Age-Related Effects in Coenzyme Binding Patterns of Rat Muscle Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: The binding of NAD^+ and of its fluorescent analogue, nicotinamide $1,N^6$-ethenoadenine dinucleotide, to glyceraldehyde-3-phosphate dehydrogenase purified from the muscles of young and old rats was studied in detail. Binding of the natural coenzyme was followed both by spectrophotometric titration of the extrinsic absorption band of the enzyme–NAD^+ complex and from the degree of quenching of fluorescence of the protein. Binding of the coenzyme analogue was monitored by using the large enhancement in its fluorescence upon forming the complex with the enzyme. Both dinucleotides showed strong negative cooperativity in binding to the enzyme, similar to that displayed in their association with the rabbit muscle enzyme. The enzyme purified from old rats displayed a markedly reduced affinity toward the two dinucleotides, compared with the enzyme isolated from young animals. The various dissociation constants of both dinucleotides from the enzyme from young rats were remarkably similar to the corresponding constants in the rabbit muscle enzyme. The degree of negative cooperativity (i.e., the ratio between the dissociation constants from high- and low-affinity binding sites) in the "young" and "old" enzyme forms was not very different. It was concluded from these results that while modifications in the subunits take place upon aging, the intersubunit interaction is not significantly affected. Increasing concentrations of ATP were found to cause a gradual decrease in the negative cooperativity of NAD^+ binding, which completely disappeared in the presence of 5 mM ATP. The observation that all four binding sites of the old enzyme display the same affinity toward NAD^+ when the negative cooperativity is removed excludes the possibility that this enzyme form is a mixture of native and inactive species. The different dissociation constants of NAD^+ from young and old enzyme forms in the presence of 5 mM ATP also demonstrate the occurrence of age-related modifications in the structure of the individual subunits.

The presence of age-dependent modifications in the properties of enzymes has been demonstrated and investigated in an ever increasing number of studies. Several recent review articles were devoted to various aspects of this phenomenon (Rothstein, 1975, 1977; Dreyfus et al., 1978; Klefenz & Zuckerman, 1978). The most common modification in "old"[1] enzymes is the partial loss of catalytic activity which was found to range between 40 and 60% in most cases. Other differences between young and old enzymes which were observed include heat inactivation patterns (Gershon & Gershon, 1970; Reiss & Rothstein, 1974; Reiss & Gershon, 1976a; Reznick & Gershon, 1977), reactivity of sulfhydryl groups (Goldstein & Khilko, 1969), K_m values (Reiss & Rothstein, 1975; Sharma et al., 1976; Orlovska et al., 1980), and some optical spectroscopic properties (Sharma & Rothstein, 1978; Demchenko & Orlovska, 1980). All these differences between young and old enzyme molecules, though significant, were not very pronounced and in most cases were even quite small.

While the origin of the age-dependent phenomena has not been identified, they may result from changes in primary structure, due to biosynthetic error or to somatic mutations (Holliday, 1975), or, alternatively, be the result of post-translational modifications (Dreyfus et al., 1978). In the latter case, the modifications are thought to accumulate in enzymes of old animals due to the slower turnover rate of proteins in the old tissues, an assumption which still lacks direct experimental support.

In the preceding paper (Gafni, 1981), we compared some properties of glyceraldehyde-3-phosphate dehydrogenase (GPDH)[2] purified from muscles of young and old rats. The two enzyme forms displayed identical properties during chromatography, had the same sedimentation coefficients in the ultracentrifuge, revealed identical amino acid composition, and displayed the same number of reactive sulfhydryl groups. Significant differences were found however between the specific activities of the two enzyme forms, indicating that subtle structural modifications do indeed exist between them.

It is well established that the binding of NAD^+ and of its fluorescent analogue, ϵNAD^+, to rabbit muscle GPDH shows strong negative cooperativity (Conway & Koshland, 1968; Schlessinger & Levitzki, 1974; Bell & Dalziel, 1975). This heterogeneity in binding indicates either that the four subunits of the enzyme are not functionally identical (despite the chemical identity of their polypeptide chains) or that sequential conformational changes take place in the enzyme upon coenzyme binding. The dramatic effects that these structural modifications have on the binding of coenzyme reveal the high sensitivity of the binding process to subtle changes in the structure of subunits in the tetrameric enzyme. Coenzyme binding patterns may therefore be expected to be very sensitive also to the modifications introduced into the enzyme's structure by age.

