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Purification and Comparative Study of Glyceraldehyde-3-phosphate Dehydrogenase from the Muscles of Young and Old Rats†

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ABSTRACT: D-Glyceraldehyde-3-phosphate dehydrogenase (GPDH) was purified from the muscles of young and old rats. A marked difference was found between the (total) activities of these two enzyme preparations which originates in their different specific activities, while the concentrations of the enzyme in young and old tissues appear to be similar. Both "young" and "old" enzyme forms show four rapidly reacting sulfhydryl groups while six additional SH groups are revealed upon longer incubation with the sulfhydryl reagent. The UV absorption spectra and sedimentation coefficients of young and old GPDH molecules are also identical, and while the two enzyme forms partially dissociate into dimers in the presence of sodium chloride, the old enzyme appears to be more dissociable. The amino acid compositions of the GPDH molecules purified from young and old rats are remarkably alike and show a great similarity to the compositions of GPDH molecules from other mammals. Small differences in composition may, however, have escaped detection due to accuracy limitations of the determination and could be responsible for the differences in enzymatic activity. Alternatively, the activity differences may originate in postsynthetic conformational changes induced in the old enzyme during its longer "dwell time" in the tissue.

Aging phenomena at the molecular level have begun to attract attention in recent years when it was found that many enzymes lose part of their (specific) activity in old animals [for recent reviews on the subject, see Dreyfus et al. (1978) and Rothstein (1977)]. Only a few of the studies were devoted to aging of enzymes from mammalian tissues, some examples being mouse and rat liver aldolase (Gershon & Gershon, 1973; Weber et al., 1976), glucose-6-phosphatase (Grinna & Barber, 1975), rat liver superoxide dismutase (Reiss & Gershon, 1976), rat heart enolase (Rothstein et al., 1980), and rat muscle phosphoglycerate kinase (Sharma et al., 1980). Some enzymes, on the other hand, were found to retain their full activity in old animals (Gupta & Rothstein, 1976; Petell & Lebherz, 1979; Steinhagen-Thiessen & Hilz, 1976; Yagil, 1976), proving that altered enzymes are not necessarily concomitant of aging.

While the partial loss of enzymatic activity is common to all enzymes that are affected by aging, changes in other properties are less consistent. In some cases, a component with increased heat sensitivity was found (Reiss & Gershon, 1976; Bolla & Brot, 1975; Epstein & Gershon, 1972). Values of

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K_m observed for "young"[1] and "old" enzymes were usually very similar (Rothstein, 1977) and so were the molecular weights, charge, and behavior during purification, indicating that the modifications induced in old enzymes are quite subtle.

The differences between young and old forms of an enzyme may result either from a change in its amino acid sequence (Orgel, 1963) or from postsynthetic events. The second possibility seems to agree better with the experimental results obtained by most workers although an unequivocal conclusion cannot be drawn on the basis of the data obtained so far.

The subtle modifications introduced by aging in an oligomeric enzyme molecule may have very pronounced effects on those molecular properties which originate in interactions among the subunits. Thus, cooperative effects in which small changes in one subunit affect the properties of neighboring subunits may be strongly expressed and easy to detect and follow. One of the most studied oligomeric enzymes, where strong cooperative effects in ligand binding have been observed, is muscle glyceraldehyde-3-phosphate dehydrogenase (GPDH).[2] The tetrameric rabbit enzyme displays a dramatic negative cooperativity in NAD^+ binding (Conway & Koshland, 1968; DeVijlder & Slater, 1968; Bell & Dalziel, 1975) and in the binding of several coenzyme analogues (Schlessinger & Levitzki, 1974; Eby & Kirtley, 1976; Henis & Levitzki, 1977). Some coenzyme analogues do, however, bind to the enzyme noncooperatively (Eby & Kirtley, 1976; Furfine & Velick, 1965).

While rabbit muscle GPDH has been studied very extensively, little is known about the rat muscle enzyme. Nagradova & Guseva (1971a,b) isolated GPDH from rat skeletal muscle and found the enzyme to differ from the rabbit muscle enzyme in its immunological properties. They also found the enzyme to be more labile than the rabbit muscle enzyme due to a reversible dissociation of the native tetramer into dimers, a process that depended strongly on the presence of certain anions in solution, and was also enhanced by mononucleotides (Nagradova et al., 1974).

