PERSISTANCE OF THE EFFECTS OF REGULATORY REAGENTS ON CONFORMATION OF CATALYTICALLY INACTIVE GLUTAMATE DEHYDROGENASE

K. Lemone Yielding, M.D.
Laboratory of Molecular Biology
University of Alabama Medical Center
Birmingham, Alabama

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Glutamate dehydrogenase has served as an interesting model for the study of small molecule regulation of enzyme activity, because it is influenced by several classes of reagents (including hormonal steroids (Yielding and Tomkins, 1960), purine nucleotides (Frieden, 1959, 1963), metal ions (Yielding, Tomkins, and Trundle, 1963), and thyroxin (Wolff, 1962)); and is readily available in highly purified form for study. Evidence that has been cited to support the idea that this enzyme possesses regulatory sites distinct from catalytic loci includes: kinetic effects; dissimilarity between regulator molecules and substrates; and interference with regulation by such reagents as organic mercurials under conditions where enzyme activity is unaffected (Bitensky, Yielding, and Tomkins, 1965).

Colman and Frieden (1966) have shown recently that acylation of the enzyme in the presence of nucleotide prevents the concentration dependent aggregation of the enzyme (which had provided the most convenient means of following changes in enzyme conformation evoked by regulatory reagents). Acylation in the absence of nucleo-

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tide also results in loss of catalytic activity. Recent studies have revealed that glutamate dehydrogenase binds the fluorescent dye anilinonaphthalene sulfonate, with a resultant large increase in fluorescence intensity and a shift in emission maximum from about 520 to 470 mμ. Furthermore, reagent induced changes in enzyme conformation were reflected in the intensity of this enzyme–dye fluorescence; thus, providing a convenient means of following such reagent effects (Thompson and Yielding; Dodd and Radda, 1967). It was thought that this technique might permit study of the effects of reagents on the conformation of the enzyme even after blocking catalytic activity and enzyme aggregation. The present communication reports the effects of regulatory reagents on enzyme–dye fluorescence using enzyme which had been inactivated by acetic anhydride.

MATERIALS AND METHODS

Glutamate dehydrogenase, type I was obtained from Sigma as a suspension in $(NH_4)_2SO_4$. After centrifugation the enzyme was dissolved in the appropriate buffer to an enzyme concentration of 1–2 mg/ml($2–4 \times 10^{-5}$M); acetic anhydride in .025 ml aliquots was added to 20 ml of the buffer solution of enzyme at 0°; and the pH carefully maintained at 7.4 by means of the Radiometer pH stat.

The ammonium salt of 8–anilinonaphthalene sulfonate was obtained from Baker Chemical Company and used without further purification. Its absorption and fluorescence spectra were identical with the recrystallized Mg salt used in previous experiments (Thompson and Yielding, 1967). Other reagents were commercial preparations of the highest purity available.

Fluorescence assays were performed with the Aminco–Bowman Spectrophotofluorometer using activation and emission wavelengths of

370 and 470 mμ. All measurements were corrected for free enzyme, free dye, and other added reagents.

RESULTS

Effect of enzyme acylation on enzyme enhancement of ANS fluorescence.

Under conditions of these experiments addition of 50λ–75λ of acetic anhydride to 20 ml of the enzyme in a concentration of 2 mg/ml resulted in more than 99 percent loss of catalytic activity. When the treated enzyme was added to a solution of ANS, there occurred the same type of increase in fluorescence intensity and shift in emission wavelength of the ANS as occurred with addition of the untreated enzyme. Acylation resulting in loss of catalytic activity did not, therefore, abolish the binding of ANS.

Effects of 'regulatory reagents' on acylated enzyme.

The effects of various reagents on the fluorescence of enzyme–ANS mixtures are shown in Table I. As described previously, diethylbesterol and Zn^{++} each increased the fluorescence of a mixture containing native enzyme and ANS; while ADP and L-leucine decreased the fluorescence (Thompson and Yielding, 1967). As shown, all these effects persisted after acylation of the enzyme. Thus, acylation, while destroying catalytic activity, did not abolish the response of the enzyme to regulatory reagents.

Effect of enzyme acylation on NADH binding.

The importance of the cofactor NADH in modifying the effects of various regulatory reagents has been emphasized (Frieden, 1959, 1963; Tomkins, Yielding, Talal, and Curran, 1963), and a separate regulatory function proposed for the nucleotide. Recent studies involving ANS fluorescence have shown that regulatory reagents

TABLE I

Effects of regulatory reagents on fluorescence of mixtures of native or acetic anhydride treated enzyme and 8-anilinonaphthalene sulfonate. Enzyme was treated as described in text and diluted to a final concentration of 0.2 mg/ml in 0.1M phosphate buffer, pH 7.4 with 10^{-3}M EDTA for experiments 1-3; and Tris HCl pH 7.4 in experiment 4.

Reagent	Percent Change in Enzyme-ANS Fluorescence on Addition of Reagent	
	Native enzyme	Treated enzyme
1. Diethylstilbestrol 4×10^{-5}M	+210	+465
2. L-Leucine 4×10^{-3}M	-65	-58
3. ADP 1.6×10^{-4}M	-45	-11
4. $ZnCl_2$ 3.2×10^{-5}M	+33	+61

can evoke changes in the enzyme in the absence of the nucleotide (Thompson and Yielding, 1967). Since the acylated enzyme is inactive but still responsive to regulatory reagents, it was of interest to see whether it could still bind NADH. Accordingly, equilibrium dialysis was performed using a concentration of NADH of 10^{-3} and enzyme at 2×10^{-4}M, and there was no evidence of nucleotide binding. Under these conditions binding by 1 enzyme chain in 25 would have been detected. These results show that NADH binding is

not required for diethylstilbesterol or Zn^{++} to influence enzyme conformation.

Effects of acylation on enzyme fluorescence.

The very interesting question arises whether acylation of the enzyme results in drastic changes in its tertiary structure. This question was explored by examining the effects of such treatment on the native fluorescence of the enzyme itself. This experiment is shown in Table II. Thus, acylation of the enzyme results in a depression of its fluorescence. Colman and Frieden (1966) had shown previously that the concentration dependent aggregation is changed by such treatment, but no detectable change in optical rotatory dispersion was observed.

TABLE II

Enzyme	Enzyme Fluorescence
Native	80.5
Acylated	62.5

Effect of acetic anhydride on fluorescence of glutamate dehydrogenase. Enzyme solution contained 2 mg/ml enzyme protein in 0.1M PO4 buffer, pH 7.4 containing 10^{-3}M EDTA. Acetic anhydride was added in 2 – 25λ increments to 10 ml of this stock solution at 10 min. intervals at 0° while maintaining the pH at 7.4. Fluorescence measurements were made using an activation and emission settings of 290 and 350 respectively; and enzyme concentrations of 1.2 mg/ml. Fluorescence is expressed in arbitrary units.

CONCLUSIONS

These experiments employing limited acylation of glutamate dehydrogenase show that its catalytic activity can be removed selectively without destroying its ability to bind a variety of regulatory reagents. These results provide additional strength for the earlier conclusion that such regulatory reagents interact at specific non-substrate sites and evoke changes in enzyme tertiary structure (Tomkins and Yielding, 1961; Frieden 1959, 1963; Yielding and Tomkins 1960, 1961). These interactions and resulting conformational changes, while not directly dependent on the catalytic center, may result in alterations in enzyme kinetic properties.

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