In the present study, the binding of both NAD^+ and the fluorescent ϵNAD^+ to young and old rat muscle GPDH was followed in detail. Dramatic differences between the affinities of the two enzyme forms toward the dinucleotides were indeed found. These differences may be exploited for the detailed investigation of the age-related changes in the GPDH molecule.

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[1] "Old" and "young" enzymes are the enzyme forms purified from old and young animals, 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; ϵNAD^+, nicotinamide $1,N^6$-ethenoadenine dinucleotide.

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Materials and Methods

Materials

ATP, NAD^+, and ϵNAD^+ were purchased from Sigma. The latter compound was further purified on a DEAE-cellulose column to remove a small amount of a fluorescent contaminant, as described by Schlessinger & Levitzki (1974). DL-Glyceraldehyde-3-phosphate diethylacetal, barium salt, from Sigma, was hydrolyzed to the free aldehyde according to the procedure recommended by the manufacturer.

Enzyme. Rat muscle glyceraldehyde-3-phosphate dehydrogenase was purified from back and hind legs muscles of young (6 months) and old (28 months) animals as described in the preceding paper (Gafni, 1981). The apoenzyme was prepared by removal of the tightly bound coenzyme with charcoal (Gafni, 1981). It had an A_{280}/A_{260} ratio of 1.85–1.95, possessed 4–4.2 rapidly reacting (within 15 s) sulfhydryl groups as determined by DTNB, and displayed 3.7–4.2 NAD^+ binding sites per tetramer as determined by Racker band titration (Racker & Krimsky, 1952). Samples of apoenzyme were used shortly after preparation due to their lability.

Unless specified otherwise, all solutions used in the present study were made in 50 mM Hepes and 10 mM EDTA buffer, pH 7.2, containing 1 mM 2-mercaptoethanol.

Methods

Enzyme concentrations were determined spectrophotometrically by using $A^{0.1\%}_{280nm} = 0.85$ mg^{-1} cm^2 for the apoenzyme and $A^{0.1\%}_{280nm} = 0.97$ mg^{-1} cm^2 for the holoenzyme (Gafni, 1981). Concentrations of nucleotides were determined spectrophotometrically by using extinction coefficients of 18 000 M^{-1} cm^{-1} for NAD^+ and 15 400 M^{-1} cm^{-1} for ATP at 260 nm (Beaucamp et al., 1974) and of 10 200 M^{-1} cm^{-1} at 266 nm for ϵNAD^+ (Barrio et al., 1972). Assays of enzymatic activity were performed as described by Bloch et al. (1971). Samples of young GPDH displayed specific activities in the range of 140–160 units/mg while the old enzyme form showed a specific activity of 80–95 units/mg.

Spectrophotometric Titrations. An extrinsic absorption band centered at 350–360 nm (the Racker band) (Racker & Krimsky, 1952) is formed upon binding of NAD^+ to apo-GPDH. Titration of this band served to follow coenzyme binding to the rat muscle enzyme and was performed by adding increments of a solution of the coenzyme to a solution of the apoenzyme and recording the absorption at 350 nm with a Zeiss model PMQII spectrophotometer. A sample of Hepes–EDTA buffer, to which the same concentration of NAD^+ was added, served as a reference. The titrations were done at a temperature of 22 °C.

Fluorometric Studies. These were carried out by using a Perkin Elmer MPF-3 fluorometer operating in the ratio mode. Artifacts due to "inner filter effects" were avoided, when necessary, by the use of a sample cell of short (ca. 2.5-mm) optical path. The stock solution of dinucleotide used in the fluorometric titrations contained apo-GPDH at the same concentration as that in the titrated solution, thus avoiding dilution of enzyme in the latter solution. All the fluorescence measurements were done at 22 °C.

