PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 385-388 (1976)

Structural Changes in Glyceraldehyde-3-Phosphate Dehydrogenase during Temperature Acclimation of Rainbow Trout (39402)

FRANK S. RANUSKA, ESTHER M. LEISE, AND IRVING GRAY

Department of Biology, Georgetown University, Washington, D.C. 20057

In recent reports it has been shown by us (1, 2) and others (3) that the kinetics of enzymes isolated from members of a species that have been acclimated to different temperatures have been altered. In addition, it has been reported by us that the changed kinetic properties may be the result of modified tertiary or quaternary structure of the enzyme (4). In the present report, immunological techniques have been used to demonstrate a change in the conformation of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), isolated from rainbow trout acclimated to 5 and 15°.

Materials and methods. Rainbow trout, *Salmo gairdneri,* were obtained from the Eastern States Fish Disease Laboratory, Leetown, West Virginia. One group was acclimated at 15°, the other at 5°, using an Instant Ocean Aquarium System. The temperatures were maintained ± 2°. The length of time of acclimation was 3 weeks for both groups.

Purification of G3PDH was carried out according to the method of Cori *et al.* (5) as modified in our laboratory by Wolfe (11). Both the 15° (warm enzyme) and 5° (cold enzyme) G3PDH were isolated at 5°. Crystals of G3PDH which gave a single protein band in acrylamide electrophoresis were obtained. The crystals were separated from the mother liquor by centrifugation at 30,000 *g*, and redissolved in triethanolamine buffer, pH 8.4. The protein concentrations were determined by the procedure of Lowry (6) and by uv absorbance at 260 and 280 nm. The enzymes were stored at pH 8.4 at 5° with the addition of dithiothreitol.

White, male New Zealand rabbits were immunized with the purified enzymes, using Freund's adjuvant for the initial injection and an alum adjuvant for the following injections (1 ml/mg enzyme). Intramuscular injections into the hindlimb near the popliteal fossa were used. Two time courses of immunization were followed: a short-term (ST) course and a long-term (LT) course. The short-term course consisted of an initial injection of 0.25 mg of enzyme, an injection of 0.50 mg 30 days later, and three injections of 1.00 mg of enzyme 4, 3, and 4 days, respectively, after the previous injections, for a total of 15 days from initial to final injection. Five days after the last injection, cardiac punctures of 20 ml were done on each rabbit as a source of ST serum for further tests. Injections were resumed 2 days later (the rabbits not having been injected for 1 week) and succeeding injections of 1.0 mg of enzyme once per week continued for 9 more weeks as an LT course of hyperimmunization. The rabbits were bled by cardiac puncture 5 days after the last injection. The serum was separated, divided into 1-ml aliquots and frozen at −10° until used. Once thawed, serum was not refrozen.

G3PDH was assayed by measuring the reduction of NAD at 340 nm using G3P and arsenate as substrates in pyrophosphate buffer at pH 8.4. The 3-ml cuvette in a Beckman, DBG recording spectrophotometer was used and the reaction initiated by the addition of 2 μg of enzyme. The reaction mixture contained 40 m*M* triethanolamine, pH 8.4, 17 m*M* $Na_2HA_SO_4$, 1.58 m*M* NAD, and G3P from 18.3 to 300 μM.

The K_m and V_{max} were determined by least Square Regression of the Lineweaver and Burke plots of the data using computer programs in the Computer Center Library.

Results and discussion. The results of the immunological tests are represented in Figs. 1 and 2. Interfacial microprecipitin tests (7) performed on the short-term (ST) antisera were specific for each enzyme, 5 and 15°, with no cross section. Precipitin tests on the long-term (LT) antisera showed limited cross reaction with both enzymes. Ouchterlony immuno-double diffusion studies (7, 8)

Copyright © 1976 by the Society for Experimental Biology and Medicine
All rights reserved.