In the present study, we prepared GPDH from the muscles of young and old rats in high yields and activity. In this paper are described the effects of aging on the activity of the enzyme, and a comparison is made of the composition and of some physical properties of young and old forms. The following paper (Gafni, 1981) is devoted to the large differences found between the affinities of young and old GPDH toward NAD^+.

Materials and Methods

Materials

DTNB, NAD^+, and DL-glyceraldehyde-3-phosphate diethylacetal, barium salt, were obtained from Sigma. The latter was deionized on Dowex 50W (hydrogen form) and hydrolyzed by heating to form the free aldehyde by using the procedure recommended by the supplier. 2-Mercaptoethanol, ammonium sulfate, and EDTA were purchased from Fluka and used without further treatment. Acid-washed activated charcoal (Norit A) was obtained from Sigma, was washed 3 times with 0.2 M aqueous EDTA (pH 7.2) followed by repeated washing with glass-distilled water, and was finally dried at 100 °C. CM-52 cation-exchange resin was purchased from Whatman, while Sephadex G-50 and G-200 were products of Pharmacia.

Methods

Purification of Glyceraldehyde-3-phosphate Dehydrogenase. The enzyme was purified from fresh back and hind legs muscles of young (6 months) and old (28 months) WF rats. Muscle tissue taken from 15 rats (300–350 g) of each age group was used to make three preparations of each enzyme form. Similar differences between the specific activities of young and old enzyme forms were observed in all these preparations. Identical purification procedures and conditions were maintained in preparing the enzyme from the two rat populations, and all the purification steps were performed at 4 °C with buffers made in glass-distilled water and containing 1 mM 2-mercaptoethanol. Enzyme preparation was based on the procedure described by Bloch et al. (1971) for the rabbit muscle enzyme. The elution profile of rat muscle GPDH from the CM-52 column was found to differ markedly from that of the rabbit muscle enzyme. Almost all the GPDH activity was recovered in a band eluted right after the column's void volume, which also contained some hemoprotein. An additional purification step was therefore introduced in the form of gel filtration through a Sephadex G-200 column. This resulted in efficient separation of the GPDH from the hemoprotein band.

Storage. The enzyme was stored as a crystalline suspension in 85% saturated ammonium sulfate containing 5 mM EDTA, 5 mM 2-mercaptoethanol, and 10^{-4} M NAD^+, pH 7.2, at 0–5 °C. It gradually lost activity at a rate of about 15% per month.

Preparation of Apo-GPDH. A solution containing about 10 mg/mL holoenzyme in 50 mM Hepes buffer, pH 7.2, containing 10 mM EDTA and 1 mM 2-mercaptoethanol was gently stirred for 20 min at 4 °C with 80–100 mg of activated charcoal which had been treated as previously described. The charcoal was removed by centrifugation and 1 mM 2-mercaptoethanol added to the solution to compensate for possible loss during the charcoal treatment. The apoenzyme obtained had an A_{280}/A_{260} of 1.85–1.95 and 3.7–4.2 NAD^+ binding sites per tetramer as determined by Racker band titration (Racker & Krimsky, 1952; Gafni, 1981). When apo-GPDH was prepared for the determination of sulfhydryl groups, the mercaptoethanol was omitted from the buffer solution.

Extinction Coefficients. The extinction coefficients of young and old enzymes were determined from the dry weight of protein in solutions of measured optical absorption. These solutions were made in dilute (3 mM) buffer, and measured portions, including a sample of pure buffer to serve as a blank, were lyophilized, dried for 24 h in a drying pistol in vacuo, at 65 °C over P_2O_5, cooled, and weighed. Repeating the drying for another 12 h did not cause further weight reduction. Evaluation of molar extinction coefficients was done by assuming a molecular weight of 145 000 for the enzyme.

Enzymatic Activity. Assay of enzyme activity was done as described by Bloch et al. (1971), at 22 °C, with a Zeiss Model PMQII spectrophotometer. Enzyme concentrations were determined spectrophotometrically by using the extinction coefficients determined in the present study.

