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Evidence for Induced Interactions in the Anticooperative Binding of Nicotinamide Adenine Dinucleotide to Sturgeon Muscle Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: The tetrameric glyceraldehyde-3-phosphate dehydrogenase isolated from sturgeon muscle shows negative cooperativity in nicotinamide adenine dinucleotide (NAD) binding. After alkylation of each of the four active sites with the sulfhydryl reagent bromotrifluoroacetone, this cooperativity is abolished and the apparent affinity is weakened about tenfold. Fluorine magnetic resonance shows a single resonance which shifts upon the addition of NAD and indicates rapid chemical exchange of NAD between the free and bound states. Using two molecules of NAD per enzyme tetramer, it is possible to direct alkylation of half of the four sites. Fluorine magnetic resonance spectra of this derivative show no change upon addition of up to two NAD molecules to the half-modified enzyme. These NAD molecules appear to bind to the nonalkylated active sites. Upon addition of between two and four molecules of NAD, two resonances are observed. The relative proportion of each varies linearly with NAD. This implies slow exchange of NAD on and off alkylated sites which are adjacent to unmodified NAD-bound sites. Since the kinetics of NAD exchange in half-alkylated material are clearly different from that observed in the fully alkylated enzyme, there appear to be induced interactions between subunits which are at least partially mediated by the active-site sulfhydryl residues.

The mechanism of cooperative allosteric transitions and cooperative binding has received considerable attention in recent years. One of the most interesting aspects of this general question is the negative cooperativity exhibited by a number of dehydrogenases in the binding of their coenzyme and substrates. This phenomenon has been most thoroughly studied using glyceraldehyde-3-phosphate dehydrogenases (GPDH)[1] from many sources. In such systems nicotinamide adenine dinucleotide (NAD) has been shown to be a participant in the oxidation of glyceraldehyde 3-phosphate, an activator of the thio-acyl bond in at least some acyl-enzyme reactions (Schwendimann et al., 1976) and a regulator of enzyme activity as a result of its anticooperative binding to the enzyme (Conway and Koshland, 1968).

It has become clear that enzymes isolated from various sources display similar qualitative behavior with respect to the roles of NAD in catalysis and regulation. The quantitative aspects of this behavior vary greatly, however, from source to source. This variability is most apparent when one considers the binding of NAD. The enzyme isolated from rabbit muscle shows a very dramatic negative cooperativity in its binding of NAD. The binding of the most tightly bound two NAD molecules to this enzyme is extremely strong with estimates of the affinity constants for these sites in the range of 10^8–10^{10} M^{-1} (Conway and Koshland, 1968). The weaker two NAD binding sites have affinities in the range of 10^5–10^6 M^{-1}. Thus, there is a several orders of magnitude difference in the apparent affinities of the strongest and weakest sites. The enzymes isolated from sturgeon or halibut also demonstrate a clear negative cooperativity in NAD binding, but only about a factor of 10 or less difference in apparent affinity of the strong and weak sites is observed (Seydoux et al., 1973). Yeast provide still another quantitative difference in the binding of NAD to their GPDH. In this case, the binding shows a mixture of apparent positive and negative cooperativity at room temperature and positive cooperativity at 37 °C (Cook and Koshland, 1970; Kirschner et al., 1971).

In addition to the various kinds of cooperativity exhibited by the GPDs from different sources, all the enzymes appear to demonstrate half of the site reactivity with respect to certain kinetic parameters and active-site alkylation by certain sulfhydryl reagents (Seydoux et al., 1974). Two alternate models have been proposed to explain both half of the site reactivity and anticooperativity in ligand binding in the enzymes. One of these supposes that the enzyme consists of four subunits which are in identical conformations in the absence of any li-

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[1] Abbreviations used are: GPDH, glyceraldehyde-3-phosphate dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; EDTA, (ethylenedinitrilo)tetraacetic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

gands. Anticooperative binding and half of the site reactivity are then the results of induced changes in the subunits (Stallcup and Koshland, 1973). The alternative suggestion considers the tetrameric four to consist of a dimer of dimers structure, such that two subunits exist in one conformation and environment while the remaining two subunits exist in a different conformation and environment (MacQuarrie and Bernhard, 1971). This $(\alpha\alpha)_2$ structure is proposed despite the identical amino acid sequences of all four subunits.

