Reversal of age-related effects in rat muscle phosphoglycerate kinase

KHE-CHING M. YUH AND ARI GAFNI*

Institute of Gerontology and Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109

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ABSTRACT Rat muscle phosphoglycerate kinase is one of several enzymes in which age-related effects have been identified. Thus, samples of this enzyme isolated from old rats display a greatly increased heat stability as compared with enzyme isolated from young animals. Previous studies detected no differences in the sequence of amino acids or in the net charge between the young and old forms of the enzyme and it was concluded that the age-related structural modifications are purely conformational. The present study was conducted with the aim of critically testing this hypothesis. To this end, samples of phosphoglycerate kinase purified from skeletal muscle of young and old rats were unfolded by an 18-hr incubation in a 2 M guanidine hydrochloride solution at 4°C, a treatment that results in extensive loss of the three-dimensional structure of the enzyme. A complete reactivation of both enzymes was achieved by dilution of the unfolded enzyme solutions into a large excess of denaturant-free buffer followed by 4 hr of incubation at 25°C. The reactivation kinetics of the unfolded young and old enzymes were practically identical and the refolded products, compared using heat-inactivation kinetics as a sensitive probe, were found to be identical. Moreover, their heat inactivation coincided with that of young untreated phosphoglycerate kinase. These results demonstrate the reversibility of age-related effects at the molecular level and provide strong support for the hypothesis that the modifications in phosphoglycerate kinase in old muscle are purely conformational and, hence, clearly postsynthetic.

Biological aging is an extremely complex process that involves a multitude of biochemical events including a modification of some enzymatic properties (1–11). The appearance of altered enzymes in old tissues may reflect changes in the primary structure resulting from programmed changes during aging in gene expression similar to the changes that occur during development (12), or it may be due to the accumulation of errors in information transfer steps such as transcription or translation (13, 14). Alternatively, the aging effects may result from postsynthetic modifications in enzyme molecules due either to changes in the cell’s environment (15, 16) or to the slower turnover rate of proteins in old tissues (17). The experimental data thus far obtained overwhelmingly support a postsynthetic modification mechanism for protein aging. However, the nature of these modifications and the mechanisms by which they develop are still not well understood.

While in most enzymes modified by aging the specific activity is reduced, this is not always the case. Thus, phosphoglycerate kinase (PGK) samples purified from young and old rat muscle show similar enzymatic activities; however, they do differ substantially in several of their spectral properties, in their stability toward heat inactivation, in the exposure of their sulfhydryl groups to certain reagents, and in their immunological activity (18). Recent studies by Hardt and Rothstein (19) strongly suggest that the age-related modifications in PGK are purely conformational and involve no covalent modifications in amino acid residues. In contrast, it was reported that when young and old PGK were inactivated by guanidine and subsequently reactivated, they refolded at different rates and to different products, implying the existence of covalent modifications in their amino acid residues (20).

The present study was conducted with the aim of critically testing the hypothesis that old and young forms of PGK are conformational isomers. To this end, enzyme unfolding-refolding experiments were performed under conditions that yield a complete unfolding of each of the two domains of the PGK molecule (21, 22). The rationale behind this experimentation is based on the recent report (21) that the C-terminal domain of muscle PGK is relatively resistant to denaturation by guanidine hydrochloride (Gdn-HCl). Potential age-related conformational modifications in this region would therefore be abolished only upon prolonged incubations in the presence of the denaturing agent; upon refolding, all the original conformational isomers should yield a common enzyme form. The results obtained indeed show unequivocally that extensively unfolded young and old forms of PGK display the same refolding pattern and that the refolded products are identical, as judged by the kinetics of heat inactivation (this property, being dramatically modified with age, was used as a sensitive parameter to determine the status of the enzyme). Moreover, these refolded products are identical to native (untreated) young PGK and significantly differ from the native old enzyme. From these experimental results we are therefore now able to unambiguously demonstrate the reversibility of aging effects at the molecular level and to provide strong direct support to the idea that young and old PGK are conformational isomers.

MATERIALS AND METHODS

Materials. The reagents used in PGK preparation and assays were supplied by Sigma. Preswollen CM-52 cellulose was purchased from Whatman.