Heat Inactivation Experiments. These were done by using samples of native GPDH containing 2–2.3 mol of firmly bound NAD^+ per tetramer. The enzyme was diluted from a cold, concentrated stock solution ($\sim 4 \times 10^{-5}$ M) into a buffer solution maintained at 46 °C by a thermostated bath, to make a final concentration of 10^{-6} M. Aliquots of this enzyme solution were removed at various times, cooled, and assayed.

Determination of Sulfhydryl Groups. Solutions of apo-

[1] "Young" and "old" enzymes are enzymes purified from young and old organisms, respectively.

[2] Abbreviations used: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); GPDH, D-glyceraldehyde-3-phosphate dehydrogenase; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; NAD and NADH, oxidized and reduced β-nicotinamide adenine dinucleotide, respectively.

Table I: Purification of Rat Muscle GPDH

purification stage	total activity units per 100 g of muscle		sp act. (units/mg)[a]		% recovery	
	young	old	young	old	young	old
65% ammonium sulfate supernatant	49 800	28 000	14.4	9.2	100	100
ammonium sulfate 65–85% precipitate	45 000	25 820	34.1	20.2	90	92
refractionation in 80% ammonium sulfate	42 420	24 540	70.4	53	85	88
CM-52 cellulose	41 200	23 940	97	62	83	85
Sephadex G-200	39 700	21 600	155	97	80	77

[a] Protein concentrations were determined spectrophotometrically by using the extinction coefficient of holo-GPDH.

GPDH were made in Hepes–EDTA buffer free of 2-mercaptoethanol. DTNB was added from a 10 mM stock solution to make a concentration of 100 μM. Fast and slow reacting sulfhydryl groups were determined from the absorption change at 412 nm after ca. 15 s and after 1 h, respectively, by using an ϵ_{412} = 13 600 M^{-1} cm^{-1} for 3-carboxy-4-nitrothiophenolate (Ellman, 1959). Buffer solutions, devoid of enzyme, that were treated with charcoal gave no reaction with DTNB and served as a blank in the spectrophotometric measurements.

Sedimentation velocity experiments were performed with a Beckman Model E analytical ultracentrifuge at a rotor speed of 56 000 rpm, at 20 °C, and by using Schlieren optics. Samples of young and old enzymes were investigated simultaneously by using a wedge cell.

Amino Acid Analysis. These were performed with a Dionex D-500 amino acid analyzer. Enzyme samples were dialyzed extensively against distilled water and lyophilized prior to hydrolysis. Cysteine was determined as cysteic acid by the procedure of Spencer & Wold (1969).

Results

The activities of young and old rat muscle GPDH at various purification stages are summarized in Table I, revealing marked differences between both total and specific activities of these two species. The reduction in activity of the old enzyme form found in the present study resembles some observations made for other aged enzymes (Dreyfus et al., 1978; Rothstein, 1977). In contrast to rat muscle phosphoglycerate kinase, where the loss of activity was found to reflect differences in the total amount of enzyme and not in its specific activity (Sharma et al., 1980), the different (total) activities of young and old GPDH stem mainly from different specific activities. In fact, the total amounts of the two enzyme species in the respective muscle tissues are seen to be quite similar, and the two forms are recovered with almost identical efficiencies.

Rat muscle GPDH differs from the rabbit muscle enzyme in its elution pattern from the CM-52 ion-exchange column. Both young and old forms of the rat enzyme are not retained by the column, while rabbit muscle GPDH is eluted only at considerable ionic strengths. It is interesting to note that Nagradova & Guseva (1971a) found significant differences between the immunological properties of the rat and rabbit muscle GPDHs and concluded that considerable differences exist between the enzyme molecules from these two sources. The specific activity of young rat muscle GPDH found in the present study is twice the value reported by Nagradova & Guseva (1971a), i.e., 155 units/mg vs. 79 units/mg. However, this difference may be partially due to the different assay conditions used in the two studies.

