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Reduced Triphosphopyridine Nucleotide Binding Sites of the Fatty Acid Synthetase of Chicken Liver*

Robert Y. Hsu and Barbara J. Wagner

ABSTRACT: Purified chicken liver fatty acid synthetase (a multienzyme complex) forms a tight binary complex with reduced triphosphopyridine nucleotide. This binding is accompanied by enhancement of nucleotide fluorescence, a small red shift of excitation maximum from 345 to 353 mμ, and a blue shift of emission maximum from 460 to 447 mμ. Stoichiometric titration of the enzyme with reduced triphosphopyridine nucleotide yields a binding weight of 119,700 (±2,400) g of protein/mole of reduced triphosphopyridine nucleotide, and a dissociation constant of 0.29 (±0.016) μM. From a molecular weight of 500,000, four reduced triphosphopyridine nucleotide binding sites were calculated for the native enzyme. Reduced diphosphopyridine nucleotide does not exhibit significant binding. Acetylcoenzyme A appears to be bound at a site different from the reduced triphosphopyridine nucleotide binding site. Potassium maleate caused dissociation of the enzyme complex into subunits approximately equal in size. Inactivation of the native enzyme by dialysis against phosphate buffer at low concentration and dissociation by treatment with maleate resulted in the loss of up to two reduced triphosphopyridine nucleotide sites. The latter treatment also produced significantly lower fluorescence yield and a tenfold increase in dissociation constant of the binary ligand complex.

Fatty acid synthetase exists as a multienzyme complex in yeast and higher animals (Lynen, 1967a,b; Hsu *et al.*, 1965; Burton *et al.*, 1968; Smith and Abraham, 1969). It contains all of the enzyme components required for the synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and TPNH. Confirmation of individual enzyme activities within this complex has been achieved through the use of model substrates (Lynen, 1961, 1967b) and characterization of acyl intermediates (Lynen, 1961, 1967b; Brodie *et al.*, 1964; Goldman and Vagelos, 1962). However, resolution of these compounds in active form has not been achieved.

Recent studies on the substrate binding sites of the yeast, pigeon, and rat liver complexes (Lynen, 1961, 1967b; Jacob *et al.*, 1968; Burton *et al.*, 1968) indicated that acetyl and malonyl moieties from respective coenzyme A thioesters are bound covalently to sulfhydryl groups on the enzyme molecule, with 4′-phosphopantetheine and cysteine playing significant roles in the binding process. Additional information suggests that the hydroxyl group of serine may also be involved in the initial phase of binding (Lynen, 1967b). TPNH, the third substrate for the overall synthetic reaction, is a coenzyme for β-keto acyl-acyl carrier protein reductase and enoyl-acyl carrier protein reductase, both components of the multienzyme (Majerus and Vagelos, 1967; Lynen, 1967b). These enzymes carry out reduction of the respective acyl intermediates with concomitant oxidation of TPNH in a manner analogous to other dehydrogenases. At the present, information on TPNH binding sites on these enzyme complexes is not available.

Fluorescence titration has been used as an effective tool in a study of binding sites for other pyridine nucleotide linked dehydrogenases (Udenfriend, 1962; Jarabak and Sack, 1969; Hsu and Lardy, 1967). Formation of the enzyme-reduced pyridine nucleotide binary complex usually results in significant enhancement of nucleotide fluorescence which can be used for the stoichiometric titration of nucleotide binding sites. These titration studies yield useful information on the number and nature of binding sites on these enzymes and provide insight to their mechanism of action. An elegant theoretical treatment of ligand binding to proteins was described by Weber (Weber and Anderson, 1965; Anderson and Weber, 1965; Weber, 1965).

