Characterization of phosphoglycerate mutase isoenzymes from free dissected facial processes

Gösta Granström, Hans Mångs, L. Peter Nilsson and Göran Zellin
Laboratory of Oral Biology, Department of Histology, Department of Oto-, Rhino-, Laryngology, University of Gothenburg, Gothenburg, Sweden

Granström G, Mångs H, Nilsson LP, Zellin G. Characterization of phosphoglycerate mutase isoenzymes from free dissected facial processes. Acta Odontol Scand 1988;46:1–8. Oslo. ISSN 0001-6357.

To characterize the three phosphoglycerate mutase (PGM) isoenzymes present in rat facial processes (types MM, BB, and MB), their sensitivity to reagents of the sulfhydryl groups and to heat treatment has been studied. Type BB PGM was not affected by the -SH group reagents; type MB PGM was inhibited about 50%, and type MM PGM was fully inhibited. Type MB PGM showed a greater heat lability than type MM PGM. There was a developmental change from type BB PGM from the 9th embryonic day to isoenzymes MB and MM on the 15th embryonic day. Isoenzyme development was first seen in mandibular processes, followed by maxillary, lateral nasal, and medial nasal processes. □ *Craniofacial embryology; enzyme biochemistry*

Gösta Granström, Laboratory of Oral Biology, Department of Histology, P.O. Box 33031, S-400 33 Gothenburg, Sweden

Glycerate-2,3-P_2-dependent phosphoglycerate mutase (PGM; EC 2.7.5.3) in mammalian tissues shows the existence of three isoenzymes (14). From patterns observed in muscle tissues at several stages of growth and on the basis of the dimer structure of PGM it was suggested that the three isoenzymes detected resulted from the dimeric combinations of two different subunits (15). It was postulated that the isoenzyme with high electrophoretic mobility (brain type, or type BB) and the isoenzyme with low mobility (muscle type, or type MM) were homodimeric species, whereas the isoenzyme with intermediate mobility (type MB) was heterodimeric. It was also suggested that both PGM subunits B and M were determined by two different gene loci. A developmental transition was observed in skeletal and cardiac muscle (1) and in facial processes (4) from a brain-type pattern to a muscle-type pattern. This transition was interpreted as a two-step process, 'turning on' the gene for type M subunit and 'turning off' the gene for type B subunit. The developmental transition in facial processes preceded obvious histologic signs of muscle differentiation.

One of the biochemical features of teratogenesis is disturbances in the composition of various isoenzymes (9, 10). The purpose of the present study was to develop biochemical testing systems to separate the MM, BB, and MB isoenzymes of PGM and to characterize them further biochemically. To characterize PGM present in facial processes, their sensitivity to reagents of the sulfhydryl groups and to heat inactivation was tested.

Materials and methods

Sprague–Dawley rats from Anticimex AB (Stockholm, Sweden) were used throughout the study. Male and female animals were housed together overnight in the proportion two males to three females. Vaginal smears were taken in the morning, and the day when sperm was present in the smears was designated day 0 of pregnancy. Pregnant dams were housed in plastic cages and kept in 12-h light/dark cycles with an ambient temperature of 21 °C. Animals were allowed free access to water and standard pellets.

Totally, 75 dams were killed on days 9, 10, 11, 12, 13, 14, and 15 of pregnancy. A total of 618 conceptuses were explanted and

examined under the dissection microscope. Staging for correct embryologic age was performed by the method of Edwards (3). Using a dental excavator, a pair of microforceps and a microhook, we dissected free different parts of the developing face. From conceptuses of 9, 10, and 11 days, mandibular and maxillary processes were obtained. From conceptuses of 12, 13, 14, and 15 days, mandibular, maxillary, lateral nasal, and medial nasal processes were obtained as described by Granström (4). The mandibular processes were defined as protruding below the stomodeum. They were dissected along a straight line extended between and below the angles of the stomodeum. The maxillary processes were dissected from the angle of the stomodeum to the lens plate. Lateral nasal processes were dissected from the lens plate to the olfactory plate. The medial nasal process was the remaining tissue between the olfactory plates and the telencephalon. During the gill stages fusion grooves between different facial processes were followed during dissection.