Recently, Bode et al. (1975a,b) have examined the rabbit muscle GPDH using magnetic resonance techniques. In particular, they demonstrated that the active-site sulfhydryl group of each of the four enzyme subunits was susceptible to specific alkylation using bromotrifluoroacetone. The result of the alkylation is a catalytically inactive subunit with a trifluoromethyl group suitable for ^{19}F magnetic resonance studies. Their studies are consistent with the $(\alpha\alpha)_2$ model to explain anticooperative binding and half of the site reactivity.

In this communication, we are concerned with the mechanism of the anticooperativity in GPD. In particular, we show that there are apparently induced changes in the affinity of a given subunit, depending on whether or not NAD is bound to adjacent subunits. Further, it appears that the active-site sulfhydryl residue plays an important role in communication between subunits.

Materials and Methods

3-Bromo-1,1,1-trifluoropropan-2-one was purchased from PCR Inc. (Gainesville, Fla.). Sturgeon muscle glyceraldehyde-3-phosphate dehydrogenase was prepared using the procedure of Seydoux et al. (1974). Enzyme grade ammonium sulfate (Schwarz/Mann) and reagent grade chemicals were used throughout. The enzyme was stored as an ammonium sulfate precipitate of the apo form (see below). Deuterium oxide (99.87%) was from Bio-Rad.

Concentrations of sturgeon muscle glyceraldehyde-3-phosphate dehydrogenase were determined from measurements of the absorbance (Cary Model 14 spectrophotometer) at 280 nm (A_{280}) and an $E_{1cm}^{1\%}$ at 280 nm which is dependent upon the A_{280}/A_{260} ratio (Seydoux et al., 1974). Either enzyme activity or number of free active-site sulfhydryl groups was used to determine the extent of enzyme modification with 3-bromo-1,1,1-trifluoropropan-2-one (see Results). The enzyme used has an A_{280}/A_{260} ratio greater than 2.0, corresponding to less than 0.1 NAD^+ per tetramer. Activities were greater than 290 IU/mg and number of active-site sulfhydryl groups per tetramer greater than 3.9.

Apo-glyceraldehyde-3-phosphate dehydrogenase reacted rapidly and stoichiometrically at pH 7 with 3-bromo-1,1,1-trifluoropropan-2-one as long as the enzyme concentration was greater than 10 mg/mL. At lower enzyme concentrations, the reaction of reagent with enzyme was slowed and base-catalyzed hydrolysis began to be a significant fate of the reagent. Often, 3-bromo-1,1,1-trifluorpropan-2-one was added in about 10% excess of the active-site sulfhydryl groups and the 3-hydroxy-1,1,1-trifluoropropan-2-one formed was used as a reference standard for ^{19}F NMR spectroscopy. Alternatively, the adduct of the reagent with 2-mercaptoethanol was used as a reference standard for ^{19}F NMR. ^{19}F NMR spectra were collected with a Varian XL-100 NMR spectrometer equipped for FT NMR operating at 94.1 MHz. A Varian 620i dedicated computer with 16K memory was used to collect and average large numbers of FT NMR spectra.

Fluorescence measurements were performed using a Schoeffel SRR-1000 spectrofluorimeter in line with a Varian 620i (BK) dedicated computer. Typically, 1000 individual fluorescent measurements were computer averaged for each value recorded. Additions of ligands to the enzyme solution were made by blowing the ligand solution into the fluorescent curvette with a Lang-Levy micropipet. NAD^+ binding isotherms were determined by following the decrease in protein fluorescence (excitation wavelength, 290–300 nm and emission wavelength, 340–350 nm) as NAD^+ was added to the enzyme solutions. This decrease in the (tryptophan) fluorescence was proportional to the degree of NAD^+ binding (see Results). The data were fit to equations similar to those of Cleland (1968) using a nonlinear regression program (O. Pfenninger, unpublished results). The data were fit using one, two, and four dissociation constants. At some point, lack of convergence indicated that the data could not be better fit by higher numbers of dissociation constants.

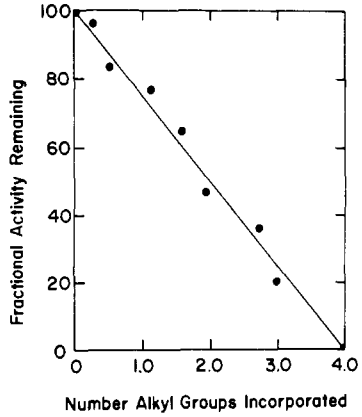

FIGURE 1: A plot of the fractional enzymatic activity remaining after alkylation vs. the number of alkyl groups incorporated.