Results

NAD^+ Binding Studies. The binding of coenzyme to rat muscle GPDH was studied both by spectrophotometric titrations of the Racker band and by following the quenching of enzyme fluorescence which accompanies this binding. The results of spectrophotometric titrations of young and old apo-GPDH with NAD^+, monitored at 350 nm, are shown in Figure 1. The increase in absorbance was found to be proportional to NAD^+ concentration up to coenzyme/enzyme ratios of 2.3 and 1.6 for young and old forms, respectively. Below these concentrations, all the NAD^+ added thus binds to the enzyme under the conditions used in the titrations. The dissociation constants of NAD^+ from the two first binding sites can therefore not be evaluated from these titrations. The molar extinction coefficient of the GPDH–NAD^+ complex at 350 nm was calculated from the initial slope and was found to be 910 M^{-1} cm^{-1} for both young and old enzyme forms. With the assumption that identical extinction coefficients characterize the binding of NAD^+ to all four sites of the tetrameric enzyme, the concentration of bound NAD^+ may be evaluated at each point along the titration. That this assumption is valid is indicated by the observation that the limiting values of A_{350}, reached at saturating concentrations of NAD^+, are exactly as predicted on the basis of this (single) extinction coefficient and the concentration of binding sites.

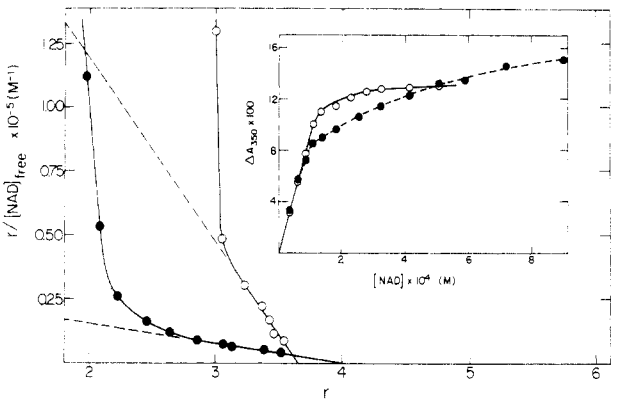

FIGURE 1: Binding of the third and fourth NAD^+ molecules to young (○) and old (●) rat muscle GPDH. The binding was followed spectrophotometrically by monitoring the increase in absorption at 350 nm as shown in the insert. The concentrations of apoenzymes were 4.0×10^{-5} M for young GPDH and 4.6×10^{-5} M for the old form. The measurements were done at 22 °C. The Scatchard plots were constructed from the titration curves as described in the text, and the dissociation constants obtained are summarized in Table I.

The binding pattern of NAD^+ to the two "low-affinity" binding sites of the young and old enzyme forms was evaluated from the spectrophotometric titrations and is presented in Figure 1 in the form of Scatchard plots. Strong negative cooperativity is evident in the binding of the coenzyme to both forms of the enzyme. The intrinsic dissociation constants of the fourth NAD^+ molecule, calculated from the limiting slopes of the Scatchard plots, were found to be 14 μM for young GPDH and 130 μM for the old form. The dissociation constant of the third NAD^+ molecule cannot be evaluated directly from the slope of the Scatchard Plot, which, in the range of $2 \leq r \leq 3$, depends on all four dissociation constants in a complex way (Dahlquist, 1978). The values of K_3 for young and old GPDH were therefore evaluated from the Adair equation for a tetramer by assuming that $K_1, K_2 \ll K_3, K_4$ as described by Henis & Levitzki (1977). The assumption made as to the relative values of the dissociation constants is justified in view of the, independently determined, values of K_1 and K_2 (see Table I). The values of K_3 thus obtained are presented in Table I, which summarizes the dissociation constants of NAD^+ and ϵNAD^+ found in the present work.

Evaluation of the dissociation constants of the first two NAD^+ molecules from the enzyme cannot be made from the spectrophotometric titrations since at the concentrations needed to produce significant absorbancies at 350 nm the binding is stoichiometric. Coenzyme binding to these first sites was therefore followed with fluorescence quenching measurements.

Table I: Intrinsic Dissociation Constants of NAD^+ and ϵNAD^+ from Rat and Rabbit Muscle GPDH

	dissociation constant of NAD^+ (μM)			dissociation constants of ϵNAD^+ (μM)		
	rat muscle GPDH		rabbit muscle	rat muscle GPDH		rabbit muscle
	young	old	GPDH[a]	young	old	GPDH[b]
K_1	ND[c]	ND	0.01	2.3	7.8	1.5
K_2	0.16	3	0.09	2.3[d]	8.0[d]	6.3
K_3	2	140	4	20	70	13-35
K_4	14	130	36	24	74	15-35

[a] Taken from Bell & Dalziel (1975). [b] Taken from Henis (1978), Luisi et al. (1975), and Schlessinger & Levitzki (1974). [c] Not determined. [d] Approximate values calculated from the extrapolated slope of the Scatchard plot to $r = 2$, assuming $K_2 \gg K_3$.