For PGM analysis facial processes were dissected free as described above and pooled separately in 50 mM tris–HCl buffer, pH 7.5. The tissues were homogenized during cooling in ice for 5 min in a glass homogenizer containing 2 ml of 50 mM tris–HCl buffer, pH 7.5, after which the samples were centrifuged in the cold for 30 min at 10,000 *g*, and the precipitate was discarded. Reference samples from homogenates of adult rat skeletal muscle and adult rat cardiac muscle were prepared in the same manner.

For isoelectric focusing (IEF), a Pharmacia flat-bed apparatus FBE 3000 was used, equipped with an ECPS 3000/150 constant power supply and a Volthour VH-1 integrator. The IEF agarose gel was prepared in accordance with the manufacturer's instructions (Pharmacia Fine Chemicals, Uppsala, Sweden). To prepare a gel with a size of 115 × 225 mm, 0.3 g agarose IEF (Pharmacia), 3.6 g sorbitol (P.A., AG Merck, Darmstadt, FRG), and 27 ml distilled water were mixed and boiled. When the agarose had dissolved completely, the mixture was cooled to 75°C, 1.9 ml Pharmalyte (pH 3–10, Pharmacia) was added, and the gel was cast in a mold. The gel was allowed to harden overnight in a humidity chamber at 4°C.

The samples were applied on paper sample applicators at volumes of 15–30 µl (equivalent to 60–120 µg protein). The samples were applied 25 mm from the anode. IEF was performed at 1500 V, 16 W, and extended to 1700 Vh at 10°C cooling temperature. The distance between the electrodes was 100 mm, and the electrode solutions consisted of 0.05 M H_2SO_4 (anode) and 1 M NaOH (cathode). After separation, the agarose gel was quickly placed face down on a reagent gel and incubated for 30 min at 37°C for color development.

To prepare the reagent gel for PGM, 0.75 g Difco Special Noble agar was dissolved in 40 ml boiling 0.1 M tris–HCl buffer, pH 8.0. After the mixture had cooled to 60°C, the following solutions were added: 1 ml 2.5 mM $MgCl_2$, 1 ml 4.2 mM ethylenediaminetetraacetic acid (EDTA), 1 ml 2.5 mM histidine hydrochloride (Sigma), 1 ml 2.5 mM glucose-1-phosphate (Sigma), 1 ml 2.5 mM nicotinamide adenine dinucleotide phosphate (NADP) (Sigma), 1 ml glucose-6-phosphate dehydrogenase (0.12 mg enzyme/ml; Sigma), 1 ml Nitro BT (10 mg/ml), and 0.2 ml phenazine methosulfate (1 mg/ml). The gel was cast in a mold the same size as the agarose gel and was allowed to harden in the dark for 2 h at room temperature (16).

After the incubation, the reagent gels were rinsed in a solution of 10% acetic acid and 10% methanol for 10 min, followed by scanning in a Schnell densitometer II (Zeiss, Jena), connected to a Vitatron UFD 100 electronic unit and a Vitatron UR 403 LIN/LOG integrating recorder.

PGM activity was measured by coupling the formation of glycerate-2-P from glycerate-3-P with the enolase-catalyzed reaction. Assays were performed in a Hitachi Spectrophotometer, model 100-20 at 30°C. The reaction mixture contained in a total volume of 3 ml: 33 mM tris–HCl buffer, pH 7.4, 3 mM $MgSO_4$, 20 mM glycerate-3-P (Sigma), 0.25 mM glycerate 2,3-P_2 (Sigma), and 1 U enolase (Sigma). One unit of PGM

gave an increase of 0.025 in optical density per minute at 240 nm (12).

Mandibular processes obtained for further analysis were homogenized in three volumes of 20 mM tris–HCl buffer, pH 7.5, containing 1 mM Na_2 EDTA and 1 mM β-mercaptoethanol (Sigma). After centrifugation at 25,000 *g* for 15 min, the supernatant was mixed with saturated ammonium sulfate, adjusted to pH 7.2. It was allowed to stand for 60 min and then centrifuged at 25,000 *g* for 30 min. The precipitate was resuspended in 1 ml extracting buffer and was percolated through a column of Sephadex G-25 equilibrated with the same buffer.

