THE MOTION OF CYTOCHROME b_5 ON LIPID VESICLES MEASURED VIA TRIPLET ABSORBANCE ANISOTROPY

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We have replaced the iron atom in the heme group with the chemically similar atom rhodium (1). Unlike iron-protoporphyrin IX, rhodium-protoporphyrin IX has a reasonable triplet yield easily measured via extinction coefficient changes in the Soret region. However, like iron-protoporphyrin, the rhodium-protoporphyrin can axially ligate to the protein, thus making it an ideal probe to study the protein motion via anisotropy decay of the triplet state.

We have inserted our rhodium-protoporphyrin IX into the intrinsic membrane protein, cytochrome b_5, and used the anisotropy decay of the triplet state excited by the pulse from a nitrogen laser-driven dye laser to measure the motion of the cytochrome b_5 molecule on the membrane surface. Possible values for orientation of the porphyrin plane relative to the membrane surface and the rotational mobility of the protein in the lipid are found.

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SPECTRAL INTERMEDIATES IN THE ACTIVATION OF GLYCERALDEHYDE-3-PO_4-DEHYDROGENASE-CATALYZED REACTIONS

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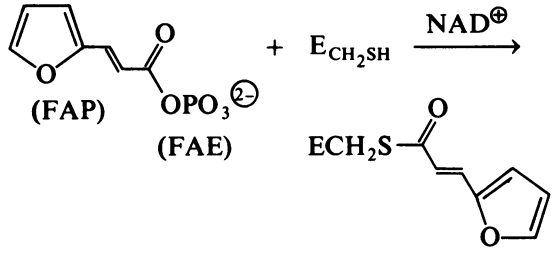
In the presence of the effector, $NAD^{\oplus}$, glyceraldehyde-3-PO_4-dehydrogenase (GPDH) reacts rapidly with the substrate (or product) analogue, β-(2-furyl)acryloyl phosphate

TABLE I

λ_m of Various Furylacryloyl Derivatives (FA-X)

X	λ_m
	(nm)
OH	310
$O^{\ominus}$	292
OCH_3	309
$NHCH_3$	301
H	322
SCH_20	337
SCH_2E (apo)	346

(FAP) to form an acyl-enzyme (FAE) (Eq. 1) (1). The electronic character of the covalently-attached ligand to the furylacryloyl chromophore contributes substantially to the energy of the first electronic transition (Table I).



Although slower in rate than the enzymic reaction with true substrate by about one or two orders of magnitude, the otherwise kinetically stable reagent (FAP) reacts rapidly and uniquely with the enzyme at the SH of Cys 149 and demonstrates kinetic biphasicity in the resultant spectral perturbations (1) (Fig. 1). The rate of the fast phase of reaction depends on the concentrations of both FAP and $NAD^{\oplus}$. The faster rate saturates in $[NAD^{\oplus}]$ at concentrations consistent with the known equilibrium dissociation constants for $E[NAD^{\oplus}]$ ($\sim 1\ \mu M$) (2). The faster rate saturates only at high concentrations of FAP (~ 10 mM). The slower phase velocity is independent of both [FAP] and $[NAD^{\oplus}]$, provided that substrate and effector are in substantial excess over enzyme sites. The amplitude of the slow phase is $[NAD^{\oplus}]$-dependent, reaching a saturation value at high $[NAD^{\oplus}]$ (~ 5 mM).

A partial resolution of these complex phenomena is derivable from studies of the equilibrium and kinetic properties of the isolated acyl-enzyme (FAE). Equilibrium studies (3) indicate that although the dissociation of $NAD^{\oplus}$ from the enzyme is small ($K_d \sim 1\ \mu M$); the dissociation of $NAD^{\oplus}$ from the acyl-enzyme is much greater ($K_d \sim 50\ \mu M$). Hence at intermediate $[NAD^{\oplus}]$ ($\sim 10\ \mu M$) the chemical reaction is approximated by Eq. 2:

$$E(NAD^{\oplus}) + FAP \rightleftharpoons EFA + NAD^{\oplus} + P_i. \qquad (2)$$

