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Protein Isomerization in the NAD$^+$-dependent Activation of β-(2-Furyl)acryloyl-glyceraldehyde-3-phosphate Dehydrogenase in the Crystal*

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Andrea Mozzarelli, Rodolfo Berni, and Gian Luigi Rossi‡
From the Institute of Molecular Biology, University of Parma, 43100 Parma, Italy

Mária Vas, Ferenc Bartha, and Tamás Keleti
From the Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, H-1502 Budapest, Hungary

Crystalline hologlyceraldehyde-3-phosphate dehydrogenase from lobster (*Palinurus vulgaris*) and pig muscle reacts with the half of the sites reagent, substrate analog, β-(2-furyl)acryloyl phosphate, to form a chromophoric acyl-enzyme intermediate. In the crystal as in solution, the reactivity of this acyl-enzyme toward phosphate or arsenate is controlled by NAD$^+$ bound to the acylated sites. In this work, activation of the catalytically inert apo-acyl-enzyme, induced by NAD$^+$ binding, has been investigated by single crystal microspectrophotometry.

In solution, the kinetics of the NAD$^+$-induced spectral changes of the acyl-enzyme, accompanying acyl bond activation, is biphasic. The fast phase is a NAD$^+$ concentration-independent first order process both in the case of the pig and of the lobster enzyme. The slow phase is a first order process in the case of the pig enzyme and a second order process in the case of the lobster enzyme. The relative amplitudes of the two phases depend in both cases on the number of acyl groups (zero to two) incorporated by the enzyme tetramer. These findings indicated the presence of two classes of apo-acyl-enzyme sites and the occurrence of protein conformational changes as necessary steps in the mechanism of activation.

The relative amplitudes of the two phases could, alternatively, be determined by measuring the much larger spectral changes accompanying deacylation under conditions in which NAD$^+$ and arsenate were concomitantly added to the acyl-enzyme and the slow phase of NAD$^+$ binding was rate determining for deacylation at the corresponding sites. These spectral changes were sufficiently large to be measured even in single crystals of glyceraldehyde-3-phosphate dehydrogenase from either lobster or pig muscle. The relative amplitudes of the two phases and the time course of the slow phase were the same in the crystal and in solution.

These results, together with previously reported microspectrophotometric data, prove that β-(2-furyl)-acryloyl-glyceraldehyde-3-phosphate dehydrogenase in the crystal can assume the various interconvertible conformations, endowed with distinct acyl bond reactivities characteristic of the acyl-enzyme in solution. The conformational changes occurring during the interconversion of species correspond to either ligand-induced intramolecular symmetry transitions or to shifts in the allosteric equilibrium between more symmetrical forms. These transformations are not prevented by the lattice forces that maintain the integrity of the holoenzyme crystal.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) is a tetrameric enzyme catalyzing the reversible oxidative phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate (1). The coenzyme, NAD$^+$, besides participating in the oxidoreduction, has an effector role in promoting phosphorolysis or arsenolysis of the 3-phosphoglyceroyl-enzyme intermediate (2–6), as well as of β-(2-furyl)acryloyl-D-glyceraldehyde-3-phosphate dehydrogenase, a chromophoric analog of the physiological acyl-enzyme (7–10).

We have taken advantage of the stability of the furylacryloylenzyme and of the large spectral changes accompanying the various elementary steps of catalysis to compare functional properties of D-glyceraldehyde-3-phosphate dehydrogenase in solution and in the crystal (11, 12). In the crystal, as in solution, the holoenzyme exhibits "half of the sites" reactivity toward β-(2-furyl)acryloyl phosphate (FAP);[1] furthermore, the spectrum and the reactivity toward arsenate of the acyl-enzyme are dependent on NAD$^+$ bound to the acylated sites. In the present paper, we report single crystal microspectrophotometric data, obtained with either lobster (*Palinurus vulgaris*) or pig muscle enzyme, that enable us to compare the quaternary conformational states and the mechanism of NAD$^+$-dependent activation of β-(2-furyl)acryloyl-D-glyceraldehyde-3-phosphate dehydrogenase in the crystal and in solution.

These results indicate that it would be extremely interesting to extend the structural studies of D-glyceraldehyde-3-phosphate dehydrogenase to include the β-(2-furyl)acryloyl derivative. This system appears to be a suitable model for x-ray crystallographic investigations of the molecular basis of both half-site reactivity and the effector role played by NAD$^+$ in catalysis.