Fractions with PGM activity eluted from the Sephadex G-25 column were pooled and applied to a column of DEAE Sephadex A-50 equilibrated with extracting buffer. The column was developed first with the same buffer and then with a linear gradient of NaCl from 0 to 250 mM.

Tissue extract in 50 mM tris–HCl buffer, pH 7.5, was incubated at 56 °C in the absence and in the presence of 2 mM glycerate-2,3-P_2. At intervals, incubation was stopped, and after centrifugation at 15,000 *g* for 15 min, PGM activity was determined in the supernatant. To identify the thermolabile and thermoresistant PGM isoenzymes, aliquots of the extracts heated for 50 min at 56 °C were analyzed by IEF.

Tissue extracts in 50 mM tris–HCl buffer, pH 7.5, were incubated at 30 °C with either 1 mM tetrathionate (potassium salt; Merck) or 0.2 mM $HgCl_2$ (Merck), in the absence and in the presence of 0.2 mM glycerate 2,3-P_2. After 5 min the incubation solutions were chilled at 2 °C, and PGM activity was measured. Dithiothreitol (10 mM, Sigma) was added, and after a further 10 min of incubation at 30 °C, PGM activity was determined again. To identify the PGM isoenzymes sensitive and resistant to Hg^{2+} and tetrathionate, aliquots were removed from the incubation mixtures before and after dithiothreitol addition and were analyzed by IEF.

The molecular weight of the PGM isoenzymes was estimated by gel filtration on Sephadex G-75. Bovine serum albumin, ovalbumin, and ribonuclease were used as standards. Samples (2 mg) of each standard protein and 25 U of PGM in 0.2 ml of 20 mM tris–HCl buffer, pH 7.5, containing 1 mM EDTA, 1 mM β-mercaptoethanol, and 100 mM NaCl, were applied to the column (1.5 × 100 cm) and eluted with the same buffer. At a flow rate of 4 ml/h, 1-ml fractions were collected and assayed for enzymatic activity and protein. Molecular weight was determined by means of the standard curve of log M_r versus elution volume.

Protein was determined by the method of Lowry et al. (11), using bovine serum albumin as standard.

Results

Fig. 1 shows the isoenzyme pattern of PGM from free dissected facial processes separated by IEF and recorded by densitometric scannings. Samples from adult rat skeletal muscle (quadriceps) and from adult rat cardiac muscle were run as reference. PGM isoenzyme BB was present from the 10th day and isoenzyme MB from the 11th day. From the 15th day isoenzyme MM was present in all facial processes. Activities of isoenzyme BB and MB increased during embryonic days 10–15. All three isoenzymes were recovered from skeletal and cardiac muscle. Isoenzyme development was first seen in mandibular processes, followed by maxillary, lateral nasal, and medial nasal processes. The isoelectric point (Ip) of the three PGM isoenzymes was 4.7, 5.2, and 6.2 for types BB, MB, and MM, respectively.

The sensitivity of PGM activity to the -SH group modification varied during development. In the earliest specimens from fetal rat, PGM from the facial processes were resistant to Hg^{2+} and tetrathionate (Table 1). After the 12th embryonic day PGM from mandibular processes became sensitive and its sensitivity increased progressively with development (Fig. 2). The PGM isoenzymes sensitive and resistant to the reagents of the -SH groups were identified by IEF. Tissue extracts from facial processes treated with tetrathionate were subjected to IEF, and their patterns were compared with those of nontreated extracts. Only the PGM band