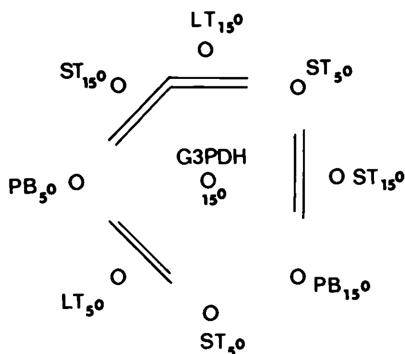

FIG. 1. Results of interfacial microprecipitin tests. G3PDH 15°, enzyme isolated from rainbow trout acclimated to 15° and used as antigen; $ST_{15°}$, $LT_{15°}$, $ST_{5°}$, $LT_{5°}$, short-term and long-term antisera to 15° and 5°, respectively; $PB_{15°}$, $PB_{5°}$, sera obtained from rabbits prior to immunization with enzyme obtained from 15 and 5° acclimated rainbow trout.

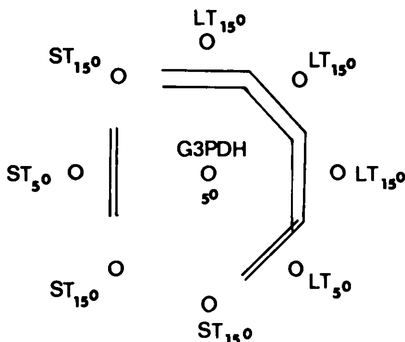

FIG. 2. Results of interfacial microprecipitin tests. G3PDH 5°, enzyme isolated from rainbow trout acclimated to 5° and used as antigen. Other descriptors as in Fig. 1.

confirmed these results. Reactions of identity were found between ST and LT antisera to cold enzyme when reacted with the cold enzyme, and also between ST and LT antisera to warm enzyme when reacted with the warm enzyme. However, reactions of nonidentity were found between LT antiserum to warm enzyme and LT antiserum to cold enzyme when reacted with cold enzyme, as well as between LT antiserum to warm enzyme and LT antiserum to cold enzyme when reacted with the warm enzyme. Thus, the LT antisera did cross react, but were distinctly different. Since antibody production is dependent on tertiary conformation, it appears that there is a difference in the tertiary structure of each enzyme. These results support the previous reports from our laboratories (4) suggesting an *in vivo* change in conformation during temperature acclimation.

Polyacrylamide gel electrophoresis of the two enzymes gave single protein bands in each case. When cochromatographed, polyacrylamide gel electrophoresis failed to separate the enzymes, using three different gel cross-linkages.

The kinetics of the enzymes in the presence of antisera were then investigated. As controls, serum of the rabbits, prior to immunization (PB), and serum of rabbits not immunized (NI) were incubated with the enzyme for 5-min before assay. No measurable effect was seen on the kinetics of the reactions when reacted with either control serum. When specific antisera were incubated with the enzymes, for the same length of time, it was found that the kinetics of the enzymes were markedly affected. A Lineweaver–Burke analysis of the data indicated an irreversible change (Table I and Figs. 3 and 4).

The cold enzyme, when reacted with PB or NI serum, yielded a control K_m of 116 μM and a V_{max} of 0.238 μmole/min/mg. When reacted with ST antiserum to cold enzyme, the K_m was unchanged by V_{max} was reduced to 0.066 μmole/min/mg, demonstrating marked inhibition. With LT anti-

TABLE I. REACTION KINETICS OF G3PDH AS AFFECTED BY ANTISERA TO "ACCLIMATED" ENZYMES.[a]

	Test enzyme			
	Warm (15°)		Cold (5°)	
Antiserum to	K_m[b]	V_{max}[c]	K_m	V_{max}
Control	208	3.4	116	0.238
Warm enzyme ST	208	0.83	116	0.201
Warm enzyme LT	208	0.84	116	0.192
Cold enzyme ST	208	3.3	116	0.066
Cold enzyme LT	208	2.5	116	0.078

[a] The standard deviation of the calculated values are ± 15% of the above value taken from the Lineweaver–Burke plots.