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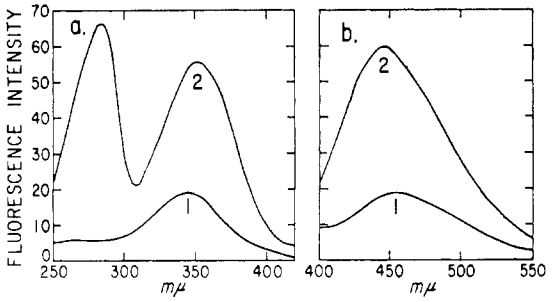

FIGURE 1: Nucleotide excitation and emission spectra of free TPNH and enzyme–TPNH binary complex. (a) Excitation spectra, emission was measured at 460 mμ. (b) Emission spectra, excitation was measured at 350 mμ. (1) Cuvet contained 100 μmoles of Tris (HCl) buffer at pH 7.0 and 2.8 mμmoles of TPNH in a total volume of 2.0 ml. (2) Enzyme (0.2 ml, 0.932 mg in 20% glycerol) was added to cuvet 1.

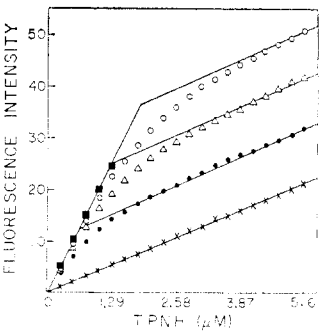

FIGURE 3: Titration of enzyme with TPNH. All cuvets contained 100 μmoles of Tris (HCl) buffer at pH 7.0, water, and the following amounts of enzyme in 20% glycerol: (×—×) 0, (●—●) 0.33 mμmoles (0.167 mg), (△—△) 0.66 mμmole (0.334 mg), (○—○) 0.99 mμmole (0.501 mg), and (■—■) 1.65 mμmoles (0.833 mg) in a total volume of 2.0 ml. Temperature 25°. TPNH was added in 0.01-ml aliquots. The initial slope was obtained from fluorescence measurements at the highest enzyme concentration where all TPNH is in bound form. Excitation was at 350 mμ; emission at 460 mμ.

Previously, it was not feasible to carry out TPNH titration on the native pigeon liver enzyme due to its inherent instability and rapid dissociation into subunits (Hsu *et al.*, 1965; Yang *et al.*, 1967). The availability of substrate amounts of chicken liver fatty acid synthetase completely stabilized in the presence of glycerol (Hsu and Yun, 1970), however, allowed us to carry out a study of the TPNH binding properties of the native enzyme with minimal contribution from inactivated as well as dissociated enzyme molecules. The present paper describes the fluorescence spectra of the protein molecule and its TPNH complex, the stoichiometry and fluorescence yield of binding. The effects of inactivation and dissociation of the enzyme complex on its TPNH sites are also examined and discussed.

Materials and Methods

The following biochemicals were purchased from the sources specified below: TPNH, DPNH, acetyl-CoA, malonyl-CoA, and dithiothreitol (P-L Biochemicals); ethylenediaminetetraacetic acid (tetrasodium salt) and Tris (Calbiochem); glycerol (Mallinckrodt); maleic acid (Sigma); and [1-^{14}C]-acetyl-CoA (New England Nuclear Corp.). Deionized, distilled water was used throughout these experiments. Nucleotide solutions were made up fresh daily in 0.01 M potassium phosphate buffer (pH 8.0), and the concentrations of TPNH and DPNH were determined spectrophotometrically with the use of molar extinction coefficient of 6.2×10^3 at 340 mμ.

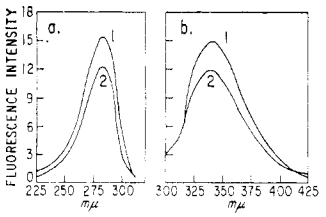

FIGURE 2: Protein excitation and emission spectra of enzyme and enzyme–TPNH binary complex. (a) Excitation spectra, emission was measured at 340 mμ. (b) Emission spectra, excitation was measured at 285 mμ. (1) Cuvet contained 100 μmoles of Tris (HCl) buffer at pH 7.0 and 0.466 mg of enzyme in 20% glycerol in a total volume of 2.0 ml. (2) TPNH solution (0.2 ml, 11.2 mμmoles) was added to cuvet 1.

Purified chicken liver fatty acid synthetase was prepared according to the accompanying paper (Hsu and Yun, 1970). Homogeneity of each preparation was checked by velocity sedimentation in the Model E ultracentrifuge before use.