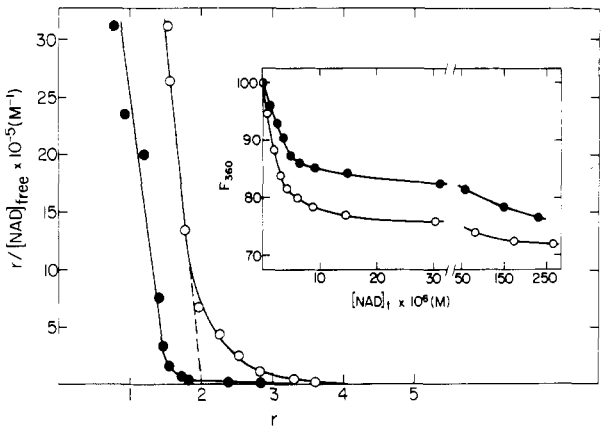

FIGURE 2: NAD^+ binding to young and old rat muscle GPDH studied by fluorescence quenching. The insert describes the fluorescence intensity at 360 nm (excited at 296 nm) of a 1.66×10^{-6} M solution of young apo-GPDH (○) and of a 3.30×10^{-6} M solution of old enzyme (●) as a function of the total concentration of added NAD^+. The Scatchard plots for the binding of coenzyme to the two enzyme forms were constructed from the titration data as described in the text. The dissociation constants evaluated from these Scatchard plots are given in Table I.

The high sensitivity of fluorometric determinations allows the use of significantly smaller enzyme concentrations than those needed for absorption measurements. We found that binding of the coenzyme leads to a significant quenching of the intrinsic enzyme fluorescence, accompanied by a slight shift (ca. 2 nm) of the emission to longer wavelengths. When a 1.8×10^{-5} M solution of apoenzyme was titrated with NAD^+, the fluorescence intensity at 360 nm (F_{360}) decreased linearly until a coenzyme to enzyme ratio of 2 was reached. The ratio between F_{360} of the GPDH–$(NAD)_1$ complex and that of the apoenzyme was 0.89 for young and 0.90 for old GPDH.

The binding of the last two coenzyme molecules to GPDH was found to induce much smaller quenching of the enzyme fluorescence (about half the effect caused by the first two NAD^+ molecules). Moreover, the dependence of F_{360} on the concentration of bound NAD^+ (based on spectrophotometric determination as described before) deviated from linearity.

The enzyme concentrations employed in the fluorometric titrations could be made small enough to allow the determination of K_2. The results of such titrations are shown in Figure 2. The degree of saturation of the binding sites by NAD^+ was evaluated from the amount of quenching, and the binding data were used to produce the Scatchard plots shown in the figure. The values of K_4 evaluated from the limiting slopes of these plots for the young and old enzymes are 20 and 100 μM, respectively, and agree reasonably well with the values derived from the spectrophotometric titrations. However, due

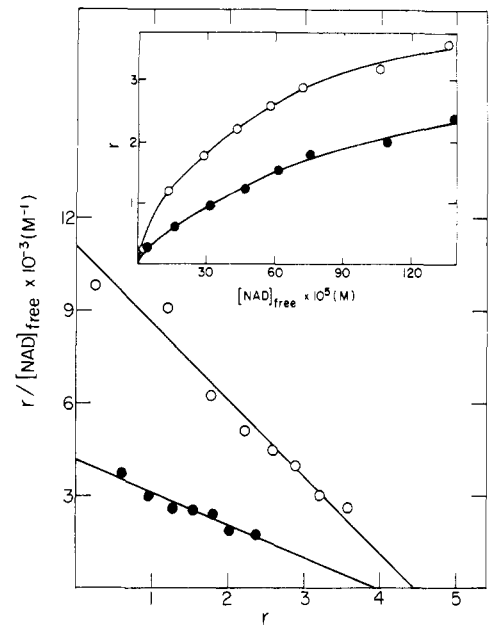

FIGURE 3: NAD^+ binding to apo-GPDH in the presence of 5 mM ATP: (○) 3×10^{-5} M young GPDH; (●) 4.3×10^{-5} M old GPDH. The insert describes the dependence of the degree of saturation of the enzyme binding sites by coenzyme (r expresses moles of NAD^+ bound per tetramer) on the concentration of free NAD^+. The data were obtained from a spectrophotometric titration similar to that described in Figure 1 and were also used to construct the Scatchard plots shown in the figure. The intrinsic dissociation constants of NAD^+ from GPDH in the presence of 5 mM ATP are 400 and 940 μM for young and old enzyme forms, respectively.

