Volume 259, number 2, 297-300 FEB 07973 January 1990

Identification of a reversible structural transition in the metal-depleted glycerol dehydrogenase from *Bacillus stearothermophilus*

P. Spencer, L.J. Paine, M.D. Scawen*, T. Atkinson* and M.G. Gore

Department of Biochemistry, School of Biochemical and Physiological Sciences, University of Southampton, Bassett Crescent East, Southampton SO9 3TU and **Biotechnology Division, PHLS Centre for Applied Microbiology Research, Porton, Salisbury SP4 OJG, England*

Received 16 October 1989

Evidence is presented to demonstrate that the Zn^{2+}-depleted, inactive form of the glycerol dehydrogenase from *Bacillus stearothermophilus* exists in one of two possible conformations in equilibrium, the position of which is temperature sensitive. The conformation of the metal-depleted enzyme favoured by higher temperatures (20–40°C) is able to bind Zn^{2+} and regain catalytic activity, whereas that favoured at lower temperatures (0–10°C) is unable to bind metal ions and is thus inactive. This equilibrium is also pH dependent with a pK of 6.6. At pH 6.0, the equilibrium lies in favour of the form of the enzyme able to bind metal ions and exhibit activity.

Glycerol dehydrogenase; Thermophile; Metallo-enzyme; Conformation

1. INTRODUCTION

Interest in glycerol dehydrogenases (GDHs) has arisen in recent years because of their possible use as clinical diagnostic reagents in the estimation of serum triglyceride. With the exception of the membrane-bound GDH from *Gluconobacter industrius*, which requires pyrroloquinine quinone as cofactor [1], all GDH enzymes studied have been found to be soluble and pyridine nucleotide dependent. The GDHs have been divided into three groups according to the site of oxidation on the glycerol molecule, and whether NAD or NADP acts as the cofactor [2]. The NAD-specific GDH (EC 1.1.1.6) from the thermophile *Bacillus stearothermophilus* is a type 1 GDH catalysing the interconversion of glycerol and dihydroxyacetone.

Although the enzyme has been isolated from several sources, bacterial [3], fungal [4] and mammalian [5], little detailed structure of this class of enzyme is known. We have recently shown that the tetrameric GDH from *Bacillus stearothermophilus* contains one Zn^{2+} ion per subunit (molecular mass = 42 000) and that removal of these ions by EDTA leads to a parallel loss in activity [6]. We have also demonstrated that the structure of the metallo-enzyme and metal-depleted enzyme are different [7]. The inactive metal-depleted enzyme can be reactivated by the addition of one of a variety of divalent cations [7], although preliminary data showed that the reactivation process is complex [6]. This paper describes studies examining the structure or structures of the metal-depleted enzyme and proposes a model to explain the complex reactivation process observed.

Correspondence address: M.G. Gore, Department of Biochemistry, School of Biochemical and Physiological Sciences, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, England

2. MATERIALS AND METHODS

2.1. *Materials*

Bacterial cells (NCIB 11400) were obtained from the Centre for Applied Microbiology and Research, PHLS, Porton, Salisbury, Wiltshire, SP4 0JG. All chemicals were obtained from BDH, Poole, Dorset.

2.2. *Preparation of enzyme*

The bacterial cells were grown as described in [8] and the enzyme was purified as described in [7].

2.3. *Assay*

GDH activity was assayed by following the increase in absorbance at 340 nm at 30°C using a Perkin-Elmer lambda 3 spectrophotometer. Reaction mixtures of 1 ml contained 0.975 ml of 200 mM triethanolamine-HCl at pH 8.5, containing 100 mM glycerol, 25 μl of 15 mM NAD and 5–10 μl of enzyme solution. 1 unit of activity was taken as the amount of enzyme needed to reduce 1 μmol of NAD/min. Protein concentrations were determined by the method of Lowry et al. [9].

2.4. *Preparation of the metal-depleted GDH*

At all stages, precautions were taken to ensure that there was no or minimal contamination by trace elements [10]. Purified enzyme was dialysed against 4 × 5 l of 50 mM potassium phosphate buffer, pH 6.0, containing 10 mM EDTA. A final dialysis against two changes of Chelex 100 (BioRad)-treated buffer was carried out in order to remove the EDTA.

