Vol. 80, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1978 Pages 593-599

HYBRID ISOZYMES OF PYRUVATE KINASE APPEAR DURING AVIAN CARDIAC DEVELOPMENT[1]

Janet M. Cardenas, Everett Bandman, and R. C. Strohman

Department of Zoology
University of California
Berkeley, California 94720

and

The Department of Biochemistry & Biophysics
Oregon State University
Corvallis, Oregon 97331

Received December 5,1977

Summary

Pyruvate kinase isozyme patterns in the ventricle of developing chicks shift gradually from one dominated by type K at ten days of embryonic development to the adult pattern, which is dominated by type M. Hybrid isozymes are apparent throughout development and are most prominent from two days before hatching until at least 14 days after hatching. These hybrid isozymes indicate simultaneous synthesis of the two subunit types in the same cells.

The complex isozyme patterns of the chick heart probably limit the usefulness of simple kinetic analyses on tissue extracts for determining isozymic compositions during development.

Tissues of the very young embryonic chicken contain predominantly type K pyruvate kinase (EC 2.7.1.40), which is sometimes regarded as a fetal form of the enzyme. Adult skeletal and cardiac muscle, as well as brain, contain mainly or only a "differentiated" isozyme, designated type M (1-4). Although the two isozymes are immunologically cross-reactive, they apparently are products of different structural genes (5). Earlier developmental studies of chick tissues are in some disagreement regarding the mechanisms by which the isozymic

[1]These investigations were supported by NIH grant AM-15645 and by NIH Program Project Grant AM-13882. JMC is recipient of NIH Career Development Award AM-00191, and EB is recipient of National Research Service Award AM-05087.

0006-291X/78/0803-0593$01.00/0

shift occurs. Strandholm et al. (2) observed only K_4 and M_4 in skeletal muscle and brain, while Ibsen et al. (3) observed a total of ten activity bands in extracts of whole chick embryos after isoelectric focusing, which he concluded to be M_4, K-M hybrids, and six variants of K_4.

We subsequently have been investigating the isozymic shifts that occur in developing chick heart ventricle using electrophoretic techniques that result in vastly improved separation of the isozymic bands. We find gradual isozymic shifts from a predominantly K-producing system to one emphasizing type M subunits; hybrid isozymes of nearly random combinations of the two subunit types occur throughout development and even in the adult state.

Materials and Methods

Substrates and lactate dehydrogenase were obtained from Sigma Chemical Company. Sucrose was Schwarz-Mann's enzyme grade, electrophoresis equipment was from Gelman, and all common chemicals were reagent grade. Fertile eggs were obtained from a local hatchery.

The lower halves of the hearts, assumed to be mainly ventricular muscle, were dissected from the animals, rinsed in saline, and blotted gently. Subsequent steps were performed at 4° unless otherwise indicated. Homogenization was accomplished in a motor-driven glass homogenizer using three ml of 20 mM tris-HCl, pH 7.5 at room temperature, 1 mM EDTA, 15 mM 2-mercaptoethanol per g tissue. Insoluble debris was removed by centrifugation for 20 min at 8,000 x g.

Enzyme assays of the supernatants were performed at 25° using the coupled assay of Bucher and Pfleiderer (6) as described by Cardenas et al. (7). Protein concentrations were determined by the Folin-Ciocalteau method as described by Clark and Switzer (8).

Samples were dialyzed 4-6 h against electrophoresis buffer (15 mM tris-HCl, pH 7.5 at room temperature, 0.5 M sucrose, 1 mM EDTA, 1 mM fructose 1,6-diphosphate, 10 mM 2-mercaptoethanol) and then diluted to 6 activity units per ml. Electrophoresis and subsequent activity detection were performed essentially as described by Susor and Rutter (9), the main difference being a lowering of the tris-HCl concentration from 20 to 15 mM. This simple alteration greatly enhanced mobility differences between the isozymes and enabled us to look for intermediate forms.

