Investigation of Reduced Nicotinamide Adenine Dinucleotide Phosphate and Acyl-Binding Sites on Avian Fatty Acid Synthase†

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ABSTRACT: The binding of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to chicken liver fatty acid synthase has been studied by using both fluorescence titrations and the direct binding method of forced dialysis. Four apparently identical sites are found per enzyme molecule, with an intrinsic dissociation constant of 0.6 μM at pH 7.0, 23 °C. The acyl-binding sites on the enzyme have been studied with a fluorescent analogue of acetyl-CoA, β-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)]alanyl coenzyme A (NBDA-CoA). The enzyme slowly transfers NBDA to acyl-binding sites. Analysis of the kinetics of binding and of the stability and hydroxylamine sensitivity of the acyl-enzyme at pH 7.5 suggests that binding occurs predominantly at two classes of sulfhydryl sites, with two sites of each class per enzyme molecule. Up to one NBDA per enzyme molecule is bound to a nonsulfhydryl site after overnight incubation of enzyme with NBDA-CoA. The acyl linkage at one class of sulfhydryl sites appears to be hydrolyzed by the thioesterase activity of the enzyme. This hydrolysis can be prevented by modifying the enzyme with tosyl fluoride. The binding of NBDA is inhibited by acetyl-CoA, malonyl-CoA, and NADPH. The NBDA–enzyme adduct is inactive, although activity can be partially restored by incubation at 35 °C. The binding of NADPH to the enzyme is not significantly altered by the binding of NBDA. Fluorescence resonance energy transfer between enzyme-bound NADPH and enzyme-bound NBDA suggests that the acyl-binding sites are 30–40 Å from the NADPH-binding sites. This distance can only be defined approximately because of the presence of multiple energy donors and acceptors and the uncertainty in the dipole–dipole orientations of the energy acceptors and donors.

Fatty acid synthase multienzyme complexes catalyze the formation of palmitic acid (or palmitoyl-CoA)[1] from malonyl-CoA and acetyl-CoA. The reaction proceeds by the successive condensation of two carbon units from malonyl-CoA on to an acetyl group that originally was esterified with CoA. Each condensation requires a series of catalytic processes: first, the malonyl group is transferred from CoA to the enzyme; second, the malonyl group is combined with the C_{2n} fatty acyl group already bound to the enzyme with concomitant loss of CO_2; next, the β-ketoacyl group is reduced with NADPH; then, the resultant intermediate is dehydrated and reduced with NADPH to yield the fully saturated acyl group [cf. Volpe & Vagelos (1973) and Bloch & Vance (1977)]; finally, the palmitoyl-enzyme is hydrolyzed and free palmitic acid is released, or the palmitoyl moiety is transferred to CoA and palmitoyl-CoA is released. The overall reaction can be written as

$$\text{acetyl-CoA} + 7\text{malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \rightarrow \text{CH}_3\text{-}(\text{CH}_2\text{CH}_2)_7\text{-COOH} + 8\text{CoA} + 7\text{CO}_2 + 14\text{NADP}^+ + 7\text{H}_2\text{O}$$

In *Escherichia coli*, the fatty acid synthase is composed of several polypeptide chains, each catalyzing an individual reaction in the total cycle (Vagelos et al., 1966). In yeast, the fatty acid synthase is composed of two different polypeptide chains, α and β, arranged in an $\alpha_6\beta_6$ structure (Kresze et al., 1976). Each polypeptide chain has multiple but different enzymatic activities (Bloch & Vance, 1977). In animals, the enzyme consists of two multifunctional polypeptide chains, each with an M_r of 220000–270000 (Smith & Abraham, 1970; Kumar et al., 1972; Yun & Hsu, 1972; Buckner & Kolattukudy, 1976). The enzyme can be dissociated to monomeric polypeptide chains, with most of the catalytic activities relatively intact (Kumar et al., 1970). However, whether the two polypeptide chains are identical or nonidentical is not yet clear. Recent evidence suggests the polypeptide chains are structurally and functionally identical (Arslanian et al., 1976; Buckner & Kolattukudy, 1976; Poulose et al., 1980; Stoops & Wakil, 1981). The structure and organization of the various catalytic sites are not known but must be quite complex.

