A NEW CATALYTIC ACTIVITY OF α-GLYCEROLPHOSPHATE DEHYDROGENASE: THE ENZYMATIC HYDROLYSIS OF p-NITROPHENYL ACETATE*

Marcelo ALFONZO** and Rafael APITZ-CASTRO
Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 1827, Caracas, Venezuela

Received 13 October 1971

1. Introduction

It has been shown that the maleimides react with α-glycerophosphate dehydrogenase (GPDH) [1]. Since histidine appears to be implicated in the reaction with *N*-ethylmaleimide, we investigated the possibility that it might also participate as a nucleophile in an acylation reaction. The present communication describes a new catalytic activity of soluble rabbit muscle GDPH, namely the enzymatic hydrolysis of *p*-nitrophenyl acetate.

2. Materials and methods

GPDH from rabbit muscle was either purchased from Boehringer-Mannheim and recrystallized in the laboratory, or was isolated from the same source using a method already described [2]. Both preparations were homogeneous in polyacrylamide gel electrophoresis and no activity toward glyceraldehyde-3-phosphate was detectable, even at very high enzyme concentration (1 mg/ml). Extensive dialysis of the enzyme against Tris-HCl, pH 7.5, or distilled water containing EDTA (1 g/l) was carried out prior to each assay of activity. Dehydrogenase activity of GPDH was measured using dihyroxyacetone phosphate as substrate [2]. Dihydroxyacetone phosphate was obtained from Boehringer-Mannheim as the dimethylketal, dicyclohexylammonium salt. D-glyceraldehyde-3-phosphate was obtained from Sigma Chemical Co., as the diethylacetal, monobarium salt. *p*-Nitrophenyl acetate (*p*-NPA), substrate grade, was a commercial preparation from Sigma Chemical Co.

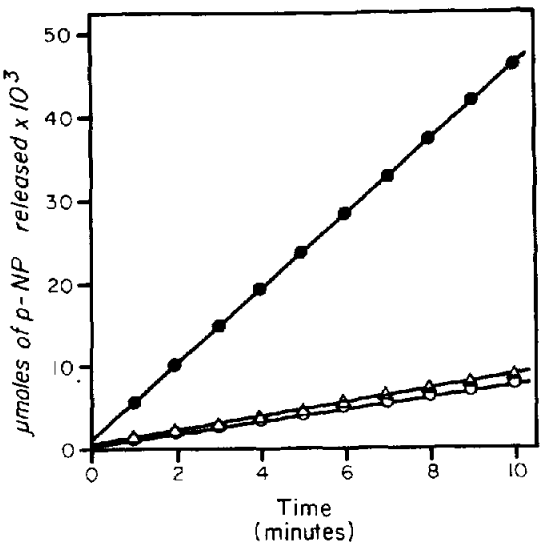

Fig. 1. Hydrolysis of *p*-NPA catalyzed by GPDH. {E} = 13 μM, *p*-NPA = 3.3 mM. Buffer Tris-HCl, 50 mM, pH = 7.13. 25°. •, Enzymatic hydrolysis; ○, spontaneous hydrolysis of *p*-NPA; △, activity after heating for 2 min at 100°.

* Presented as part of a Symposium on "The Molecular Basis of Biological Activity", First Meeting of the Pan-American Association of Biochemical Societies (PAABS), Caracas, 11–17 July (1971).