Results and Discussion

Electrophoretic patterns obtained with extracts from chick ventricle at various developmental ages are shown in Figure 1. The relative intensities of the bands of a given electrophoretic pattern are approximately proportional to the relative enzyme activities, but quantitative comparisons from one electrophoretic pattern to another are less reliable due to differences in exposure and development times. As shown in Figure 1, K-type subunits predominate during the earlier stages of cardiac development, but type M is the adult form. Furthermore, forms having electrophoretic mobilities intermediate between types K and M are apparent at all stages of development.

Chicken type M pyruvate kinase has been demonstrated to be a tetramer (10). As chicken type K pyruvate kinase has yet to be purified, its subunit structure has not been determined. However, the distribution of enzyme activities among the bands shown in Figure 1 is consistent with random, or at least nearly random, combinations of two subunit types among tetrameric hybrids. This suggests that chicken type K, like all other native pyruvate kinases so characterized to date, is a tetramer and that chicken type K and M subunits, like their bovine counterparts (11) demonstrate no subunit type preference during tetramer formation.

While we feel that the intermediate bands in chick heart are due to the formation of hybrid isozymes, clearly other explanations are possible. It is likely that our tissue samples contain more than one cell type, and multiple bands could possibly represent different isozymes that arise from these different cell types. This is an especially important consideration since the whole heart is known to undergo changes in cell

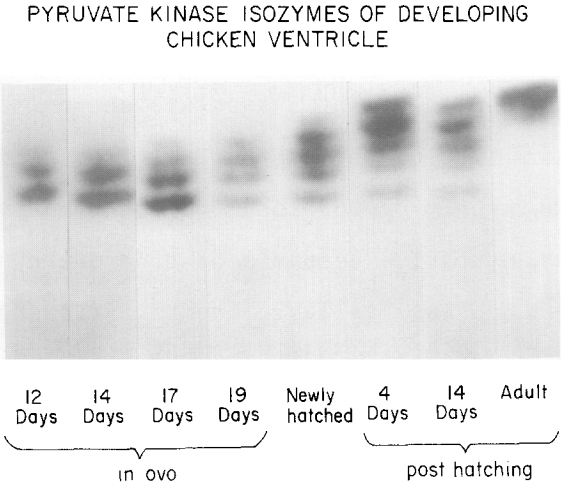

Fig. 1. Electrophoretic separation of pyruvate kinase isozymes in developing heart. Electrophoresis was performed as described in the text, utilizing a potential difference of 200 v and a total electrophoresis time of 30 h. The origin is located at the lower edge of the zymograms. The slowest moving band, seen most clearly in the 12- and 14-day fetal samples, is designated as K_4, while the fastest moving band, seen most clearly in the 14-day post-hatch and adult samples, is M_4. The intermediate bands, in order of increasing electrophoretic mobilities, are K_3M, K_2M_2, and KM_3, respectively. Although minor differences in electrophoretic mobility can be seen from one zymogram to another, the patterns are remarkably reproducible.

population with development; previous to five days of development, the heart contains only myocytes, but fibroblasts, vascular endothelial, smooth muscle, and circulatory blood cells are added during development (12,13). Hence, further studies will be necessary to clarify this point, although such a mechanism for cardiac pyruvate kinase isozymic changes would require very uniform structural differences among the isozymes in order to produce the extremely uniform distribution of electrophoretic mobilities that we see.

An alternate possibility would be the occurrence of interconvertible forms of the type seen by Ibsen *et al.* (3). However, our patterns appear to be stable under our storage

Table 1. Determination of pyruvate kinase activity concentrations in developing chick muscle. Determinations were performed as described in the text and are expressed as units per g wet wt and as units per mg of extracted protein. The ages of the fetal chicks are given in days since incubation of the eggs began. Chicks hatched on approximately the 21st day of incubation.

Age	Pyruvate Kinase Activity	
	units/g tissue	units/g protein
Cardiac ventricular muscle:		
12 day fetal	25.3	0.95
14 day fetal	34.8	1.00
17 day fetal	33.1	1.08
19 day fetal	34.6	0.99
Newly hatched	32.5	0.84
4 days after hatching	38.5	0.95
14 days after hatching	51.1	0.62
Adult	71.7	1.28
Pectoralis:		
13 day fetal	11.0	0.39
Adult	421.0	7.61

and handling conditions and do not interconvert. Also, mixtures of extracts containing mainly the two parental forms produce additive electrophoretic patterns and do not result in the formation of additional bands.