EXPERIMENTAL PROCEDURES

Materials—Glyceraldehyde-3-phosphate dehydrogenase was isolated and crystallized from the Mediterranean lobster *P. vulgaris* (11,

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‡ To whom correspondence should be addressed.

[1] The abbreviations used are: FAP, β-(2-furyl)acryloyl phosphate; FA-, β-(2-furyl)acryloyl-.

13) and from pig (14). The molecular weight of the tetramer was taken to be 145,000 (15, 16) for both pig and lobster muscle D-glyceraldehyde-3-phosphate dehydrogenase.

Enzyme concentrations were determined spectrophotometrically by using the molar absorption coefficients for pig and Atlantic lobster enzymes reported earlier (17, 18). The amount of NAD^+ bound/tetramer was determined on the basis of the A_{280}/A_{260} ratio by using an appropriate calibration curve (19).

Crystals of Mediterranean lobster muscle holoenzyme, shaped as rhombic plates, were grown at pH 6.2 by the method described earlier (11). By following a similar crystallization procedure, crystals of pig holoenzyme, rhombic plates in shape, were grown at pH 8.5. Mediterranean lobster D-glyceraldehyde-3-phosphate dehydrogenase crystals have been characterized crystallographically (13). The quality of the pig enzyme crystals has not yet allowed this type of characterization, although the crystals were suitable for single crystal microspectrophotometry.

The substrate analog, β-(2-furyl)acryloyl phosphate was synthesized according to Malhotra and Bernhard (7). β-NAD^+, grade I, was a Boehringer product. Ammonium sulfate was a special quality for enzymology from Serva. All other chemicals were reagent grade commercial preparations.

Preparation and Spectral Characteristics of β-(2-Furyl)acryloyl-D-glyceraldehyde-3-phosphate dehydrogenase—The chromophoric acyl-enzyme from both lobster and pig muscle D-glyceraldehyde-3-phosphate dehydrogenase in the crystal was prepared, as described earlier, by allowing FAP to diffuse into pregrown holoenzyme crystals (11, 12). The number of acyl groups incorporated per tetramer varied between zero and two, depending on the reagent concentration in the crystal-suspending medium (12). After the reaction, the remaining FAP and the NAD^+ loosely bound to the acylated sites were removed by washing with 70% saturated ammonium sulfate solution. The remaining NAD^+ was mainly bound to the unacylated sites (8).

The absorption spectrum of this acyl-enzyme has a maximum at $\lambda = 345$ nm both in solution and in the crystal (8, 11). This species is called apo-acyl-enzyme. After addition of NAD^+, at concentrations sufficient to saturate all of the acylated sites, the spectrum appeared to be red shifted (8, 11), with $\lambda_{max} = 360$ and 365 nm for the lobster and the pig enzyme, respectively. This species is called holoacyl-enzyme. The isosbestic point of the difference spectrum between apo- and holoacyl-enzyme is approximately $\lambda = 360$ nm. In solution, the number of acyl groups bound per tetramer was calculated on the basis of the molar absorption coefficient of the acyl-band at the isosbestic point ($\epsilon_{360} = 24{,}000\ M^{-1}\ cm^{-1}$) (8, 11). In the crystal, the number of acyl groups bound per tetramer was calculated from the ratios A_{280}/A_{360} and $\epsilon_{280}/\epsilon_{360}$, assuming that the extinction coefficients are the same as determined in solution (11).

For solution experiments, FA-D-glyceraldehyde-3-phosphate dehydrogenase crystals were dissolved either in Tris-HCl buffer, pH 7.0, 0.05 ionic strength, or in water, containing 1 mM EDTA and an appropriate amount of ammonium sulfate to reach 50% saturation.

Kinetic Measurements—All experiments were carried out at pH 7.0 in the presence of 1 mM EDTA and of ammonium sulfate (50% saturation and 70% saturation in solution and in the crystal, respectively) at 20 °C.

The kinetics of spectral perturbation caused by NAD^+ binding to FA-D-glyceraldehyde-3-phosphate dehydrogenase was followed at 380 nm, *i.e.* at the maximum of the difference spectrum between apo- and holoacyl-enzymes (8, 12). The kinetics of arsenolysis of the acyl bond was monitored at 360 nm following the disappearance of the characteristic acyl-enzyme absorption band.