All experiments were performed at 10 mM ethylenediamine, 1.0 mM EDTA, and 100 mM KCl, pH 7.0.

Specific Half-alkylation of GPD. The procedure for specifically alkylating half of the GPDH active-site sulfhydryl groups makes use of the pronounced inhibition by bound NAD^+ of the reaction of the active-site sulfhydryl groups with Nbs_2 (Seydoux and Bernhard, 1974). Thus, if exactly 2 equiv of NAD^+ was added to the enzyme solution at relatively high enzyme concentrations ($>10^{-4}$ M) followed by 2 equiv of Nbs_2, an enzyme species is formed which has thionitrobenzoic acid groups at the two active sites which do *not* bind NAD^+ tightly. Treatment of this enzyme species with 2 equiv of 3-bromo-1,1,1-trifluoropropanone, followed by treatment with excess mercaptoethanol, passage through a Sephadex G-25 column, and concentration by ultrafiltration, gives an enzyme species which is alkylated at the two "tight" NAD^+-binding active sites. Preparations in this fashion routinely gave enzyme with 2.0 reactive sulfhydryl groups remaining.

Results

Alkylation of GPD. Sturgeon glyceraldehyde-3-phosphate dehydrogenase was mixed with bromotrifluoroacetone at various reagent to enzyme ratios and left to stand 1 h. Then each mixture was assayed for enzyme activity and number of sulfhydryl groups reacting with Nbs_2. Seydoux and Bernhard (1974) have shown that in aqueous solution only the four active-site sulfhydryl groups react with Nbs_2, so that assay with Nbs_2 indicates the number of active-site sulfhydryl groups which have not been alkylated with the reagent. Figure 1 shows the enzyme activity as a function of the number of free, unalkylated active-site sulfhydryl groups. Complete alkylation of the active-site sulfhydryl groups with bromotrifluoroacetone inactivates the enzyme and, more importantly, enzyme activity

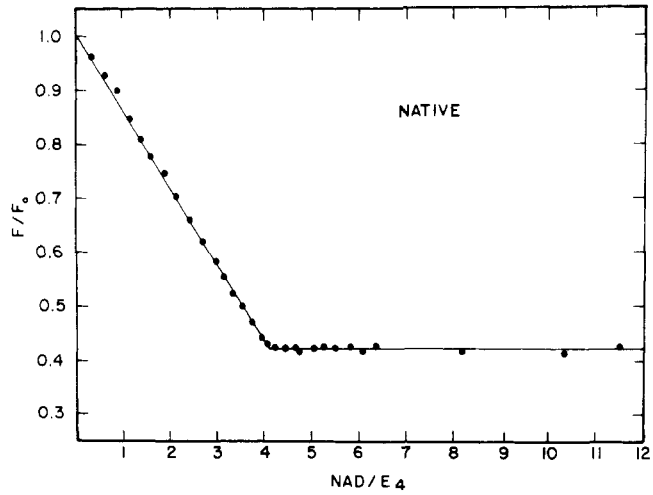

FIGURE 2: A fluorescence titration of sturgeon GPDH at high enzyme concentration (1.0×10^{-5} M sites) with NAD. The exciting wavelength was 305 nm and emission was monitored at 350 nm.

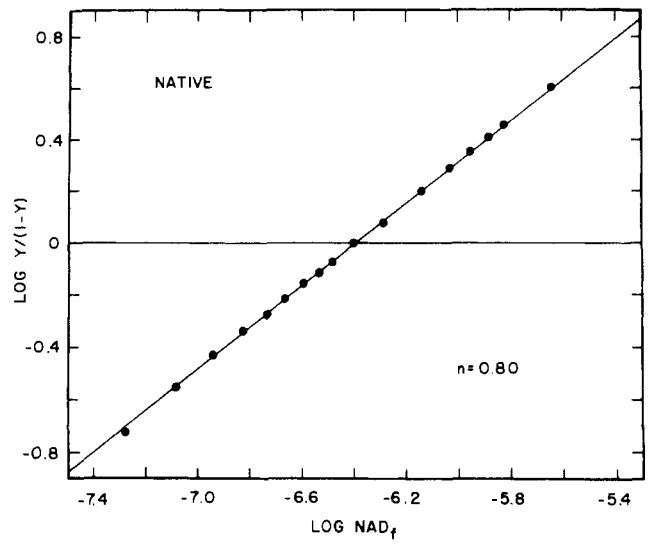

FIGURE 3: A Hill plot of the fluorescence binding data obtained at low native enzyme concentration (1.0×10^{-7} M sites) using the quenching of protein fluorescence caused by NAD binding. The temperature was 25 °C.