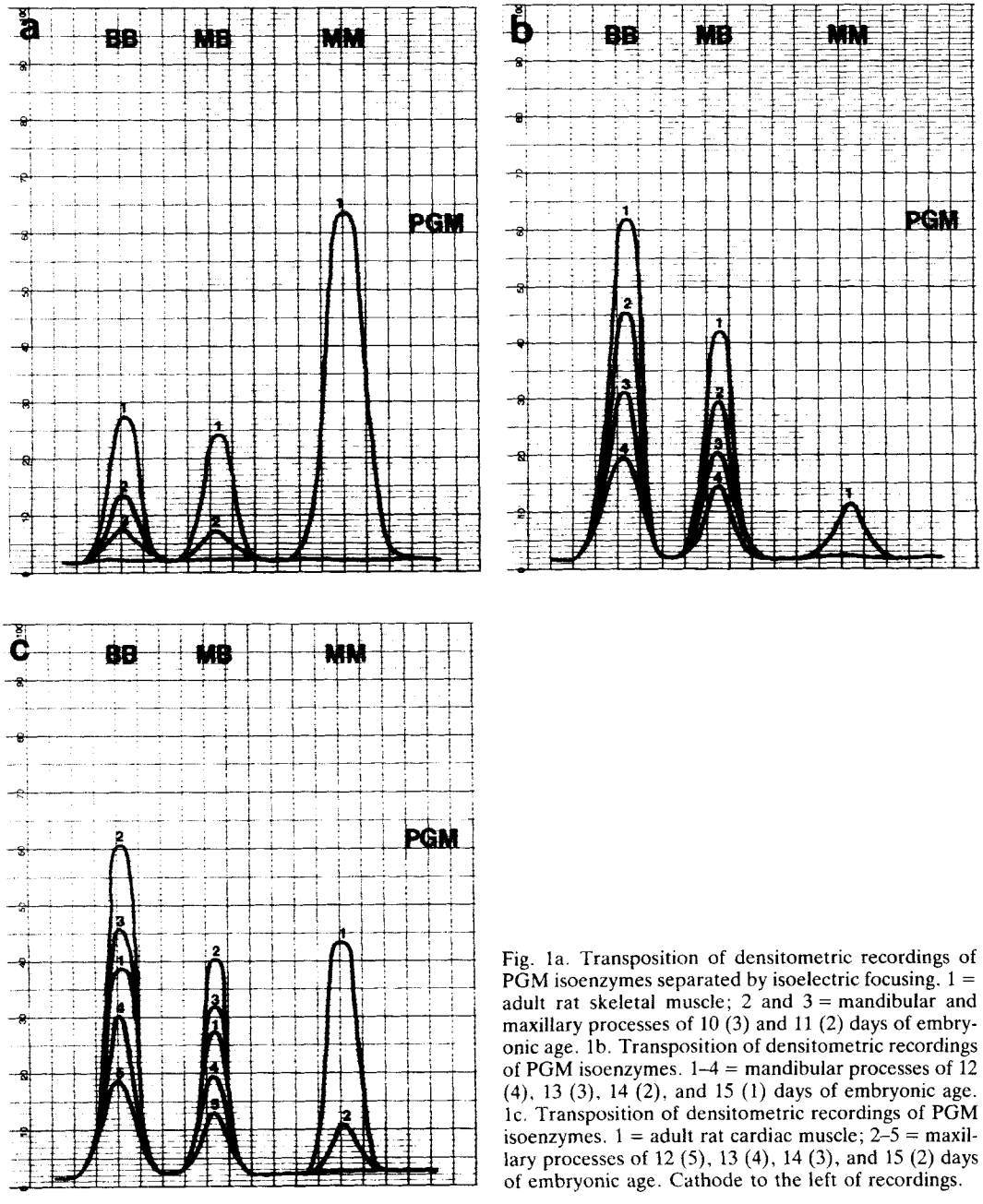

Fig. 1a. Transposition of densitometric recordings of PGM isoenzymes separated by isoelectric focusing. 1 = adult rat skeletal muscle; 2 and 3 = mandibular and maxillary processes of 10 (3) and 11 (2) days of embryonic age. 1b. Transposition of densitometric recordings of PGM isoenzymes. 1–4 = mandibular processes of 12 (4), 13 (3), 14 (2), and 15 (1) days of embryonic age. 1c. Transposition of densitometric recordings of PGM isoenzymes. 1 = adult rat cardiac muscle; 2–5 = maxillary processes of 12 (5), 13 (4), 14 (3), and 15 (2) days of embryonic age. Cathode to the left of recordings.

that corresponded to the MM isoenzyme disappeared completely after tetrathionate treatment. The band with intermediate mobility (MB isoenzyme) decreased, and the type BB isoenzyme was not affected. From the results it was concluded that the activity of the BB isoenzyme is resistant to the modification of the -SH groups, the activity of the MB isoenzyme is partially sensitive, and the activity of the MM isoenzyme is fully sensitive.

