HALF-OF-THE-SITES REACTIVITY OF YEAST GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE

W. B. Stallcup and D. E. Koshland, Jr.

Department of Biochemistry
University of California, Berkeley, California 94720

Received September 28,1972

SUMMARY: Yeast glyceraldehyde 3-phosphate dehydrogenase is shown to exhibit a half-of-the-sites reactivity with both alkylating and acylating reagents. The shape of the inactivation profiles indicates that disubstituted enzyme is inactive and that monosubstituted enzyme has approximately 10% of the activity of native enzyme.

INTRODUCTION

A significant number of enzymes have now been shown to exhibit a half-of-the sites reactivity (1), a phenomenon in which only half of the apparently identical subunits in a polymeric protein react with a given substrate or inhibitor. The explanation of this phenomenon requires an understanding of protein design and subunit interactions, and is inter-related with the regulatory properties of proteins. Preliminary evidence for such half-of-the-sites behavior in yeast glyceraldehyde 3-phosphate dehydrogenase (GPDH) (2, 3) was particularly puzzling in view of the fact that nicotinamide-adenine dinucleotide (NAD) is known to bind to all four subunits (4, 5, 6), and that all four of these sites participate in the catalysis (7, 8). Moreover, the work of Bernhard and co-workers (9, 10) has established a half-of-the-sites relationship for the rabbit muscle enzyme, and it was of interest to compare the phenomenology of these two closely related GPDH's. So much is known about the chemistry and biochemistry of this enzyme that it is a particularly attractive example for the study of half-of-the-sites phenomena. This communication describes studies which reveal a strong half-of-the-sites relationship in yeast GPDH for both alkylating and acylating agents.

MATERIALS AND METHODS

Yeast GPDH was prepared and assayed as described previously (11). The

enzyme had a specific activity of 100 units/mg in the forward reaction and had four functional sites as measured by burst titrations with p-nitrophenyl acetate. The A_{280}/A_{260} was 2.0 - 2.1, indicating little or no bound nucleotide The absorbance of a 1 mg/ml solution of enzyme was taken to be 0.93 at 280 nm.

All absorbance measurements were taken on a Zeiss PM Q II spectrophotometer, and all enzyme assays and other kinetic data were obtained using a Gilford 2000 recording spectrophotometer.

Protein determination was performed using a micro-Folin method (12). In a typical experiment the labelled protein was precipitated with 10% trichloroacetic acid, centrifuged, and finally washed three times in cold 10% trichloroacetic acid. It was then redissolved in 0.1 M Na_2CO_3 for Folin determination and scintillation counting.

Scintillation counting was performed in a Packard TriCarb model 3375 using 10 ml of Bray's solution (13) and 1 ml of water.

Enzyme was centrifuged from ammonium sulfate suspension immediately prior to use, and was incubated for ten minutes at pH 8.5 in 0.05 M Tris-Cl, 0.01 M EDTA, and 0.001 M dithiothreitol. The sample was then passed through a small Sephadex G-50 column equilibrated with the standard pH 8.5 Tris-EDTA buffer without dithiothreitol.

Furyl acryloyl phosphate (FAP) was synthesized according to Malhotra and Bernhard (9). ^{14}C-fluorodinitrobenzene (FDNB) and ^{14}C-iodoacetamide were purchased from Amersham-Searle.

RESULTS

The course of inactivation of the enzyme by iodoacetamide and FDNB is given in Figure 1. It can be seen that the enzyme is essentially completely inactivated when two alkyl groups have been incorporated into the tetramer. The specific modification of cysteine-149 (14, 15) appears to be the decisive factor in the half-of-the-sites inactivation, since the aromatic dinitrophenyl group and the aliphatic carboxyamidomethyl group give virtually indistinguishable inactivation patterns.

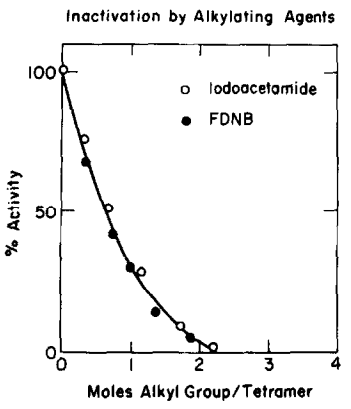

Fig. 1.