For kinetic experiments carried out on single enzyme crystals, a Leitz UV-MP microscope photometer with a flow reaction cell was used. Individual crystals were placed in the observation cell with quartz windows separated by polyethylene walls; the cell volume was about 30 μl and the crystal-suspending medium could be completely replaced with a solution containing the reagents in about 20–30 s without any serious crystal movement. Absorption of unpolarized monochromatic light was calculated as $A = -\log I/I_0$ where I and I_0 are the intensities of the light transmitted by the crystal and by the suspending medium, respectively. Time-dependent spectral changes were measured every 5 or 10 s.

For kinetic measurements in solution, a Cary 118 spectrophotometer and a Durrum-Gibson stopped flow (model D110) were used. The temperature of the cuvette house or of the reaction chamber was kept at 20 °C.

In the case of stopped flow measurements, FA-D-glyceraldehyde-3-phosphate dehydrogenase and NAD^+ solutions were separately introduced from the two syringes. The dead time of the instrument was about 3 ms.

RESULTS

As previously noted (11), neither acylation of the holoenzyme by FAP nor removal of the loosely bound NAD^+ from the acylated sites caused apparent physical damage to D-glyceraldehyde-3-phosphate dehydrogenase crystals.

Kinetics of the Spectral Perturbation of FA-D-glyceraldehyde-3-phosphate dehydrogenase by NAD^+—Following the studies carried out on the lobster (*P. vulgaris*) enzyme (11, 12), we investigated the kinetics of spectral perturbation of acylated pig muscle D-glyceraldehyde-3-phosphate dehydrogenase by saturating concentrations of NAD^+ (*i.e.* by concentrations sufficient to maximally red shift the spectrum of the acyl-enzyme) both in solution and in the crystal. As shown in Fig. 1*A*, in solution biphasic kinetics was found. Both the fast (Fig. 1*B*) and slow (Fig. 1*A*) parts of the process were first order reactions, indicative of enzyme isomerization steps. The difference spectra for the fast and slow spectral changes have practically the same isosbestic point, excluding the possibility that the fast reaction corresponds to the formation of a spectrally distinct transient intermediate (Fig. 2). In the crys-

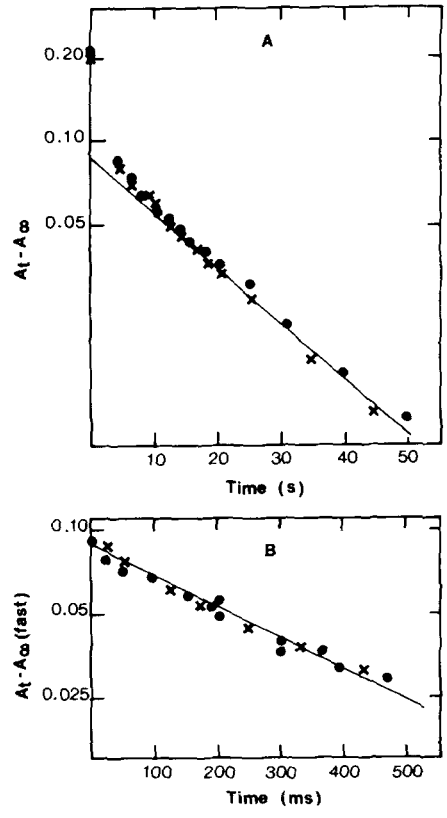

FIG. 1. **Kinetics of spectral perturbation caused by NAD^+ binding to pig FA-D-glyceraldehyde-3-phosphate dehydrogenase in solution.** To the solution of 10^{-2} mM pig FA-D-glyceraldehyde-3-phosphate dehydrogenase containing 1.74 acyl groups/tetramer, 0.5 mM (×——×) or 5 mM (●——●) NAD^+ was added. Initially the acyl-enzyme contained 2.1 mol of NAD^+/tetramer. The kinetics was followed at 380 nm in solution containing 50% saturated ammonium sulfate, at pH 7.0, in the presence of 1 mM EDTA at 20 °C. *A*, from the slow part of the semilogarithmic plot of the biphasic kinetics, a rate constant of 2.7 min^{-1} was calculated independently of the NAD^+ concentrations used. *B*, the fast part of the biphasic kinetics was followed by stopped flow measurements. This phase was also independent of the NAD^+ concentration used. A rate constant of 2.6 s^{-1} was calculated from the shown semilogarithmic plot, the data points of which were obtained after subtraction of the slow reaction.