Table II: Amino Acid Compositions of Young and Old Rat Muscle Glyceraldehyde-3-phosphate Dehydrogenase

amino acid	no. of residues in subunit[a]	
	young	old
aspartic acid	43	42
threonine	19	19
serine	18	19
glutamic acid	28	30
proline	15	15
glycine	32	31
alanine	32	32
cysteine	5	5
valine	23	22
methionine	8	8
isoleucine	14	13
leucine	22	22
tyrosine	9	9
phenylalanine	13	13
histidine	7	8
lysine	28	28
tryptophan	3	3
arginine	11	11

[a] Calculated by assuming a total of 330 residues per subunit. The numbers presented (except for tryptophan) are averages of values obtained in two analyses.

Amino acid analyses of young and old GPDH are summarized in Table II. No significant difference between the two enzyme forms is evident, and the small differences found are within the accuracy of the determination. We therefore conclude that if differences in amino acid composition do exist these must be very limited. A high degree of similarity is also found between the amino acid composition of the rat muscle GPDH and those of GPDH purified from other organisms, in line with the great extent of sequence conservation known to exist in GPDH of various species (Harris & Waters, 1976; Allison & Kaplan, 1964).

Titration of reactive sulfhydryl groups by DTNB revealed the existence of 4–4.2 fast-reacting (within ca. 15 s) thiol groups in the tetrameric enzyme molecule. These sulfhydryl groups most likely belong to the essential cysteine (Cys-149) residues in the active site, which are conserved in all known GPDH molecules and which are known to be highly reactive (Harris & Waters, 1976). Longer incubation with DTNB resulted in further reaction, and the total number of cysteine residues titrated by the reagent after 1 h was 9.3–10. This number is considerably smaller than the total number of cysteine residues found in the amino acid analysis (i.e., 20 per tetramer), indicating that part of these residues are inaccessible to the reagent. The young and old forms of the enzyme behaved identically and revealed the same numbers of both fast- and slow-reacting sulfhydryl groups.

The different activities displayed by old and young GPDH forms are therefore not reflecting partial oxidation of cysteine residues in the active site. In this connection, it is interesting to note that enzyme suspensions stored in the absence of mercaptoethanol for several weeks revealed only 2.6–3 fast-reacting sulfhydryl groups per tetramer, and their specific activities were reduced about 3-fold. Incubation for 12 h with 5 mM mercaptoethanol at 0–4 °C restored almost full activity.

Ultracentrifugation experiments with young and old GPDH are shown in Figure 1. In 50 mM Hepes and 10 mM EDTA buffer (pH 7.2), both young and old enzyme forms sedimented in single peaks with a sedimentation coefficient of 7.3 S. This value is in agreement with the one reported by Nagradova & Guseva (1971b) and is characteristic of GPDH tetramers from various organisms (Allison & Kaplan, 1964; Harrington &

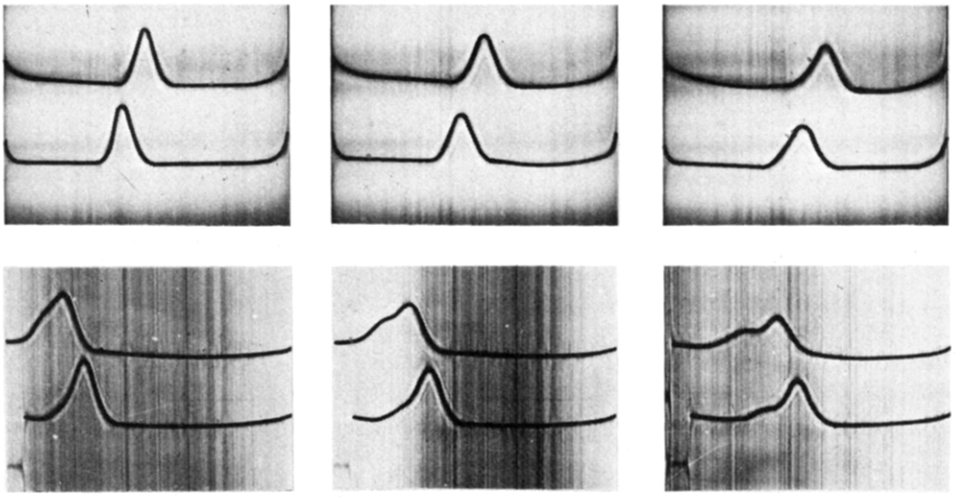

FIGURE 1: Schlieren boundaries observed during the sedimentation of rat muscle GPDH. The upper trace in each photograph is of the old enzyme while the lower trace is that formed by the young enzyme. The concentrations of enzymes were 6.2–6.4 mg/mL, and the temperature was 20 °C. An experiment performed in pure buffer (see Materials and Methods) is shown in the upper three photographs while the lower photographs represent an experiment done in the presence of 0.15 M NaCl. Photographs were taken (from left to right) at 20, 32, and 44 min after attaining a rotor speed of 56 000 rpm.

