

MECHANISM OF AGING OF RAT MUSCLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE STUDIED BY SELECTIVE ENZYME-OXIDATION

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SUMMARY

Controlled oxidation of rat muscle glyceraldehyde-3-phosphate dehydrogenase (GPDH) was carried out in an attempt to simulate age-related effects observed in enzyme samples purified from old animals. A comparative study of the "simulated aged" and of native young and old GPDH forms was done using fluorescence techniques. The present work is based on our previous findings that the locus of the age-related modifications in GPDH is in the nicotinamide-binding site, where the catalytically active Cys-149 residue is located, and that an increase in oxidation potential occurs in old animal tissues which may enable various oxidizing agents to play a significant role in the inactivation of certain enzymes. Thus it has been suggested that the loss of specific activity observed in old GPDH may be due to subtle and irreversible conformational changes caused by reaction of Cys-149 with these agents.

The circularly polarized luminescence (CPL) spectrum emitted by the fluorescent sulfhydryl reagent I-AEDANS covalently bound to GPDH through Cys-149 at the nicotinamide binding site, revealed a significant difference in conformation between these sites in young and old GPDH forms. Large differences were also observed between corresponding spectra when the binding sites were saturated with NAD⁺, reflecting the development of marked conformational changes in both young and old GPDH species upon coenzyme binding.

The oxidizing reagents employed in the current study (hydrogen peroxide, superoxide radical and atmospheric dioxygen) are all expected to be more commonly encountered in the less reducing environment of old animal tissues. All of them, though to a different extent, caused a significant inactivation of the

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enzyme dependent on the initial oxidant concentration. Although the original enzymatic activity could be partially restored by incubation with a reducing agent, the prior oxidation was found to induce some irreversible structural changes as expressed in a decrease in the number of fast reacting SH groups. The extent of irreversible inactivation was a function of both oxidant concentration and the duration of exposure to the oxidant.

The affinity of the oxidized GPDH species (termed "aged") toward coenzyme, as monitored by fluorometric titrations, was markedly lower than that observed for both the native young and old GPDHs. In addition, the CPL spectra of the "aged" enzymes were different from those obtained for both native forms. This indicates that the structural modifications induced by the oxidation reactions tested differ from those present in native old GPDH, although in each case the changes are localized within the nicotinamide binding site.

Key words: Rat muscle; Enzyme aging; Oxidation; Free radicals

INTRODUCTION

Recent studies of aging phenomena at the molecular level have revealed the presence of significant age-dependent modifications in the properties of enzymes isolated from old animals [1—7]. These modifications frequently involve a marked reduction of 40—60% in the catalytic activity as well as changes in a variety of physical and spectroscopic properties of the enzymes involved.

Preparations of glyceraldehyde-3-phosphate dehydrogenase (GPDH) purified from the muscles of young and old rats were found to differ in their catalytic efficiencies [6], in their affinities toward NAD^+ [7], in their heat stabilities and subunit dissociation pattern [6—8], as well as in some spectroscopic properties [9]. Based on the observation that only the affinity of GPDH towards NAD^+ , but not the negative cooperativity of coenzyme binding, was affected by aging, it was suggested that age-dependent modifications in rat muscle GPDH occur in the nicotinamide binding site, where the catalytically active Cys-149 residue is located [7,8]. It is well documented that this residue in GPDH molecules from various sources is highly reactive towards oxidizing and alkylating agents, and that its interactions with such agents lead to inactivation of the dehydrogenase [10].

The origin and nature of the age-related modifications in enzymes have been the subject of considerable speculation and there are at present a number of theories trying to provide a molecular basis for the process of aging [11]. Accumulated evidence show that the age-related changes in proteins are postsynthetic in nature. It has been proposed that these could partially arise due

to a less reducing environment found in old animal tissues and it has indeed been recently shown that the levels of oxidized glutathione and NAD^+ (NADP^+) are increased in old rat muscle at the expense of the reduced forms [12]. The role of reduced glutathione in keeping sulfhydryl groups in their reduced form, thus serving as a general protective agent against oxidation, may thus be hampered at old age allowing oxidizing agents and free radicals to play a significant role in the inactivation of enzymes in old cells.

Free radical reactions are initiated continuously throughout cells by both enzymatic and nonenzymatic mechanisms. Enzymatically-generated free radicals are usually produced as short lived intermediates in enzymatically controlled processes. However, these intermediates occasionally "escape", providing a small background flux of free radicals over the lifetime of the organism [13—16]. Due to the high chemical reactivity of free radicals it could be expected that all components of the body would be constantly subjected to some degree of chemical change in a random manner, somewhat like the effects produced by the free radicals formed by ionizing radiation [17]. It has also been reported that in aged rats superoxide dismutase displays markedly reduced activity, thus enabling enhanced accumulation of superoxide radicals [18].

In order to test whether the oxidation of Cys-149 is involved in the aging of muscle GPDH, aging simulation experiments were conducted by controlled oxidation and subsequent reduction of this amino acid residue of the enzyme. These experiments were performed employing stoichiometric amounts of iodine, an oxidizing agent which preferentially reacts with Cys-149 [19]. The enzyme so treated (termed "aged") indeed displayed a remarkable similarity to enzyme samples purified from old rats, both in its catalytic activity and NAD^+ -binding patterns and in its spectroscopic properties [19]. Based on these findings, a model for the aging process in GPDH was proposed which involves conformational changes in the enzyme, which develop following the oxidation of Cys-149 and which are irreversible upon subsequent reduction. GPDH aging was thus explained as a consequence of the less reducing environment found in cells of old animals. While oxidation of GPDH by iodine resulted in valuable results, this oxidizing agent is obviously of no physiological significance.