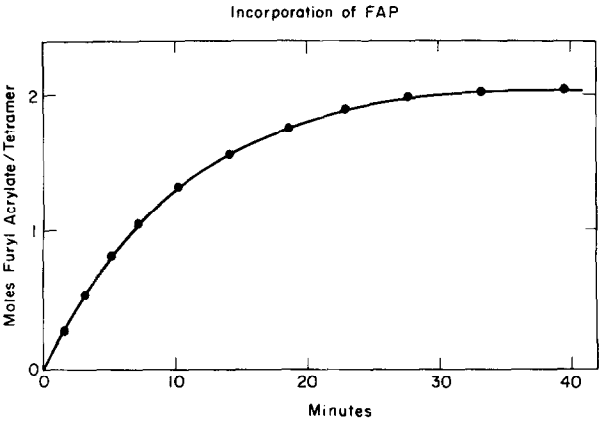

Fig. 2.

Figure 1. Inactivation by alkylating agents. The enzyme was prepared for the experiment as detailed in Methods. After the initial enzymatic activity was determined, 1 µl of either the ^{14}C-FDNB or ^{14}C-iodoacetamide (10^{-2} M in ethanol) was added to 2 ml of enzyme (5×10^{-6} M in pH 8.5 Tris-EDTA). After a 5 minute incubation, the activity was determined again and a small aliquot was removed and precipitated with cold TCA for quantitation of the label as described in Methods. This procedure was then repeated several more times until the enzyme was totally inactivated.

Specific radioactivity of the ^{14}C-FDNB was determined by hydrolysis to dinitrophenol in 0.01 M NaOH followed by determination of the absorbance at 360 nm ($\varepsilon = 1.48 \times 10^4$ at 360 nm) and scintillation counting.

Specific radioactivity of the ^{14}C-iodoacetamide was determined by weighing an exact quantity of the recrystallized reagent and making a standard solution for scintillation counting.

Figure 2. Incorporation of furyl acryloyl phosphate. 50 µl of 60 mM FAP was added to 0.45 ml of enzyme in a cuvette (5×10^{-6} M enzyme in pH 8.5 Tris-EDTA buffer), and the reaction was followed at 360 nm. The stoichiometry of FAP incorporation was calculated using an extinction coefficient of 2.4×10^4 (7), a value which was verified by checking the absorbance of guanidine-HCl denatured enzyme at 336 nm where the extinction coefficient is known to be 3.0×10^4.

In view of the fact that the enzymatic mechanism involves an acyl intermediate at cysteine-149 (16, 17), the effects of acylation were studied using furyl acryloyl phosphate (9). As shown in Figure 2 only two moles of this acyl group could be incorporated into the tetramer. Since under assay conditions the furyl acryloyl enzyme hydrolyzes about 100 times more slowly than the normal phosphoglyceroyl intermediate (10), the inhibitory effects of the group could be determined in the normal assay procedure, as shown in Figure 3. This inacti-

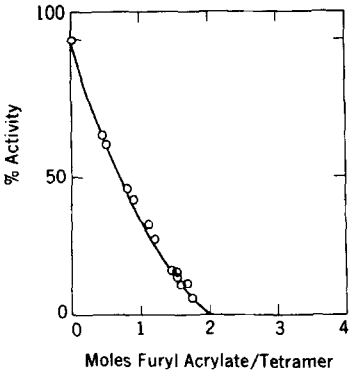

Figure 3. Inactivation by furyl acryloyl phosphate. In parallel to the kinetic experiment of Figure 2, an identical run was carried out. At various times small aliquots were taken from this second sample and assayed for the normal GPDH reaction on a second Gilford. Thus, at these chosen times both the activity and the extent of labelling could be calculated, and an inactivation curve plotted. The experimental points shown here are the result of two separate trials.

---

vation curve is essentially identical to those of Figure 1, showing that in some cases acylation of cysteine-149 can produce the same effects as alkylation in yeast GPDH. Not all acylating agents behave identically, since it has been shown that p-nitrophenyl acetate gives a burst of 4 sites in the initial turnover (11, 18).