Animals. Young (2 months) and old (28 months) Sprague-Dawley rats were obtained from Harlan Sprague Dawley (Indianapolis) and fed ad libitum with Purina Laboratory Chow. Animals were killed by decapitation. Skeletal muscles from the hind legs were removed and washed in 20 mM Tris Mops buffer, pH 7.0/10 mM 2-mercaptoethanol/1 mM EDTA (sodium salt). Muscles not used immediately were stored at –70°C. Enzyme samples purified from fresh or frozen tissue show the same recovery during purification as well as identical physical and enzymatic properties (18).

Enzyme Isolation and Purification. PGK was isolated as described by Sharma et al. (18) with slight modifications: Ammonium sulfate fractionation was performed by removing

Abbreviations: PGK, phosphoglycerate kinase; Gdn-HCl, guanidine hydrochloride.

*To whom reprint requests should be addressed.
the protein precipitate formed at 50% saturation and adding ammonium sulfate to bring the supernatant to 85% saturation, where the enzyme was precipitated. The pellet was dissolved in a minimal volume of 5 mM Tris Mops buffer (pH 6.7) containing 10 mM 2-mercaptoethanol and dialyzed against the same buffer overnight with three buffer changes. The dialyzed solution was centrifuged at 12,000 × g for 30 min and the supernatant was applied to a CM-52 column (2.5 × 12 cm for 20 g of tissue), which had been previously equilibrated with 5 mM Tris Mops, pH 6.7/10 mM 2-mercaptoethanol.

The column was developed with 75 ml of the same buffer and fractions of 2.5 ml were collected. PGK was then eluted with 100 ml of a 5 mM sodium d-3-phosphoglycerate solution in the buffer described above. Protein concentration and enzyme activity were monitored, and all fractions containing 8 enzyme units or more were pooled.

**Protein Assays.** Determination of protein concentration was done by the method of Bradford (23) using fraction V bovine serum albumin (Sigma) as a standard.

**Enzyme Activity Assay.** PGK activity was assayed at 25°C in a reaction mixture containing 50 mM Tris acetate (pH 7.3), 25 mM MgCl₂, 10 mM d-3-phosphoglycerate, 5 mM ATP, 0.2 mM NADH, and 7 units of glyceraldehyde-3-phosphate dehydrogenase in a total vol of 1 ml. The enzyme preparation was tested first and the rate of decrease in the absorbance at 340 nm was recorded using a Shimadzu UV-260 Spectrophotometer. One unit of enzyme is defined as the amount of protein catalyzing the phosphorylation of 1 μmol of d-3-phosphoglycerate per min at 25°C.

**Heat-Inactivation Experiments Using Native (Untreated) Enzymes.** The young and old forms of PGK were heat inactivated at 42°C, 44°C, and 47°C in a 70 mM Tris acetate buffer (pH 7.3) containing 35 mM MgCl₂. The incubation at 47°C was also carried out in the same buffer system but in the presence of 10 mM 2-mercaptoethanol. The final concentration of enzymes used in these experiments was 30 μg/ml and a total vol of 1 ml was used. Aliquots of 0.1 ml were taken out at various time intervals and assayed for PGK activity.

**Unfolding and Refolding Experiments.** Young and old forms of PGK were unfolded by incubation with 2 M Gdn-HCl at 4°C for 18 hr to ensure complete unfolding of the enzymes (21, 22). The unfolded enzymes were then subjected to reactivation by diluting their solutions into 40-fold excess of denaturant-free buffer [either 5 mM Tris (pH 6.7) or 100 mM sodium phosphate (pH 7.5)], in the presence of 10 mM 2-mercaptoethanol, 1 mM NaEDTA, and incubating for 24 hr or 25°C for 4 hr. The final concentration of reactivated enzyme was 30 μg/ml in both cases.

**Heat Inactivation of Unfolded–Refolded Enzymes.** The unfolded-refolded young and old forms of PGK were subjected to heat inactivation either at 47°C in 70 mM Tris acetate (pH 7.3) containing 35 mM MgCl₂ and 10 mM 2-mercaptoethanol or at 52°C in a 100 mM sodium phosphate buffer (pH 7.5) containing 10 mM 2-mercaptoethanol and 1 mM NaEDTA. The final concentrations of enzymes were 21.6 μg/ml and 30 μg/ml in Tris and phosphate buffer, respectively. Solutions of native (not unfolded-refolded) young and old PGK were also heat inactivated under the conditions described above to serve as the controls in these experiments.