For studies on the native enzyme, stabilization was accomplished by the addition of 20% (v/v) glycerol to the purified preparation, and stored under N_2 in a tightly capped tube at 4°. Glycerol had no effect on the fluorescence of TPNH or enzyme. Activity assays were performed on the stored enzyme at frequent intervals to assure complete stabilization. Glycerol, however, was not added to enzyme used in inactivation and dissociation studies.

Sedimentation velocity experiments were carried out in the Spinco Model E analytical ultracentrifuge at 20° and 52,000 rpm unless otherwise specified. Fatty acid synthetase activity was measured according to Hsu *et al.* (1965). Protein concentration was determined spectrophotometrically at 279 mμ in matched cuvets using an extinction coefficient of 0.965 for a 0.1% protein solution (Hsu and Yun, 1970).

Fluorescence studies were carried out with the Aminco spectrofluorometer equipped with a thermojacketed cuvet holder. Temperature was controlled with a constant-temperature circulator to give 25°. Sensitivity setting of the instrument was varied between experiments according to the type and concentration of fluorescent material (*i.e.*, protein or nucleotide); therefore, direct comparison of fluorescence yield was made only on experiments employing the same sensitivity and slit settings. Fluorescence readings from titration studies were corrected for volume changes.

Results

Fluorescence Spectra of Free TPNH, Free Enzyme, and Enzyme–TPNH Binary Complex. The nucleotide excitation and emission spectra of free TPNH (cuvet 1) and enzyme–TPNH binary complex (cuvet 2) are shown in Figure 1a,b. The spectra for bound TPNH were determined with an excess of enzyme (ratio of TPNH equivalents: enzyme/TPNH =

TABLE I: Binding Weight and Dissociation Constant of Native Fatty Acid Synthetase–TPNH Binary Complex.

Expt	Enzyme Preparation	Titrant	Other Compounds	Binding Weight (10^5 g of protein/mole TPNH)	Dissociation Constant (μM)
1	I	TPNH		1.24	
				1.24	0.33
2	II	TPNH		1.02	
				1.07	0.33
				1.18	
3	II	TPNH		1.03	
				1.05	0.33
				1.23	
4	II	TPNH		1.18	0.29
				1.34	
5	II	TPNH		1.29	
				1.30	0.25
				1.34	
6	III	TPNH		1.18	
				1.18	0.22
				1.31	
7	III	TPNH		1.14	0.29
				1.22	
				Mean 1.197 ± 0.0242[a]	Mean 0.29 ± 0.016[a]
8	I	TPNH	Acetyl-CoA (5 μM)	1.12	0.33
				1.10	
				1.18	
9	III	Enzyme		1.08	
10	IV	Enzyme		1.40	0.33
11	III	Enzyme		1.17	
				1.14	
				Mean 1.194 ± 0.054[a]	

[a] Mean ± standard error.

2.7, calculated on the basis of four TPNH binding sites, see Discussion) so that free TPNH concentration was minimal. Free TPNH exhibits an excitation peak at 345 mμ. Upon the addition of enzyme, a small red shift occurred and the new maximum was at 353 mμ. Fluorescence intensity was enhanced about threefold. A new excitation peak appeared at 285 mμ. The emission maximum for free TPNH was at 460 mμ. Enzyme binding was accompanied by enhancement of fluorescence and a small blue shift to a new emission maximum at 447 mμ. The fluorescence effect was specific for TPNH. When DPNH was used at similar concentrations no fluorescence enhancement was observed (DPNH, 3.13 μM; enzyme, 0.5 μM).

The protein excitation and emission spectra of fatty acid synthetase (cuvet 1) and its binary TPNH complex (cuvet 2) are presented in Figure 2a,b. Cuvet 2 contained TPNH in excess (ratio of TPNH equivalents: TPNH/enzyme = 3.0) so that free enzyme concentration was minimal. The free protein had an excitation maximum at 285 mμ and an emission maximum at 340 mμ. Addition of TPNH caused a slight quenching of fluorescence (20%) but no change in either the excitation or emission maximum.