Of particular interest is the shape of the inactivation profiles. In all three cases presented, enzyme with an average of one modified SH group per tetramer has significantly less than 50% of the native activity. Such behavior is understandable if the monosubstituted species ER of Equation 1 has less than

$$\underset{\text{activity = 1.0}}{E} \xrightarrow{k_1} \underset{\text{activity = } \phi}{ER} \xrightarrow{k_2} \underset{\text{activity = } \theta}{ER_2} \qquad (1)$$

50% of the native activity and the disubstituted species ER_2 has 0% activity. At any point along the inactivation profile there will be a mixture of these three species of the enzyme. The activity relationships can be analyzed by solving the differential equations for Equation 1 (19) and substituting various values for ϕ and θ. Such calculations have been made in Figure 4, and a family of curves has been drawn for $\theta = 0$ and $\phi = 0$, 0.2, and 0.5. It is apparent

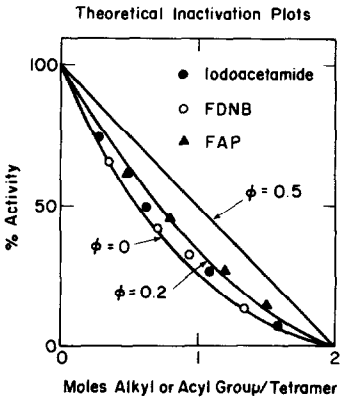

Fig. 4.

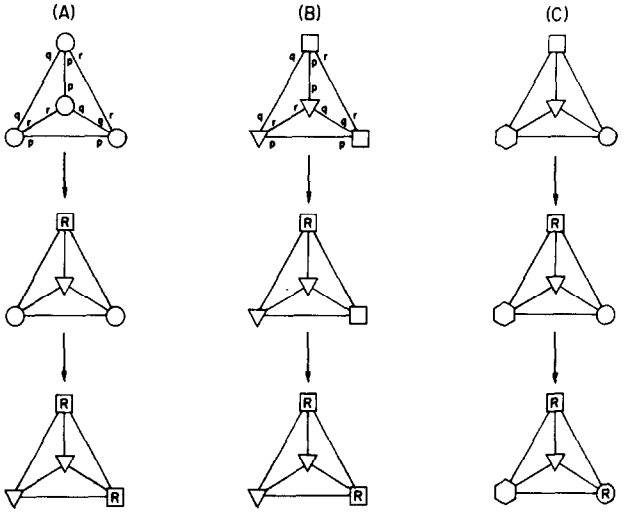

Fig. 5.

Figure 4. Theoretical inactivation plots. Assuming that the first two moles of inhibitor react at the same rate and that there is no further reaction, Equation 1 is solved (17) to give the concentrations of E, ER, and ER_2. $E = E_o e^{-k_1 t}$, $ER = \frac{E_o k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t})$, and $ER_2 = E_o - E - ER$, where $k_1 = 2k_2$. Activity is calculated as $A = 1.0[E] + \phi[ER] + \theta[ER_2]$ where $\theta = 0$ and $\phi = 0$, 0.2, and 0.5. The experimental data is from Figures 1 and 3.

Figure 5. Schematic representations of the reaction of some possible GPDH tetramers with an inhibitor.

(A) An α_4 tetramer in which all the subunits are initially identical. Reaction of R induces a conformational change across the pp domain only.

(B) An $\alpha_2\alpha'_2$ tetramer in which there is a pre-existing asymmetry within the pp domain such that only two subunits react with R. This reaction induces no further conformational changes.

(C) An $\alpha\alpha'\alpha''\alpha'''$ tetramer in which pre-existing asymmetry extends across all domains and gives each subunit a unique conformation.

---

that the experimental data lie between the curves in which $\phi = 0$ and 0.2. Thus the monosubstituted enzyme must be less than 20% as active as native enzyme, and in fact is of the order of 10% active.

DISCUSSION

Previously, four mechanisms were listed as possible explanations for the half-of-the-sites phenomenon (1): (a) a pre-existing asymmetry due to non-identical primary structure of the subunits, such as is found in isozymes or