To quantify the PGM isoenzymes, these

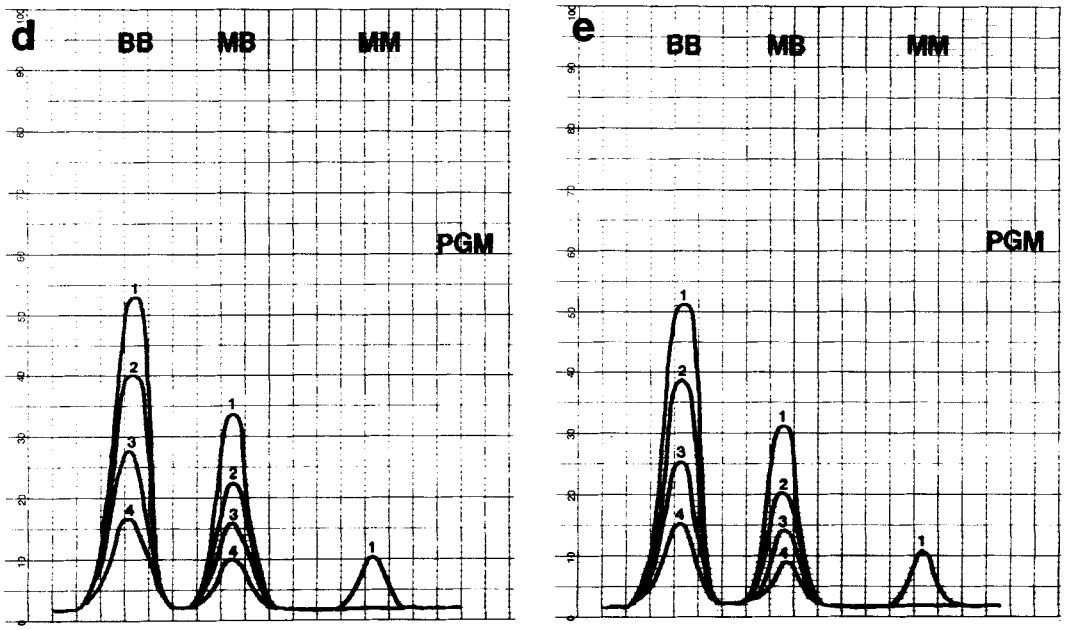

Fig. 1d. Transposition of densitometric recordings of PGM isoenzymes. 1–4 = lateral nasal processes of 12 (4), 13 (3), 14 (2), and 15 (1) days of embryonic age. 1e. Transposition of densitometric recordings of PGM isoenzymes. 1–4 = medial nasal processes of 12 (4), 13 (3), 14 (2), and 15 (1) days of embryonic age. Cathode to the left of recordings.

were isolated by ion-exchange chromatography on DEAE Sephadex A-50. As shown in Fig. 3, three peaks were eluted from 15-day mandibular processes. By IEF, the first peak was identified as type MM isoenzyme, the second peak as type MB isoenzyme, and the third peak as type BB isoenzyme. The molecular weight of the purified isoenzymes was estimated by gel filtration. All three isoenzymes emerged

Table 1. Inactivation by tetrathionate and $HgCl_2$ of the phosphoglycerate mutase activity in rat facial processes. The figures in parentheses refer to the activity after reactivation with dithiothreitol

		Residual activity	
Age	Tissue	Tetrathionate	$HgCl_2$
12 days	Mandibular process	100	97 (100)
12 days	Maxillary process	100	96 (100)
12 days	Lateral nasal process	100	97 (100)
12 days	Medial nasal process	100	96 (100)
15 days	Mandibular process	80 (100)	69 (97)
15 days	Maxillary process	81 (100)	70 (96)
15 days	Lateral nasal process	79 (100)	69 (96)
15 days	Medial nasal process	81 (100)	68 (97)
Adult	Skeletal muscle	1 (72)	1 (100)
Adult	Cardiac muscle	56 (100)	36 (100)