falls linearly with the extent of alkylation. As a further check of the specificity of the reagent for the active-site sulfhydryls, the tetraalkylated enzyme was assayed with Nbs_2. This agrees with the published value of 12.0 total sulfhydryl groups per tetramer and, in fact, we have also found that for native enzyme 12.0 sulfhydryls react with Nbs_2 in 8.0 M urea. Thus, there is no half of the site reactivity apparent for this alkylating reagent.

NAD Binding to Native Sturgeon GPD. The binding of NAD to sturgeon muscle GPD quenches the tryptophan fluorescence of the protein. This is similar to the effect observed in GPDs from other sources. The protein fluorescence is observed by excitation at 295 nm. At this wavelength, essentially no light is absorbed by the NAD and the observed decrease in fluorescence truly represents the changed fluorescence of the enzyme–NAD complex. This change can be used to quantitate the binding of NAD provided a linear relationship exists between the change in fluorescence at a particular wavelength and the number of NAD molecules bound. This is shown in Figure 2 as a plot of the observed fluorescence vs. the number of NAD molecules added to the solution per mole of tetrameric GPD. The observed curve consists of two intersecting straight lines, indicating essentially stoichiometric binding. There is a linear decrease in fluorescence intensity as NAD is added until 4.0 molecules of coenzyme have been added per mole of enzyme. At higher concentrations of NAD there is no further change in fluorescent intensity of the enzyme as NAD is added. Thus, four molecules of NAD bind per tetramer. Further, the linear dependence of the change in fluorescence with moles of NAD bound suggests that the fluorescence truly represents the fractional saturation of the enzyme sites.

At lower enzyme concentrations, where the binding is no longer stoichiometric, the thermodynamics of the binding may be examined. This is shown in Figure 3, as a Hill plot of the fractional saturation of the enzyme deduced from the fluorescence data. The Hill coefficient obtained from this data is 0.80 ± 0.03 with half-saturation occurring at 4.0×10^{-7} M NAD. The binding has a very apparent anticooperativity. The data may be adequately fit to a binding polynomial characterized by two dissociation constants using values of 1.8×10^{-7} and 1.4×10^{-6} M.

Thermodynamics of NAD Binding to Fully Alkylated Sturgeon GPD. The tryptophan fluorescence of this fully alkylated material shows the same linear dependence of quenching vs. fractional occupancy of the alkylated sites by NAD as exhibited by the native material. A Hill plot of the binding of NAD deduced from fluorescence data using the tetraalkylated enzyme at low concentration gives a slope of 0.99 ± 0.03, suggesting no cooperativity in NAD to this enzyme derivative. The midpoint of the binding curve of the derivative has been shifted by a factor of more than 10 toward weaker binding of a value of 2.8×10^{-6} M NAD.

^{19}F NMR Studies. The ^{19}F nuclear magnetic resonance spectrum of tetrakis(methylcarbonyltrifluoromethyl)glyceraldehyde-3-phosphate dehydrogenase, $E_4(CF_3)_4$, is shown in Figure 4. The peak due to the enzyme-bound trifluoromethyl group is a broad singlet characteristic of a nucleus bound to a molecule of high molecular weight (in this case 140 000) located 229 Hz downfield from an internal standard (the adduct of mercaptoethanol and bromotrifluoroacetone). The broad singlet is shifted upfield 40 Hz when NAD^+ is completely bound to the enzyme. The shift in frequency of this peak can be used as an indicator of the interaction of modified enzyme with NAD. Figure 5 shows the NAD titration of the enzyme at pH 7.0. This titration shows a linear change in chemical shift, as up to 4 equiv of NAD is added to the enzyme; further additions caused no change in the total chemical shift of 0.4 ppm.