** Student assistant under the training program of the Centro de Estudios Avanzados (C.D.E.A.), I.V.I.C.

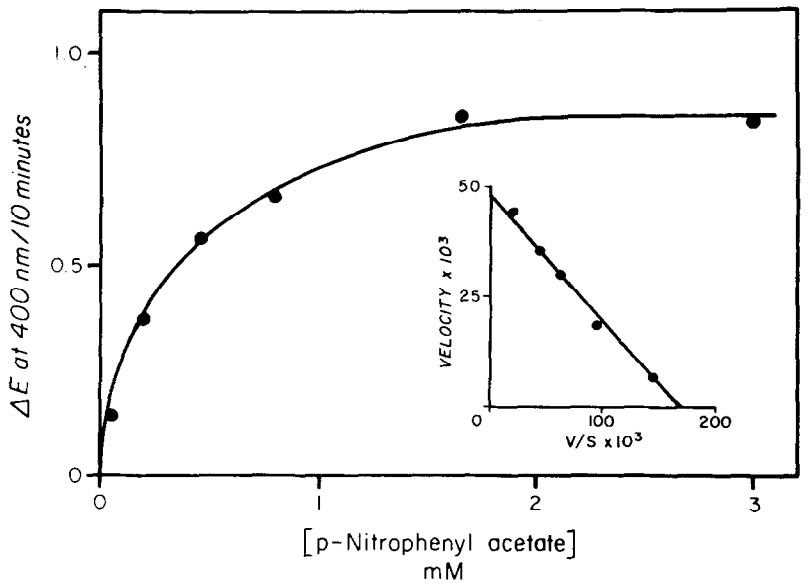

Fig. 2. Dependence of the esterolytic activity of GPDH on *p*-NPA concentration. Same conditions as in fig. 1. Hofstee plot shown in the insert: velocity expressed as μmoles of *p*-NP produced in 10 min.

Rose Bengal 2B, obtained from Edward Gurr, was purified before use [3]. Norit A was obtained from Pfanstiehl Lab. Inc., Illinois, and *p*-chloromercuribenzoate (*p*-CMB) from Sigma Chemical Co.

Hydrolysis of *p*-NPA was measured by monitoring the formation of *p*-nitrophenoxide ion at 400 nm as a function of time [4]. Calculation of total product concentration (phenol + phenolate) was carried out with an $\epsilon_{400} = 1.84 \times 10^4$ for the *p*-nitrophenoxide ion and pKa value of 7.13 for *p*-nitrophenol [5].

Protein was determined by the method of Waddell [6, 7]. All other reagents were of the best commercial grade available.

3. Results and discussion

The enzyme showed catalytic activity towards *p*-nitrophenyl acetate when assayed in Tris buffer, pH 7.13, at enzyme concentrations ranging from 2–14 μM. The same results were obtained both in Veronal and maleate buffer.

Fig. 1 shows the time course of hydrolysis of *p*-NPA catalyzed by GPDH. Under the experimental conditions employed, the rate of the spontaneous hydrolysis of *p*-NPA amounts only to 16% of that of the enzymatic reaction. It is also shown in fig. 1 that heating the enzyme for 2 min at 100° completely abolishes the esterolytic activity of GPDH. The residual activity after this treatment amounts to less than 1% of the original. This result indicates that the esterolytic activity of GPDH requires the three-dimensional integrity of the enzyme molecule.

In fig. 1 one also notes the absence of a detectable initial "burst" of product, characteristic of many other esterases, including the esterolytic activity of glyceraldehyde-3-phosphate dehydrogenase [8]. A similar result is obtained when the reaction is carried out at 4°.

These results suggest that the rate of enzyme deacylation, if an acyl-enzyme intermediate occurs, is at least as fast as the rate of acylation. A similar situation has been described for the esterolytic activity of hen's egg white lysozyme, known to involve histidine-15 [9].

The dependence of the esterolytic activity of GPDH on the concentration of *p*-NPA is shown in fig. 2. This plot suggests normal Michaelis-Menten behaviour for this reaction. From a Hofstee plot of the data, a value of 2.8×10^{-4} M for K_m app. is obtained.

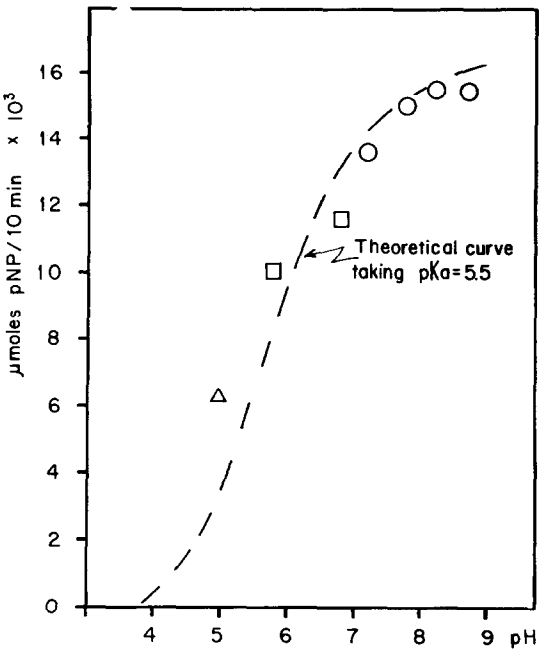

Fig. 3. pH dependence of the rate of hydrolysis of *p*-NPA by GPDH. △, Acetate; □, maleate; ○, Veronal buffers.