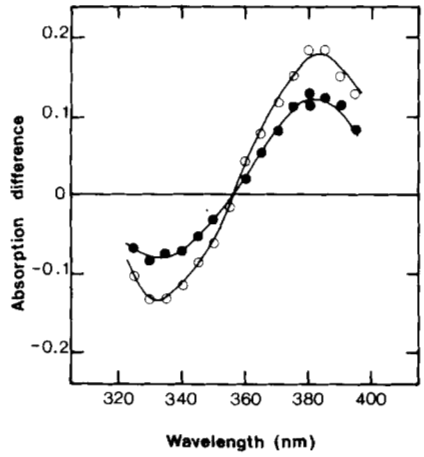

FIG. 2. **Difference spectra between apo- and holo- pig FA-D-glyceraldehyde-3-phosphate dehydrogenase in solution.** The amplitudes of fast (○——○) and slow (●——●) spectral changes induced by NAD^+ binding to 1.4×10^{-2} mM pig FA-D-glyceraldehyde-3-phosphate dehydrogenase, containing 1.55 acyl groups/tetramer, were determined from individual measurements of spectral perturbation upon addition of 1 mM NAD^+. The time courses were followed at different wavelengths, indicated on the abscissa. The same experimental conditions were used as described in the legend to Fig. 1.

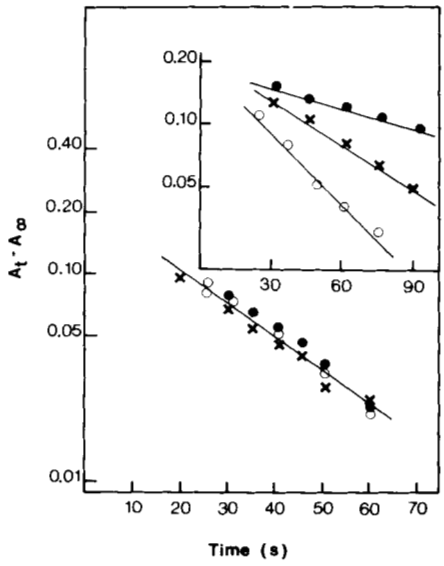

FIG. 3. **Kinetics of spectral perturbation caused by NAD^+ binding to pig FA-D-glyceraldehyde-3-phosphate dehydrogenase single crystals.** To single crystals of pig muscle FA-D-glyceraldehyde-3-phosphate dehydrogenase containing 1.9 FA groups/tetramer, 0.5 mM (×——×), 5 mM (●——●), or 15 mM (○——○) NAD^+ was added. The kinetics was followed at 380 nm in the presence of 70% ammonium sulfate and 1 mM EDTA, at pH 7.0 and 20 °C. A first order rate constant of 2.25 min^{-1} corresponds to the straight line of the semilogarithmic plot. The *inset* shows similar experiments carried out with crystals of lobster FA-D-glyceraldehyde-3-phosphate dehydrogenase, containing 1.8 ± 0.2 FA groups/tetramer exposed to solutions containing 1 mM (●——●), 2 mM (×——×), and 3 mM (○——○) NAD^+. The second order rate constant is 0.4 mM^{-1} min^{-1}, close to the value previously reported (12).

tal (Fig. 3), only one first order process was detected, the rate constant of which was practically identical with that of the slow phase in solution (*cf.* Fig. 1*A*).

Similarly, in the crystal of lobster acyl-enzyme, only one second order process was previously detected (12), as also shown in the *inset* of Fig. 3. Neither in the case of lobster enzyme nor in the case of pig enzyme were we able to reassuringly demonstrate the occurrence of fast phases of spectral perturbation in the crystalline state, possibly due to errors inherent to the determination of zero time and to the small amplitude of the signal.

To overcome these difficulties, we took advantage of the observation that arsenolysis of the acyl-enzyme only takes place at sites that are activated by bound NAD^+ (8). Since the spectral change associated with deacylation is much larger than the spectral change associated with NAD^+ binding to the acyl-enzyme, by measuring the first one under appropriate conditions, we expected to obtain a better resolution of the two phases of acyl-enzyme activation. By this way, the presence and the relative amplitudes of fast and slow phases of acyl-enzyme activation could indeed be determined even in the crystal state, as shown in the following section.