In the present study we compare properties of native young and old GPDHs with several preparations of "aged" GPDH produced by controlled oxidation of Cys-149 with hydrogen peroxide, superoxide radical and molecular oxygen, the major oxidizing agents expected to be found in the living cells of old animals. The main question addressed is whether these oxidizing agents could bring about the postsynthetic structural changes leading to loss of activity in old GPDH. In the comparative structural study the technique of circularly polarized luminescence (CPL) spectroscopy was used taking advantage of the extreme sensitivity of this method to subtle conformational changes in biological molecules [20—22]. As a fluorescent probe we used *N*-iodoacetyl-*N'*-(5-sulfo-1-

naphthyl)-ethylenediamine (I-AEDANS), a sulfhydryl reagent that binds covalently to Cys-149 of GPDH, thus probing the nicotinamide binding site, where the structural changes are expected. Since the probe is covalently bound to the enzyme one can eliminate background fluorescence, which otherwise can interfere with CPL spectra, by dialysis or gel filtration.

MATERIALS AND METHODS

Materials

DTNB, catalase, NAD⁺ and D,L-glyceraldehyde-3-phosphate diethyl-acetal, barium salt, were obtained from Sigma. The latter compound was deionized on Dowex 50 W (hydrogen form) and hydrolyzed by heating to form the free aldehyde using the procedure recommended by the supplier. Ammonium sulphate, 2-mercaptoethanol and EDTA were purchased from Fluka. Tetrasodium pyrophosphate and sodium dihydrogen orthophosphate were obtained from B.D.H., England. Acid washed activated charcoal (Norit A), obtained from Sigma, was washed three 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. Cellulose phosphate cation-exchange resins were purchased from Sigma while Sephadex G-25 (fine) was a product of Pharmacia. *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)-ethylene diamine (I-AEDANS) was obtained from Aldrich Chemical Co., Potassium superoxide was a product of Fluka and nitroblue tetrazolium (NBT) and hydrogen peroxide were purchased from Sigma. DMSO and 18-crown-(1,4,7,10,13,16-hexaoxacyclooctadecane) were products of Aldrich Chemical Co.

Purification of glyceraldehyde-3-phosphate dehydrogenase

The enzyme was purified from fresh back and hind leg muscles of young (3–6 months) and old (28–31 months) Wistar–Furth rats [6]. In a typical preparation muscle tissue was taken from 6 rats (100–150 g tissue) of each age group and identical purification steps and conditions were maintained in preparing the enzyme from the two age populations. All the purification steps were performed at 4°C with buffers made in glass-distilled water and containing 2 mM 2-mercaptoethanol. Enzyme preparation was based on the procedure described by Bloch *et al.* [24] for the rabbit muscle enzyme with slight modifications. Thus, extensive dialysis for 8 h was performed instead of elution through a Sephadex G-200 column and cellulose phosphate cation-exchange column was used in place of the CM-52 column. The major modification introduced into the preparation procedure, however, was the replacement of the sodium chloride, used to generate a salt gradient in the ion-exchange chromatography, by sodium phosphate. This modification was introduced based on the finding by Nagradova and Guseva that the activity and the partial dissociation of rat muscle GPDH strongly depend on the anionic composition of the buffer used [25]. The order of

effectiveness of various anions in dissociating the GPDH tetramer conformed to the Hoffmeister series of ions. The phosphate ion, being at the end of this series, could be expected to destabilize GPDH much less than chloride and was thus used in our preparations. Indeed we found that the specific activities of young and old GPDHs eluted from the ion-exchange column were 190–250 units/mg and 90–140 units/mg, respectively, which is significantly higher than was reported previously [6]. The fractions exhibiting specific activity in excess of 20% of the activity at the peak were pooled, the enzyme was saturated by NAD^+ (1 mM) and precipitated in 85% ammonium sulphate solution containing 5 mM 2-mercaptoethanol, 5 mM EDTA (pH 7.3) and stored at 4°C.

Alkylation of GPDH by I-AEDANS

GPDH was collected from its suspension in ammonium sulphate by centrifugation (20 000 *g* for 5 min at 4°C) and the precipitate was dissolved in 50 mM phosphate buffer containing 10 mM pyrophosphate (used as chelating agent instead of EDTA which was found to react with hydrogen peroxide) at pH 7.3 and passed through a Sephadex G-25 fine column to remove both ammonium sulphate and 2-mercaptoethanol. The solution of holoenzyme (20–40 μM) was gently stirred for 20 min at 4°C with 5–6 mg of activated charcoal/mg of protein. The charcoal was then removed by centrifugation. The apoenzyme obtained in this way had a A_{280}/A_{260} ratio of 1.82–2.04, and displayed 3.75–4.2 NAD-binding sites/tetramer, as determined by Racker-band titration [26]. Absorption coefficients for holo-GPDH and apo-GPDH are $1.41 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and $1.23 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$, respectively [6]. The apoenzyme was then reacted with a 10-fold excess of I-AEDANS and the alkylation carried out in the dark as the reagent is light sensitive (although the product of its reaction with sulphhydryl groups is stable). After 10 min at 20°C, the solution was passed through a Sephadex G-25 fine column (1.5 \times 10 cm) pre-equilibrated with phosphate/pyrophosphate buffer, to remove excess reagent. The number of I-AEDANS residues bound per tetramer was computed from the absorbance at 337 nm $\epsilon_{337} = 6000 \text{ M}^{-1} \text{ cm}^{-1}$ for the dye [27] and from the absorbance at 280 nm using $\epsilon_{280} = 1.23 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ for the enzyme.

