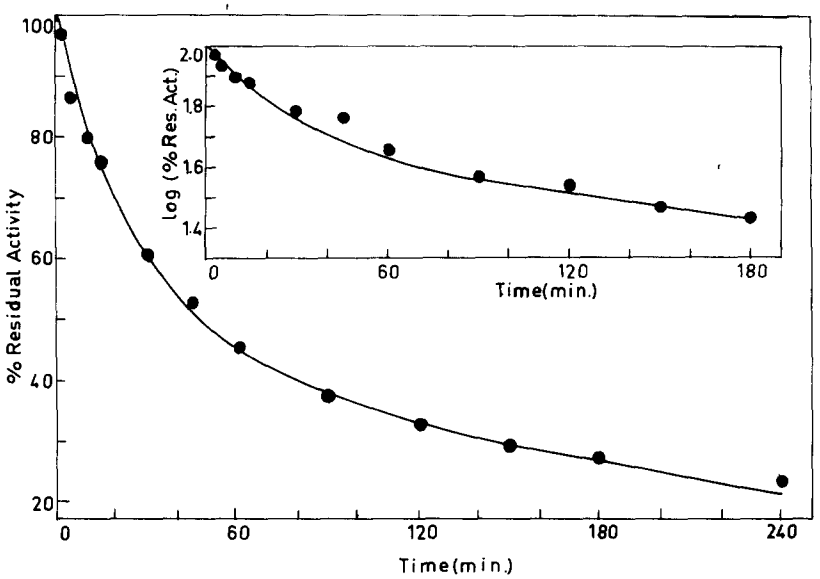


Fig. 1. Heat inactivation of crude pea glyceraldehyde-3-phosphate dehydrogenase at 55°C (in extraction buffer, pH 7.2). Protein concentration was 15 mg/ml. The curve is calculated on the basis of Eqn. 1 (see text) with rate constants of Table II and compared with experimental points. The inset is a semilog plot of the experimental points.

2 min, when the first aliquot was withdrawn for testing. In Figs. 1 and 3 the experimental points are compared with theoretical curves calculated on the basis of Eqn. 1 and rate constants of Table II. In each case, the correspondence is remarkable.

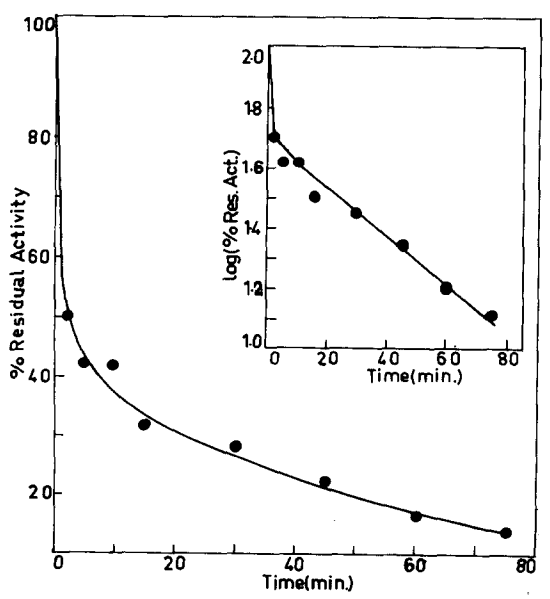


Fig. 2. Heat inactivation of purified pea glyceraldehyde-3-phosphate dehydrogenase at 55°C (in extraction buffer, pH 7.2). Protein concentration was 350 μg/ml. The fast reaction in this case is almost complete within 2 min. The inset shows a semilog plot.