Kinetics of Arsenolysis of FA-D-glyceraldehyde-3-phosphate Dehydrogenase Limited by NAD^+ Activation—We showed previously that *after preincubation* of lobster FA-D-glyceraldehyde-3-phosphate dehydrogenase with saturating concentrations of NAD^+, arsenolysis occurred as a monophasic process with a second order rate constant $k = 0.075 \pm 0.010$ mM^{-1} min^{-1} (12) both in solution and in the crystal. In the following experiments, we added *concomitantly* NAD^+ and arsenate to the apo-acyl-enzyme. By choosing the appropriate concentrations of NAD^+ and arsenate, we could assure that while the fast step of the biphasic spectral perturbation was fast in comparison with arsenolysis, the slow step was slow enough to become rate limiting. The acyl-enzyme population was, therefore, expected to decay, via arsenolysis, in two readily separable steps, the relative amplitudes of which could be measured as shown in Fig. 4*A*. The ratio of amplitudes of the fast and slow phases of acyl-enzyme activation, obtained by measuring either arsenolysis or spectral perturbation, was indeed found to be identical and to exhibit the same dependence on the number of acyl groups per tetramer as shown in Fig. 5.

The same measurements of arsenolysis limited by NAD^+ activation were carried out in the crystal, and even in this case, it was possible to separate the two phases, as shown by the data in Fig. 4*B*. Both in solution (Fig. 4*A*) and in crystal (Fig. 4*B*), as it was expected, the fast phase of arsenolysis is independent of NAD^+ concentration and the second order rate constant with respect to arsenate is $k = 0.060 \pm 0.010$ mM^{-1} min^{-1}, corresponding to the value previously determined for the activated arsenolysis (12). This result implies that diffusion of NAD^+ through the crystal is fast enough not to become rate limiting for activated arsenolysis.

In contrast to the fast phase, the slow phase of arsenolysis is second order with respect to NAD^+ ($k = 0.37 \pm 0.05$ mM^{-1} min^{-1}) both in solution (Fig. 4*A*) and in the crystal (Fig. 4*B*), as it was expected for lobster FA-D-glyceraldehyde-3-phosphate dehydrogenase if the slow phase of spectral perturbation ($k = 0.33 \pm 0.05$ mM^{-1} min^{-1}, *cf.* Ref. 12 and Fig. 3, *inset*) was indeed the rate-limiting step.

Experiments performed with pig FA-D-glyceraldehyde-3-phosphate dehydrogenase gave results similar to those reported for the lobster acyl-enzyme (data not shown). In this case, however, the slow phase of arsenolysis was independent of NAD^+ concentration, in agreement with the data of Fig. 1*A* and of Fig. 3, indicating that in the case of this enzyme species, activation is limited by an isomerization step.

When the experiments were carried out on enzyme (from either lobster or pig muscle) containing two β-(2-furyl)acryloyl groups per tetramer, the relative amplitudes of the fast and the slow components of the biphasic kinetics of spectral perturbation (in solution) and of arsenolysis (both in solution and in the crystal) were almost equal. As the number of acyl groups incorporated per tetramer decreased, the relative con-