to the nonlinear dependence of the quenching on the degree of saturation of the last two binding sites, as mentioned above, the values adopted for K_3 and K_4 were those obtained in the spectrophotometric titration. The values of K_2 for both young and old enzyme forms, as calculated from the Scatchard plots of Figure 2, are summarized in Table I.

Henis (1978) found that ATP binds noncooperatively to rabbit muscle GPDH and, moreover, that in the presence of saturating concentrations of this nucleotide the negative cooperativity of NAD^+ binding is removed. A similar behavior is observed for the rat muscle enzyme. Figure 3 presents spectrophotometric titrations of young and old GPDH with NAD^+ in the presence of 5 mM ATP. The Scatchard plots evaluated from the data are linear, indicating a single intrinsic dissociation constant from all four subunits of the enzyme. Moreover, the linear Scatchard plots exclude the possibility of heterogeneity in the old enzyme preparation, i.e., that this enzyme form is a mixture of active and denatured protein, since in this case biphasic Scatchard plots are expected. This observation is of great importance and will be further dealt with under Discussion. The dissociation constants from young and old enzyme forms obtained from the data of Figure 3 are 400 and 940 μM, respectively. The difference between the affinities of the two enzyme forms toward NAD^+ is thus markedly reduced in the presence of 5 mM ATP.

The dependence of the degree of negative cooperativity in NAD^+ binding on ATP concentration is shown in Figure 4, which presents Hill plots of coenzyme binding to young and old GPDH forms in the presence of several ATP concentrations. The Hill coefficients, as derived from the slopes of these plots at half-saturation by NAD^+, are summarized in Table II. It is clear from this table that the negative cooperativity in coenzyme binding is reduced with increasing ATP concentrations and indeed disappears in the presence of 5 mM ATP.

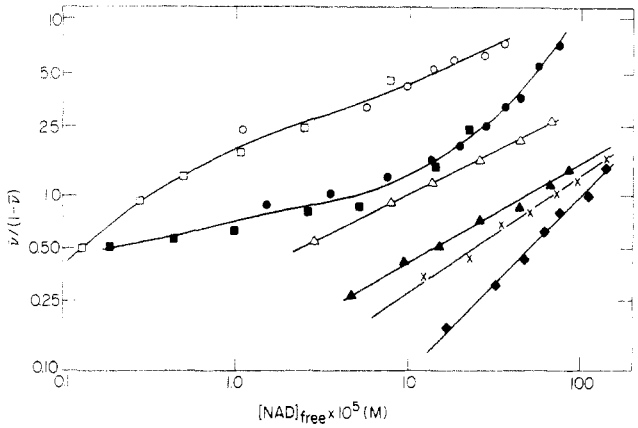

FIGURE 4: Hill plots of NAD^+ binding to rat muscle GPDH in the presence of several concentrations of ATP. The binding data were obtained from spectrophotometric titrations as described in Figure 1. Open symbols represent young enzyme while closed symbols represent old enzyme. The following ATP concentrations were used: 1 mM (△); 2.5 mM (×, the results are for old enzyme); 5 mM (♦). Hill plots of the binding in the absence of ATP are also shown, based on spectrophotometric (○) and fluorescence quenching (□) titrations. $\bar{\nu}$ is the fraction of occupied binding sites.

Table II: Hill Coefficients for the Binding of NAD^+ to Young and Old Rat Muscle GPDH in the Presence of ATP[a]

ATP concn (mM)	$\eta_H(NAD^+)$	
	young GPDH	old GPDH
0	0.56	0.36
1	0.55	0.57
2.5	0.94	0.70
5.0	0.98	1.00

[a] Calculated from the slopes of the Hill plots (Figure 4) at half-saturation of the enzyme by NAD^+.