Steady state fluorescence experiments

The fluorescence spectrum of the $\text{GPDH}:(\text{AEDANS})_4$ complex at 22°C was recorded on a Hitachi-Perkin Elmer MPF 44A spectrofluorometer, equipped with a thermostated cell holder and operated in the ratio-measurement mode. The fluorescence excitation wavelength was 330 nm. To record the excitation spectrum the fluorescence intensity was monitored at the emission maximum (482 nm), as a function of the excitation wavelength.

The determination of the association constants of different $\text{GPDH}:(\text{AEDANS})_4$ forms with NAD^+ was carried out by automated titrations of the

protein (3–6 μM tetrameric enzyme) with concentrated coenzyme solutions (1.0×10^{-2} M) and following the quenching of the fluorescence of the GPDH: (AEDANS)₄ derivatives.

Circular polarization of luminescence measurements

CPL measurements were performed using an instrument built in our laboratory and described elsewhere [28]. In this instrument the intensity of the circularly polarized component of the luminescence light is selectively modulated at a chosen frequency by a photoelastic light modulator, while the unpolarized component of the luminescence is unaffected. The modulated light beam is then translated into a modulated electric current, the AC and DC components of which correspond to the circularly polarized light intensity and the total light intensity, respectively, emitted by the sample. The AC and DC signals are each amplified and monitored, the relation between them yielding, after suitable calibration, the extent of circular polarization of the luminescence light. The light used for excitation of the sample and the luminescence light are monochromated.

The excitation wavelength of I-AEDANS fluorescence in the CPL measurements was 325 nm. The spectral resolution for the excitation beam was 30 nm while the fluorescence was studied using a spectral resolution of 15 nm. The CPL spectra were measured using cells having an optical path of 1 mm to allow the use of high enzyme concentration. The samples were changed several times during measurements in order to avoid artefacts due to protein denaturation. Also, the spectra were taken within 1 h after the labeling with I-AEDANS. The CPL spectra are presented through the emission anisotropy factor g_{em} [20,21]. All the measurements were done at room temperature (22 °C).

Oxidation by hydrogen peroxide

Holo-GPDH was incubated at room temperature with various concentrations of H₂O₂, which were determined by a method described elsewhere [29]. After the desired incubation time (usually 4 h), excess reducing agent (2-mercaptoethanol) was added and 15–30 min later removed by passing the sample through a Sephadex G-25 fine column. The enzyme was then treated with charcoal to obtain the apo-form, which was subsequently alkylated with I-AEDANS. Samples prepared in this way also served in NAD⁺ titration experiments or were used for CPL measurements.

Oxidation by superoxide radical

The superoxide radical was generated by the method described by Valentine and Curtis [30]. KO₂ was ground to a fine powder with a mortar and pestle in a glove bag under a dry nitrogen atmosphere. The powder was then added to dry DMSO in a test tube and 18-crown-6, at a concentration double that wished for O₂⁻, was added. 18-crown-6 is required to solubilize alkali metal ions in

nonpolar solvents. The contents of the tube (approx. 10 ml) were vigorously vortexed for at least 5 min and the solution was then centrifuged for about 1 min at low speed.

The concentration of superoxide, prepared as described above, was estimated by measuring the reduction of nitroblue tetrazolium following the procedure of Beauchamp and Fridovich [31]. KO_2 in DMSO (5 μl) was added slowly to 2.1 ml of 0.1 mM NBT dissolved in 50 mM carbonate buffer and 0.1 mM EDTA (pH 10.2), with rapid stirring and the absorbance at 560 nm was measured. An absorbance change of 0.26 reflects 50 nmol O_2^- .

GPDH was incubated with the desired concentration of previously prepared superoxide (in the presence or absence of catalase) for 1 h at room temperature and pH 8.75. This higher pH ensures that the rate of disproportionation of the superoxide radical is very slow [32]. It has indeed been shown that above pH 5 the rate constant for disproportionation decreases by a factor of 10 for each unit increase in the pH. The oxidized enzyme was then reduced and treated with charcoal before being used for titration or CPL spectra measurements.

Oxidation by atmospheric oxygen

This was achieved by incubating the enzyme at 4°C in well aerated buffer for 1–14 days in the absence of 2-mercaptoethanol. The latter was then added in order to reactivate the enzyme. Activity was determined before and subsequent to the addition of 5 mM 2-mercaptoethanol followed by DTNB assay or labeling with I-AEDANS. Samples of GPDH incubated for 14 days under the same conditions, but in the presence of 10 mM mercaptoethanol, lost less than 10% of their activity, and served as the reference in these experiments.