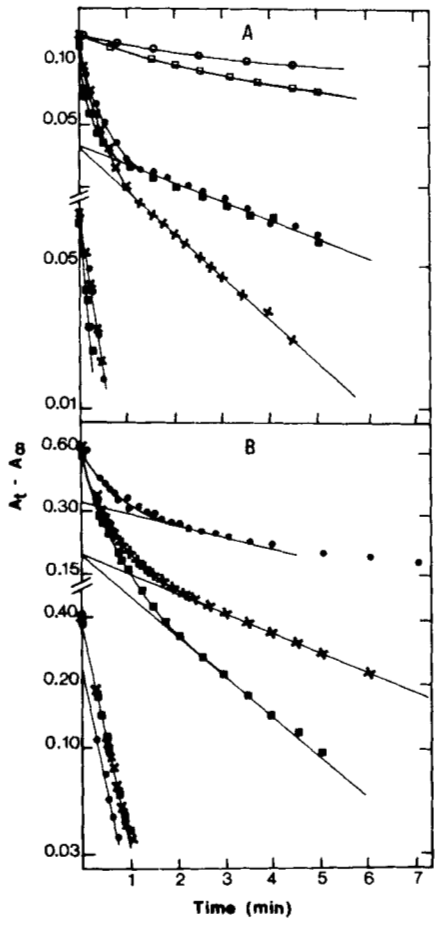

FIG. 4. **Deacylation via arsenolysis of lobster FA-D-glyceraldehyde-3-phosphate dehydrogenase limited by NAD^+ activation.** Deacylation was monitored at 360 nm both in solution (*A*) and in the crystalline state (*B*) under the conditions given in the legends to Figs. 1 and Fig. 3, respectively. *A*, the solution of 4.3×10^{-3} mM lobster FA-D-glyceraldehyde-3-phosphate dehydrogenase contained 1.4 FA groups and 2.2 mol of NAD^+/tetramer. Arsenate and NAD^+ were added simultaneously to the solution: 50 mM arsenate + 0.5 mM (●——●) or 1.3 mM (×——×) NAD^+, and 100 mM arsenate + 0.5 mM NAD^+ (■——■). In control experiments, 50 mM (○——○) and 100 mM (□——□) arsenate were applied without adding NAD^+ to show that there are no fast reacting sites in the apo-acyl-enzyme. In the presence of added NAD^+, the slow phase is independent of arsenate concentration; the second order rate constant with respect to NAD^+ is 0.37 mM^{-1} min^{-1}. The fast part is independent of NAD^+ concentration; the second order rate constant with respect to arsenate is 0.066 mM^{-1} min^{-1} (*cf.* the lower part of the figure). *B*, 50 mM arsenate was added to a single crystal of lobster FA-D-glyceraldehyde-3-phosphate dehydrogenase, obtained as described in the text, which contained 2.1 FA groups/tetramer. ●——●, Control without addition of NAD^+; ×——×, arsenate added concomitantly with 0.5 mM NAD^+; ■——■, arsenate added concomitantly with 1.0 mM NAD^+. With excess of NAD^+ (latter cases), the second order rate constant with respect to arsenate for the fast part is 0.06 mM^{-1} min^{-1}. The second order rate constant with respect to NAD^+ for the slow part is 0.4 mM^{-1} min^{-1}. The control experiment indicates that a significant fraction of acylated sites are preactivated by bound NAD^+ that has not been removed during crystal washing; however, there are no well defined fast and slow parts of the process due to the continuous decrease of rate (12). Therefore, to obtain the intercept on the ordinate corresponding to the amplitude of fast reaction, the rate constant of activated arsenolysis (0.06 mM^{-1} min^{-1}) was taken into account and the *solid straight line* was found to be the best fit to represent the slow phase. The amplitude of the fast phase observed in the control experiment was subtracted from the total amplitude of the fast phase in the experiment with added NAD^+ in order to correct for the presence of pre-activated molecules.

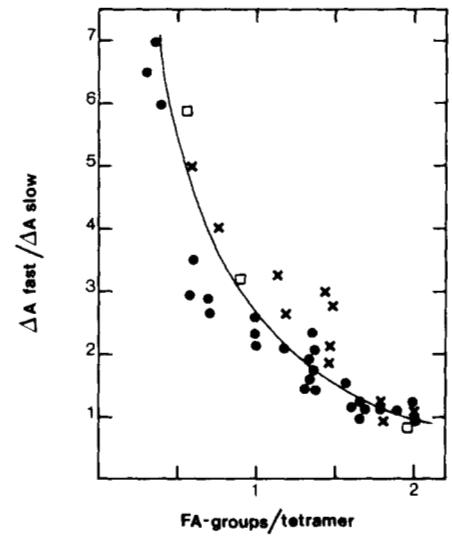

FIG. 5. **Dependence of the relative amplitudes of the fast and slow phases on acyl content in the biphasic activation process of FA-D-glyceraldehyde-3-phosphate dehydrogenases.** Lobster and pig FA-D-glyceraldehyde-3-phosphate dehydrogenases containing different numbers of acyl groups per tetramer were prepared as described under "Experimental Procedures." In the *crystal* of lobster FA-D-glyceraldehyde-3-phosphate dehydrogenases, the kinetics of arsenolysis was followed as described in the legend to Fig. 4*B*. The ratio of amplitudes of the fast and slow phases (□——□) was calculated by taking into account the contribution of the fast part in the control experiment due to the NAD^+ initially present as indicated in the legend for Fig. 4. The data given for *solution* comprise the NAD^+-limited arsenolysis (*e.g.* Fig. 4*A*) and spectral perturbation (*e.g.* Fig. 1*A*) experiments both with pig (●——●) and lobster (×——×) acylenzymes. Data points obtained by measurements of either arsenolysis or spectral perturbation of acylenzyme in solution are not characterized by different symbols because the two methods were invariably found to give results identical within the experiment error.

tribution of the slow component progressively decreased, as shown by the data reported in Fig. 5. This indicates that the heterogeneity of the molecular population may be somehow correlated with the amplitudes of the fast and slow processes.