The spectrum of specifically half-alkylated enzyme shows a similar fluorine magnetic resonance signal to the fully alkylated material. Figure 6 shows the spectra obtained in the presence of varying amounts of NAD. In the presence of zero to two NAD molecules per molecule of enzyme tetramer, there is no change in spectrum. The fluorine resonance stays at the ligand free position until more than two NAD molecules have been bound per enzyme tetramer. The addition of between 2 and 4 equiv of NAD causes a change in the fluorine spectrum of the protein. Under these conditions a new spectral line appears 0.6 ppm upfield of the position of the enzyme resonance in the absence of NAD. This new chemical shift is the same as that of the fully alkylated material. The differences between the observed effects of NAD on the fully alkylated and half-alkylated material is due to the rate of chemical exchange between free and bound conditions. In the fully alkylated material the exchange rate is rapid, giving rise to a single, averaged resonance whose chemical shift is the population av-

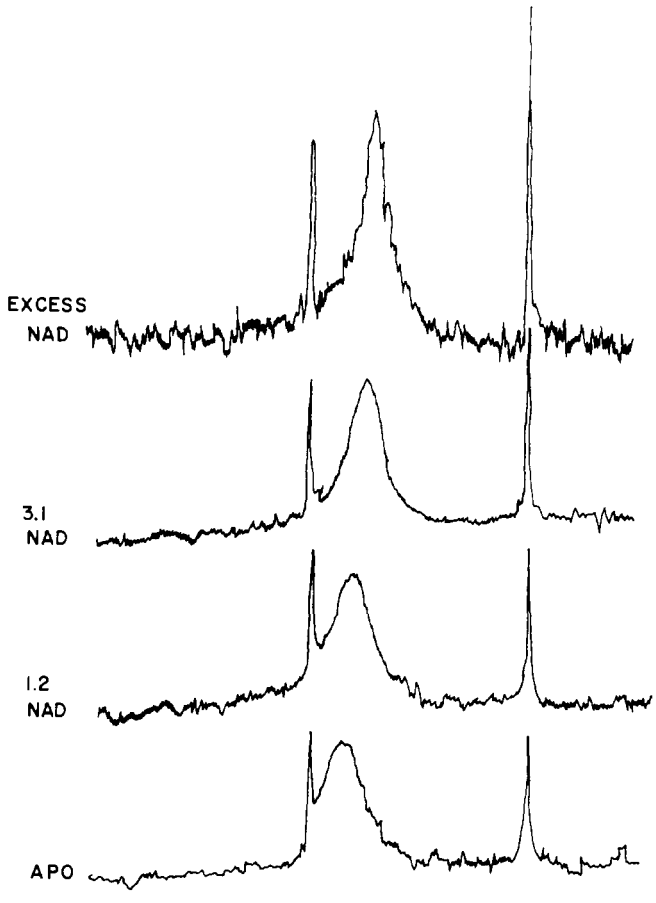

FIGURE 4: The ^{19}F magnetic resonance spectrum of tetraalkylated GPDH in the presence of various concentrations of NAD. The sharp peak on the right (high field) is an internal standard of the adduct of mercaptoethanol and bromotrifluoroacetone. The sharp peak to low field is hydrolyzed bromotrifluoracetone. The separation between these two sharp lines is 275 Hz. The temperature was 20 °C.

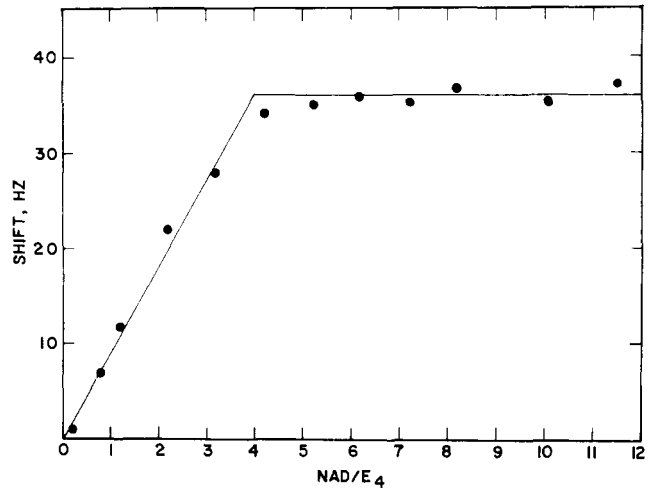

FIGURE 5: A plot of the shift of the trifluoromethyl resonance (measured from internal standards of hydrolyzed bromotrifluoroacetone and the mercaptoethanol bromotrifluoroacetone adduct) of fully alkylated GPDH vs. equivalents of NAD added.

erage of those of the free and bound states. In the case of the half-alkylated material this exchange rate is slow such that the free and bound states give rise to distinct lines.