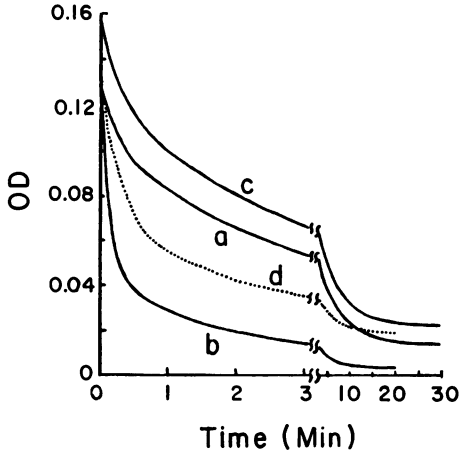


FIGURE 1 Kinetics of deacylation of FA-GPDH. In every case the reaction was started with a small volume of acceptor and monitored at 360 nm. Protein and $NAD^{\oplus}$ were preincubated. Concentration conditions are as follows: (*a*) 2.4 μM protein with 2.08 FA groups per mole enzyme, 25 μM $NAD^{\oplus}$, and 0.5 mM arsenate; (*b*) 2.4 μM protein with 2.08 FA groups per mole enzyme, 250 μM $NAD^{\oplus}$ and 0.5 mM arsenate; (*c*) 4.1 μM protein with 2.08 FA and two carboxymethyl groups per mole enzyme, 65 μM $NAD^{\oplus}$, and 0.5 mM arsenate; (*d*) 3.1 μM protein with 1.83 FA groups per mole enzyme, 131 μM $NAD^{\oplus}$, and 0.5 mM phosphate (some of the curves have been shifted along the vertical scale for clarity).

At higher [$NAD^{\oplus}$], the binding of $NAD^{\oplus}$ to the acyl (FA)-enzyme can be detected by its effect on the FAE absorption spectrum (3) (Table I). [$NAD^{\oplus}$] influences (increases) the amplitude, but not the velocity, of the slow step of acylation, suggesting that this slow phase is a consequence of the reaction of the sequence of Eq. 3.

$$\text{FAE} \overset{\text{SLOW}}{\rightleftharpoons} \text{FA*E} \overset{\text{FAST}}{\rightleftharpoons} \text{FA*E (NAD}^{\oplus}\text{)}. \qquad (3)$$

These speculations can be tested directly by studying the kinetics of interaction of the acyl-enzyme with the effector [$NAD^{\oplus}$] in the absence of any acyl acceptor (HPO_4^{2-}, $HAsO_4^{2-}$, or NADH). The kinetics of spectral perturbation upon addition of $NAD^{\oplus}$ to the FA enzyme show biphasic behavior. The fast phase of reaction requires stopped-flow techniques for its quantitative measurement, whereas the slow phase reaction has a half-life of the order of 1 min. The amplitudes for each step are wavelength-dependent (Fig. 2). The overall equilibrium of Eq. 3 has been investigated spectrophotometrically as well (Fig. 2). At equilibrium, the reaction shows only two spectrally discernible species (Table I) with a single isobestic point at 360 nm (3). Indeed, the acylation of GPDH by FAP exhibits monophasic pseudo-first-order kinetics at this wavelength (1). From the wavelength dependence of stopped-flow experiments on the interaction of apo-FA enzyme with $NAD^{\oplus}$, the kinetic isobestic point wavelengths can be determined for each of the two readily separable steps of acyl enzyme perturbation. These isobestic points differ from one another (Fig. 2) and from the equilibrium isobestic point. The spectually identifiable species in the interaction of

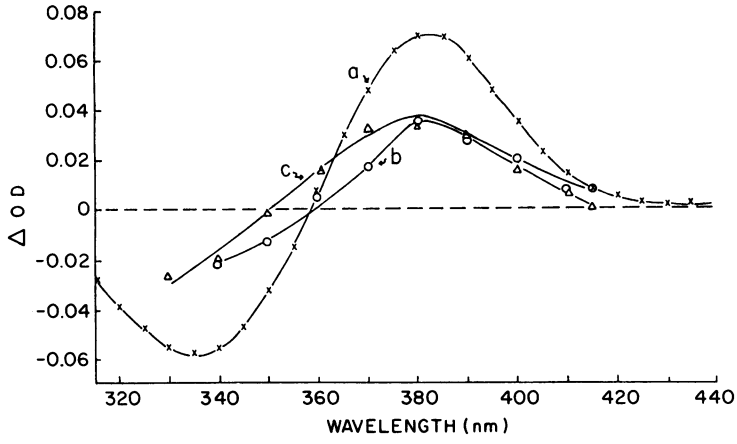


FIGURE 2 (*a*) Total ΔOD FAE + 1 mM $NAD^{\oplus}$ vs. FAE, after equilibrium (X). (*b*) ΔOD after the fast step after $NAD^{\oplus}$ addition (o—o). (*c*) ΔOD after the slow step; OD after slow step – OD after fast step (Δ).

