Activation of a Covalent Enzyme-Substrate Bond by Noncovalent Interaction with an Effector

(NAD binding/structural asymmetry/acyl glyceraldehyde 3-phosphate dehydrogenase/negative cooperativity)

O. P. MALHOTRA* AND SIDNEY A. BERNHARD†

Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, Ore. 97403

Communicated by V. Boekelheide, April 9, 1973

ABSTRACT The absorption spectrum of an active-site-specific chromophoric acyl enzyme, sturgeon β-(2-furyl)-acyroyl-1-glyceroyl-3-phosphate dehydrogenase, is reported. This acyl enzyme undergoes all of the catalyzed reactions characteristic of the intermediate of the physiological acyl enzyme, 3-phospho-D-glyceraldehyde-3-phosphate dehydrogenase. The rates of reactions of both these acyl enzymes depend strongly on the extent of interaction of the acyl enzyme with the oxidized coenzyme, NAD, even where the "redox" properties of the coenzyme are not required. Likewise, the spectral properties of chromophoric acyl enzyme are affected by the extent of bound NAD. Under the pseudo-physiological conditions reported herein, there is a stoichiometric limitation of two furfurylacyloyl-acyl groups per enzyme molecule containing four covalently-equivalent subunits. The binding of NAD both to the apoenzyme and to the diacyl enzyme is heterogeneous: at low extents of NAD occupancy, NAD binding is stronger. The binding to acyl enzyme can be quantitatively described by an enzyme model involving a tetramer with 2-fold symmetry, and consequently containing equal numbers of two classes of sites. NAD binding to difurfuralcyloyl-enzyme occurs virtually discretely, first to the two unmodified (tight-binding) sites, followed by looser binding to the two acyl-sites. NAD occupancy at these latter sites transforms the chromophoric acyl enzyme spectrum from that characteristic of a model furfurylacyloyl-thiol ester in H2O to a highly perturbed furfuralcyloyl spectrum characteristic of monomeric native "active-thiol" furfuralcyloyl-enzymes. Likewise the acyl reactivity towards arselenophosphate depends on the extent of NAD bound to the loose sites. Elimination of the tight binding of NAD to the difurfuralcyloyl enzyme tetramer by alkylation of the remaining two free SH groups with iodoacetate has no apparent influence on the NAD-dependent furfuralcyloyl-spectral perturbation at the "two equivalent acyl sites," even though it eliminates the apparent "negative cooperativity" in NAD binding.

Models for allosteric activation and inhibition have centered primarly on the premise that this type of regulation involves variation in the effectiveness of ligand binding (1–3). According to these models, allosteric effectors function by regulating the distribution among quaternary conformational states (and consequently the fraction of states with high affinity for substrates). In various enzyme-catalyzed reactions, formation of covalent enzyme–substrate intermediates is essential to the reaction pathway (4). The subsequent chemical transformation of such intermediates is often the rate-limiting step in catalysis. An example, par excellence, of the formation of a stoichiometrically significant covalent intermediate is for muscle glyceraldehyde-3-phosphate dehydrogenase (GPDH; EC 1.2.1.12), a tetrameric molecule composed of identical subunits. In this case an acyl (3-phosphoglycerol)-enzyme can be isolated in stoichiometrically significant yield (5, 6). Consistent with this finding are kinetic inferences that the rate-limiting step in the oxidative phosphorylation catalyzed by muscle GPDH (Eq. 1) near neutral pH is the phosphorolysis of the acyl enzyme (7). For such oligomeric enzymes, particularly when rate-control occurs subsequent to intermediate formation, we should consider the effect of allosteric effectors not only on the unsubstituted enzyme, but on the stoichiometrically significant enzyme–substrate covalent intermediates.

O
R—C—H + EH
NAD
O
R—C—E
HPO
O
R—C—OPO
EH

n-Glyceraldehyde NADH Acyl
3-phosphate enzyme
3-Phospho
phosphate

R—C—H + EH
HPO
O
R—C—OPO
EH

$\beta$(2-furyl)-acyroyl-1-glyceroyl

We have reported the isolation and spectrophotometric identification of a chromophoric acyl enzyme with properties analogous to that of the physiologically isolable 3-phosphoglyceroyl GPDH (8). This $\beta$(2-furyl)-acyroyl enzyme (FA-enzyme), like the physiologically important acyl enzyme, undergoes phosphorolysis, arselenolysis, and reduction by NADH. Catalytic reactions involving acyl enzyme exhibit an absolute requirement for bound NAD even where the oxidative potential of the coenzyme is not required (8–11). Under appropriate conditions, acyl enzymes can be obtained with stoichiometric limits of acylation of 2 or of 4 acyl groups per tetramer (8, 12, 13). Due to concentration limitations in acyl phosphate and to other factors, the stoichiometric limitation of two acyls per tetramer is more stringent with FA-enzyme than with the 3-phosphoglyceroyl-enzyme.