Discussion

There have been two fundamental types of mechanisms proposed to account for both negative cooperativity in ligand

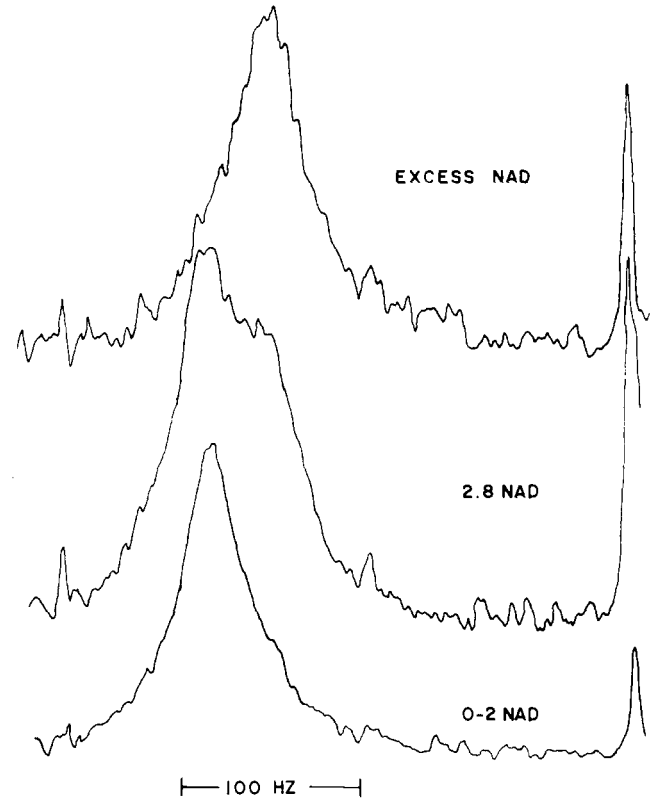

FIGURE 6: The ^{19}F magnetic resonance spectrum of specifically half-alkylated GPDH in the presence of various amounts of NAD. The sharp peak at the right of the spectra is the mercaptoethanol-bromotrifluoroacetone adduct. The temperature was 20 °C.

binding and the related phenomenon of half of the site reactivity in polymeric proteins. One of these, the induced model, has a number of variations which have been proposed (Levitzki and Koshland, 1971). These all share several common features. The most important of these is that in the absence of ligands all polypeptide chains are identical. In the case of GPDH the four binding sites for NAD located on each of the four subunits would be fully equivalent. Anticooperativity then results from the induced differences in the subunits once the first ligand is bound. The second model presupposes that, even though the four polypeptide chains of GPDH are identical in amino acid sequence, the architecture of the tetramer is such that the polypeptides are not equivalent. In its most extreme representation, there is absolutely no interaction between subunits, and anticooperative binding is simply an indication of the differing affinities of the ligands for the different sites. MacQuarrie and Bernhard (1971) proposed that sturgeon GPDH consists of two different kinds of environments of its polypeptides. This proposal accounts for both half of the site reactivity and anticooperative binding. The magnetic resonance experiments of Bode et al. (1975a,b) on rabbit muscle GPDH have been interpreted in terms of the preexistent asymmetry model.

The binding of NAD to native sturgeon apo-GPDH shows a substantial anticooperativity. We have quantitated this both by least-square fit procedures and by measurement of the Hill coefficient. The measured value of 0.80 is a clear indicator of anticooperativity in the binding. The least-square procedure shows a good fit of the observed binding data to an equation derived from consideration of two independent noninteracting binding sites. The data are well fit to the binding polynomial which is derived from a dimer of dimers model, in which only two phenomenological constants are needed to describe the

TABLE I: A Summary of the Interpretations of the Binding Data of NAD to Native Sturgeon GPDH Assuming a Dimer of Dimers Model.[a]

	K_{tight} (M)	K_{loose} (M)	K_{tight}/K_{loose}
Preexistent asymmetry model	0.18×10^{-6}	1.4×10^{-6}	8
Induced model	3.4×10^{-7}	7.4×10^{-7}	2.2

[a] Both models are described by the binding polynomial for the fraction of sites bound, $\bar{y}$, where $\bar{y} = (\psi_1 N + 2\psi_2 N^2)/(1 + \psi_1 N + \psi_2 N^2)$ and N is the free NAD concentration. $\psi_1 = [(1/K_{tight}) + (1/K_{loose})]$ and $\psi_2 = 1/K_{tight}K_{loose}$ for the preexistent asymmetry model; $\psi_1 = 1/2K_{tight}$ and $\psi_2 = 1/K_{tight}K_{loose}$ for the induced model.

binding. The data fit an equation derived from four phenomenological constants equally well. We shall employ the constants derived from consideration of only two constants for the sake of simplicity only.