DISCUSSION

As shown previously for the lobster (*P. vulgaris*) enzyme (12), the kinetics of acylation of pig muscle holo-D-glyceraldehyde-3-phosphate dehydrogenase by FAP and the kinetics of arsenolysis of the acyl-enzyme at saturating concentrations of NAD^+ were essentially the same in the crystal and in solution in the presence of high salt concentrations. Although the salt decreased the rates of all investigated reactions, it did not influence the basic features of the overall reaction mechanism. A salt-induced structural change of the enzyme was excluded and the rate-reducing effect was attributed to specific binding of anions to the enzyme-active centers (12).

The results presented in this paper describe the NAD^+-dependent activation mechanism of the acyl-enzyme, *i.e.* the process linking the two elementary steps of acylation and deacylation previously investigated. We present the results on both the pig and lobster muscle enzymes because the two species exhibit different kinetic behaviors. The biphasic character of acyl-enzyme activation, reflected both by the kinetics of spectral perturbation by NAD^+ and by the kinetics of arsenolysis, indicates that at least two distinct classes of acylated sites are present in solutions of pig (Fig. 1) and lobster (Fig. 4*A* and *cf.* also Ref. 12) enzymes. The slow component of the process is NAD^+ concentration independent in the case of pig enzyme, as in the case of sturgeon FA-D-

glyceraldehyde-3-phosphate dehydrogenase (20). Thus, a slow protein isomerization is kinetically demonstrated in at least two enzyme species. The second order character of the slow component, observed earlier with lobster FA-D-glyceraldehyde-3-phosphate dehydrogenase (12), indicates that for this enzyme species, NAD^+ binding becomes the rate-limiting step in the activation process.

The stopped flow experiments show that the fast component of spectral perturbation by NAD^+ corresponds to an NAD^+ concentration-independent first order process (fast protein isomerization) not only in the case of pig (Fig. 1*B*), but also in the case of lobster enzyme (data not shown). This means that NAD^+ binding to this class of sites must be even faster than the isomerization. Therefore, the difference in reactivity of acylated sites toward NAD^+, in the case of both species, should be pre-existing rather than NAD^+ induced. The possibility that the first NAD^+ bound interferes with the binding of a second NAD^+ molecule because of steric hindrance seems out of question in view of the known quaternary structure of the enzyme (21–23).

In principle, the observed biphasicity could be explained on the basis of two alternative models by assuming either acylation-induced site heterogeneity within the tetramer or an allosteric equilibrium between tetramer conformations.

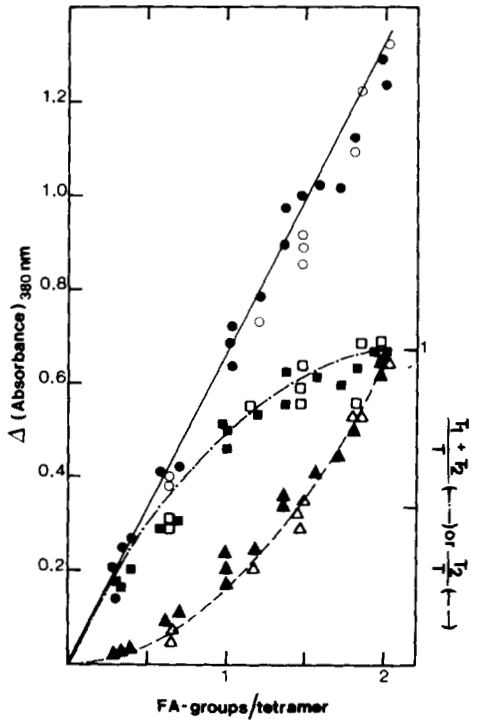

FIG. 6. **A comparison of the calculated distribution of mono- and diacylated tetramers with the amplitudes of the fast and slow phases of spectral perturbation caused by NAD^+ on FA-D-glyceraldehyde-3-phosphate dehydrogenases at different average degrees of acylation.** Amplitudes of the fast (□·—·□, ■·—·■) and slow (△- - -△, ▲- - -▲) parts of spectral perturbation as well as the total (○——○, ●——●) spectral change were determined in solutions of both lobster (*open symbols*) and pig (*filled symbols*) FA-D-glyceraldehyde-3-phosphate dehydrogenase containing different numbers of acyl groups per tetramer. The experimental conditions are given in the legend to Fig. 1. Essentially identical values for the relative amplitudes of fast and slow phase could be obtained by measuring arsenolysis limited by NAD^+ activation of acyl-enzyme in solution and in the crystal (Fig. 5). The relative concentration of the sum of mono- (T_1) and diacylated (T_2) tetramers with respect to the total (T) tetramer concentration (*i.e.* $T_1 + T_2/T$; ·—·) as well as the relative concentration of diacylated tetramers (T_2/T; - - -) was calculated at different average degrees of acylation, assuming a binomial distribution of acylated subunits.