Stoichiometric Titration of Enzyme with TPNH. The fluorescence enhancement of TPNH resulting from the formation of binary enzyme–TPNH complex was very large (300%) and was used to determine the stoichiometry of binding. Titrations were made by the addition of small aliquots of TPNH to cuvets containing either buffer or the enzyme solution. Figure 3 shows a typical titration experiment. In this experiment, three levels of enzyme were used. Addition of TPNH to buffer alone (lower line) yielded linear increases in fluorescence intensity. In the presence of enzyme, the addition of aliquots of TPNH initially produced large increases in fluorescence value, owing to the higher fluorescence yield of bound TPNH. When the titration was complete, further increments of TPNH were not bound, and the slope became parallel to the free TPNH line. At high protein concentrations a linear, limiting initial slope was found, indicating that added TPNH was totally bound. The intercept points between this limiting slope and final slopes for different protein concentrations were measured. TPNH equivalents estimated from the extrapolated intercepts were proportional to the amount of protein in each case. Binding weights (grams of protein per mole of TPNH) were calculated to be 129,000, 130,000, and 134,000 for 0.33, 0.66,

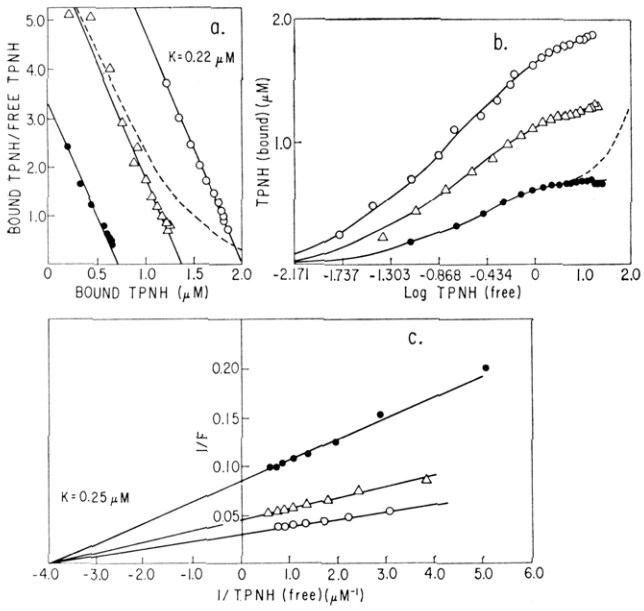

FIGURE 4: (a) Scatchard plot of data from Figure 3. (b) Log plot of data from Figure 3. (c) Adelstein plot of data from Figure 3.

and 0.99 mμmoles of enzyme, respectively. Titrations were repeated with different enzyme preparations. Results of these titrations are shown in Table I. The mean binding weight was calculated to be 119,700 ± 2,420.

Experimental data from Figure 3 were further analyzed for binding sites of different affinities by several plotting methods (Edsall and Wyman, 1958) as shown in Figure 4. These methods are selected to amplify the nonequivalence of binding sites. Theoretical curves for two sets of binding sites with different dissociation constants are shown for both the Scatchard and logarithmic plots (dotted lines, Figure 4a,b). The experimentally determined points did not conform to these theoretical curves, but were fitted into linear Scatchard plots (Figure 4a) giving 4.1 binding sites with a dissociation constant of 0.22 μM or simple titration curves by the logarithmic method (Figure 4b), therefore suggesting a set of equivalent binding sites for TPNH. The dissociation constant can be

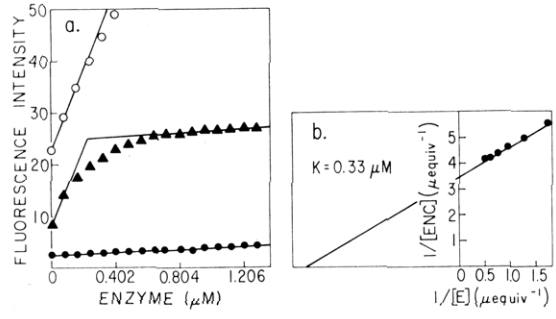

FIGURE 5: Titration of TPNH with enzyme. All cuvets contained 100 μmoles of Tris (HCl) buffer (pH 7.0), water, and the following: (●—●) no TPNH, (▲—▲) 1.507 mμmoles of TPNH, and (○—○) 5.03 mμmoles of TPNH in a total volume of 2.0 ml. Titration was carried out with purified enzyme in 20% glycerol. Excitation was at 350 mμ; emission at 460 mμ. (a) Titration curve. (b) Reciprocal plot.