2.5. *Determination of the metal content of the protein*

A Perkin-Elmer 280 Atomic Absorption spectrophotometer was calibrated by sampling solutions of known concentration of the metal ion under investigation. The concentration of metal ions in the buffer

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50 © 1990 Federation of European Biochemical Societies

or protein solution (minimum concentration = 2 μM) was measured and calculated from the appropriate standard curve.

3. RESULTS AND DISCUSSION

Metallo-enzyme (GDH containing 1 mol equivalent of Zn^{2+} per subunit of molecular mass = 43 000) loses its activity if placed in the presence of a chelating agent such as EDTA or hydroxyquinoline [11]. This loss of activity can be reversed by the removal of the chelating agent and the re-addition of Zn^{2+} ions. However, recent experiments have shown that regeneration of catalytic activity is highly dependent upon the conditions in which the metal-depleted enzyme is stored prior to the re-addition of metal ions. Metal-depleted enzymes left at temperatures close to 0°C for 1 h in 50 mM potassium phosphate buffer, pH 7.4, were found to yield little activity on addition to an assay solution (see section 2) containing 20 μM Zn^{2+} ions, whereas solutions of the same metal-depleted enzyme stored at room temperature (21°C) or above were found to give higher levels of activity. This loss of ability of the cold equilibrated metal-depleted enzyme to rapidly regain activity in such assay conditions was found to be time dependent, reversible, and sensitive to both pH and temperature. In the experiment shown in fig.1, samples of metal-depleted enzyme were stored either at 0°C or 40°C for 2 h at pH 7.4 in 50 mM potassium phosphate buffer and samples were then taken for assay (see section 2) in the presence of Zn^{2+} ions. The enzyme sample stored at 40°C regained (within the response time of the spectrophotometer, approximately 5 s) 53% of the activity exhibited by an equivalent amount of native metallo-enzyme under the same conditions, whereas that stored at 0°C showed an immediate activity of only 28%. A rapid switch in temperature was then effected for both solutions from 40°C to 0°C or vice versa and the activity of each sample monitored with time. The enzyme previously stored at 40°C suffered a loss in its ability to recover activity on addition to the assay solution containing Zn^{2+} ions whereas the sample stored at 0°C prior to the temperature change, demonstrated a time-dependent increase in the activity regained in the presence of Zn^{2+} ions. Thus, it appears possible that higher temperatures support a form of the enzyme able to rebind Zn^{2+} and therefore exhibit immediate activity and that lower temperatures stabilise a form unable to bind the metal and which therefore remains inactive. An equilibrium exists between the two forms, the position of the equilibrium being temperature sensitive.

Experiments where the pH of a solution of metal-depleted enzymes was rapidly changed (at a fixed temperature) and samples taken at various times for assay in the presence of Zn^{2+} demonstrated that this equilibrium is also pH sensitive. Fig.2 illustrates the results of one such experiment where metal-depleted enzyme was stored for 2 h at pH 6.0 or pH 8.4 in 50 mM potassium phosphate buffer at 10°C. Samples taken before the pH was rapidly changed showed that at pH 6.0, the metal-depleted enzyme was able to immediately regain 50% of the equivalent amount of native enzyme, whereas the metal-depleted enzyme equilibrated at pH 8.4 showed an immediate regain of only 26%. Thus the lower pH would appear to act in a similar manner to a higher temperature and favour a conformation of the metal-depleted enzyme which is able to exhibit activity on addition to an assay containing metal ions. After a rapid change of the pH of both enzyme incubations (pH

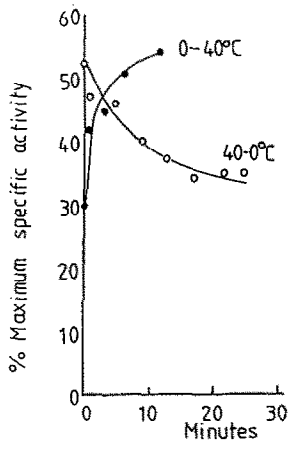

Fig.1. Effect of temperature on the conformation of metal-depleted GDH. Solutions of metal-depleted enzyme (9.2 μM) in 50 mM potassium phosphate buffer pH 7.4 (at the appropriate temperature) were equilibrated at 40°C or 0°C. A rapid change in temperature from 40°C to 0°C or vice versa was then carried out by transfer of 200 μl into an equal volume of the same buffer system at the desired temperature. The activity of the enzyme on addition to an assay containing 20 μM Zn^{2+} was monitored with time.