ϵNAD^+ Binding Studies. Binding of ϵNAD^+ to dehydrogenases is accompanied by an increase in the intensity of its fluorescence, due to unfolding of the stacked conformation of the dinucleotide (Luisi et al., 1975; Gafni, 1977). Such a large enhancement in the fluorescence was also observed upon binding of ϵNAD^+ to rat muscle GPDH. When excited at 330 nm, the fluorescence at 400 nm increased 7.4- and 7.3-fold, respectively, for young and old enzyme forms. A slight blue shift (ca. 5 nm) in the emission wavelength of bound coenzyme analogue was also observed.

Fluorometric titrations of young and old GPDH by ϵNAD^+, monitored at 400 nm, are shown in Figure 5 and compared with the fluorescence of the free dinucleotide. Reciprocal plots of the titration data (i.e., plots of $1/\Delta F_{400}$ vs. $1/[\epsilon NAD^+]$, where ΔF_{400} is the amount of fluorescence enhancement observed at a given ϵNAD^+ concentration) when extrapolated to zero values of $1/[\epsilon NAD^+]$ yielded the maximal emission enhancement to be expected upon complete saturation of the enzyme. These values were evaluated for the two enzyme forms and were identical with the ones expected from the fluorescence enhancement factors given above. We therefore conclude that the same increase in fluorescence accompanies ϵNAD^+ binding to all four subunits of the enzyme. ΔF_{400} may therefore serve to follow the binding of the coenzyme analogue. Figures 5 and 6 show Scatchard plots of the fluorescence titration data describing ϵNAD^+ binding to young and old GPDH. Both plots are clearly biphasic, revealing a reduction in the affinity of the enzyme toward the coenzyme analogue above half-saturation. The negative cooperativity displayed in the bindng of ϵNAD^+ is, however, significantly smaller than that found in the binding of the natural coenzyme as evidenced

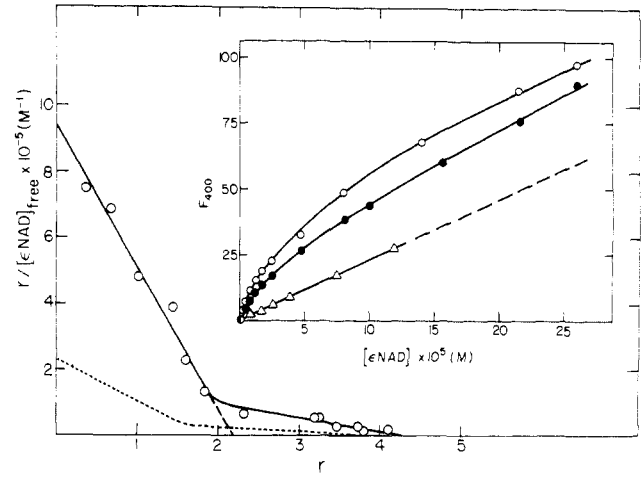

FIGURE 5: Binding of ϵNAD^+ to rat muscle apo-GPDH. The binding was followed by monitoring the fluorescence intensity of the coenzyme analogue at 400 nm, as shown in the insert. The excitation wavelength was 330 nm, and the spectral resolution in both excitation and emission was 5 nm. The solutions studied contained (○) 5.5×10^{-6} M young enzyme, (●) 5.8×10^{-6} M old enzyme, and (△) free ϵNAD^+. The titration data were used to construct the Scatchard plots as described in the text. The Scatchard plot of ϵNAD^+ binding to the old enzyme is only indicated in this figure (broken line) due to the low affinity of this enzyme form toward the coenzyme analogue. This plot is shown, on an extended scale, in Figure 6.

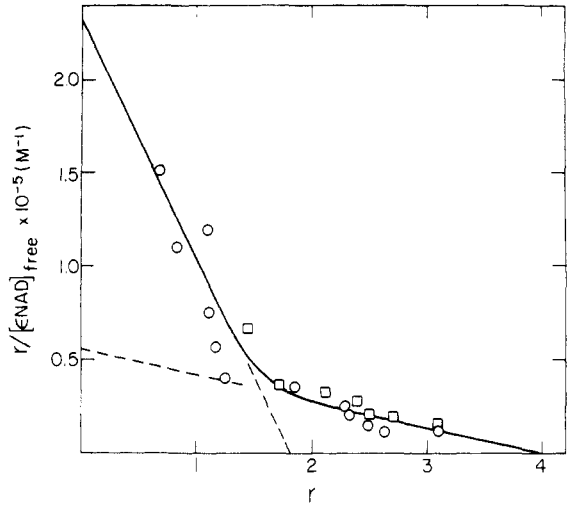

FIGURE 6: Scatchard plot describing the binding of ϵNAD^+ to old rat muscle apo-GPDH. (○) Data taken from the fluorometric titration described in Figure 5; (□) data taken from a similar titration in which a concentration of 8.2×10^{-6} M apoenzyme was used. The dissociation constants evaluated from this plot are summarized in Table I.

by the smaller ratio between the dissociation constants from the high- and low-affinity sites. Also, the difference between the dissociation constants of ϵNAD^+ from a given site in young and old GPDH is significantly smaller than the corresponding difference observed for NAD^+. The consequences of these observations will be discussed below.