Isolation of FA-enzyme from a large excess of acyl phosphate results in a diacyl tetramer containing two equivalents of bound NAD (8). The UV-visible absorption spectrum of this acyl enzyme species is consistent with that of a model compound FA-thiol ester in water, and exhibits only a small change in spectrum when the enzyme protein is denatured (8, 14). In contrast, a monomeric enzyme that, like GPDH,
contains a cysteine sulphydryl at the active site and functions by formation of a native enzyme thiol ester intermediate, exhibits highly perturbed ("red-shifted") chromophoric acyl enzyme thiol ester spectra (14, 15). The denatured chromophoric acyl enzyme spectra correspond to those for the chromophoric thiol esters in water. Likewise, chromophoric acyl enzyme intermediates formed with serine oxygen at the active site are highly "red-shifted" relative to the corresponding model esters and denatured acyl enzymes (16, 17). For "active serine" acyl-enzymes, where a wide variety of enzyme species have been studied, the $\lambda_{max}$ for any particular chromophoric acyl group in the native acyl enzyme is enzyme-species invariant, hence suggesting that the chemical-bond structure of the oxygen ester (and presumably the thiol ester) are altered from the usual transplanar configuration (16-18).

Since the native FA-GPDH is catalytically convertible to all of the products characteristic of the catalytic true substrate reactions (Eq. 1), it was surprising to us, on the basis of our past experience with chromophoric acyl enzymes, that FA-GPDH was spectrally unperturbed. A potential explanation lies in the known effect of NAD on the thermodynamic stability of this acyl enzyme (8). As seen by chemical equilibrium measurements, the free energy of formation of the acyl enzyme bond in the FA-enzyme is strongly affected by the presence of bound NAD. The unperturbed acyl enzyme spectrum reported previously was for a species containing two or less bound NAD per tetramer. In the experiments that follow, we report on the effect of higher concentrations of NAD (concentrations in the physiological range) on the spectral and chemical properties of the FA-enzyme. There is a profound effect of noncovalently bound NAD on the bonding properties of the acyl enzyme thiol ester, as indicated by changes in the FA absorption spectrum and the reactivity of the acyl-enzyme bond.

MATERIALS AND METHODS

The procedure of Allison and Kaplan (19) for purification of sturgeon muscle GPDH was modified, yielding a higher specific activity. The DEAE-cellulose chromatography step was replaced by a DEAE-Sephadex chromatography step and was followed by an additional step involving carboxymethyl cellulose chromatography. The activity was estimated according to Ferdinand (20) and protein concentration was obtained from absorbance at 280 and 260 nm. The specific activity of our enzyme preparation was 310–320 Ferdinand units/mg protein, the highest muscle GPDH activity reported. The reaction of FA-phosphate (FAP) with enzyme was done as described (8). The molecular weight of the enzyme was assumed to be $1.4 \times 10^7$. FA-apoenzyme was prepared by passing a solution of the FA-enzyme through a small column of charcoal (washed according to Krimsy and Racker (12)). The 280/260 ratio of the FA-apoenzyme was in the range 2.02–2.10 and was invariant to further charcoal treatments. Carboxymethylated FA-enzyme was prepared by treatment of the FA-enzyme (without charcoal treatment) with a 10- to 20-fold excess of iodoacetate solution (pH about 7), and isolated by passage through a Bio-gel (P-30) column. The 280/260 ratio at this stage was about 1.8 and increased to 2.06 on charcoal treatment. The isolated carboxymethylated FA-enzyme was deacylated (1–2 mM arsenate, pH 7.0) and free reactive SH groups were estimated with Ellman's reagent [5,5'-dithio-bis-(2-nitrobenzoic acid)]. The number of such groups was invariably equal to the number of FA groups that were initially present, indicating that the rest of the reactive SH groups (4 — number of FA groups per tetramer) had reacted with iodoacetate. For rate studies, the time-dependent disappearance of the FA-GPDH absorption band was monitored at 360 nm after addition of 10 µl of 40 mM arsenate solution (pH 7.0) to a solution of FA-enzyme (total volume 0.8 ml).