in hemoglobin, (b) a pre-existing asymmetry due to conformational changes induced by the association of the subunits, as postulated for the rabbit muscle GPDH (9, 10) and observed in insulin crystals (20), (c) a steric or electrostatic interaction between adjacent active sites, and (d) a negative cooperativity in which one ligand induces conformational changes that affect the reactivity of adjacent subunits (21, 22). Alternative (a) and (c) appear very unlikely in the light of the sequence studies on yeast GPDH by Jones and Harris (23) and the crystallographic studies of Rossman *et al.* (24) (insofar as X-ray data for the lobster GPDH can be extrapolated to the yeast enzyme). Such studies do not allow us to decide conclusively on alternative (b), however, and thus two alternative structures for the initial apo-enzyme are drawn in Figure 5A and B, using the previously described nomenclature for quaternary interactions (25). In these two structures it is assumed that the ligand-induced asymmetry (Figure 5A) or pre-existing asymmetry (Figure 5B) is confined to the dimer, i.e. across the pp domain, and hence the unmodified dimer should be unaffected, leaving the monosubstituted enzyme 50% active. This, of course, does not fit the experimental facts. The data could be explained by a pre-existing structure such as that in Figure 5C in which each of the subunits has a unique conformation, but this seems unsatisfactory in view of the X-ray data.

It seems necessary, therefore, that a suitable model include an induced conformational change across the qq and/or rr domain. This latter alternative will allow the first mole of inhibitor to affect more than one of the adjacent subunits and is capable of explaining the concave inactivation plots of Figures 1 and 3. This reduced reactivity could be caused either by affecting the innate reactivity of a qq or rr partner or by affecting the binding properties of the coenzyme NAD. It remains to be resolved whether the half-of-the-sites reactivity is caused by ligand-induced changes across the pp domains of an α_4 enzymes (all subunits initially identical) or by a pre-existing $\alpha_2\alpha'_2$ structure, but it is clear that within either of these frameworks the inactivity of the one-fourth modified enzyme must be caused by ligand-induced changes across the qq or rr domains.

REFERENCES

1. Levitzki, A., Stallcup, W. B., and Koshland, D. E., Jr., Biochemistry 10, 3371 (1971).
2. Halsay, Y. D., J. Biol. Chem. 214, 589 (1955).
3. Givol, D., FEBS Letters 5, 153 (1969).
4. Velick, S., Hayes, J. E., and Harting, J., J. Biol. Chem. 203, 527 (1953).
5. Kirschner, K., Gallego, E., Schuster, I., and Goodall, D., J. Mol. Biol. 58, 29 (1971).
6. Cook, R. A., and Koshland, D. E., Jr., Biochemistry 9, 3337 (1970).
7. Trentham, D. R., Biochem. J. 122, 71 (1971).
8. Kirschner, K., J. Mol. Biol. 58, 51 (1971).
9. Malhotra, O. P., and Bernhard, S. A., J. Biol. Chem. 243, 1243 (1968).
10. Macquarrie, R. A., and Bernhard, S. A., J. Mol. Biol. 55, 181 (1971).
11. Stallcup, W. B., Mockrin, S. C., and Koshland, D. E., Jr., J. Biol. Chem. (in press).
12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).
13. Bray, G. A., Anal. Biochem. 1, 279 (1960).
14. Harris, J. I., and Perham, R. N., J. Mol. Biol. 13, 876 (1965).
15. Shaltiel, S., and Tauber-Finkelstein, M., FEBS Letters 8, 345 (1970).
16. Velick, S. F., and Furfine, C., in P. D. Boyer, H. Lardy, and K. Myrback (Editors), The Enzymes, Vol. VII, Academic Press, New York, p. 243 (1963).
17. Perham, R. N., and Harris, J. I., J. Mol. Biol. 7, 316 (1963).
18. Behme, M. T. A., and Cordes, E. H., J. Biol. Chem. 242, 5500 (1967).
19. Frost, A. A., and Pearson, R. G., Kinetics and Mechanism, Ed. 2, John Wiley and Sons, Inc., New York (1961).
20. Adams, M. J., Blundell, T. L., Dodson, E. J., Dodson, G. G., Vijayan, M., Baker, E. M., Harding, M. M., Hodgkin, D. C., Rimmer, B., and Sheat, S., Nature 224. 491 (1969).
21. Conway, A., and Koshland, D. E., Jr., Biochemistry 7, 4011 (1968).
22. Levitzki, A., and Koshland, D. E., Jr., Proc. Nat. Acad. Sci. U.S. 62 1121 (1969).
23. Jones, G. M. T., and Harris, J. I., FEBS Letters 22, 185 (1972).
24. Rossman, M. G., Ford, G. C., Watson, H. C., and Banaszak, L. J., J. Mol. Biol. 64, 237 (1972).
25. Cornish-Bowden, A., and Koshland, D. E., Jr., J. Biol. Chem. 245, 6241 (1970).