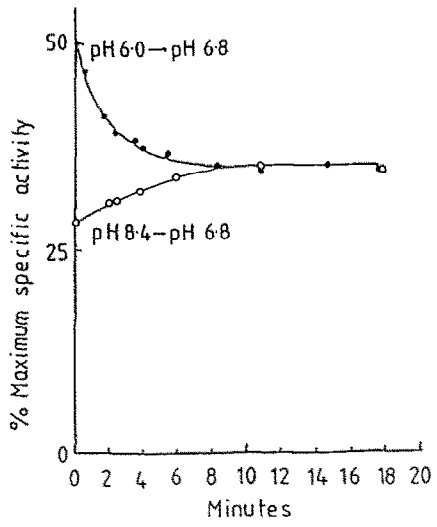

Fig.2. Effect of pH on the conformation of metal-depleted GDH. 50 μl of metal-depleted GDH (60 μM) pre-equilibrated in 50 mM potassium phosphate buffer (pH 6.0 or 8.4 at 10°C) was added to 200 μl of 200 mM potassium phosphate buffer with a final pH of 6.8. Aliquots were taken at various times and the activity determined in an assay containing 20 μM Zn^{2+} ions.

6.0 to 6.8 and pH 8.4 to 6.8) samples were assayed as before. As was expected, metal-depleted enzyme previously at a lower pH (6.0) suffered a pseudo first-order decrease of 0.57/min in the activity exhibited, whereas the metal-depleted enzyme previously equilibrated at the higher pH (8.4) demonstrated a first-order increase in activity (0.25/min) after the change in pH. The end point of each pH change was identical, 34% of maximum specific activity, as would be expected for a non-denaturing reversible change between two conformations and is in agreement with the equilibrium position calculated from the forward and reverse rate constants. The data from a series of these experiments is collated for fig.3 which shows that pH and temperature both play an important role in the balance between the two forms of the metal-depleted enzyme. There is a measurable pK around 6.6 for the transition at 40°C, this is an ionisation on the enzyme and is not due to an ionisation change in the buffer since other buffer systems give identical results. At 20°C there is a smaller change in the amount of activity regained over the same pH range, although a pK around 6.6 is still apparent, however, at 0°C the change in regain of activity is too small to provide adequate data for a pK to be measured. These experiments provide evidence for synergistic effects of temperature and pH described by the postulated equilibria shown below.

metallo-enzyme $\rightleftharpoons$ metal-depleted enzyme $\rightleftharpoons$ metal-depleted enzyme $\rightleftharpoons$ monomers

Zn^{2+}-enzyme	**E***	**E**	
Stable 0–60°C	High temp. (20–40°C)	Low temp. (0–15°C)	
pH 6.0–8.5	Low pH < 6.6	High pH > 6.6	pH > 8.5

The species described above have been shown to be tetrameric under the conditions of the experiments described in this paper. Samples were passed down an HPLC gel permeation column (TSK G3000 SW, 7.5 mm × 600 mm) and the elution of protein was monitored at 230 nm. However, using the same chromatographic system we have shown that when the protein concentration is very low (< 1 μM) and is kept at pH 8.4 or above, dissociation into subunits occurs.

Atomic absorption studies were used to determine whether E*, the form of the metal-depleted enzyme favoured by low pH, or higher temperatures, is able to bind more Zn^{2+} ions per subunit than E, the form favoured by high pH and low temperature, or, whether both forms are able to bind the metal but only E* is in a conformation which is catalytically activity. Enzyme (53 μM) totally depleted of metal with no activity in the absence of Zn^{2+} in the assay mixture, was pre-equilibrated for 2 h at pH 8.0, 10°C and then mixed with 100 μM Zn^{2} ions at pH 8.0. Samples of the mixture

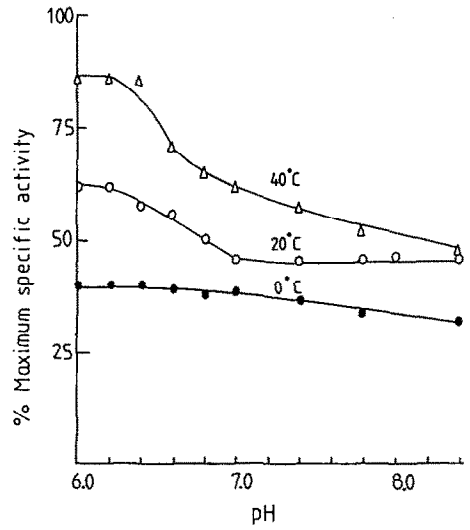

Fig.3. The synergistic effect of pH and temperature on the conformation of metal-depleted GDH. Metal-depleted GDH (6.7 μM) was pre-equilibrated in 200 mM potassium phosphate buffer at various pH values and temperatures. Aliquots from each incubation were assayed for activity in standard assay conditions in the presence of 20 μM Zn^{2+} ions.