In this work, a fluorescent analogue of acetyl-CoA, NBDA-CoA, is used to characterize functionally important acyl-binding sites on the chicken liver enzyme. The binding of NADPH to the enzyme also has been reexamined by using both forced dialysis and fluorescence titrations. The distance between acyl-binding sites and NADPH-binding sites has been estimated by resonance energy transfer measurements, with NADPH as the energy donor and NBDA as the energy acceptor.

Materials and Methods

Chemicals. NADPH, NBD-Cl, dithiothreitol, poly(ethylene glycol) (M_r 6000), acetyl-CoA, malonyl-CoA, and CoA were from Sigma. Dicyclohexylcarbodiimide and β-alanine were obtained from Eastman. β-[^{14}C]Alanine was from New England Nuclear. All other reagents were high-purity commercial grades, and all solutions were prepared with deionized distilled water.

Preparation of Fatty Acid Synthase. Six-week-old chickens were fasted for 3 days and then fed a fat-free diet for 3 days prior to killing. The livers were removed immediately and a high-speed centrifugation supernatant was prepared as previously described, except that the homogenization buffer was at pH 7.5 rather than at pH 8.0 (Hsu & Yun, 1970). The high-speed supernatant had a protein concentration of about

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[1] Abbreviations: NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; NBDA, β-[*N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)]alanine; EDTA, ethylenediaminetetraacetic acid; NADPH, reduced nicotinamide adenine dinucleotide phosphate; CoA, coenzyme A; DEAE, diethylaminoethyl.

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60 mg/mL and an enzyme activity of about 2.5 units/mL. This material was stored under N_2 at −20 °C. Fatty acid synthase was prepared from the high-speed supernatant by a modification of the method of Linn (1981). Unless otherwise noted, all operations were performed at 0 °C, and all solutions contained 10% (w/v) glycerol, 1 mM dithiothreitol, and 1 mM EDTA. All centrifugations were done at 17500*g* for 15 min.

The high-speed supernatant (80 mL) was thawed and glycerol was added to 10% (w/v). A stock solution of poly(ethylene glycol) (25% w/v) in 50 mM potassium phosphate, pH 7.2, was added dropwise to bring the solution to 9% (w/v) poly(ethylene glycol). After 15 min, this mixture was centrifuged, the pellet discarded, and the supernatant brought to 14.5% poly(ethylene glycol) by the further addition of the stock solution. After 15 min, this mixture was centrifuged, the supernatant discarded, and the pellet suspended in 45 mL of 50 mM potassium phosphate, pH 7.5. To this solution was added a stock solution of saturated ammonium sulfate (containing 5 mM potassium phosphate, pH 7.5, and no glycerol) to bring the mixture to 38% saturation. The precipitate, obtained by centrifugation, was suspended in 25 mL of 50 mM potassium phosphate, pH 7.2. The stock solution of poly(ethylene glycol) then was added to a final concentration of 7.5% poly(ethylene glycol). After 15 min this mixture was centrifuged and the pellet discarded. The supernatant was brought to 14% poly(ethylene glycol) by the addition of a 40% (w/v) poly(ethylene glycol) stock solution in 50 mM potassium phosphate, pH 7.2. After 15 min the mixture was centrifuged, and the pellet was suspended in a minimal volume of 20 mM potassium phosphate, pH 7.5; the protein concentration was 30–40 mg/mL. This solution was applied to a DE-52 column (1.5 × 12 cm) and eluted with a 20–150 mM potassium phosphate gradient (90 mL), pH 7.5. Fractions with activities greater than 1.3 units/mg were pooled and brought to 40% saturation by the addition of saturated ammonium sulfate as described above. After centrifugation, the pellet was suspended in 2–3 mL of 0.2 M potassium phosphate, pH 7.2, and applied to a Bio-Gel A-1.5m column (1.5 × 25 cm) equilibrated with the same buffer. Fractions with activities greater than 1.4 units/mg were collected, and dithiothreitol was added to 10 mM. The enzyme was stored at −20 °C in aliquots of approximately 1 mg each. The activity was typically 1.5–1.6 units/mg, and a total of about 70 mg of the purified enzyme was obtained. Prior to use, the enzyme was thawed and passed through a Sephadex G-50 centrifuge column (Penefsky, 1977) or a small (1.5 × 6 cm) Sephadex G-50 column to remove dithiothreitol and/or to change the buffer.