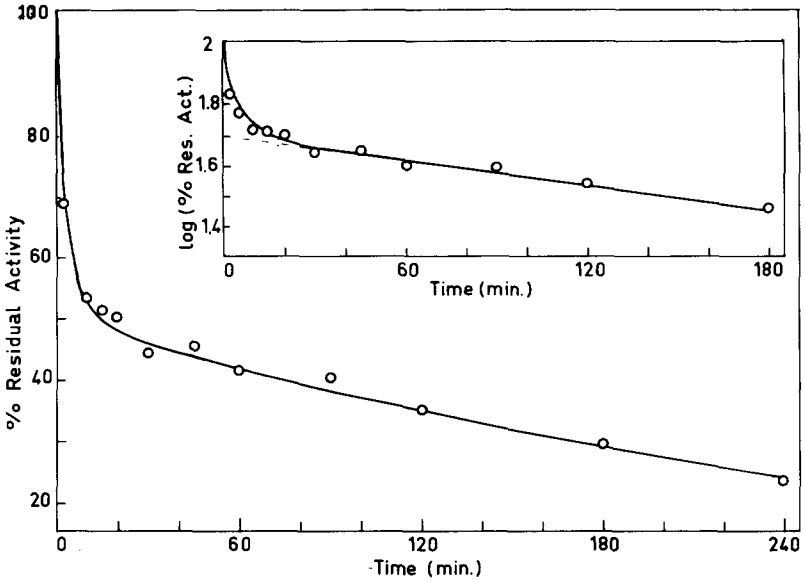


Fig. 3. Heat inactivation of purified pea glyceraldehyde-3-phosphate dehydrogenase at 50°C (in extraction buffer, pH 7.2). The curve is calculated on the basis of Eqn. 1 (see text) with the rate constants of Table II and compared with the experimental points. The inset is a semilog plot of the experimental points.

Biphasic thermal inactivation has been reported earlier for acetoacetate decarboxylase obtained from *Clostridium acetobutylicum* [17]. In that case, however, the two phases do not account for equal proportions of the initial activity. Further, the relative contributions of the two phases change with temperature. In our case, each phase accounts for eactly half of the initial activity and this behaviour is independent of (i) the temperature (50—55°C), (ii) the state of purification and (iii) the presence of excess NAD^+. These three parameters alter the values of the two rate constants without changing the overall pattern or the applicability of Eqn. 1.

In the above experiments, the enzyme aliquot was chilled and immediately transferred to the assay mixture (at 30°C) and thus, there was practically no time lag between heating and activity measurement. If, however, the enzyme

TABLE II

RATE CONSTANTS FOR THE BIPHASIC HEAT INACTIVATION OF PEA GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

Protein concentration was 350 μg/ml for the purified enzyme and 15 mg/ml in the crude extract.

Enzyme preparation	Temperature (°C)	Rate constants (min^{-1})	
		For fast phase (k_1)	for slow phase (k_2)
Pure enzyme	50	0.28	0.003
Pure enzyme	55	—	0.018
Pure enzyme + 1.2 mM NAD^+	55	0.14	0.0025
Crude extract	55	0.039	0.0035

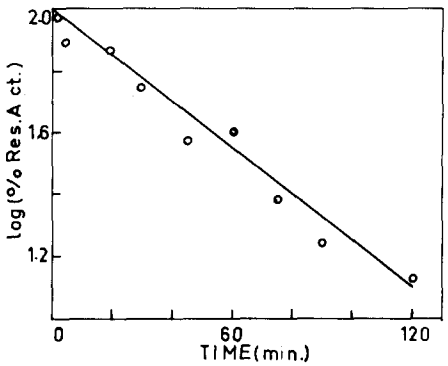


Fig. 4. Semilog plot of percent residual activity after 'annealing' versus initial periods of heating of purified glyceraldehyde-3-phosphate dehydrogenase at 55°C (see text).

solution after heating is allowed to stand in ice bath prior to the activity estimation, there is a time-dependent partial recovery of enzyme activity ('annealing'). The final activity after 'annealing' depends on the extent of damage to the protein during heating. If the inactivation on heating is 50% or less, the recovery is more or less quantitative. As the inactivation proceeds further, lesser and the lesser activity can be restored on cooling. When the final activity after 'annealing' is plotted against the initial period of heating, simple first-order kinetics are observed (Fig. 4). This is in sharp contrast to the biphasic kinetics obtained if the activity estimation is carried out without allowing any 'annealing' to occur. The rate constant for activity loss obtained in this case (0.017 min^{-1} for heating at 55°C, followed by 'annealing' at 0°C) correspond very closely to k_2 for thermal inactivation at the corresponding temperature (0.018 min^{-1} at 55°C).