Determination of sulfhydryl groups (DTNB-assay)

Solutions of apo-GPDH in phosphate/pyrophosphate buffer free of 2-mercaptoethanol were prepared by dialysis. DTNB was added from a 10-mM stock solution to make a final concentration of 100 μM . The numbers of fast and slow reacting sulfhydryl groups were determined from the absorption change at 412 nm after approx. 15 s and 1 h, respectively, by using an $\epsilon_{412} = 13\,600\text{ M}^{-1}\text{ cm}^{-1}$ for 3-carboxy-4-nitrothiophenolate [33]. Buffer solution, devoid of enzyme, containing the same concentration of DTNB served as a blank in the spectrophotometric measurements.

RESULTS

Purification of GPDH from young and old rats

In spite of the well documented sensitivity of GPDH to monovalent anions, and in particular to chloride ions [25], sodium chloride has been routinely used to

generate the salt gradient used in ion-exchange chromatography of the enzyme during purification.

The modification in this part of the purification procedure, as described in the Materials and Methods section, indeed resulted in preparations of GPDH with markedly increased specific activities. Thus, our purified enzymes displayed specific activities as high as 245 and 160 units/mg for young and old species of enzyme, respectively, compared to 150 and 90 units/mg described previously for the same enzyme forms [6].

Enzyme oxidation experiments

Several oxidizing agents likely to be of physiological significance were used in these experiments.

Hydrogen peroxide: This potent oxidant attacks the sulfur containing amino acids cysteine and methionine in proteins, thus quickly inactivating many enzymes [32]. Figure 1 presents the residual activity of GPDH treated with increasing concentrations of H_2O_2 and incubated for 1 h at room temperature. The inactivation under these conditions is complete only when a 2–3-fold excess of oxidant over enzyme subunits is used. In contrast, no inactivation is observed in the presence of catalase showing that H_2O_2 is removed at a rate much faster than its rate of interaction with GPDH. When oxidation by H_2O_2 was carried out for prolonged periods of time we found that the degree of inactivation reached a limiting value (that depended on H_2O_2 /GPDH ratio) after 3–4 h at room temperature. Thus, when 4 mol H_2O_2 /mol GPDH were added and the sample

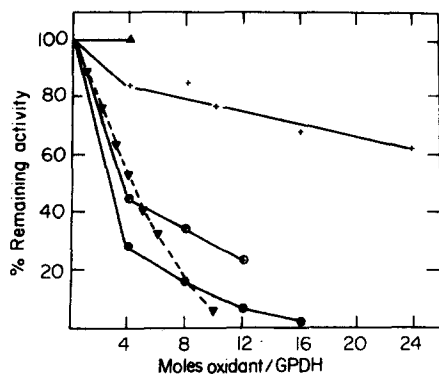


Fig. 1. Inactivation of rat muscle GPDH by various oxidizing agents. Remaining activity after oxidation with: (∇ — ∇), H_2O_2 in 50 mM phosphate and 10 mM pyrophosphate, potassium salts (pH 7.4); (\bullet — \bullet), KO_2 /DMSO in the same buffer but at pH 8.9; (+ — +), same in the presence of catalase; (\circ — \circ), recovery of activity of O_2 -oxidized enzyme after 1 h incubation with 5 mM 2-mercaptoethanol at 4°C; (— \blacktriangle), activity remaining following treatment with 4 equivalents of H_2O_2 in the presence of catalase. All measurements, except where indicated, were performed after 1 h incubation with reagents at room temperature. More details are given in the Materials and Methods section.

incubated for 4 h the activity was reduced to about 40% of the original value (Table I). Addition of excess 2-mercaptoethanol to the oxidized enzyme brought about only a partial restoration of activity to a value similar to that of native old GPDH (see Table I). The H_2O_2 -inactivation was also found to be associated with a decrease in the number of SH groups which rapidly react with DTNB (Table I). These results are in accordance with the ones reported by Little and O'Brien [35].

The results of inactivation experiments carried out using superoxide radical (O_2^-) as the oxidizing agent are also presented in Fig. 1. In order to isolate the effect of superoxide from that of the dismutation product, H_2O_2 , we used an identical concentration of potassium superoxide with and without catalase, which as discussed above is an efficient scavenger of H_2O_2 . As is depicted in Fig. 1 we found that in absence of catalase GPDH was inactivated to a much higher extent than when catalase was present, indicating that the O_2^- by itself is not as efficient an oxidizing agent as H_2O_2 and that GPDH is modified mostly by directly interacting with the latter oxidant.

The inactivation of GPDH by atmospheric oxygen was found to proceed relatively slowly and the enzyme lost 60–70% of its specific activity only after being incubated in air saturated buffer at 4°C for 2 weeks. Upon adding 2-mercaptoethanol, most of the original activity was recovered for both young and old enzyme forms (70–80%). The partial irreversibility of dioxygen oxidation was reflected also in the retention of only two fast reacting SH groups, as