Protein concentrations were determined by using an extinction coefficient for fatty acid synthase of 0.965 cm^2/mg at 279 nm (Hsu & Yun, 1970). An M_r of 500 000 was assumed to calculate the protein molarity (Hsu & Yun, 1970).

Fatty acid synthase activity was measured spectrophotometrically at 25 °C in 0.2 M potassium phosphate, pH 7.0, 1 mM EDTA, 100 μM NADPH, 100 μM acetyl-CoA, and 200 μM malonyl-CoA by observing the decrease in absorbance at 340 nm. The reaction was initiated by the addition of a mixture of acetyl-CoA and malonyl-CoA. One unit is the amount of enzyme that will oxidize 1 μmol of NADPH/min at 25 °C.

The enzyme was modified with tosyl fluoride by the method of Srinivasan & Kumar (1976). Fatty acid synthase (1–5 mg/mL) in 50 mM potassium phosphate, pH 7.0, and 1 mM EDTA was incubated at 30 °C. Five equal aliquots of tosyl fluoride (100 mM in 2-propanol) were added at 30-min intervals to a final concentration of 2 mM. Unreacted tosyl fluoride was removed after 2.5 h by passing the solution through a Sephadex G-50 centrifuge column. Less than 5% of the original catalytic activity remained.

Synthesis of NBDA-CoA. NBDA was synthesized as described by Lancet & Pecht (1977). Ten millimoles of NBD-Cl in 120 mL of ethanol was mixed with 50 mmol of β-alanine in 80 mL of 0.25 M $NaHCO_3$ in H_2O at 50 °C. The pH of the mixture was maintained near 8.0 by the occassional addition of solid $NaHCO_3$. After 2 h, the mixture was centrifuged at 12000*g* for 20 min. The supernatant was evaporated to about 50 mL and the pH adjusted to 8.0. About 15 mL was applied to a DEAE-Sephacel column (2.5 × 30 cm) equilibrated with 50 mM $NaHCO_3$ and eluted with the same buffer. The eluant was monitored by observing the absorbance at 475 nm. The first (minor) peak was discarded and the second (major) peak collected and acidified with HCl to pH 2.5. This was extracted 3 times with ethyl acetate (1 volume each time), and the ethyl acetate phase was dried over $MgSO_4$ and evaporated to dryness. NBDA has a molar extinction coefficient of 24 000 M^{-1} cm^{-1} at 475 nm in water (Lancet & Pecht, 1977).

NBDA was esterified to CoA by the procedure of Rutkoski & Jaworski (1978). Forty micromoles of NBDA was dissolved in about 2 mL of absolute ethanol and dried under N_2. The powder was then dissolved in 1 mL of dry redistilled tetrahydrofuran containing 0.2 M thiophenol, and the test tube was sealed with a serum stopper. Then 1 mL of 0.2 M dicyclohexylcarbodiimide in tetrahydrofuran was added with a syringe over a period of 1 h while purging continuously with N_2. After 2 h, 25 μmol of CoA in 0.6 mL of 1:1 tetrahydrofuran and 0.8 M $KHCO_3$/0.2 M Na_2CO_3 (pH 9.2) was added dropwise with a syringe. Additional CoA was added 2 (15 μmol of CoA in 0.4 mL) and 3 h (10 μmol of CoA in 0.25 mL) after the first aliquot. The reaction was quenched by the addition of 0.25 mL of glacial acetic acid 3.5 h after the addition of the first CoA. The mixture then was dried under N_2 to a minimal volume, applied to Whatman No. 3MM chromatography paper (5 × 18 in.), and developed overnight in butanol–acetic acid–H_2O (5:3:2). The brightly colored slow-moving band was cut out and eluted at 4 °C with 10 mM acetic acid. The final purification step, primarily to remove free CoA, was high-pressure liquid chromatography on a μC_{18} column with acetonitrile as the stationary phase and 10 mM acetic acid brought to pH 4.5 with ammonium hydroxide as the mobile phase. The ratio of absorbance at 260 nm to that at 475 nm was used to determine how much free CoA was left. Since the extinction coefficient of CoA at 260 nm is 14 600 M^{-1} cm^{-1} (Dugan & Porter, 1970) and that of NBDA is about 2000 M^{-1} cm^{-1} at the same wavelength, the expected ratio is 0.69. All preparations of NBDA-CoA gave absorbance ratios in the range of 0.65–0.70. The NBDA-CoA was evaporated to a minimal volume and stored at −20 °C at a concentration of 2–5 mM. The concentration was determined by using the extinction coefficient at 475 nm given above.