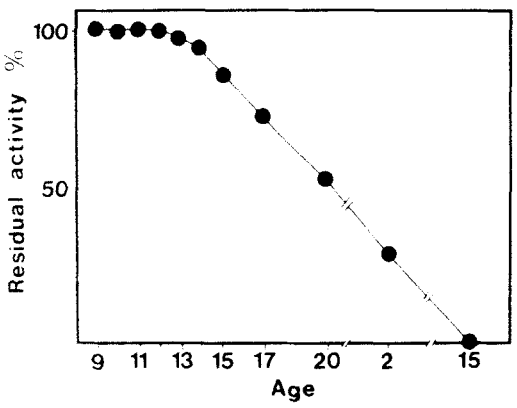

Fig. 2. Inactivation by tetrathionate of the PGM activity in mandibular processes of 9–20 days of embryonic age, 2 weeks and 15 weeks after birth. Mandibular processes were dissected as described in Materials and Methods. From the 2- and 15-week animals, tissue of the masseter muscle was obtained.

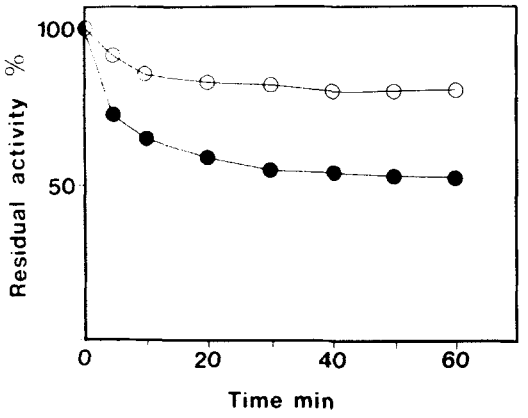

Fig. 4. Thermal lability of PGM activity in extracts from embryonic day 15 mandibular processes in the absence and presence of glycerate-2,3-P_2. Solid symbols correspond to the extracts heated in the absence of glycerate-2,3-P_2. Open symbols correspond to the extracts heated in the presence of glycerate-2,3-P_2.

between bovine serum albumin and ovalbumin, and an apparent molecular weight of 57,000 ± 1000 was calculated.

To compare the thermal lability of PGM, tissue extracts from 15-day mandibular processes were heated at 56°C in the presence and the absence of glycerate-2,3-P_2. In the absence of glycerate-2,3-P_2, extracts from mandibular processes retained about 50% activity after 1 h of incubation. The presence of glycerate-2,3-P_2 protected the thermal inactivation, since about 85% activity was retained after 1 h of incubation (Fig. 4).

Aliquots of tissue extracts from embryonic day 15 mandibular processes were analyzed by IEF at different times after incubation at 56°C (Fig. 5). Types BB and MB isoenzymes were more rapidly inactivated than the MM isoenzyme.

Discussion

Accumulating information indicates that the early phases of craniofacial development of all vertebrate embryos are considerably

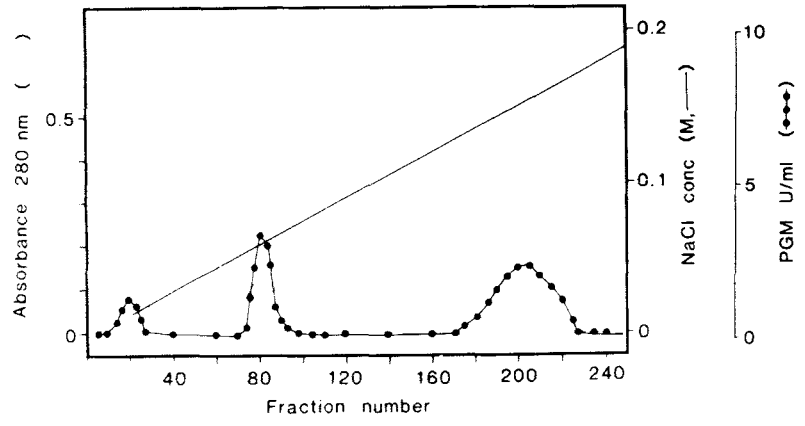

Fig. 3. DEAE Sephadex A-50 chromatography of extract from embryonic day 15 mandibular processes. Five milliliters of the extract obtained as described in Materials and Methods were applied to a column (1.5 × 20 cm) of DEAE Sephadex A-50 equilibrated with extracting buffer. The column was first eluted with 50 ml of equilibrating buffer and then with a 200-ml linear gradient of NaCl ranging from 0 to 250 mM in the same buffer. Fractions of 1 ml were collected and assayed for protein and PGM activity.