As Fig. 5 shows, the relative amplitudes of the fast and slow phases depend on the number of acyl groups bound per tetramer. Since the amplitude of the fast phase dominates at low acyl content and the amplitudes of the two phases become equal when two sites are acylated per tetramer (Fig. 5), the following hypothesis was formulated. The monoacylated tetramers (T_1) react with NAD^+ in a single fast reaction, but only one of the two acyl sites of the diacylated tetramers (T_2) possesses the same high reactivity, while the other site reacts slowly. This hypothesis was tested by the plot shown in Fig. 6. While the total change of spectral perturbation is obviously proportional to the average acyl content, the values of the amplitudes of the fast and slow phases are clearly related to the calculated binomial distribution of the molecular species acylated to various extents. The assumption of a binomial distribution of acyl groups within tetramers is justified by the observation that acylation occurs as a monophasic process even when two acyl groups per tetramer are incorporated (12). The amplitude of the fast phase follows the same dependence on the average acyl content as the sum of the concentrations of mono- and diacylated molecules and the amplitude of the slow phase is proportional to the concentration of the diacylated molecules. In accordance to this model, it appears, therefore, that the acylated sites within the diacylated tetrameric molecule are not equivalent with respect to the NAD^+-dependent activation. The first acyl group incorporated into the tetrameric molecule is more easily activated by NAD^+ than the second one. This unequivalence (site heterogeneity) might be due to distinct conformational changes taking place at the two sites in the course of acylation.

The allosteric model, based on the assumption of NAD^+-dependent interconversion between active and inactive conformations of symmetric acyl-enzyme molecules, with distinct equilibrium constants for the mono- and diacylated species has been proposed and discussed by Malhotra and Bernhard (24, 25).

According to our data on activated arsenolysis (12), it appears that after saturation of acyl sites with NAD^+, deacylation is monophasic both in the case of lobster and pig acyl-enzyme. Thus, if all of the sites are saturated with NAD^+, no cooperativity (or heterogeneity) between the acylated sites can be detected. It follows that binding of NAD^+ is the important factor in controlling either the intermolecular equilibrium between different conformations or the intramolecular symmetry transitions. In this connection, it should be noted that site heterogeneity with respect to arsenolysis and in the uv and resonance Raman spectra has been reported for the NAD^+-saturated furylacryloyl enzyme prepared from rabbit (*but not sturgeon*) muscle D-glyceraldehyde-3-phosphate dehydrogenase (26).

As the biphasicity in the mechanism of activation of the furylacryloyl enzyme by NAD^+ (Fig. 4*B*) and the acyl content dependence of the relative amplitudes of the fast and slow steps (Fig. 5) are detected also in the crystal, the same conformational changes are expected to take place in the crystalline state. In fact, in the crystal of pig FA-D-glyceraldehyde-3-phosphate dehydrogenase, the observed spectral perturbation (Fig. 3) was a NAD^+ concentration-independent first order process. Thus, the lattice forces do not prevent the conformational changes accompanying NAD^+ binding, at least to the acyl-enzyme formed upon reaction of D-glyceraldehyde-3-phosphate dehydrogenase with the substrate analog FAP.

Although in the case of crystalline lobster D-glyceraldehyde-3-phosphate dehydrogenase we do not observe a first order process, directly suggesting a NAD^+-induced protein isomer-

ization, the above data (biphasic activation kinetics and acyl content dependence of the relative amplitudes of the two phases) show the presence of site heterogeneity and an activation mechanism basically similar to that observed for the pig enzyme. It is noteworthy that the difference in the kinetic behavior of pig and lobster D-glyceraldehyde-3-phosphate dehydrogenases, possibly due to dissimilar intersubunit interactions operating in D-glyceraldehyde-3-phosphate dehydrogenase tetramers isolated from different sources, is maintained in the crystalline state.

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