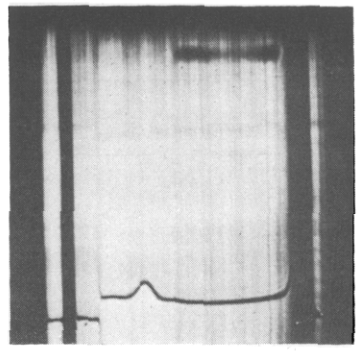

FIGURE 6: Schlieren pattern of maleate treated enzyme, speed 44,770 rpm. The picture was taken 46 min after reaching speed. The purified enzyme (1 ml, 6.4 mg) was dialyzed against 500 ml of potassium maleate buffer (0.1 M) KCl (0.1 M) (pH 6.9) for 65 hr and stored at 4° for 48 hr before use.

estimated with more precision from an Adelstein plot (Adelstein, 1965; Figure 4c). Lines for different enzyme concentrations converge at the abscissa giving a dissociation constant of 0.25 μM. An average value of 0.29 ± 0.016 μM, estimated from seven different experiments, is presented in Table I. The linearity of Adelstein plots further demonstrates the equivalency of binding sites.

In one titration experiment, the effect of acetyl-CoA was examined by inclusion of this compound (5 μM) in the reaction mixture. However, neither the number nor the dissociation constant of TPNH binding was affected (Table I).

Titration of TPNH with Enzyme. Coenzyme titration was also carried out in the reverse direction with enzyme as the titrant. The binding curve is shown in Figure 5a. An average binding weight of 119,400 ± 5,400 (Table I) was calculated from five separate measurements. Assuming four independent binding sites, a plot of the reciprocals of concentrations of binary complex versus free enzyme should yield a straight line intersecting at a point equal to $-1/k$ on the abscissa (Hsu and Lardy, 1967). As shown in Figure 5b and Table I, a straight line was obtained giving a dissociation constant of 0.33 μM. The binding weight and dissociation constant are in close agreement and further substantiate values obtained previously.

TPNH Binding Characteristics of Inactivated and Dissociated Enzyme. In the absence of glycerol native chicken liver fatty acid synthetase inactivates and dissociates in a manner comparable with the pigeon enzyme (Butterworth *et al.*, 1967), but at a slower rate.

Inactivation of the overall synthetic activity was obtained by aging of freshly purified enzyme (without added glycerol) and by dialysis against phosphate buffer of low ionic strength. Irreversible dissociation was obtained by maleate treatment. Figure 6 shows the sedimentation pattern of the purified enzyme after dialysis against maleate buffer. A single, slowly sedimenting peak (s = 7.5) was obtained at the expense of the native 11.85S peak. Results of these experiments are summarized in Table II, together with the mean values of native enzyme for comparison.

As shown in Table II, all three treatments resulted in the complete loss of overall synthetic activity. TPNH binding properties of the aged enzyme were not altered. Dialysis and maleate treatment, however, produced decreases in fluorescence value. This decrease may be due to a reduction in

TABLE II: Titration of TPNH Binding Sites on Inactivated and Dissociated Chicken Liver Fatty Acid Synthetase.

Enzyme	Expt	Number of TPNH Binding Sites	k (μM)	Activity (% of Native Enzyme)	Sedimentation Pattern
Native[a]	I	4.2	0.29	100	One peak, 11.8 S
Aged[b]	II	3.8, 4.0, 4.0	0.29	<1	One peak, 12.7 S
Dialyzed (1)[c]	III	3.0, 3.2	0.4	<1	
Dialyzed (2)[c]	IV	2.7	0.4	5	
Dialyzed (3)[c]	V	2.4	0.33	<1[e]	Two peaks, 80% 14.0 S, 20% 7.4 S
Maleate treated[d]	VI	3.1, 3.0	4.0	<1	Two peaks, 6% 10.1 S, 94% 6.2 S