The buffered solvent used in all the experiments contained 0.01 M ethylenediamine chloride–0.1 M KCl–1 mM EDTA at pH 7.0. The temperature was 25° unless stated otherwise. The spectra were recorded with a Cary model 14 spectrophotometer interfaced to a Varian 620i computer. They consist of measurements at 2.0-nm intervals, the value at each wavelength having been obtained by averaging of multiple absorbance measurements. For precise low absorbance measurements, a Cary 16 spectrophotometer was used.
RESULTS

The absorption spectra of a native FA-enzyme in the presence of varying amounts of NAD are shown in Fig. 1a. The diacyl enzyme isolated by Sephadex chromatography contains two tightly bound NAD, which can be removed by charcoal treatment. The spectra in the presence of NAD are potentially complicated to a small extent by formation of a new broad electronic absorption band of low extinction (the "Racker band") due to interaction of NAD with unmodified active-site cysteine SH. Indeed, the loss of absorbance on treatment of the diacyl-di FA-enzyme with charcoal (Fig. 1a) can be quantitatively accounted for by the loss of two equivalents of "Racker band" per enzyme tetramer; hence no further "Racker band" absorption is anticipated on further NAD binding to the di NAD-di FA-enzyme. NAD binds to sites where the active-site SH groups are modified. Large spectral changes characteristic of perturbation in the chromophoric acyl (FA) group are observed at higher NAD concentrations where more than two NADs per enzyme are bound (Fig. 1a). Note the "red-shift" of $\lambda_{\text{max}}$. This NAD-induced perturbation of the FA-enzyme chromophore is readily distinguished from spectral changes due to formation of NAD-enzyme "Racker-band" by the spectral peculiarities of the latter. The spectral properties of the diacyl "Racker band" have been well characterized. The $\pi \rightarrow \pi^*$ transition of the FA-chromophore is a narrow single electronic absorption band. Thus, the NAD-binding sites responsible for the "Racker band" can be distinguished from the NAD-binding sites that perturb the acyl spectrum, if such discrete sites exist. From the NAD concentration dependence of the absorbance at 390 nm (Fig. 2) compared to that at 420 nm (Fig. 3), the sites responsible for the "Racker band" are occupied at concentrations much lower than that required to perturb the FA-thiol ester enzyme spectrum.

The $\lambda_{\text{max}}$ of acyl enzyme at high concentrations of NAD is at about 360 nm (Fig. 1a). This is also the $\lambda_{\text{max}}$ of the FA chromophore when it is attached to the active-site cysteine of native papain, a monomeric, nonallosteric enzyme (14). Spectra of FA-enzymes containing different amounts of bound NAD, greater than two NAD per tetramer, are shown in Fig. 1a. As can be seen in Fig. 1a, and more clearly in Fig. 1b, there is no discernible intermediate acyl spectrum differing from both the FA-apoenzyme spectrum and that obtained under conditions of nearly complete NAD saturation. Rather, the distribution between the two spectral species is affected by the extent of NAD binding. Also, appreciable perturbation of the acyl enzyme spectrum, from that anticipated for a model thiol ester, occurs only when there are more than two NAD bound per tetrameric enzyme.

Distinctions among NAD binding sites become clear when the remaining two active-site SH groups of the di FA-tetramer are carboxymethylated with iodoacetate. In this way (Eq. 2) a di FA-dicarboxymethyl-enzyme can be prepared in which there is no possibility for "Racker band" formation upon NAD binding.

$$(\text{FA-S})_{\text{n}}\text{E(SH)}_{\text{n}} + 2 \text{I-CH}_{\text{2}}\text{COO}^\text{0} \rightarrow$$

$$(\text{FA-S})_{\text{n}}\text{E(SCH}_{\text{2}}\text{COO}^\text{0})_{\text{n}} + 2\text{H}^\text{0} + 2\text{I}^\text{0} \ [2]$$

In the presence of higher concentrations of NAD, the FA-dicarboxymethyl-enzyme (Eq. 2) shows a perturbation in the FA-enzyme spectrum identical to that observed with the nonalkylated FA-enzyme, provided that due account of "Racker band" formation is taken in the nonalkylated case (Fig. 1b). This is substantiated by an identical NAD-concentration dependence for the spectral perturbation in the alkylated and nonalkylated FA-enzymes (Fig. 2). Carboxymethylation merely destroys the tight binding to two sites, and consequently the apparent "negative cooperativity" in NAD binding. These results illustrate the inherent intramolecular structural asymmetry of the diacyl enzyme.