The above results, specially the extent of recovery on annealing, suggest that the activity lost due to the fast phase of thermal inactivation only can be regained on 'annealing'. The extent of recovery decreases as the slow phase progresses.

The data on thermal inactivation and 'annealing' taken together suggest Scheme 1 for the thermal denaturation of pea glyceraldehyde-3-phosphate dehydrogenase.

$$\text{Active enzyme} \overset{k_1}{\rightleftarrows} \text{Half active enzyme} \overset{k_2}{\rightarrow} \text{Inactive enzyme}$$

Scheme 1.

A possible alternative explanation would be that our enzyme preparation may contain two different proteins of unequal thermal stability but making equal contribution to the overall activity both in the crude extract and purified enzyme. The less stable protein would then be presumed to be reactivated on cooling. This explanation may be ruled out on more than one considerations: (1) The ratio of the two proteins must be the same in the crude extract and the purified enzyme, which is very unlikely. (2) Only a single protein band is observed in polyacrylamide gel electrophoresis (with and without SDS) of the purified enzyme, suggesting not only the presence of a single oligomeric protein

(after purification) but also that it is made up of only one type of subunit. (3) According to this explanation, the activity after 'annealing' should be independent of the extent of progress of the slow phase and in no case it should be less than 50% of the initial activity. Our experiments show that the extent of recovery on 'annealing' is very much dependent on the extent of progress of the slow phase. The activity after 'annealing' may approach zero if the slow phase goes almost to completion (Fig. 4).

Inactivation with SH reagents

Pea glyceraldehyde-3-phosphate dehydrogenase contains some very reactive SH groups, which can be easily titrated with 5,5′-dithio-bis-(2-nitrobenzoic acid). A total of 3.8 reactive SH groups are titrated per mol of enzyme (molecular weight 152 000) which corresponds to approx. 1 SH group/subunit. These SH groups appear to be closely involved in the enzyme activity. Thus, the enzyme is inactivated in the presence of even small concentrations of SH reagents, like *N*-ethylmaleimide, iodoacetate and *p*-chloromercuri benzoate. The results of treatment of pea glyceraldehyde-3-phosphate dehydrogenase with excess iodoacetate and *N*-ethylmaleimide are shown in Fig. 5. The rate constants of reaction with 30 μM iodoacetate and 20 μM *N*-ethylmaleimide are 0.139 and 0.099 min^{-1}, respectively, at 33° C. All the SH groups apparently have equal reactivities against these reagents.

However, differences in the order of reactivities of SH groups are observed in the reaction of pea glyceraldehyde-3-phosphate dehydrogenase with *p*-chloro-

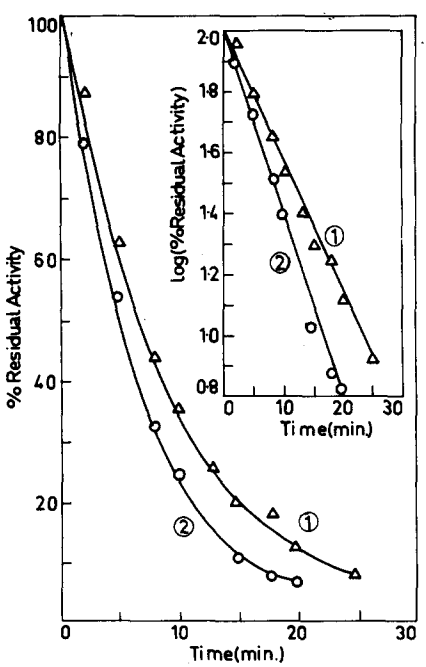


Fig. 5. Inactivation of purified pea glyceraldehyde-3-phosphate dehydrogenase (4.0 μg/ml) with 20 μM *N*-ethylmaleimide (curve 1) and 30 μM iodoacetate (curve 2) at pH 8.6 and 33°C in assay buffer in the presence of 0.625 mM NAD^+.