TABLE I

TREATMENT OF RAT MUSCLE GPDH WITH NATURAL OXIDANTS

GPDH form	Oxidizing reagent	Spec. act. ^a (%) ^b	DTNB assay (reactive SH groups)	AEDANS/mol GPDH	CPL $g_{em} \times 10^4$ at 490 nm
Young	None	225 ± 20	4.1 ± 0.2	4.4 ± 0.5	6.7
Old	None	125 ± 35 (55 ± 12)	4.1 ± 0.2	4.2 ± 0.3	4.7
Young	H_2O_2 (4 eq)	(42 ± 8)	1.3 ± 0.2	3.2 ± 0.3	—
Young	H_2O_2 + 2-ME ^c	(58 ± 7)	2.4 ± 0.3	4.2 ± 0.3	3.3
Young	O_2^- (4 eq)	(28 ± 2)	—	—	—
Young	O_2^- + catalase	(80 ± 5)	—	3.3 ± 0.2	3.4
Young	O_2^- + 2-ME	(50 ± 8)	—	5.0 ± 0.4	3.6 ± 0.2
Young or old	O_2 ^d	(34 ± 2)	2.7 ± 0.6	3.0 ± 0.5 ^e	4.3–6.5 ^e
Young or old	O_2 + 2-ME	(75 ± 5)			

^aSpecific activity in units/mg.

^bBracketed values correspond to percent specific activity as compared to the untreated young control.

^cOxidation followed by incubation with 5 mM 2-mercaptoethanol (2-ME).

^dAir saturated buffer, 2 weeks incubation at 4°C.

^eThe specific value obtained within the given range in any experiment depended on the duration of exposure of GPDH solutions to atmospheric oxygen.

revealed by DTNB assay, as well as in the binding of 2—2.5 I-AEDANS/mol tetramer only (Table I). Shorter exposure to O₂ allowed more of the original activity to be recovered and a larger number of fast reacting SH groups to react with DTNB.

Spectroscopic studies

In order to study both the age-related and oxidation induced structural changes in GPDH, which are expected to occur at the nicotinamide binding site of the enzyme, a fluorescent probe, I-AEDANS, which binds covalently to Cys-149 was employed. Alkylation of the apoenzyme, both in young and old enzyme forms, by a 10-fold molar excess of the dye over the active sites, resulted in the binding of 4 mol I-AEDANS/mol tetramer, indicating that only the reactive SH-groups in the active site of the enzyme were labeled by the alkylating reagent. Oxidized enzyme forms were in all cases unable to bind 4 mol probe/ tetramer, as is shown in Table I, due to the loss, upon oxidation, of Cys-149 resulting in the formation of mercaptide oxidation products [36]. Upon reduction the number of AEDANS labeling sites went back to four. In the case of GPDH oxidized by H₂O₂, there was a discrepancy between the number of fast reacting SH groups detected by DTNB assay and the maximal number of AEDANS molecules bound per enzyme. This may be explained in terms of the rapid reaction between 5-mercapto-2-nitrobenzoic acid (MNB), the colored product of the reaction of DTNB with sulfhydryl groups, with sulphenic acid abolishing the absorption at 412 nm [37]. Thus, if previously oxidized GPDH is more susceptible to oxidation than the native enzyme it may become partially reoxidized when excess 2-mercaptoethanol is removed prior to DTNB titration and may thus contain some sulphenic acid that will react with DTNB (but not with I-AEDANS).

The alkylation of apo-GPDH by I-AEDANS is accompanied by a loss of enzymatic activity [22]. Also, GPDH:(AEDANS)₄ binds NAD⁺ with lower affinity and in a non-cooperative manner. The binding of NAD⁺ to derivatized GPDH is accompanied by a significant quenching of AEDANS fluorescence thus providing a tool for examining the differential NAD⁺ binding affinity for different GPDH species. The binding of the coenzyme to GPDH:AEDANS conjugates prepared with young, old and "aged" enzymes, as monitored by steady state fluorescence titration with NAD⁺, is presented in Fig. 2. The enzyme saturation curves evaluated from these fluorescence quenching data are presented in Fig. 3. In calculating these saturation curves two assumptions were made: (a) that the degree of fluorescence quenching, relative to the maximal quenching at infinite NAD⁺ concentration, is proportional to the fraction of enzyme sites occupied by NAD⁺, and (b) that NAD⁺ binding to the GPDH:(AEDANS)₄ conjugate is non-cooperative (i.e. that binding to each of the four GPDH subunits is characterized by only one intrinsic association constant). An iterative least-squares analysis was then performed to calculate the maximal quenching