Both the induced model and the preexistent asymmetry models predict *identical* binding polynomials and are therefore indistinguishable by that method (Mueggler et al., 1975). Table I summarizes the interpretations of the phenomenological constants in terms of the specific microscopic constants of the two models. The induced model interprets the anticooperativity to be due to a factor of a 2.2 decrease in the affinity of the second class of sites which is induced by binding to the initially unliganded enzyme. The preexistent asymmetry model interprets the anticooperativity to be due to a factor of 8 difference in the affinity of two classes of preexistently different binding sites.

Alkylation of the active-site sulfhydryl residue of the protein with the NMR probe bromotrifluoroacetone provides a convenient method to investigate which of the two extreme models is more likely. Total alkylation, which occurs in an apparently random process (Figure 1), results in the substitution of each of the four subunits with the trifluoroacetonyl residue at the active-site sulfhydryl residue. Interestingly, this material no longer displays anticooperativity in its NAD binding. This was judged by two methods. Measurements using the quenching of protein fluorescence by NAD showed that binding now was essentially a perfect hyperbola. The NMR spectrum of the derivatized protein also showed no cooperativity in binding. The fully alkylated material showed one fluorine resonance at pH 7 and this was seen to shift with no discernible broadening as NAD was added. The shift was complete after four NAD molecules were added per enzyme tetramer. Thus, each of the four trifluoromethyl groups was fully equivalent to the other three, even in the presence of NAD. Therefore, no site had any higher affinity for NAD than any other, since no differential shift or broadening was observed.

A lower limit can be placed on the NAD exchange rate in the fully modified enzyme. No broadening of the fluorine signal was observed even when half the enzyme sites were free and half were bound. Under conditions of fast exchange, a contribution to the observed line width due to chemical exchange between two environments different in chemical shift by 40 Hz and equal populations free and bound would be given by (Carrington and McLachlan, 1967)

$$\Delta V_{exchange} = \frac{1}{8\pi}(\Delta\omega)^2\tau_{bound}$$

$$= \frac{1}{8\pi}(2\pi 40)^2\tau_{bound}$$

If we limit the accuracy of our line-width measurements to ±10 Hz, then the lifetime of the bound state, τ_{bound}, must be 4 ms or less.

While the fact that the alkylated protein no longer demonstrates anticooperativity in ligand binding does not allow us to distinguish between an induced or preexistent asymmetry model, it does, however, provide information about the region of the binding site which must be involved in expressing either the induced or preexistent differences. It would appear that the specific interaction of the active-site sulfhydryl group with the coenzyme is responsible for the interaction. It is interesting to note that the alkylated material no longer has the Racker band, a broad, weak absorption at 340–350 nm usually associated with the binding of NAD (Racker and Krimsky, 1952). This band must somehow be associated with the presence of a free SH group at the active site. This sulfhydryl group may be directly responsible for formation of the new absorption. Alternatively, it may be that, upon alkylation, the orientation of the bound NAD is sufficiently different that the Racker band no longer appears because of steric considerations.

In their work concerning the trifluoroacetonylated rabbit muscle GPDH, Bode et al. (private communication) found that the fully alkylated material retained some of its dramatic anticooperativity in NAD binding. The binding was weakened by alkylation and the binding isotherm did not appear to have the sharp breaks characteristic of the native rabbit muscle GPDH. The Hill coefficient for NAD binding to fully alkylated rabbit muscle GPDH was about 0.7. This suggests that the rabbit muscle enzyme must have more interactions which establish the induced or preexistant differences between the binding sites. This probably explains the much more pronounced anticooperativity displayed by the native rabbit muscle enzyme as compared to that from sturgeon muscle.

Our experiments concerning specifically half-alkylated material allow us to rule out a preexistent asymmetry model in which absolutely no induced changes to other subunits are allowed. The chemical shift of the trifluoromethyl resonances from this material is independent of NAD binding until two NAD molecules are added per mole of enzyme. Furthermore, the chemical shift is the same as that of fully alkylated material in the absence of NAD. Therefore, it is clear that the resonance shifts only when NAD binds to the specific subunit where the trifluoromethyl is located. No shift occurs for the first two NADs added because nonalkylated sites have higher affinity than alkylated ones. Thus, NAD preferentially binds to the nonalkylated sites first.