were taken at various times after the addition of metal ions, 2.5 mM EDTA was rapidly added, and the mixture was immediately separated from excess Zn^{2+} using an HPLC gel permeation column (described above) equilibrated in 50 mM potassium phosphate buffer, pH 8.0. It should be noted that the loss of Zn^{2+} ions from the metallo-enzyme by the presence of EDTA has been shown to be too slow to have an effect over a period of a few minutes [11]. The protein peak collected was assayed for activity in an assay solution without metal ions present and containing 1 mM EDTA, so that only the metallo-enzyme from the column would contribute to the activity observed. These samples were also subjected to analysis by atomic absorption spectroscopy for determination of their Zn^{2+} content. The data show that the enzyme activity is regained following the addition of the Zn^{2+} ions in a pseudo first-order process of rate 0.076/min. This rate is slower than that observed at pH 6.8 (above) and reflects the shift in the position of the equilibrium in favour of enzyme species E. This is accompanied by a simultaneous increase in the metal content of the enzyme. Enzyme incubated at pH 8.0 prior to the addition of Zn^{2+} ions was able to bind 0.46 ± 0.02 mol Zn^{2+} per mol enzyme at time zero after addition of the metal and 0.99 ± 0.13 mol equivalent after 70 min. For controls samples of metal-depleted enzyme at pH 8.0 were again used, but 2.5 mM EDTA was added immediately after the 100 μM Zn^{2+} ions and the samples were then treated as before. This control experiment showed that the amount of metallo-enzyme regenerated did not increase with time and was that which would be expected at time 0, due to the initial level of species E* at this pH. This experiment

strongly supports the proposal that the conformational change which occurs in the metal-depleted enzyme when a solution of the protein is cooled or adjusted to a pH above 6.6 ($E^* \rightarrow E$) produces a species unable to bind Zn^{2+}. The regeneration of metallo-enzyme is rate limited by a conformational change in the reverse direction, $E \rightarrow E^*$. Determination of the rates of the activation ($E \rightarrow E^*$) and inactivation ($E^* \rightarrow E$) processes at pH 7.4, induced by changes in temperature alone, show that the activation energy for the $E \rightarrow E^*$ transition is 72 kJ per mol whereas that for the reverse transition is only 32 kJ per mol suggesting a difference in free energy between E^* of 40 kJ per mol at this pH. The slow rates of conversion of $E \rightarrow E^*$ or vice versa caused by changes in temperature or pH show that this transition is not rate limited by a simple ionisation of a residue, although such an event may be the initiator.

REFERENCES

[1] Ameyama, M., Shinagawa, E., Kazunobu, M. and Adachi, O. (1985) Agric. Biol. Chem. 49, 1001–1010.
[2] Kong, Y.-C., May, J.W. and Marshall, J.H. (1985) J. Gen. Microbiol. 131, 1571–1579.
[3] Scharshmidt, M., Pfleiderer, G., Metz, H. and Brummer, W. (1983) Hoppe-Seylers Z. Physiol. Chem. 364, 911–921.
[4] Ben-Amotz, A. and Arvon, M. (1973) Plant Physiol. 51, 875–878.
[5] Kormann, A.W., Hurst, R.O. and Flynn, T.G. (1972) Biochim. Biophys. Acta 258, 40–55.
[6] Spencer, P.D., Bown, K., Scawen, M., Atkinson, T. and Gore, M.G. (1988) Biochem. Soc. Trans. 16, 975–976.
[7] Spencer, P.D., Gore, M.G., Bown, K., Scawen, M. and Atkinson, T. (1987) Biochem. Soc. Trans. 15, 1099–1100.
[8] Sargeant, K., East, D.N., Whitaker, A.R. and Elsworth, R. (1971) J. Gen. Microbiol. 65, iii.
[9] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[10] Thiers, R.E. (1957) Methods Biochem. Anal. 5, 273–335.
[11] Spencer, P.D., Bown, K., Scawen, M., Atkinson, T. and Gore, M.G. (1989) Biochim. Biophys Acta 994, 270–279.