An inherent problem here is our finding that when young PGK is incubated in solution, it undergoes a gradual increase in its heat stability, which, after prolonged incubation, approaches that of the old enzyme (see Fig. 2). To avoid ambiguity when using young untreated PGK as the control, we therefore used freshly purified enzyme samples (i.e., within a few hours after purification). The implication of this protocol on the comparison between native young PGK and the unfolded-refolded forms of the enzyme is discussed below.

**RESULTS**

In agreement with Sharma et al. (18) we found that the young and old forms of PGK significantly differ in their heat-inactivation kinetics. Thus, when the enzymatic activities were followed as a function of incubation time at 42°C, 44°C, and 47°C, the young form of PGK was significantly more heat labile than its old counterpart (Fig. 1A). The differences observed between the heat stabilities of these two enzymes increased with temperature in the range indicated above. Therefore, all subsequent heat-inactivation experiments in the Tris acetate buffer were conducted at 47°C.

Fig. 1B depicts the heat-inactivation pattern of young and old PGKs at 47°C in the same Tris acetate buffer but in the presence of 10 mM 2-mercaptoethanol. This reducing agent greatly stabilizes both forms of PGK and the time needed for 50% inactivation of the young enzyme is increased from ~1 min to ~12 min, while for old PGK the corresponding change is from 7 to ~27 min. These results provide a strong indication that oxidation of PGK may play a role in its inactivation at elevated temperatures. The observation that the young enzyme is much more susceptible to oxidation than its old counterpart (as reflected in the 12-fold increase in its rate of heat inactivation when the reducing agent is removed, as compared with the 4-fold increase found for old PGK) is supported by the finding that old PGK reacts with sulphydryl reagents slower than the young enzyme (18).

![Fig. 1. Heat inactivation of native forms of PGK. (A) Solutions (30 μg/ml) of young (open symbols) and old (solid symbols) PGKs inactivated at 42°C (●, ■), 44°C (○, ●), and 47°C (○, ●) in 70 mM Tris acetate (pH 7.3), including 35 mM MgCl₂. (B) Solutions (30 μg/ml) of young (○) and old (●) PGKs inactivated at 47°C in the same 70 mM Tris acetate buffer (pH 7.3), including 35 mM MgCl₂ but in the presence of 10 mM 2-mercaptoethanol. (Inset) Inactivation of young PGK under the same experimental conditions as in B but carried out for 60 min. The inactivation data in this and in subsequent figures are expressed in relative enzyme activity—i.e., the enzyme activity of untreated samples was normalized to 100%.](image-url)
incubation with the denaturing agent and represent equilibrium values for the inactivation reaction (21, 22). The residual enzymatic activity critically depends on Gdn-HCl concentration and a sharp transition occurs around 0.6 M denaturant. Both young and old forms of PGK displayed very similar inactivation profiles and were completely inactivated by an 18-hr incubation in 1 M Gdn-HCl. The inactivation profile shown in Fig. 3 is in excellent agreement with the one reported by Desmadril et al. (21), who investigated the unfolding of horse muscle PGK in a study that did not address aging effects in the enzyme. These authors found that 1 M Gdn-HCl, while totally inactivating the enzyme, did not completely unfold its polypeptide chain (as judged by tryptophan fluorescence changes, circular dichroism at 220 nm, as well as UV difference spectra). At a Gdn-HCl concentration of 2 M, however, the unfolding was complete as judged by all criteria. While at this concentration of denaturant the inactivation of young and old PGKs at 4°C was relatively rapid (50% of the enzyme activity being lost in <20 min in Tris as well as in phosphate buffers), enzyme unfolding was nevertheless routinely performed for a period of 18 hr to ensure its completion.

Reactivation of the unfolded enzymes was performed by diluting their solutions in 2 M Gdn-HCl into an excess of denaturant-free buffer. Fig. 4 shows the reactivation kinetics at 4°C and at 28°C. At both temperatures, we found the reactivation reaction to be biphasic, in good agreement with the reactivation kinetics reported for horse muscle PGK (22). This pattern is indicative of the existence of intermediate(s) along the folding pathway. It is also clear from Fig. 4 that the reactivation rates are strongly temperature dependent. Thus, while only 75% of young and old PGK activities were restored after 24 hr of incubation at 4°C, 100% reactivation of

The heat-inactivation pattern of young PGK was found to follow first-order kinetics down to below 5% of its original activity (Fig. 1B, *Inset*), demonstrating its freedom from any significant amounts of the old form. The inactivation of old PGK did not display a fast-decaying initial component, indicating that the old enzyme is also homogeneous. Thus, aging of PGK appears to reflect a change (to higher heat stability) in all the old enzyme molecules.