[a] Mean values obtained from Table I. [b] Purified enzyme stored under N_2 at 4° in 0.25 M potassium phosphate buffer, 1 mM EDTA, and dithiothreitol (pH 7.0), for 1 month. [c] Purified enzyme dialyzed at 4° against 0.005 M potassium phosphate buffer (pH 7.0), 1 mM EDTA with no dithiothreitol. (1) Dialysis time, 24 hr. The dialyzed enzyme was stored at 4° for 48 hr. (2) Dialysis time, 32 hr. The dialyzed enzyme was stored at 4° for 18 hr. (3) Dialyzed enzyme from 2 was stored at 4° for a total of 10 days. [d] Purified enzyme (1.9 ml, 8.1 mg/ml) was dialyzed against 0.1 M potassium maleate–0.25 M potassium phosphate–1 mM EDTA (pH 7.0) for 25 hr at 4°, and stored in the cold for 13 days before use. [e] Forty-three per cent of original activity was restored by incubating the inactive enzyme solution (0.0342 mg/ml) in 0.25 M potassium phosphate buffer, 1 mM EDTA (pH 7.0) containing 6 mM dithiothreitol at 37° for 1 hr. Reassociation of the 7.45S peak was observed.

binding sites, lower affinity of TPNH for the enzyme, or a decrease in fluorescence yield of the binary complex. To further investigate these possibilities, the above binding parameters were calculated (Table II and Figure 7): Fluorescence yield was calculated according to Tomkins *et al.* (1962). The dialyzed enzyme lost one binding site rapidly (2 days). Further reaction was slower and an additional 0.6 site was lost after 10 days, suggesting a difference in the rate of inactivation of these binding sites. The fractional binding site represents an average of enzyme molecules at different stages of inactivation. Binding affinity was not changed. Dialyzed enzyme also slowly dissociated. This process was reversible, and incubation with 6 mM dithiothreitol for 1 hr caused reassociation of subunits concomitant with partial restoration of overall activity. Dissociation by maleate was accompanied by loss of one binding site, and more than tenfold decrease in the affinity for TPNH. Furthermore, a significant decrease in fluorescence yield was observed (Figure 7).

Discussion

Free TPNH exhibits major excitation and emission peaks at 345 and 460 mμ, respectively. The excitation peak corresponds to the absorption maximum at 340 mμ. These excitation and emission maxima differ as much as 20 mμ from previously reported wavelengths (Langan, 1960; Hsu and Lardy, 1967) probably reflect differences in instrumentation. The new excitation maximum at 285 mμ accompanying formation of a tight protein–nucleotide complex has been observed previously (Hsu and Lardy, 1967). This was contributed by protein absorption. The resulting emission at 460 mμ could be due either to intramolecular energy transfer from the protein to the reduced nucleotide, or to an intermolecular process, as the protein emitted strongly at the excitation wavelength of TPNH, or both. In these titration experiments, free TPNH concentration was minimal and the former must be the predominant phenomenon. The fluorescence enhancement and the small blue shift of fluorescence emission maximum due to the formation of the binary complex are characteristic of many pyridine nucleotide linked enzymes (Udenfriend, 1962) and indicated higher fluorescence efficiency for the enzyme-bound nucleotide.

Chicken liver fatty acid synthetase carries out the synthetic reaction with DPNH at a much slower rate (unpublished results) analogous to the pigeon enzyme (Hsu *et al.*, 1965). Titration of enzyme with DPNH, however, did not produce enhancement of nucleotide fluorescence. This is probably due to a low affinity of DPNH for the enzyme resulting in the lack of significant binding under the conditions of the experiment.

Chicken liver fatty acid synthetase exhibits strong protein fluorescence at excitation and emission wavelengths of 285

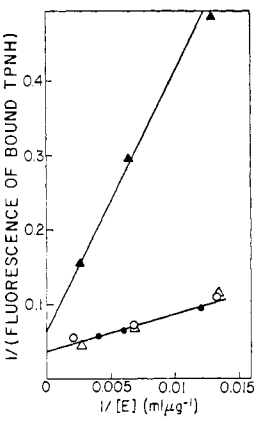

FIGURE 7: Fluorescence yield of binary complexes: (●—●), native enzyme, (○—○) aged enzyme, (△—△) dialyzed enzyme, and (▲—▲) maleate-treated enzyme. Data used in these calculations were from experiments presented in Table II.

and 340 mμ, respectively. The 285-mμ excitation band corresponds to its 279-mμ absorption maximum. Formation of the nucleotide binary complex did not alter the excitation or fluorescence maxima. The fluorescence quenching was also observed for other dehydrogenases (Hsu and Lardy, 1967; Velick, 1958). It could be the result of energy transfer to the reduced nucleotide which fluoresces maximally at 460 mμ (Figure 1b) or from a change in protein conformation upon coenzyme binding (Shifrin *et al.*, 1959).