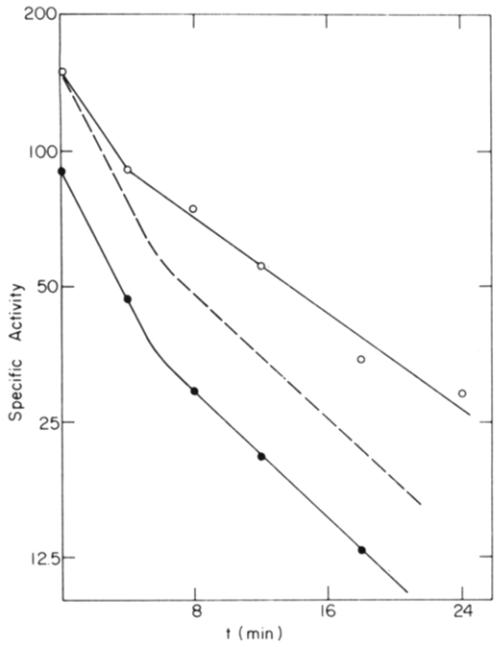

FIGURE 2: Kinetics of the inactivation of young (○) and old (●) GPDH at 46 °C, as described in detail under Materials and Methods. The broken line describes the activity data of the old enzyme when normalized to the initial activity of the young sample to allow a better comparison of the two patterns.

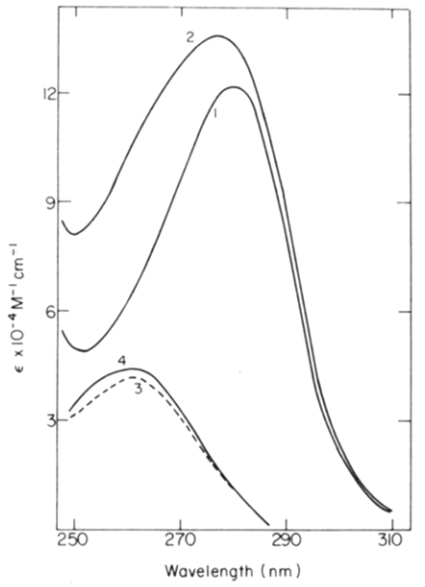

FIGURE 3: Absorption spectra of apo-GPDH (1) and of an enzyme–NAD^+ complex containing 2.4 mol of bound coenzyme (2) in 50 mM Hepes and 10 mM EDTA buffer (pH 7.2). Curve 3 is the difference spectrum between the absorption of the enzyme–NAD^+ complex and that of the apoenzyme, while curve 4 represents the absorption spectrum of a 2.4 M solution of free NAD^+.

Karr, 1965). Intact GPDH molecules from various sources are known to dissociate to (inactive) dimers in the presence of certain anions (Nagradova & Guseva, 1971b; Constantinides & Deal, 1970). Indeed, in the presence of 0.15 M sodium chloride, the sedimentation pattern of both enzyme forms showed two peaks with sedimentation coefficients of 7.3 and 4.9 S. The latter peak represents a fragment believed to be the dimer formed upon dissociation of the tetrameric molecule. It may be seen in Figure 1 that while both young and old enzymes are partially dissociated in the presence of sodium chloride the old form is somewhat more labile since its (slow moving) dimer peak is more pronounced. This observation is supported by heat inactivation experiments performed with young and old GPDH and shown in Figure 2. The inactivation patterns of both enzyme species at 46 °C are biphasic. The fast inactivation step originates in the reversible dissociation of the tetrameric enzyme to inactive dimers, following its dilution, while the second phase of the inactivation reaction represents the irreversible unfolding of the polypeptide chain. The amplitude of the fast inactivation step is seen to be considerably larger in the old enzyme sample, showing this enzyme form to be more dissociable. The rate constants for the second phase of inactivation calculated from the data presented in Figure 2 are 0.06 min^{-1} for young GPDH and 0.08 min^{-1} for the old enzyme form.