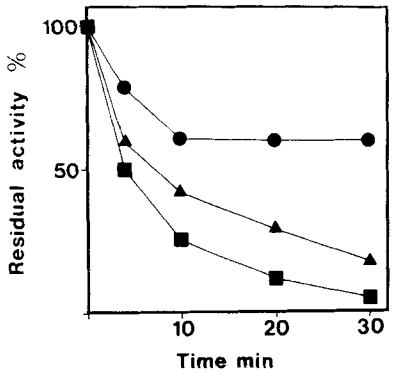

Fig. 5. Thermal lability of the PGM isoenzymes isolated by DEAE Sephadex A-50 chromatography from embryonic day 15 mandibular processes. Type MM PGM, type MB PGM, and type BB PGM. The peaks isolated from the DEAE Sephadex A-50 column were heated at 56°C in 20 mM tris–HCl buffer, pH 7.5, containing 1 mM EDTA in the presence of glycerate-2,3-P_2 (6 mM). The protein concentration was 1 mg/ml. At intervals incubation was stopped and PGM activity was determined.

more complex than was earlier believed. In spite of their complexity, the development can be broken down into a step-by-step sequence involving extensive migrations of cells and biochemical interactions among various cell groups. By combining microdissection with biochemical analysis, we can obtain further knowledge of the complex field of craniofacial development.

By following the natural groove formation between the facial processes, distinctive areas for dissection were obtained. As judged morphologically, the tissues consisted of a surface epithelium and a cellular part of multipotent cells that during later development differentiated into osteogenic, chondrogenic, myogenic, and fibrogenic cells (4).

The time interval used (9–15 days) was selected because during this time important embryogenic changes occur in the developing face, in that the facial processes appear, fuse, and at the end of the observation period start to differentiate into future tissues (4). Earlier studies have shown that the tissue metabolism in facial processes of 9-day embryos is of an anaerobic type. With increasing age, a shift to a metabolism of aerobic type parallels the formation of new blood vessels in the facial processes (5).

The development of hard tissues in facial processes starts on the 14th embryonic day with the formation of mandibular osteoid. Nonspecific alkaline phosphatase, which is a marker for mineralization, undergoes isoenzymic development during embryonic days 15–20 (6).

Phosphoglycerate mutase, like creatine phosphokinase and fructose diphosphate aldolase, can be detected in facial processes (4) and provides a good marker for investigation of differentiation of muscle tissue (15).

To characterize the three PGM isoenzymes (MM, MB, and BB) detected in facial processes by IEF, their sensitivity to the modification of the sulfhydryl groups with tetrathionate and Hg^{2+} and their heat lability were determined. The existence of PGM isoenzymes with different sensitivities to Hg^{2+} was first suggested by Grisolia et al. (8), who found that the PGM activity from different mammalian tissues was differently affected by Hg^{2+} treatment and reported a developmental increase of Hg^{2+} sensitivity in PGM from human and chicken skeletal muscle. The different heat labilities of human PGM isoenzymes were detected by Omenn et al. (14, 15), who found that PGM from human brain was much less stable to heating than PGM from skeletal muscle.

In mammals and reptiles three PGM isoenzymes have been found whose sensitivity to tetrathionate and Hg^{2+} varies from tissue to tissue and also during embryonic and postnatal development (12).