The same binding weight and dissociation constant were obtained by stoichiometric titration of TPNH whether TPNH or enzyme was used as the titrant. From a molecular weight of 500,000 (Hsu and Yun, 1969) and a binding weight of 119,700, the number of TPNH binding sites was estimated to be 4. The small number of tightly bound TPNH molecules is consistent with specific binding at the active site of the enzyme. It is reasonable to assume that in the present study, both reductase components in the enzyme complex were titrated, since active sites of analogous enzymes in the yeast complex are accessible for TPNH in activity measurements (Lynen, 1961). The possibility of nonequivalent binding sites from two distinct enzymes was further examined by various plotting methods. The Scatchard, logarithmic, and Adelstein plots were in agreement with each other and were consistent with a single set of four independent and equivalent sites. Contribution of binding sites from two distinct enzymes, however, precludes the exact equivalency of these sites. Precision of the present technique is reasonably good and should pick up differences in affinity within a twofold range. Lack of detectable differences, therefore, must lead to the conclusion that differences in these binding sites are small and within the limits of detection mentioned above.

Acetyl-CoA covalently binds sulfhydryl groups on the pigeon liver synthetase in the absence of TPNH (Brodie *et al.*, 1964). Present study indicates that TPNH binding is independent of acetyl-CoA since the latter did not alter TPNH binding properties. This observation is in accord with separate and noninteracting binding sites for these two substrates.

Dialysis of freshly purified enzyme against phosphate buffer at low concentration or against maleate buffer resulted in the dissociation of the native complex into subunits with s values of 6.2–7.4 S. This implies that the native enzyme may be composed of two subunits similar in size as found for other animal enzymes (Butterworth *et al.*, 1967; Burton *et al.*, 1968; Smith and Abraham, 1969). The simplest arrangement compatible with two subunits would be for each subunit to contain a complete set of enzyme components including β-keto acyl-acyl carrier protein reductase and enoyl-acyl carrier protein reductase, and that the active site of each reductase binds one TPNH molecule. The existence of two subunits and only one 4′-phosphopantetheine (Jacob *et al.*, 1968) are not necessarily mutually exclusive. Nevertheless, they point out a molecular arrangement more complex than indicated above.

In the absence of glycerol as a protective agent, fatty acid synthetase activity was completely lost during aging, dialysis, and maleate treatment. Aged enzyme retained full TPNH binding capability indicating that loss of overall activity was not due to loss of TPNH binding. For the dialyzed enzyme, progressive loss of binding sites was observed during storage of the dialyzed enzyme prior to dissociation, and nearly two binding sites were lost after 10 days. Alteration of protein structure was most extensive for the maleate-treated enzyme. Dissociation of the enzyme into subunits was extensive and irreversible as compared with a reversible process for the aging and dialysis treatments. In addition to the loss of one binding site, significant decrease in binding affinity and fluorescence yield occurred suggesting extensive changes in the conformation at the active site. It may be pointed out that initial inactivation for all three treatments probably involves the alteration of a limited number of sulfhydryl groups, as titration of less than ten sulfhydryl groups with DTNB (Ellman, 1959) resulted in complete loss of activity (unpublished data). Loss of TPNH sites, however, occurred at a much slower rate. This could be due to the destruction of buried, and less available sulfhydryl groups responsible for TPNH binding. It also could be the result of a secondary and nonspecific denaturation process as a consequence of alteration of sulfhydryl groups. During all these inactivation treatments, at least two TPNH binding sites remained. Therefore, these four equivalent binding sites reported here may, nevertheless, differ in their stability characteristics.