In the presence of more than 4 equiv of NAD, the trifluoromethyl resonances have the same chemical shift as is observed when excess NAD is added to the completely trifluoroacetonylated material. Therefore, we conclude that the bound and free environments of the trifluoromethyl groups are the same in half-alkylated and fully alkylated material. The difference arises when one examines the trifluoromethyl group resonance in the presence of 2.8 NAD per partially alkylated material (Figure 6). Here resonances for both free and bound species are observed. This corresponds to a substantially slower exchange time for the conversion of free and bound signals. Again the measured line widths in Figure 6 show no significant broadening. If the error in line width is 10–15 Hz, we might miss a contribution of 10–15 Hz to the line width from exchange effects. Thus, we may estimate the lower limit of the bound lifetime using the equations describing the slow-exchange condition (Carrington and McLachlan, 1967).

$$\tau = \frac{1}{\pi 15\ \text{Hz}} = 20\ \text{ms}$$

Thus, the lifetime of the NAD complex on an alkylated subunit

is 20 ms or longer. The fully alkylated material gave a bound lifetime of 4 ms or shorter. Thus, at least a factor of 5 difference in bound lifetime exists between the two kinds of alkylated subunit one observed. If there were no interactions between subunits, the alkylated sites should be described by the same binding constant and bound lifetime independent of the conditions on adjacent subunits. Our results clearly demonstrate that this is not the case.

It is tempting to equate a longer bound lifetime with tighter binding. Of course this is not a rigorously valid conclusion. Unfortunately, it is essentially impossible to make the direct binding measurements on the partially alkylated material because there are too many affinities and differential fluorescence yields to be considered in fitting the data. We are left with the firm conclusion, however, that some sort of induced interactions between subunits must occur.

It is worthwhile considering the distribution of trifluoroacetonylated subunits in our specifically alkylated GPDH. This was prepared by adding two molecules of NAD for each tetramer of sturgeon GPDH. The remaining two subunits were then reacted with a stoichiometric amount of Nbs_2. Seydoux and Bernhard (1974) showed that NAD inhibits the reaction rate of the sulfhydryl groups of sturgeon GPDH by several orders of magnitude. Therefore, only those subunits without NAD could react. If NAD binding were infinitely anticooperative, all the enzyme molecules would be substituted by 2.0 TNB residues. Less extreme anticooperativity would produce relatively more randomness in the distribution of TNB-substituted molecules. To complete specific partial trifluoroacetonylation, a slight excess of bromotrifluoroacetone was added and allowed to react. Then the reaction was quenched with excess mercaptoethanol which also removed the TNB residues. Thus, alkyl groups were placed in the sites originally occupied by NAD. It is difficult to predict what the various bound species of GPDH should be when an average of two NAD molecules are bound per tetramer. Our binding data allow such a calculation only if we really believe that two phenomenological constants are sufficient to describe the binding process. However, the experimental results show there is not a preponderance of tetraalkylated material present.

It is interesting to compare our results using sturgeon muscle GPDH to those of Bode et al. (1975a,b) using rabbit muscle GPDH. There are several similarities. We both find that the essential sulfhydryl residue reacts rapidly and specifically with bromotrifluoroacetone. The resonance shifts upfield by about 40 Hz when NAD binds. In the rabbit enzyme, slow exchange conditions hold even with the fully alkylated material, however. Bode et al. found a strongly pH-dependent fluorine spectrum which showed the two resonances separated by ~600 Hz of equal intensity at high pH in the rabbit muscle preparation. A similar effect was found in sturgeon enzyme, but it was not freely reversible as it is in the rabbit muscle preparation and as a result we cannot discuss it in any detail. The differences are also very interesting and quite dramatic. The trifluoroacetonylated rabbit enzyme was found to be reducible using NADH to reduce the trifluoroacetonyl residue. This did not occur at all using the sturgeon enzyme. We found that alkylation of sturgeon GPDH completely destroyed anticooperative binding of NAD. In the rabbit enzyme, partial anticooperativity was retained.

This comparison of results in the two systems further demonstrates that, while GPDHs isolated from different sources have many features in common, there are clear and distinct quantitative and qualitative differences between them. A great deal of care must be taken in extrapolating results obtained on a GPDH from one source to another source.

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