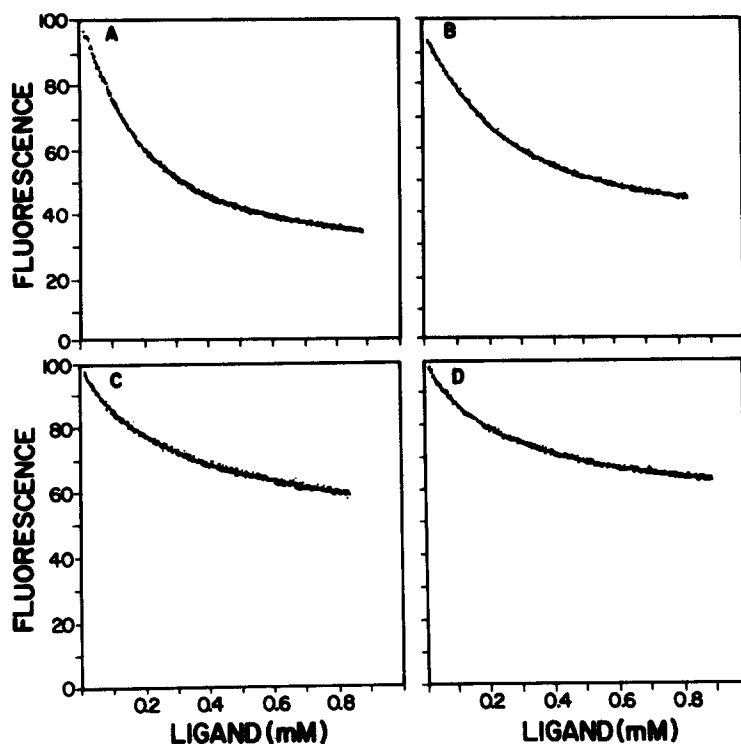


Fig. 2. Volume-corrected fluorometric titration curves of GPDH:(AEDANS)₄ with NAD⁺ at 25°C. Initial tetrameric enzyme concentrations were 3–6 μM, the starting volume was 1.0 ml, and titration was performed by continuously adding NAD⁺ from a 10⁻² M stock solution containing GPDH at the same concentration as in the sample being titrated. Both ligand and protein were in 50 mM phosphate, 10 mM pyrophosphate, potassium salts (pH 7.4). The titration curves shown are those of (A), young form; (B), old form; (C), young form treated with 4 equivalents H₂O₂/mol enzyme; (D), oxidized as in (C) but subsequently reduced with 5 mM of 2-mercaptoethanol. For details see Materials and Methods. The experimental data were best fitted with the calculated curves (—) assuming non-interacting identical binding sites as described in the text. The maximal degree of fluorescence quenching (i.e. when all sites are saturated with NAD⁺) was derived in each case, the values being: (A), 82.2%; (B), 71.8%; (C), 52.9%; (D), 49.9%.

and the association constant for each of the GPDH forms studied. The excellent fits between the experimental data and the calculated saturation curves (Fig. 3) provide strong support for the validity of the assumptions described above. The values for the association constants calculated from the data in Figs. 2 and 3 were: $5.0 \times 10^3 \text{ M}^{-1}$, $4.4 \times 10^3 \text{ M}^{-1}$, $3.6 \times 10^3 \text{ M}^{-1}$, $4.7 \times 10^3 \text{ M}^{-1}$ for young, old, hydrogen peroxide oxidized and H₂O₂ oxidized-2-mercaptoethanol-reduced enzyme forms, respectively.

The CPL spectra of GPDH:(AEDANS)₄ complexes of different GPDH forms are presented in Fig. 4. Each spectrum is an average of several repeated measurements. A comparison of the CPL spectra of native young and native old

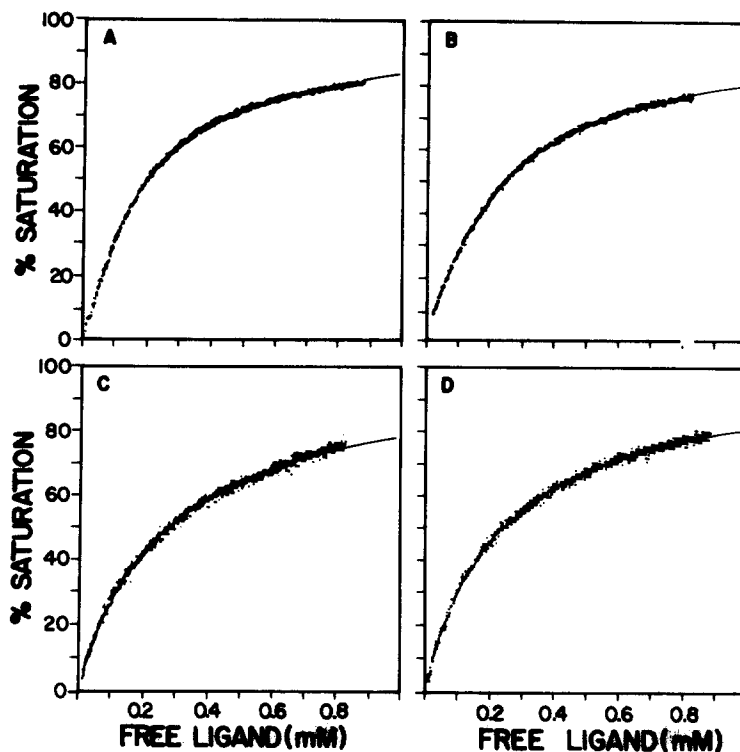


Fig. 3. Binding of NAD^+ to the $\text{GPDH}:(\text{AEDANS})_4$ forms described in Fig. 2. The saturation curves (.....) were evaluated for each of the four conjugates from the corresponding fluorescence quenching data shown in Fig. 2. The calculated binding curves (——) depict the best fit to the experimental data assuming binding to non-interacting, identical binding sites. The association constants evaluated from the data are given in the text.

$\text{GPDH}:(\text{AEDANS})_4$ complexes both in NAD^+ -free and NAD^+ -saturated solutions, shows that pronounced structural differences exist between the nicotinamide binding sites of the two enzyme forms. This difference was not detected by circular dichroism (data not shown). In the presence of an excess of NAD^+ , the spectra of both species change markedly, reflecting conformational changes which occur in the vicinity of the probe upon binding the coenzyme. It is noteworthy to mention that the CPL spectrum of AEDANS bound to rat muscle GPDH differs largely from the corresponding spectrum of the same probe bound to rabbit muscle GPDH [22], demonstrating the sensitivity of the CPL to small structural differences between the nicotinamide sites of these two homologous enzymes.