Discussion

The binding of NAD^+ to rat muscle GPDH is characterized by strong negative cooperativity, similar to the one observed in the binding of the coenzyme to the rabbit muscle enzyme. The dissociation constants of NAD^+ from young GPDH, as found in the present study, are indeed quite similar to the values reported for rabbit muscle GPDH by Bell & Dalziel (1975). A similar relation exists between the binding pattern of ϵNAD^+ to GPDH from the two mammalian sources as may

be seen in Table I. Both young rat and rabbit muscle enzymes reveal two classes of binding sites, each with its characteristic affinity toward the coenzyme analogue, and, moreover, the dissociation constants from the two enzymes are remarkably similar. This finding is of great importance since the binding of dinucleotides to rabbit muscle GPDH has been studied extensively, yielding a deep insight into the enzymes mechanism of action and control as well as into its molecular structure and subunit interactions (Conway & Koshland, 1968; MacQuarrie & Bernhard, 1971; Schlessinger & Levitzki, 1974 Gafni, 1977; Henis & Levitzki, 1977; Henis et al., 1979). The similarity between the binding patterns of both NAD^+ and ϵNAD^+ to rat and rabbit GPDHs indicates that a large degree of structural similarity exists between these two enzymes. It may be reasonably assumed that the two enzymes share the same functional properties (i.e., mechanism of action, allosteric effects like "half-sites reactivity", control mechanism, etc.). The detailed information available on rabbit muscle GPDH may therefore be used in the interpretation of results obtained for the rat muscle enzyme.

Undoubtedly the most prominent finding of the present study is the dramatic decrease in affinity toward the coenzyme displayed by old GPDH. In fact, the dissociation constants of NAD^+ from young and old rat muscle GPDH differ more than the corresponding constants of young rat and rabbit muscle enzymes. A similar behavior is observed in the binding of ϵNAD^+ to these three enzyme species (i.e., similar dissociation constants from young rat and rabbit enzymes as opposed to the significantly larger K_D values associated with binding to the old enzyme).

The large effects induced by age in the coenzyme binding properties of GPDH are uncommon. All the enzymes in which age-related changes have been studied showed relatively small differences between the K_m, or K_i, values of their young and old forms. In fact, these differences were usually smaller than those observed in the specific activities (Dreyfus et al., 1978; Rothstein, 1977; Orlovska et al., 1980; Sharma et al., 1980). To the best of our knowledge, the differences between young and old forms of GPDH reported here are considerably larger than any age-related effect in enzymes found thus far. Such large effects in the binding affinity undoubtedly disclose marked modifications to have taken place in the coenzyme binding sites of the old enzyme form, modifications which appear to make the old binding sites differ from the young ones more than the latter differ from the NAD^+ binding sites in rabbit GPDH.