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Ethoxyformylation of Proteins. Reaction of Ethoxyformic Anhydride with α-Chymotrypsin, Pepsin, and Pancreatic Ribonuclease at pH 4*

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ABSTRACT: The use of ^{14}C-labeled ethoxyformic anhydride has shown that this reagent rapidly acylates "accessible" imidazole and amino groups in proteins at pH 4. *N*-Ethoxyformylimidazole has a half-life of 55 hr at pH 7, 25°. At pH 4, one ethoxyformyl group is introduced into pepsin at all reagent concentrations tested. The site of modification is the α-amino group. There is no reaction with the single histidine residue and no change in enzymic activity. In ribonuclease, three of the four histidine residues react rapidly at about the same rate. The fourth histidine residue appears to be completely inaccessible.

The ethoxyformyl groups can be removed in minutes at pH 7 with 0.1 M hydroxylamine, with recovery of enzymic activity. At pH 4 several amino groups in ribonuclease are as reactive as the imidazole groups. At pH 4 and 10^{-4} M reagent, one ethoxyformyl group is introduced into α-chymotrypsin at the active-site serine residue. At higher concentrations amino groups are ethoxyformylated. The reactivity of the two histidine residues in chymotrypsin is very much depressed compared with that of the histidines in ribonuclease.

Ethoxyformic anhydride is at the same time an ester and an anhydride; because of resonance involving the ester function, it is less reactive than many anhydrides. Ethoxyformic anhydride has been introduced recently as a food preservative due to its bactericidal action. This anhydride is ideal for this purpose because in aqueous solution it hydrolyzes to ethanol and carbonic acid with a half-life of about 25 min near neutrality.

This paper presents results of an exploratory study of the reaction of ethoxyformic anhydride with bovine α-chymotrypsin, bovine pancreatic ribonuclease, and swine pepsin at pH 4. We were led to these experiments by the report of Fedorcsák and Ehrenberg (1966) that ethoxyformic anhydride inactivates ribonuclease and trypsin. Recently ethoxyformic anhydride has been employed to inactivate nucleases in the preparation of RNA (Solymosy *et al.*, 1968), to inactivate arginine and creatine kinases (Pradel and Kassab, 1968), and to modify actin (Mühlrad *et al.*, 1969). We have investigated the reaction of radioactive ethoxyformic anhydride with proteins in more detail and find that ethoxyformic anhydride has a specificity of reaction which, although similar, is significantly different from other acylating agents used in protein modification studies.

Experimental Section

[1-^{14}C-ethyl]Ethoxyformic Anhydride. Xylene and ether were dried over sodium wire. Small pieces of sodium were melted in dry refluxing xylene to remove the oxide coating. Sodium sand was prepared immediately before use by shaking the desired quantity of cleaned sodium in about 20 ml of hot xylene. Absolute ethanol was dried by reaction of water with magnesium ethoxide (Fieser, 1955).

Sodium sand (467 mg, 20.3 mmoles) was washed twice with dry ether and transferred in 15 ml of ether to a dry 50-ml flask placed in an ice bath and outfitted with a dropping funnel, gas inlet tube, and a Dewar condenser filled with ice and protected with a $CaCl_2$ drying tube. Dry ethanol (974 mg, 21.1 mmoles) was used in portions to transfer 1 mCi of [1-^{14}C]ethanol (ICN, 20 mCi/mmole) to the dropping funnel. Magnetic stirring and a stream of carbon dioxide through a concentrated sulfuric acid bath and then into the ether were started. The ethanol–ether was added over a period of 4 min and the dropping funnel was washed with two 2-ml portions of ether. The reaction mixture was then allowed to stand at room temperature for 4 hr. After the ether had been removed at reduced pressure, freshly

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[1] Abbreviations used are: *N*-Ac-L-TyrEt, *N*-acetyl-L-tyrosine ethyl ester; RNase, pancreatic ribonuclease. Although *Chemical Abstracts* lists ethoxyformic anhydride, European chemists tend to use the name diethyl pyrocarbonate for this compound. In view of the pattern of reactions with nucleophiles, *i.e.*, acylation of nucleophiles or ethoxyformylation, the name ethoxyformic anhydride is more appropriate.