When solutions of young PGK were incubated at 4°C with 2-mercaptoethanol for up to 6 days, the specific activity of the enzyme was unaffected; however, the thermal stability of the enzyme increased with time, as shown in Fig. 2, and gradually approached the heat-inactivation profile of old PGK. This process was faster when the incubation was done in the absence of 2-mercaptoethanol; however, under these conditions the enzyme also displayed a reduction in its specific activity. The fact that upon incubation in solution young PGK is spontaneously converted to a form that resembles old enzyme in its heat inactivation may have significant effects during the performance of lengthy experiments (i.e., when using samples of enzyme as controls in unfolding–refolding experiments).

The degrees of inactivation of young and old forms of PGK as a function of Gdn-HCl concentration are shown in Fig. 3. These fractional activities were measured after 18 hr of incubation with the denaturing agent and represent equilibrium values for the inactivation reaction (21, 22). The residual enzymatic activity critically depends on Gdn-HCl concentration and a sharp transition occurs around 0.6 M denaturant. Both young and old forms of PGK displayed very similar inactivation profiles and were completely inactivated by an 18-hr incubation in 1 M Gdn-HCl. The inactivation profile shown in Fig. 3 is in excellent agreement with the one reported by Desmadril et al. (21), who investigated the unfolding of horse muscle PGK in a study that did not address aging effects in the enzyme. These authors found that 1 M Gdn-HCl, while totally inactivating the enzyme, did not completely unfold its polypeptide chain (as judged by tryptophan fluorescence changes, circular dichroism at 220 nm, as well as UV difference spectra). At a Gdn-HCl concentration of 2 M, however, the unfolding was complete as judged by all criteria. While at this concentration of denaturant the inactivation of young and old PGKs at 4°C was relatively rapid (50% of the enzyme activity being lost in <20 min in Tris as well as in phosphate buffers), enzyme unfolding was nevertheless routinely performed for a period of 18 hr to ensure its completion.

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both young and old PGKs, at each of the two temperatures, is of great significance and strongly suggests a common form for the unfolded enzyme, in support of the conformational isomers model for PGK aging.

The heat-inactivation patterns of unfolded-refolded young and old PGKs are presented in Fig. 5 and are compared with the inactivation kinetics of the native (untreated) forms of these two enzymes. The inactivation experiments were conducted both in Tris acetate buffer in the presence of MgCl₂ and in phosphate buffer. The heat-inactivation rates depend on the buffer system used and are considerably slower in the phosphate buffer. Most importantly, in each buffer system the inactivation pattern of the unfolded-refolded old enzyme shows a striking resemblance to that of its young counterpart as well as to the inactivation kinetics of native young PGK (while the native old enzyme is significantly more heat stable).

**DISCUSSION**

The hypothesis that the age-related effects observed in rat muscle phosphoglycerate kinase originate in alterations in the enzyme's conformation only and do not involve covalent modifications in amino acid residues is supported by a significant body of experimental evidence—all of which, however, being of negative nature (i.e., based on the failure to detect any covalent modifications). Thus, the observation that both young and old PGKs show identical migration patterns in isoelectric focusing was used to exclude charge differences between these two enzyme forms (18), ruling out the existence of differences in the content of charged amino acids or of covalent modifications that change the charge of any amino acid (phosphorylation, alkylation, deamidation, etc.). Both the amino- and carboxyl-terminal amino acids were found to be unmodified by age, excluding the possibility of loss of amino acid residues (18). The number of cysteine residues titratable by 5,5'-dithiobis(2-nitrobenzoic acid) is also not modified by age (18), thereby excluding the possibility that the formation of disulfide bridges leads to the development of structural modifications in the enzyme. More recently, Hardt and Rothstein (19) made a detailed comparison, by HPLC, of the peptide fragments obtained from young and old PGKs following enzyme digestions with three proteases. They found that the peptide patterns obtained from the young and old enzymes were identical and concluded that there are no changes in the sequence of old PGK, once more supporting the hypothesis that the age-related changes involve conformational modifications only.