$NAD^{\oplus}$ with acyl (FA) enzyme at equilibrium are summarized in Table II. The qualitative and quantitative kinetic data allow an estimation of the number and chemical bonding properties of addition transient acyl-enzyme intermediates. These analyses identify three spectral components; EFA (λ_m = 340 nm) EFA* (λ_m = 350 ± 10 nm), and EFA*(NAD^+) (λ_m = 360 nm). The equilibrium apo acyl-enzyme (λ_m = 346 nm) is a mixture of EFA and EFA*. The fast component of reaction is the reversible formation of EFA*($NAD^{\oplus}$) from EFA*, and the slow NAD^+-independent rate is the rate of the isomerization reaction (Eq. 2). "Red-shifts" in the longest wavelength absorption bands of the furylacryloyl chromophore are associated with increased polarity of the carbonyl (4) [(+)C—O(–)] and hence with greater susceptibility to nucleophilic attack, for example by HPO_4^{2-}. This enhanced nucleophilicity is inherent in the conformational isomerization of the acyl enzyme to a state (EFA*), which is stabilized by the noncovalent interaction with the effector ($NAD^{\oplus}$), only the red-shifted acyl-enzyme (EFA*) is catalytically competent towards nucleophilic attack.

The conformational isomerization (Eq. 3) limits the overall turnover of the enzyme.

TABLE II
IDENTIFICATION OF BOUND DINUCLEOTIDE TO VARIOUS SITES OF THE DIACYL ENZYME TETRAMER (FA_2E_4)

Dinucleotide	Site	Diagnostic
$NAD^{\oplus}$	Free SH	Racker band[6] λ_m = 360 nm
$NAD^{\oplus}$	$FASCH_2E$	λ_m shifts from 340 to 360 nm
NADH	Free SH	Spectral shift at 275 nm Fluorescence of NADH quenched
NADH	$FASCH_2E$	λ_m shifts from 240 to 328 nm

There is a stoichiometric limitation of one FA group per two subunits (1).

Hence, the addition of HPO_4^{2-}, AsO_4^{2-}, or NADH[5] to FA enzyme at suboptimal concentrations shows a common slow phase of reaction velocity with a specific rate or approximately 0.01 s^{-1}, as in the slow phase of spectral perturbation. A common limiting rate of turnover has been observed in the saturated rates with glyceraldehyde-3-PO_4 or 1,3 diphosphoglycerate (2) presumably due to a corresponding conformational change in the intermediate 3-phosphoglyceroyl enzyme. In this case, the specific rate is approximately 10^3-fold faster, thus indicating a synergistic effect of acyl-structure and protein tertiary structure.

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STUDIES OF THE ACTIVATION OF YEAST ENOLASE BY METALS USING A "TRANSITION STATE ANALOGUE"

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Yeast enolase, a dimeric protein, binds up to 2 mol of "conformational" Mg, which enables up to 2 mol of substrate or competitive inhibitor to bind, which in turn enables more Mg to bind. The latter Mg produces the actual catalysis. A putative "transition state analogue," aminoenolpyruvic acid-2-phosphate (AEP), synthesized by Spring and Wold, exhibits a large 295-nm difference spectrum upon binding to enolase with Mg present, permitting the monitoring of binding of AEP, conformational Mg, "catalytic" Mg, and other metals. Spectrophotometric titrations and stopped-flow measurements have led to some tentative conclusions:

(*a*) The strength of catalytic Mg and AEP binding is interdependent, consistent with an ordered sequence of addition. With saturating AEP, about two-thirds of the 295 nm absorbance change occurs on addition of "conformational" Mg, the rest on adding catalytic Mg (Fig. 1).

(*b*) The nonactivating metals Ca, Hg, and Ba do not give this 295-nm absorbance change though at least Ca binds at the same sites as Mg, with a similar affinity and effect on the protein. Ni (a weak activator), Mn (intermediate), and Mg (best) give