Figure 3 presents UV absorption spectra of rat muscle GPDH. The spectra obtained for the old and young forms were strictly identical throughout the wavelength range studied (i.e., 250–320 nm). Enzyme fractions coming off the G-200 column at the last stage of purification had A_{280}/A_{260} ratios of 1.1–1.2, indicating the presence of bound coenzyme in large amounts. Extensive dialysis against NAD^+-free buffer resulted in only a slight increase of this ratio to 1.25–1.30, the wavelength of maximal absorption being 277 nm. When the native

enzyme was repeatedly treated with charcoal, A_{280}/A_{260} increased to a limiting value of 1.95, and the peak wavelength shifted to 280 nm. When enzyme–NAD^+ complexes were denatured by heating in boiling water for 3 min, followed by centrifugation to remove the precipitated protein, practically all their NAD^+ content was found in the supernatant and was quantitatively determined from the absorption at 260 nm. Solutions of apoenzyme showing $A_{280}/A_{260} = 1.95$, when subjected to the heat denaturation treatment, were found to have zero NAD^+ content. We therefore conclude that these apoenzyme preparations are completely free of bound coenzyme.

The extinction coefficient of apo-GPDH, based on dry weight determination, was found to be $E^{0.1\%}_{280nm} = 0.85$ cm^2/mg. With the assumption of a molecular weight of 145 000, the molar extinction coefficient was evaluated to be 1.23×10^5 M^{-1} cm^{-1}. The corresponding values for the holoenzyme are $E^{0.1\%}_{280nm} = 0.97$ cm^2/mg and $\epsilon_{280} = 1.41 \times 10^5$ M^{-1} cm^{-1}.

The absorption spectrum of native enzyme presented in Figure 3 has $A_{280}/A_{260} = 1.30$. The number of coenzyme molecules bound to GPDH in this sample was evaluated from the absorption of NAD^+ released during heat denaturation to be 2.4. The difference spectrum between the molar absorption of the sample of native enzyme and that of the apoenzyme is also shown in Figure 3 and is remarkably similar to the absorption of free NAD^+ at a concentration the same as that for NAD^+ bound to the enzyme. From this result, it may be concluded that absorption by rat muscle GPDH–NAD^+ complexes may to a good approximation be assumed to be the sum of absorptions by the two constituents (this is especially true for wavelengths above ca. 270 nm). In this respect, rat muscle GPDH differs somewhat from the rabbit muscle enzyme where such a relation was not found (Fox & Dandliker, 1956).

Discussion

The main observation made in the present study is that pure young and old rat muscle GPDHs differ considerably in their specific activities while showing a high degree of similarity in most other properties (amino acid composition, sedimentation velocity, UV spectra, number of reactive sulfhydryl groups, etc.). In this respect, rat muscle GPDH appears to conform to the pattern found for most other old enzymes (Dreyfus et al., 1978; Rothstein, 1977). Obviously, the substitution of a limited number of amino acids in the polypeptide chain of the enzyme, which contains some 330 residues, could have readily escaped detection in the amino acid analysis and cannot be ruled out. It is well established that changing even a single amino acid residue may dramatically affect the activity of an enzyme molecule. Indeed, complete inactivation of galactose-1-phosphate uridyltransferase in human red blood cells has been suggested to occur as a result of a single amino acid substitution (Dale & Popjak, 1976). A change of one amino acid in hemoglobin is known to lead to a defective molecule (sickle cell anemia).