In contrast to the results described above, an early attempt to test the mechanism of PGK aging by unfolding-refolding experimentation produced disconcerting results (20). Under the conditions used, the unfolded young and old PGKs displayed different refolding kinetics and, moreover, refolded to form different products. These results imply that a covalent modification is likely to be present in the old form of PGK. Indeed, Fucci et al. found that PGK is readily oxidized by mixed function oxidation systems (24). More recently, Oliver et al. (25) reported that this oxidation involves a histidine residue and leads to the introduction of a carbonyl group, a process that may leave the enzyme's charge unaffected and be undetectable by isoelectric focusing. A second enzyme where a histidine residue is readily oxidized by mixed function oxidation systems is enolase (25). It is therefore interesting to note that when unfolding-refolding experiments were performed with nematode enolase, the young and old forms of the enzyme refolded to a product similar (but not identical) to old enolase (26), leaving open the possibility that a charge-conserving covalent modification exists in the old enzyme and is introduced into the young form during treatment.

The major goal of the present study was to critically test the hypothesis that the age-related modifications in rat muscle PGK are purely conformational and to demonstrate unambiguously that the aging effects in this enzyme may be successfully reversed. The experimental conditions were chosen so as to ensure complete unfolding of the young and old forms of the enzyme—a condition apparently not met in the previous study mentioned above (20). To this end, the rapid loss of enzymatic activity in 2 M Gdn-HCl was not assumed to reflect efficient unfolding of the polypeptide chain; rather, the incubation was continued for prolonged periods of time previously shown to result in the loss of tertiary and secondary structure of muscle PGK (21, 22). The denatured enzymes were then refolded, taking advantage of the fact that completely unfolded muscle PGK is able to regain full activity upon removal of the denaturing agent (21, 22). Both the reactivation kinetics and the prominent differences between the heat stabilities of the young and old forms of PGK were used in our investigation to determine the status of the enzyme being tested.

The results obtained provide direct positive evidence that old and young rat muscle PGKs are conformational isomers. Thus, the refolding kinetics of the two unfolded enzymes coincide and, moreover, both enzymes refold to a common product identical with young PGK. The rejuvenation of old PGK reported here successfully demonstrates that aging effects at the molecular level may be reversed. These results attest to the fact that the age-related effects in PGK result
from postsynthetic modifications rather than from a synthesis controlled by different genes or from errors in sequence.

Several explanations have been proposed as to why enzymes are modified in vivo during the aging process. Rothstein and coworkers proposed that the structural changes could be brought about by a slowing of protein turnover (1, 20), a phenomenon that was indeed demonstrated in Turbatrix aceti (27). Hiremath and Rothstein (28) found that in regenerating liver in old rats only the young form of PGK is present during the first 2 days following hepatectomy and that the enzyme is gradually modified with increasing time, becoming old again by 9 days after hepatectomy. Our data (Fig. 2) show that this process occurs in vitro as well. As young PGK is incubated in solution it is transformed to a form similar to old enzyme over a period of several days (this transformation involving no reduction in specific activity). It thus appears that a long dwell time in the cell can indeed bring about the development of aging effects in PGK.

In addition to slow protein turnover, the aging effects may result from changes in the old cells’ environment. Harman (15) suggested that the combined free radical reactions that continuously occur in living cells and tissues are a major contributor to the aging process. More recently, Noy et al. (16) demonstrated that a significant increase in the relative concentration of the oxidized forms of glutathione, NAD, and NADP, at the expense of the reduced forms, occurs in old muscle tissue providing a less reducing environment in the tissues of old animals and potentially leading to an increase in the concentration of H2O2, molecular oxygen, superoxide radicals, and other potent oxidants. Evidence that such an increase in oxidation potential may be involved in the aging of PGK in rat muscle is provided by our observation that solutions of PGK incubated in the absence of 2-mercaptoethanol rapidly lose their enzymatic activity and that when excess thiol is added the activity recovers but the enzyme displays increased heat stability. It seems very likely that cysteine residues participate in the initial phase of PGK aging (i.e., the oxidation) in view of their known high reactivity toward oxidizing and alkylating agents. However, even when these residues are subsequently fully reduced by excess thiol (as evidenced by the fact that the total number of cysteines titratable by 5,5′-dithiobis(2-nitrobenzoic acid) is restored) some structural modifications in the enzyme persist and are reflected in the modified properties characteristic of old PGK. The present study proves these structural modifications to be purely conformational; however, the identification of their location as well as their mechanism of development are left for future work.

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