Neither EDTA, nor dithiothreitol produce any significant change in the esterolytic activity of GPDH toward *p*-NPA. Treatment of the enzyme with charcoal, in order to separate the non-protein component [10], does not affect the esterolytic activity.

p-CMB inhibits the activity of the enzyme towards *p*-NPA, but, as has been described for the dehydrogenase activity [11], the effect appears to be non-specific. That is, incubation of the enzyme with a concentration of *p*-CMB sufficient to block 15 -SH groups in the enzyme yields only 60% inhibition of esterase activity. This result suggests again that -SH groups in GPDH are mainly engaged in the maintenance of an active conformation of the enzyme but are not directly involved in catalysis. At the same time, it suggests that the esterolytic and dehydrogenase activities probably do not share precisely the same active site. This follows from the fact that similar chemical alterations introduced by the action of *p*-CMB produce somewhat different effects on the dehydrogenase and esterase activities: complete inhibition of the former [11] but only 60% inhibition of the latter.

Photo-oxidation of the enzyme in the presence of Rose Bengal also leads to inhibition of the esterolytic activity. As described for the inhibition by *p*-CMB, the behaviours of the dehydrogenase and esterolytic activities are quite different after photoinactivation in the presence of Rose Bengal. After 20 min of illumination, the degree of inactivation for the dehydrogenase reaction is 75%, while for the esterolytic activity is 95%.

A plausible interpretation of the results just described is that the group or groups involved in the esterolytic activity are exposed on the enzyme surface; hence, conformational changes induced by reaction of *p*-CMB with -SH groups [11] do not strongly affect this already exposed residue. On the other hand, being easily accessible probably renders it more susceptible to destruction by photo-oxidation.

The dependence of the reaction rate on pH, shown in fig. 3, suggests the involvement of the basic form of a group with a pKa near 5.5. The facts that values of pH lower than 5 denature the enzyme [12], and that beyond pH 8.5 difficulties arise from the rapid rate for the spontaneous hydrolysis of *p*-NPA, make it difficult to calculate a precise value for this pKa.

These results, in agreement with those obtained with N-ethylmaleimide [1], suggest that GPDH possesses a highly reactive and relatively exposed histidinyl residue which may function, in the basic form, as a nucleophile and, thus, be responsible for the esterolytic activity of GPDH.

The lack of an initial "burst" of product during the reaction, suggests that the catalysis might operate through a simple bimolecular reaction as has been described for the esterolytic activity of hen's egg white lysozyme [9]. Coincidentally, an unusually low pKa (5.2) has also been found for histidine-15 of lysozyme [9].

Previous studies [13] have shown that, at the active center of GPDH, there are two histidine residues of about equal reactivity towards 5-diazo-1-H-tetrazole, at least one of which is essential for the dehydrogenase activity. The results reported here, although not conclusive, suggest that the site on the enzyme involved in the esterolytic activity of GPDH is different from that responsible for the dehydrogenase activity.

Acknowledgements

The authors are deeply indebted to Dr. Jane Harting Park for helpful discussion and Dr. Eugene H. Cordes for interesting suggestions and reviewing of the manuscript.

References

[1] R. Apitz-Castro and Z. Suárez, Biochim. Biophys. Acta 185 (1969) 258.
[2] R. Apitz-Castro and K. Gaede, Acta Cient. Venezolana 16 (1965) 137.
[3] L. Brand, J.R. Gohlke and D.S. Rao, Biochemistry 6 (1967) 3510.
[4] T. Spencer and J.M. Sturtevant, J. Amer. Chem. Soc. 81 (1959) 1874.
[5] M.T.A. Behme and E.H. Cordes, J. Biol. Chem. 242 (1967) 5500.
[6] W.J. Waddell, Lab. Clin. Med. 48 (1956) 311.
[7] J.B. Murphy and M.W. Kies, Biochim. Biophys. Acta 45 (1960) 382.
[8] J.H. Park, B.P. Meriwether, P. Clodfelder and L.W. Cunningham, J. Biol. Chem. 236 (1961) 136.
[9] D. Piszkiewicz and Th.C. Bruice, Biochemistry 7 (1968) 3037.
[10] H. Ankel, Th. Bucher and R. Czok, Biochem. Z. 332 (1960) 315.
[11] R. Apitz-Castro and K. Gaede, Enzymologia 37 (1969) 119.
[12] J. van Eys, J. Judd, J. Ford and W.B. Womack, Biochemistry 3 (1964) 1755.
[13] R. Apitz-Castro and Z. Suárez, Biochim. Biophys. Acta 198 (1970) 176.