[b] K_m in μM.

[c] V_{max} in μmole/min/mg.

sera to cold enzyme, the same K_m and a V_{max} of 0.078 μmol/min/mg resulted. While not statistically different from each other, both experimental results are different from controls ($P < 0.05$). Reaction of the cold enzyme with the ST antiserum to warm enzyme yielded the same K_m and a V_{max} of 0.201 μmole/min/mg, results not different from the control assay. However, reaction with the LT antiserum to warm enzyme yielded the same K_m but a V_{max} Of 0.192 μmole/min/mg, indicating only limited inhibition of the reaction (not statistically significant).

Qualitatively, similar results were obtained with the assays of the warm enzyme. When this enzyme was incubated with NI serum, a K_m of 208 μM and a V_{max} of 3.4 μmole/min/mg were obtained. With ST antiserum to warm enzyme, the same K_m, but a V_{max} of 0.83 μmole/min/mg resulted, demonstrating significant inhibition ($P < 0.05$). Reaction with the LT antiserum to 15° G3PDH yielded the same K_m and a V_{max} of 0.84 μmole/min/mg. Incubation with the ST antiserum to cold enzyme yielded the same K_m and a V_{max} of 3.3 μmole/min/mg, not different from controls. However, incubation with the LT antiserum to cold enzyme yielded the same K_m, but a V_{max} of 2.5 μmole/min/mg, indicating some limited degree of inhibition.

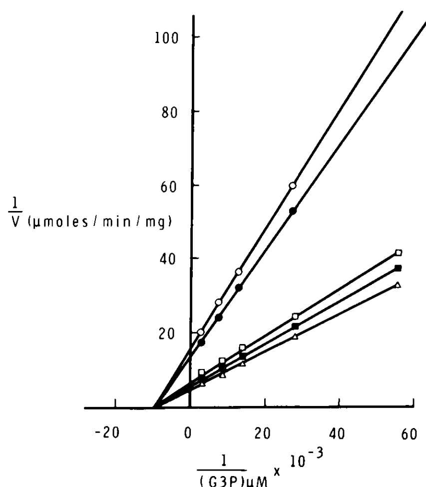

FIG. 4. Lineweaver-Burke analysis of cold acclimated G3PDH activity. The 5° enzyme has been allowed to react with ST (○) or LT (●) antiserum to 5° G3PDH, or ST (□) or LT (■) antiserum to 15° G3PDH, or PB or control (△) serum. K_m and V_{max} values with respect to G3PDH were calculated by iterative curve fitting using weighted least squares. The standard deviation of the points with respect to the calculated lines is less than ±15%.

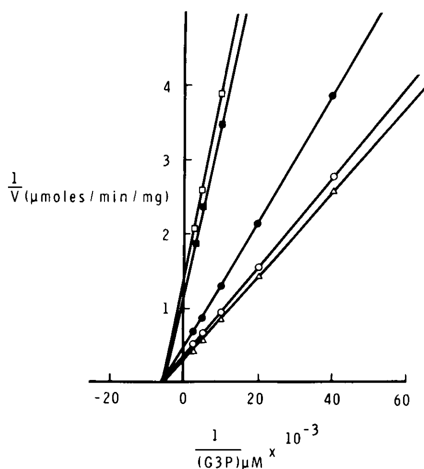

FIG. 3. Lineweaver-Burke analysis of control G3PDH activity. The 15° enzyme has been allowed to react with ST (□) or LT (■) antiserum to 15° G3PDH, or ST (○) or LT (●) antiserum to 5° G3PDH, or PB or control (△) serum. K_m and V_{max} values with respect to G3P were calculated by iterative curve fitting using weighted least squares. The standard deviation of the points with respect to the calculated lines is less than ±15%.