Analysis of the CPL spectra of AEDANS bound to GPDH previously oxidized with hydrogen peroxide or with superoxide radical, shows that both oxidizing agents cause structural changes at the nicotinamide binding site. The extent of the modification depends on the concentration of the oxidant. A comparison between

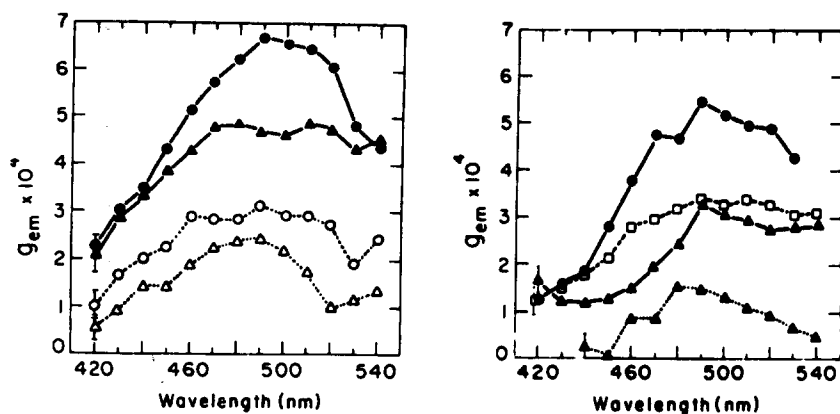
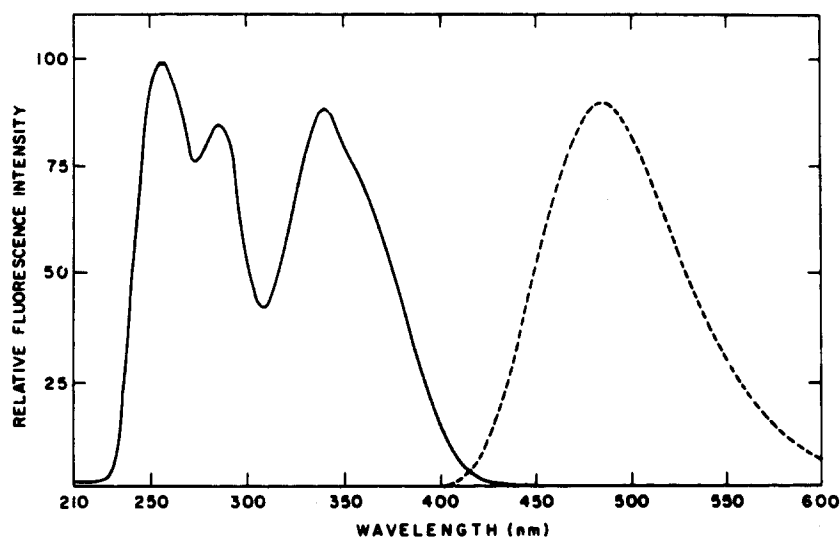


Fig. 4. Fluorescence and CPL spectra of GPDH:(AEDANS)₄ conjugates prepared as described in the Materials and Methods section. Upper part, excitation (—) and emission (-----) spectra. The excitation spectrum was monitored for the emission at 482 nm while the emission spectrum was measured upon excitation at 337 nm. Lower part left, CPL spectra of GPDH:(AEDANS)₄ derivatives of young (●—●) and old (▲—▲) enzyme forms and the spectra of young (○—○) and old (△—△) enzymes in presence of 1 mM NAD⁺. Lower part right, CPL spectra of GPDH:(AEDANS)₄ derivatives of "aged" enzyme forms obtained upon oxidation of young GPDH with 2 mol (●—●) or 4 mol (▲—▲) of H₂O₂; 4 mol of O₂⁻ (□—□), and with 4 mol of H₂O₂ followed by addition of 1 mM NAD⁺ (△—△). The enzyme concentration was 5–10 μM and the buffer used was 50 mM phosphate, 10 mM pyrophosphate, potassium salts (pH 7.4). The excitation wavelength was 315 nm with a band width of 30 nm. The CPL spectra were obtained with a 15 nm resolution and with the accuracy indicated by the error bars.

the spectra of the "aged" and native old enzyme, however, reveals significant differences (Fig. 4) between the two enzyme forms. It may therefore be concluded from our data that the structural changes induced upon controlled oxidation of GPDH by the reagents employed do not mimic exactly the conformation of the native old enzyme. However, when GPDH was oxidized with only 2 mol H_2O_2 /mol enzyme, the resulting CPL spectrum exhibited more resemblance to the spectrum of the old enzyme. Interestingly, the CPL spectra of GPDH:AEDANS conjugates prepared with enzyme previously oxidized with atmospheric oxygen do not differ much from the one obtained using the native form (data not shown).