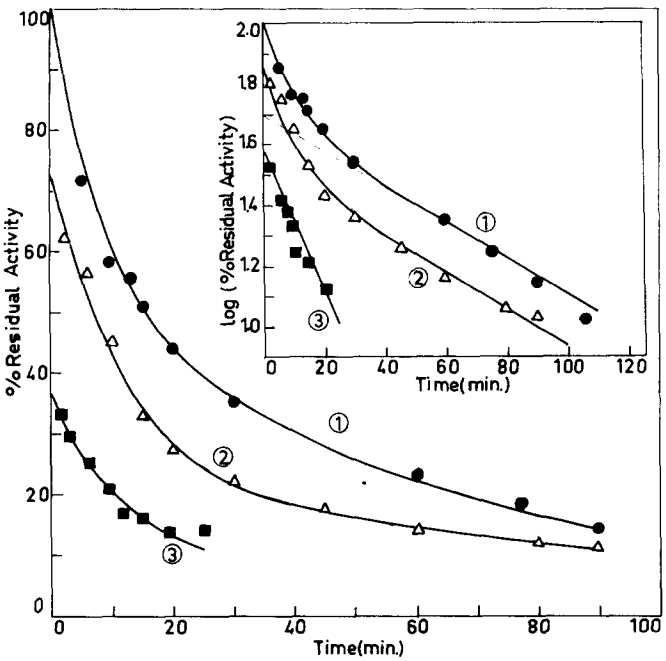


Fig. 6. Inactivation of purified pea glyceraldehyde-3-phosphate dehydrogenase (4.0 μg/ml) with 0.6 μM *p*-chloromercuri benzoate at pH 8.6 and 33°C in the presence of 0.625 mM NAD^+. Curve 1 is calculated on the basis of Eqn. 1 and is compared with experimental points. Curve 2 shows the reaction of *p*-chloromercuri benzoate with an enzyme sample which was first subjected to heat inactivation (at 55°C, residual activity 29%) and allowed to 'anneal' in an ice bath (final activity 70.8% of the original) before treatment with *p*-chloromercuri benzoate. Curve 3 shows the reaction of *p*-chloromercuri benozate with an enzyme sample which was inactivated to 37.5% residual activity by heating at 55°C and then treated with *p*-chloromercuri benzoate without allowing the enzyme to 'anneal'.

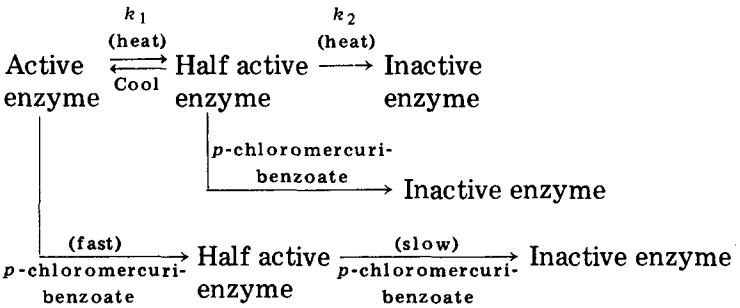
mercuri benzoate. Kinetics of inactivation with 0.6 μM *p*-chloromercuri benzoate at 33°C are shown in Fig. 6. Note the biphasic character of the reaction, as is the case with heat inactivation. In Fig. 6 experimental points are compared with the calculated curve (curve 1) based on Eqn. 1. and a close correspondence is evident. The rate constants for the fast and the slow reactions in this case are 0.107 and 0.0139 min^{-1}, respectively.