It is interesting to note that the dramatic age-induced changes in affinity have a relatively small effect on the negative cooperativity of the binding. Thus, the K_4/K_1 ratios observed in the binding of ϵNAD^+ to young and old GPDH are 10.4 and 9.5, respectively, while the K_4/K_2 ratios in NAD^+ binding are 88 for young and 43 for the old enzyme. The negative cooperativity displayed in coenzyme binding to GPDH is known to be intimately connected with the enzyme's catalytic properties and mechanism of regulation and has been analyzed in terms of two possible models. One model assumes that the negatively cooperative binding results from successive conformational changes induced in the four identical subunits upon ligand binding (Conway & Koshland, 1968; Henis & Levitzki, 1977). In the second model, the binding phenomena are explained by preexisting asymmetry, i.e., by assuming two classes of sites in the tetrameric enzyme (Seydoux et al., 1973; Bernhard & MacQuarrie, 1973). Henis & Levitzki (1979) made a theoretical evaluation of the effects that arise in the negative cooperativity of binding of a ligand when a non-cooperatively binding ligand is added. They concluded that the preexisting asymmetry model does not allow a change in the cooperativity of binding, while such a change is allowed by the "induced-fit" model. However, as pointed out by the authors, this treatment applies only to enzyme molecules that do not undergo ligand-dependent dissociation. Rat muscle GPDH, on the other hand, has been shown to dissociate to (inactive) dimers in the presence of various mononucleotides (Nagradova et al., 1974). This could explain the disappearance of negative cooperativity in NAD^+ binding without excluding the possibility of preexisting asymmetry in the intact tetramer. While the findings of the present study thus do not allow one to distinguish between the two alternative models of binding cooperativity, they, nevertheless, lead to some interesting conclusions. If the preexisting asymmetry model is assumed, then the large increase in the dissociation constants, while the strong negative cooperativity is being retained, can be explained only if the intrinsic affinities of the two classes of subunits toward coenzyme are reduced by the same amount. This requirement has to be fulfilled for both NAD^+ and ϵNAD^+ in order to explain our experimental results, and while not impossible, this does not seem to be very likely. In the alternative "induced-fit" model, the experimental observations may be concluded to indicate that the age-related modifications in GPDH affect the structure of the coenzyme binding sites but not the interaction among the subunits. Schlessinger & Levitzki (1974) suggested that these intersubunit interactions are due to changes in the adenine binding domains that occur upon coenzyme binding and which are transmitted to neighboring subunits, thereby decreasing their affinity toward further coenzyme binding. If this approach is accepted, then the adenine binding domains appear to be relatively unaffected by age, and the large decrease in the dissociation constants must have its origin in other parts of the coenzyme binding site.

It is interesting to consider the number of subunits in each old enzyme molecule that have to be modified in order to explain its reduced affinity. Henis et al. (1979) showed that modification of only one subunit in GPDH, by a sulfhydryl reagent, is sufficient to cause a large reduction in the affinities of the other three subunits toward NAD^+ and also to lead to a much weaker negative cooperativity in the binding. It may seem plausible, therefore, that aging of muscle GPDH involves modifications in only a fraction of the subunits. Moreover, assuming gradual posttranslational modifications to be the origin of the aging effects, one would expect the degree of modification to vary among individual enzyme molecules depending on their respective "dwell times" in the tissue. Heterogeneous populations of enzyme molecules, ranging from fully active to completely inactive forms, have indeed been reported in several studies to be the result of enzyme aging (Gershon & Gershon, 1970; Reiss & Gershon, 1976a,b; Rothstein, 1977; Dreyfus et al., 1978). Such heterogeneity of binding sites requires the individual subunits to differ in their properties and should not be confused with that assumed by the preexisting asymmetry model of cooperativity in GPDH. In the latter model, all four subunits are a priori identical, and the asymmetry arises from their mode of assembly in forming the tetrameric molecule. The identical intrinsic affinities toward NAD^+ displayed by all four subunits of old GPDH in the presence of 5 mM ATP clearly demonstrate that under these conditions the subunits are identical and noninteracting. These results, while being compatible with the preexisting asymmetry model of negative cooperativity, if one assumes subunit dissociation, unequivocally exclude heterogeneity of

the individual subunits. Such heterogeneity would have been revealed in markedly different affinities of modified and unmodified subunits toward NAD^+, even when the negative cooperativity is removed, by the presence of ATP. This conclusion is verified by the observation that differences of the type described above are indeed observed when one compares the dissociation constants of NAD^+ from young and old GPDH in the presence of 5 mM ATP. The different intrinsic affinities of old and young subunits toward the coenzyme undoubtedly reflect structural differences between the binding sites in the two enzyme forms.

The structure and interactions of NAD^+ binding sites in rabbit muscle GPDH have been studied very extensively by a variety of spectroscopic techniques. Circular polarization of luminescence (Schlessinger & Levitzki, 1974; Schlessinger et al., 1975) and nanosecond fluorescence decay measurements (Gafni, 1977) of bound ϵNAD^+ were found to be extremely sensitive tools in these investigations, allowing one to detect and follow very subtle conformational changes. Application of these techniques to the study of age-related changes in the structure of GPDH will, undoubtedly, contribute significantly to the identification and characterization of the modifications involved.

Acknowledgments

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