![Fig. 2. Effect of NAD on the catalytic and spectral properties of FA-GPDH. Spectral perturbation titration of apo FA-GPDH (4.1 μM protein containing 1.9 FA-groups per tetramer) with NAD at 390 nm (○); spectral perturbation titration of carboxymethylated FA-GPDH (2.25 μM protein containing 1.5 FA-groups and 2.5 carboxymethyl groups per tetramer) with NAD at 390 nm (●); initial rates of arsenate-catalyzed deacylation of FA-GPDH (1.65 μM protein containing 2.1 FA-groups per tetramer) in the presence of 0.5 mM arsenate (×). The NAD-saturated concentration-independent maximal rate of deacylation is 4.8 acyl groups per acyl site per min.](image-url)
It is possible to terminate the acylation reaction at stoichiometries less than two acyl groups per tetramer and to alkylate the remaining free SH groups with iodoacetate. Acyl-alkyl enzymes were prepared as indicated in Table 1, and the extent of perturbation of the acyl-spectrum by a nearly saturating NAD concentration was measured. At a fixed protein concentration, the NAD-induced absorbance changes are proportional to the acyl content (Table 1), further confirming that the acyl spectral perturbations are independent of acylation at the remaining SH sites.

In contrast to acylation of GPDH with FAP, which follows first-order kinetics, the removal of acyl groups with specific acceptors (phosphate, arsenate, or NADH) exhibits more complex kinetics (8). The initial slope of time-dependent deacylation, however, was first order in FA-enzyme concentration (unpublished data). We have now measured these initial rates of deacylation with 0.5 mM arsenate (pH 7.0), at different NAD concentrations. The NAD concentration dependence of the acyl-enzyme bond reactivity, as measured by the rate of deacylation, is the same as that obtained in the spectral perturbation experiments (Fig. 2).

**DISCUSSION**

We believe that these results are significant in the following ways: (i) an intermediate in enzyme catalysis has been demonstrated to be "activatable" by the noncovalent binding of a ligand that does not participate in the subsequent catalysed reaction process. (ii) The acyl enzyme so activated exists in an alternate chemical configurational state, as indicated by the unique, activated-acyl absorption spectrum.

During our work, several interesting conclusions regarding acyl-enzyme stability and enzyme–NAD affinity were uncovered: Diacyl apoenzyme contains two classes of NAD binding sites. The sites of high NAD affinity are those in which the SH groups are unmodified. Activation of the acyl bond is mediated by NAD binding to the subunit containing the acyl group. This is the NAD binding site of lower affinity in the diacyl enzyme. Chemical modification of the tight binding NAD sites by carboxymethylation of the SH groups has little or no effect on the NAD-induced activation of the acyl-enzyme bond. Rather, alkylation results in the loss of the "ineffective" tight NAD binding.

**Activation of Chemical Bonds by Ligand Binding.** The spectrum of FA-GPDH in the presence of higher concentrations of NAD (Fig. 1a) is identical to the spectrum of the FA-enzyme formed by specific acylation of the active-site cysteine of papain. This enzyme is monomeric. Denaturation of the FA-enzymes, including FA-GPDH, leads to a spectrum identical to that obtained for model FA-thiol esters in aqueous solution. There are essentially only two spectra for FA-enzyme; the spectrum obtainable for native FA-papain and native FA-GPDH at high [NAD] and the spectrum obtainable for denatured FA-enzymes. This result might be contrasted with that obtained by measuring the spectra of model FA chromophores in various solvents, a process that leads to a continuum of spectra dependent on the changing solvent polarity (17). Previously one of us has proposed that the characteristic perturbed acetyl-enzyme spectrum results from a higher-energy configurational ground state of the acyl ester bond in native acyl enzymes. This proposal was based on the spectra of acyl chromophores at the active-sites of enzymes from both "active-serine (oxygen)" and "active-cysteine (thiol)" esters. For "active-serine" acyl enzymes, we have suggested a discrete configuration for the perturbed acetyl enzyme bond (17) based on the known three-dimensional structure of indole-3-acryloyl chymotrypsin (21). This suggestion is consistent with the spectrum of the acyl enzyme, and, furthermore, presents a plausible route for catalysis by facilitation of nucleophilic attack at a more electrophilic carbonyl-carbon. Our knowledge of the electronic properties and electronic spectra of sulfur compounds is not so extensive as to make such definitive mechanistic proposals. Nevertheless, we note the similar, and even larger, perturbation to higher $\lambda_{\text{max}}$ of the acyl enzyme spectrum for "active-cysteine" acyl enzymes. An oligomeric enzyme–substrate intermediate requires bound effector for activation, whereas the corresponding monomeric intermediate does not require any bound ligand for its presumably identical activation.