Removal of the COOH-terminal tyrosine residue in mouse liver aldolase results in partial inactivation of the enzyme. Cleavage of this residue, by limited proteolysis during storage of liver extracts, has been reported to be the origin of the defective aldolase molecules observed in extracts of old mouse liver (Petell & Lebherz, 1979). This mechanism of producing defective enzyme molecules was excluded in the present study on the basis of the following arguments: The partial proteolysis reported by Petell and Lebherz became significant only after incubation of the liver extracts for periods longer than a day, while in the present study the ammonium sulfate fractionation stage, in which proteolytic enzymes are removed, was reached 3–6 h after tissue extraction. Moreover, muscle aldolase samples prepared from the same young and old tissues used for GPDH extraction had identical specific activity, proving that no proteolysis had occurred during purification. Also, identical lactate dehydrogenase activities were observed in the young and old muscle extracts after removal of the GPDH by $(NH_4)_2SO_4$ fractionation. We therefore conclude that if differences between the amino acid composition of young and old GPDHs do exist these originate in synthetic "errors" and not in modifications that occur during enzyme purification. To what extent a limited change in amino acid composition can affect the enzymatic properties of GPDH is of great importance in determining the origin of the age-related phenomena. GPDH molecules purified from a large variety of organisms show a high degree of similarity, not shared by other groups of dehydrogenases, in their amino acid content and sequence as well as in the structures of coenzyme and substrate binding domains (Jörnvall, 1973; Harris & Waters, 1976). Residues which participate in the active sites are especially conserved. Allison & Kaplan (1964), who studied the properties of a series of GPDH molecules purified from various sources, found significant differences among them in the numbers of reactive sulfhydryl groups, in electrophoretic mobilities, and in immunological properties. They found, however, that pure preparations of all the muscle GPDHs studied had the same specific activity and that most enzymes isolated from vertebrate species showed a remarkable similarity in the degree of their inhibition by pyridine-3-aldehyde adenine dinucleotide. From these findings, it appears that the enzymatic activity of GPDH is not sensitive to the changes introduced by evolution in its amino acid composition. The latter changes may, however, have been selected by evolution so as not to affect the enzymatic activity, while the effects caused by random substitution of some amino acid residues (as assumed by the "error theory") may be very pronounced (it is very likely that replacement even of one of the residues in the active site, which directly participates in coenzyme or substrate binding or in the catalytic steps, would greatly affect the activity).

While limited errors in amino acid composition or sequence in the old GPDH molecule cannot be ruled out, based on the present study, as the origin of the aging effects, there is an increasing amount of evidence in the literature that age-related alterations of proteins are due to postsynthetic modifications (Rothstein, 1977; Dreyfus et al., 1978). Sharma & Rothstein (1978) showed that young enolase may be converted in vitro to an enzyme form similar to old enolase and suggested that enzyme aging was due to conformational rather than to covalent changes (Sharma et al., 1980). The structural modifications proposed may be due to a slower protein turnover in old organisms leading to a longer "dwell time" of enzymes in the tissues, thus permitting them to undergo partial denaturation. While this hypothesis lacks direct experimental evidence of slowed protein turnover in old mammals, the notion that subtle conformational changes may be responsible for the inactivation of old GPDH is very appealing. GPDH from various sources, and in particular the mammalian muscle enzyme, is an exceptionally labile enzyme. The essential cysteine residue (Cys-149) in the catalytic site is readily oxidized by various reagents, leading to inactivation of the enzyme. This is the origin of the low activity shown by GPDH purified in the absence of EDTA and thiols (Cori et al., 1948). At the first stage, this inactivation is reversible, and incubation of the enzyme with thiols leads to reactivation (Velick, 1955).

Incubation in a nonreducing medium at room temperature, and especially upon partial unfolding by urea, leads to the formation of a disulfide bond with Cys-153 which is close to Cys-149 in the tertiary structure. Subsequent reduction, with thiols, does not restore the enzymatic activity, indicating that formation of the S–S bond leads to an irreversible conformational change in the enzyme (Harris & Waters, 1976).

The fact that all four reactive cysteine residues were found to be in the reduced state in both young and old GPDH does not prove unequivocally that these residues had not been already modified before the isolation of the enzyme (and reduced by the mercaptoethanol during purification). If the reduction in the activity of the old enzyme is indeed due to posttranslational structural modifications, it is very likely to occur via alteration of Cysteine-149, which, in light of the previous discussion, appears to be the most susceptible residue. As a further test of this possibility, the conformational changes (as well as changes in other parameters, like coenzyme binding) induced by the formation and subsequent reduction of the S–S bond ought to be studied under controlled conditions and compared with the effects observed in old GPDH. These studies are now being carried out.

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