DISCUSSION

The main goal of the present study was to examine the possibility that the partial loss of specific activity in GPDH samples isolated from old animals could be attributed to oxidizing-agents mediated alterations of the enzyme's active site. Several previous observations have provided evidence that this might be the case:

First, age-related modifications in rat muscle GPDH, as detected by CPL studies using the fluorescence of bound NADH were clearly shown to reside at the nicotinamide binding site [9]. This is the site where the catalytically active Cys-149 is located, a residue known to rapidly react with oxidizing and alkylating agents leading to inactivation of the dehydrogenase [10]. Second, it has been demonstrated that by the controlled oxidation of Cys-149 in young GPDH with iodine it is possible to simulate the aging effects [19]. Artificially aged GPDH thus produced showed remarkable similarity to enzyme samples purified from old rats, both in its activity and in NAD^+ binding patterns under various conditions. Third, the study of the redox status of several biological components which undergo oxidation-reduction reactions, like glutathione, NAD^+ and NADP^+ , has shown that the environment in old rat muscle cells is significantly less reducing than that found in young animals [42]. Moreover, it has been reported that the specific activity of superoxide dismutase in old rats is greatly diminished [18], leading to enhanced accumulation of superoxide radical which in aqueous solution can serve as a one electron oxidant if a proton is available to neutralize the negative charge transferred from the substrate to O_2^- . However, these reactions must effectively compete with the pH-dependent, very rapid dismutation reaction [33,39] which is known to be catalyzed by transition metal ions. O_2^- produced by myoglobin or by an oxidase, might therefore have a sufficiently long lifetime to oxidize nearby proteins before encountering a second superoxide molecule. This, in turn, could affect catalase, which was found to be inhibited by a flux of superoxide generated by the aerobic xanthine/xanthine oxidase reaction [38]. It can therefore be

assumed that enzymes with oxidation-sensitive sulfhydryl groups, like GPDH, may be affected by the exposure to various oxidizing agents likely to accumulate in the more oxidizing environment of old animal tissues.

Here we present comparative spectroscopic and biochemical analysis of native young and old, as well as "aged" rat muscle GPDH. "Aged" forms of the enzyme were obtained by controlled oxidation with hydrogen peroxide, superoxide radical and molecular oxygen. A fluorescent sulfhydryl reagent, I-AEDANS, which covalently and specifically binds to Cys-149 at the nicotinamide binding site, enabled us to monitor, using CPL, subtle conformational changes around the dyes binding domain.

The results presented confirm our previous finding that the major site of age-related structural modifications in GPDH, leading to the markedly lower specific activity of enzyme purified from old rats, is the nicotinamide binding site. Thus, the CPL spectra of bound AEDANS clearly reveal a significant difference in conformation of this site between young and old GPDH forms. Also, large changes in the CPL spectra were found to occur upon coenzyme binding to GPDH:(AEDANS)₄ conjugates reflecting the marked conformational changes that occur upon NAD⁺ binding. In addition, the steady state fluorescence titrations with the coenzyme showed that the young and old forms of the enzyme exhibit different binding affinities toward NAD⁺.

The controlled and partially reversible oxidation of native young GPDH with stoichiometric amounts of H₂O₂ or superoxide radicals produced species of the enzyme with a reagent-dependent decrease in specific activity. In neither case could the activity of the enzyme be fully restored upon the addition of a reducing agent, reflecting some irreversible structural changes induced by oxidation. The CPL spectra of treated enzyme samples showed pronounced conformational changes at the NAD⁺ binding site in GPDH thus "aged". However, in both cases, the observed spectroscopic and biochemical characteristics did not match those found in the native old enzyme. Moreover, significant differences were observed between the two oxidized forms, indicating that the mechanism of superoxide inactivation (presumably milder) may be different from the one resulting from oxidation by hydrogen peroxide. This may be due to possible intervention of radical reactions produced by O₂⁻. On the other hand, oxidation with atmospheric oxygen was found to proceed relatively slowly, resulting in significant loss of original activity only after incubation for many days. Although a large fraction of activity could be recovered upon addition of 2-mercaptoethanol, the enzyme revealed 1—2 missing fast reacting SH groups, potentially oxidized to sulfonic acid which could not be reduced under the conditions employed [36]. As may be expected, the structural modifications induced by dioxygen were found to be different from those caused by H₂O₂ and O₂⁻. The CPL spectrum of AEDANS conjugated with O₂-oxidized GPDH was

similar to the spectrum of the dye bound to native young GPDH, suggesting that the formation of a sulfonic acid derivative of Cys-149 did not cause much conformational change in the remaining unoxidized subunits of the enzyme.

Finally, a comparison of our present experiments with previous results obtained by oxidation with iodine leads to the conclusion that H_2O_2 and O_2^- induce modifications in GPDH which are different from those induced by iodine. In contrast to I_2 and to ICI the reagents employed here may also produce radical chain reactions. Fenton-like reactions may produce the hydroxyl radical ($\text{HO}\cdot$) which is a more potent oxidant than either H_2O_2 or O_2^- . In this way, the reaction initiated by H_2O_2 , O_2^- or O_2 , may, on propagation, lead to the accumulation of different oxidants which can in turn cause additional damage to the protein environment.

From the experiments described in the present study it is apparent that enzymes such as GPDH are susceptible to mild oxidizing agents to a significant extent even when exposed for a relatively short time. The effect of a long-term low dosage exposure to such oxidants is still unknown. There is a considerable interest concerning superoxide-mediated oxygen toxicity and the experimental evidence for it [39—41]. The present work provides strong support for the existence of such damage. Moreover, it is clear that even after reduction of the oxidized enzyme forms some structural and functional modifications persist which, though not identical to the modifications in the native old enzyme, support the view that GPDH aging is triggered by enzyme oxidation.

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