Shown in Table 1 are the specific activities of chick cardiac and skeletal muscle pyruvate kinase during development. It is interesting to note that cardiac muscle undergoes only a threefold increase in pyruvate kinase specific activity from the twelfth day of embryonic development until adulthood, in spite of the major isozymic shift that occurs during this time.

These results are consistent with a decrease in the synthesis of type K subunits and a concomitant, probably larger, increase in the synthesis of type M.

Small quantities of K-M hybrids probably occur in skeletal muscle during development, but hybrid isozymes are found in much lower concentrations in developing skeletal muscle than in cardiac ventricular tissue from the same animals (Cardenas, Bandman, and Strohman, manuscript in preparation). Moreover, skeletal muscle undergoes at least a fortyfold increase in specific activity during the same period that cardiac muscle undergoes only a threefold increase (see Table 1). Thus, the two muscle types experience the same pyruvate kinase isozymic shift but differ considerably in the timing and magnitude of this change.

Harris et al. (4) have suggested utilization of the fact that since types K and M pyruvate kinase isozymes are differentially affected by specific metabolites, kinetic analyses may be used to determine isozymic compositions. We feel that any simple kinetic assay of the type proposed by Harris et al. must be used with extreme caution, in view of the now demonstrated widespread presence of hybrid isozymes (see, for example, refs. 11, 14,15). The necessity of such caution is especially true in view of evidence that hybrid isozymes of pyruvate kinase probably have unique kinetic properties that are inconsistent with a simple summation of expected kinetic contributions based on the properties of the two homotetrameric forms (16,17).

References

1. Cardenas, J.M., Dyson, R.D., and Strandholm, J.J. (1975) in Isozymes: Molecular Structure (C.L. Markert, ed.), pp. 523-541, Academic Press, New York.

2. Strandholm, J.J., Cardenas, J.M., and Dyson, R.D. (1975) Biochemistry 14, 2242-2246.

3. Ibsen, K.H., Murray, L., and Marles, S.W. (1976) Biochemistry 14, 1064-1073.

4. Harris, W., Days, R., Johnson, C., Finkelstein, I., Stallworth, J., and Hubert, C. (1977) Biochem. Biophys. Res. Commun. 75, 1117-1121.

5. Harkins, R.N., Black, J.A., and Rittenberg, M.B. (1977) Biochemistry 16, 3831-3837.

6. Bucher, T. and Pfleiderer, G. (1955) Meth. Enzymol. 1, 435-440.

7. Cardenas, J.M., Dyson, R.D., and Strandholm, J.J. (1973) J. Biol. Chem. 248, 6931-6937.

8. Clark, J.M., Jr. and Switzer, R.L. (1977) Experimental Biochemistry, 2nd ed., p. 12, W.H. Freeman & Co., San Francisco.

9. Susor, W.A. and Rutter, W.J. (1971) Anal. Biochem. 43, 147-155.

10. Cardenas, J.M., Blachly, E.G., Ceccotti, P.L., and Dyson, R.D. (1975) Biochemistry 14, 2247-2252.

11. Strandholm, J.J., Dyson, R.D., and Cardenas, J.M. (1976) Arch. Biochem. Biophys. 173, 125-131.

12. Manasek, F.J. (1970) Amer. J. Cardiol. 25, 149-168.

13. Doyle, C.M., Zak, R., and Fischman, D.A. (1974) Develop. Biol. 37, 133-145.

14. Susor, W.A. and Rutter, W.J. (1970) Fed. Proc. 29, 729 abs.

15. Osterman, J., Fritz, P.J., and Wuntch, T. (1973) J. Biol. Chem. 248, 1011-1018.

16. Dyson, R.D. and Cardenas, J.M. (1973) J. Biol. Chem. 248, 8482-8488.

17. Ibsen, K.H. and Marles, S.W. (1976) Biochemistry 15, 1073-1079.