It is important to note the reactive chemical properties of FA-papain as contrasted with an ordinary thiol ester (14). Likewise the conditions of NAD concentration required for optimal reactivity of FA-GPDH, according to Eq. 1, are those that lead to the same perturbed FA-enzyme spectrum ($\lambda_{\text{max}}$ at 360 nm) (Fig. 2). Hence the term "activated acyl enzyme" is equally applicable to the spectral and the chemical properties.

A charge transfer complex has been proposed to give rise to a unique NAD–enzyme electronic absorption band (the "Racker band") (22). Our results show that those NAD binding sites that give rise to "Racker band" do not perturb the FA-enzyme spectrum. It is also of interest to note the lower affinity for NAD at the acyl-perturbing sites. Such apparently weaker interactions might be due to an "imperfect" fit be-

<table>
<thead>
<tr>
<th>Number of acyl groups per mole of enzyme</th>
<th>Initial $A$</th>
<th>$\Delta A$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.0121</td>
<td>0.016</td>
</tr>
<tr>
<td>0.8</td>
<td>0.0185</td>
<td>0.026</td>
</tr>
<tr>
<td>1.1</td>
<td>0.0218</td>
<td>0.037</td>
</tr>
<tr>
<td>1.5</td>
<td>0.0307</td>
<td>0.044</td>
</tr>
</tbody>
</table>

The reaction of GPDH with FAP was terminated at different submaximal extents of acylation, and the remaining free SH groups of the enzyme were exhaustively alkylated with iodoacetate (see Methods). NAD (1.1 mM) was added to a fixed concentration of protein (5.2 $\mu$M) and the absorbance changes were noted at 390 nm.
tween the coenzyme and the enzyme site. Alternatively, it might reflect a coupling of a relatively strong NAD-site interaction with an energetically unfavorable quaternary structural change, thus using part of the ligand-binding free energy to stabilize that conformation that results in chemical-bond activation.

On the Existence of 2-Fold Symmetry in Tetrameric Muscle GPDH. We (8, 23, 24) and others (25) have suggested that the subunit structure of muscle GPDH has maximal 2-fold symmetry (C2) and hence functions as a pair of dimers (a dimer of dimers). This is apparent for the stoichiometrically-limited di FA-enzyme tetramer. However, we have argued strongly in the past that unmodified apoenzyme also has a maximal 2-fold symmetry (22, 26). The results presented herein add further substance to the argument. A 2-fold symmetry tetramer might a priori be anticipated to have two distinct kinds of binding sites (27): in the extreme, only two of the four potential enzyme sites might be catalytically reactive, thus leading to "half-site reactivity." The fact that the two tight NAD-binding sites in the diacyl enzyme can be blocked by alkylation without changing the effector role of NAD at the two acyl sites suggests that the ligands bound to the tight binding sites do not affect NAD binding at the "weaker" sites. Previously, the existence of "negative cooperativity," particularly for NAD binding to muscle GPDH (28), has been cited in refutation of the allosteric model of Monod et al. (1). This allosteric model in its simplest formulation did not consider the possibility of 2-fold symmetry in tetramers, or of any other subunit arrangements in oligomers in which the symmetry was suboptimal. Phenomenologically, "negative cooperativity" is possible if a quaternary conformational state has more than one kind of binding site for ligand, as for example, a tetramer with maximal 2-fold symmetry. "Negative homotropic" interactions are not allowed by the allosteric model. The present results give no evidence for "negative homotropic" interactions in sturgeon muscle GPDH; the "negative cooperativity" is a result of site heterogeneity consequent to the maximal 2-fold symmetry of the tetramer.

The results presented herein do not give the molecular details by which the noncovalent interaction of an effector with an oligomeric enzyme results in the activation of chemical bonds. Nevertheless, it is abundantly clear from our results that a set of weak interactions between ligand and enzyme site can lead to strong perturbation of chemical bonds, a mechanism that has often been proposed without direct evidence. The present results also provide a basis for invoking a coupling of weak ligand-site interactions to a chemical-bond perturbation in chemoreceptors.

This work was supported by Public Health Service Grant GM10451-09 and National Science Foundation Grant GB313-75X1.