In the present study the extent of inhibition by tetrathionate and Hg^{2+} correlated with the proportion of type MM PGM present in the specimen, suggesting that this isoenzyme was the one sensitive to the modification of the -SH groups. IEF analysis of extracts treated with tetrathionate confirmed that type BB PGM was not affected by treatment, whereas type MB isoenzyme was partially sensitive and type MM isoenzyme fully inhibited. It was also confirmed that type MM and type BB PGM from facial processes differ in their thermal lability. As in humans (14, 15), rat facial process PGM types BB

and MB showed much greater heat lability than type MM PGM. These results strongly support the homodimeric and heterodimeric structure suggested for PGM isoenzymes (1, 13–15) and favor their genetic origin. The experiments with heat inactivation demonstrate that PGM subunits type M and B from rat facial processes differ in their thermal lability. The experiments of inhibition with tetrathionate and Hg^{2+} show that both subunits differ in the presence of sulfhydryl groups, the modification of which causes the loss of enzymatic activity. The protection of glycerate-2,3-P_2 against inactivation could indicate that the -SH groups sensitive to modification are located at the cofactor binding site. However, glycerate-2,3-P_2 binding could also develop the protective effect at a distance, through conformational changes.

The molecular weight values obtained are similar to those reported for purified PGM, which are known to have dimeric structures (7). The Ip of the three isoenzymes suggests a relative predominance of the acidic amino acid residues in a type B subunit, which agrees with the amino acid composition of PGM from pig heart (2), human brain, and skeletal muscle (15).

Acknowledgements.—This study was supported by grants from the Anna Ahrenberg foundation, the Magnus Bergvall foundation, Kungl. and Hvitfeldtska Stipendieinrättningen, O.E. and Edla Johanssons Foundation, and by the Swedish Dental Society.

References

1. Adamson ED. Isoenzyme transitions of creatine phosphokinase aldolase and phosphoglycerate mutase in differentiating mouse cells. J Embryol Exp Morphol 1976;35:355–67.
2. Bartrons R, Carreras J. Purification and characterization of phosphoglycerate mutase isozymes from pig heart. Biochim Biophys Acta 1982; 708:167–77.
3. Edwards JA. The external development of the rabbit and rat embryo. Adv Terat 1968;3:239–63.
4. Granström G. Isoenzyme changes during rat facial development. Scand J Dent Res 1986;94:1–14.
5. Granström G, Magnusson BC. Lactate dehydrogenase isoenzyme changes during facial development. J Anat 1986;148:183–92.
6. Granström G, Magnusson BC. Changes in alkaline phosphatase isoenzymes of hard tissue origin during facial development in the rat. Arch Oral Biol 1986;31:513–9.
7. Grisolia S, Carreras J. Phosphoglycerate mutase from yeast, chicken breast muscle and kidney (2,3-PGA-dependent). Meth Enzymol 1975;42:435–50
8. Grisolia J, Diederich D, Grisolia S. Developmental increase in activity and sensitivity to mercury of human and chicken skeletal muscle phosphoglyceromutase. Biochem Biophys Res Commun 1970;41:1238–43.
9. Johnson EM. Electrophoretic analysis of abnormal development. Proc Soc Exp Biol Med 1965;118:9–11.
10. Johnson EM, Lambert C. Effects of N-nitroso-N-methylurea on enzymatic ontogeny associated with teratogenesis. Teratology 1968;1:192–7
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.
12. Mezquita J, Carreras J. Phylogeny and ontogeny of the phosphoglycerate mutases. I. Electrophoretic phenotypes of the glycerate-2,3-P_2 dependent phosphoglycerate mutase in vertebrates. Comp Biochem Physiol 1981;70B:237–45.
13. Mezquita J, Bartrons R, Pons G, Carreras J. Phylogeny and ontogeny of the phosphoglycerate mutases. II. Characterization of phosphoglycerate mutase isozymes from vertebrates by their thermal lability and sensitivity to the sulfhydryl group reagents. Comp Biochem Physiol 1981;70B:247–55.
14. Omenn GS, Cheung SCY. Phosphoglycerate mutase isozyme marker for tissue differentiation in man. Am J Hum Genet 1974;26:393–9.
15. Omenn GS, Hermodson MA. Human phosphoglycerate mutase: isozyme marker for muscle differentiation and for neoplasia. In: Markert CR, ed. Isozymes. Vol. 3. New York: Academic Press, 1975:1005–18.
16. Sutton JG, Burgess R. Genetic evidence for four common alleles at the phosphoglycomutase-1-locus (PGM) detectable by isoelectric focusing. Vox Sang 1978;34:97–103.

Received for publication 21 April 1987