According to Scheme I, if partially thermally inactivated enzyme is allowed to regain its activity, we should get a mixture of fully active native enzyme and completely inactive protein. When such a mixture is treated with *p*-chloromercuri benzoate, and the activity monitored at different times, the kinetics must be identical with those observed with untreated enzyme. Results of such an experiment are shown in Fig. 6 (curve 2). The enzyme was heated at 55°C for 20 min (residual activity 29%), cooled in ice bath for 'annealing' (activity recovered to 70.8% of the original) and then treated with *p*-chloromercuri benzoate. The loss in activity follows biphasic kinetics, with more or less the same rate constants (k_1 = 0.099 min^{-1}, k_2 = 0.0144 min^{-1}) as are observed with the untreated enzyme.

Banas et al. [18] have also demonstrated similar differences in reactivities of SH groups (Cys 149) of rabbit and pig muscle glyceraldehyde-3-phosphate dehydrogenase against 5,5′-dithio-bis-(2-nitrobenzoic acid). On the other hand,

the biphasic reaction of active site SH groups of rabbit muscle enzyme with a fluorogenic reagent (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) seems to be complicated by concomitant reaction of neighboring SH groups [19].

The above experiments show that the active sites of pea glyceraldehyde-3-phosphate dehydrogenase can be differentiated on the basis of heat and *p*-chloromercuri benzoate inactivation. The relationship between these two modes of inactivation can be shown by a mixed inactivation experiment. In this experiment the enzyme is first partially inactivated by heating (residual activity <50%) and then treated with *p*-chloromercuribenzoate immediately (without 'annealing'). Results of such an experiment are shown in Fig. 6 (curve 3). This reaction exhibits single first-order kinetics with a rate constant equal to 0.051 min^{-1} which must be the rate constant for the reaction of thermally modified enzyme with excess *p*-chloromercuri benzoate. The value of the rate constant is different from the values for the fast (0.107 min^{-1}) and the slow phases (0.0139 min^{-1}) of the reaction of native purified enzyme with this reagent. Scheme II given below is an extension of Scheme I and gives a summary of the studies of inactivation of pea glyceraldehyde-3-phosphate dehydrogenase with heat and *p*-chlorormercuri benzoate.



(Scheme II)

Discussion

Pea glyceraldehyde-3-phosphate dehydrogenase is made up of apparently identical subunits. Some of the properties of these subunits in the tetrameric enzyme are identical as in the reaction with iodoacetate and *N*-ethylmaleimide. The subunits, however, are not identical in all respects. The molecular asymmetry is clearly evident from the biphasic kinetics of inactivation by heat and *p*-chloromercuri benzoate. The two sets of sites are also differentiated by the 'annealing' experiment, which shows that only the fast phase of heat inactivation is reversible. The equilibrium is temperature dependent; lower temperatures favour the active form.

The unique quantitative relationship in the biphasic kinetics of inactivation suggests that the two types of site must make equal contributions to the overall enzyme activity. A simple assumption will be that there are an even number of sites (in the case of a tetrameric protein, two or four) of equal activity, half of which are destroyed at a faster rate than the rest. This suggests a pairwise arrangement of monomers in the tetrameric enzyme molecule.

The enzyme preparation used in these experiments is apparently free from any bound nucleotide or from any other known effector. The molecular dissymmetry observed under these conditions (specially in the heat inactivation) is significant, because this must arise from a unique feature of the quaternary structure and cannot be said to have been 'induced' by any effector. The association of subunits in this case must, therefore, be heterologous or pseudo-isologous [20].

Acknowledgements

Financial assistance of the University Grants Commission and Council of Scientific and Industrial Research, New Delhi, are gratefully acknowledged.

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