Each antiserum reacted primarily in an irreversible manner with its specific enzyme. The degree of cross reactivity seen with the LT antisera indicates that the structures of the enzymes have areas of similarity. However, the kinetic data indicate that the enzymes are quite different in activity. The values obtained for the K_m and V_{max} of each enzyme are in agreement with those of Wolfe and Gray (4), the 5° enzyme having a greater affinity for the substrate, but a lower V_{max} than the 15° enzyme. The subunits of the G3PDH molecule are probably identical in composition (9) with differing *in vivo* conformations in the two altered environments. Antibodies formed to these enzymes were capable of distinguishing them, indicating such a structural change. These alternate forms of the enzyme are also quite stable, retaining their acquired differences through the purification process.

It would seem that this multimeric enzyme, composed of identical subunits, is ca-

pable of achieving different conformations as a result of chronic exposure to altered environmental temperature. This could result in changed NAD^+ binding to cause the different activities observed. Perhaps the actual mechanism of the altered enzyme conformation is dependent upon another temperature-dependent metabolic process, e.g., one leading to differences in *in vivo* salt concentrations at different temperatures. Still, our data do not rule out the possibility that the subunits of the acclimated enzymes are actually different, and that current methods have been unsuccessful in distinguishing them. Such changes in subunit structure would also incite different antibody production. However, the fact that long-term antibodies gave some cross reactions *in vitro* indicates that the subunit differences may be quite subtle. One might suggest new protein synthesis in cold acclimation as a result of inducer–repressor mechanisms with temperature change, where a gene capable of producing an enzyme with but slightly different subunit amino acid sequence, becomes turned on in the cold. Such gene-related changes may be mainly the result of changes in the concentration of cell metabolites and ions previously reported (10, 11).

Summary. G3PDH was isolated from rainbow trout acclimated to 15 and 5°. Antisera to each of the enzymes were prepared in rabbits. Microprecipitin tests showed that the short-term (ST) antiserum reacted only with its specific antigen. However, with the long-term (LT) antisera, there was some cross reaction. Ouchterlony double diffusion confirmed these results. Kinetic studies of each of the G3PDH enzymes in the presence of its specific antiserum were carried out. Specific ST and LT antisera significantly inhibited the activity of the enzyme. No significant effect was seen with the antisera to the noncomplementary enzyme. Some cross reaction was seen with the LT antisera in both instances. The Lineweaver–Burke analyses demonstrated no change in the K_m while the V_{max} was reduced, indicative of noncompetitive inhibition by the specific antisera.

1. Robert, M. S., and Gray, I., Comp. Biochem. Physiol. **42B,** 389 (1972).
2. Audilet, D. O., and Gray, I., Cryobiology **11,** 92 (1974).
3. Somero, G. N., and Hochachka, P. W., Biochem. J. **110,** 395 (1968).
4. Wolfe, J. K., and Gray, I., Cryobiology **10,** 295 (1973).
5. Cori, G. T., Slein, M. W., and Cori, C. F., J. Biol. Chem. **173,** 605 (1948).
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
7. Campbell, D. H., Garvey, J. S., Cremer, N. E., and Sussdorf, D. H., *in* "Methods in Immunology." 2nd ed., pp. 3, 234. W. A. Benjamin, New York (1970).
8. Ouchterlony, E., ACTA Pathol. Microbiol. Scand. **26,** 507 (1949).
9. Harris, J. I., and Perham, R. N., J. Mol. Biol. **13,** 876 (1965).
10. Hickman, C. P., McNabb, R. A., Nelson, J. S., Van Breekman, E. D., and Comfort, D., Canad. J. Zool. **42,** 577 (1964).
11. Beurisch, H. W., and Hochachka, P. W., Biochem. J. **111,** 287 (1969).

Received January 19, 1976. P.S.E.B.M. 1976, Vol. 152.