The preparation of [^{14}C]NBDA followed essentially the same procedure. Forty milligrams of NBD-Cl in 1.2 mL of ethanol was added to 0.8 mg of β-[^{14}C]alanine (sp act. 57 mCi/mmol) and 2.4 mg of β-alanine in 2 mL of 0.25 M $NaHCO_3$ at 50 °C. This reaction mixture was treated as above to obtain [^{14}C]NBDA. The [^{14}C]NBDA then was reacted with about a 2-fold excess of CoA (32 μmol) by using the procedure described above, and the final product yielded 2.6 μmol of [^{14}C]NBDA-CoA with a specific activity of 1.9 × 10^4 cpm/nmol. The ratio of absorbances at 260 and 475 nm was 0.68.

Direct Binding Measurements. The binding of NADPH to enzyme was studied by the method of forced dialysis (Cantley & Hammes, 1973). The conditions were the same as those in the fluorescence titration experiments. Various amounts of NADPH (100–200 μM) were added to 0.3–0.5 mL of fatty acid synthase (0.5–1.2 μM). These solutions were then transferred to a forced dialysis apparatus, and aliquots of 25–50 μL were forced through Amicon XM-50 membrane filters with N_2. The aliquots were diluted to 200 μL, and the concentration of free NADPH was determined by comparison of the fluorescence emission with those of a series of standards of known NADPH concentrations.

Fluorescence Measurements. Fluorescence measurements were carried out on a Hitachi Perkin-Elmer MPF-44B fluorometer. The NADPH fluorescence was excited at 345 nm and the emission observed at 460 nm. Corrected spectra were obtained with the differential corrected spectra unit accessory. The quantum yield of enzyme-bound NADPH was determined by using the relationship (Parker & Rees, 1960)

$$Q_2/Q_1 = F_2A_1/(F_1A_2) \quad (1)$$

where Q_2 is the quantum yield, F_2 is the area of the corrected emission spectrum, and A_2 is the absorbance at the exciting wavelength for bound NADPH; Q_1, F_1, and A_1 are the corresponding quantities for the quantum yield standard, quinine bisulfate in 0.1 N H_2SO_4, which was assumed to have a quantum yield of 0.70 (Scott et al., 1970). Fluorescence polarization measurements were carried out for enzyme-bound NADPH and NBDA as previously described (Azumi & McGlynn, 1962; Cerione & Hammes, 1982).

The interaction of the enzyme with NADPH was monitored by fluorescence measurements. The enzyme was prepared as described and clarified by centrifugation (12000*g*, 30 min). Small aliquots (2–10 μL) of NADPH at various concentrations (10–200 μM) were added to 0.5 mL of fatty acid synthase (0.2–1.0 μM). One complete titration took about 30 min. All titrations were done at 23 °C in 50 mM potassium phosphate and 1 mM EDTA, pH 7.0, unless otherwise noted. As a control, aliquots of NADPH also were added to buffer without enzyme to determine the fluorescence of free NADPH.

Binding of NBDA to Fatty Acid Synthase. An aliquot of [^{14}C]NBDA-CoA was added to an enzyme solution (2–10 μM) at 35 °C in 100 mM potassium phosphate and 1 mM EDTA, pH 7.5. At various times, 50 μL of the mixture was removed and quenched by adding it to 1 mL of 0.5 M perchloric acid on ice to precipitate the protein. This suspension was filtered on a GF/F glass fiber filter, the filter was washed with 20 mL of 0.5 M perchloric acid and 10 mL of ethanol, and the radioactivity was determined in ACS scintillation fluid after the filter was dried. Alternatively, an aliquot of unlabeled NBDA-CoA was added to the enzyme. The reaction was stopped by passing a 50-μL aliquot through a 1-mL Sephadex G-50 centrifuge column. The amount of NBDA bound per mole of enzyme was determined spectrophotometrically by the ratio of the absorbance at 475 nm to that at 280 nm (by using the extinction coefficients given previously). When the amount of bound NBDA was determined both spectrally and by scintillation counting on the same sample, the results differed by less than 5%. A third method used to determine the amount of NBDA bound to the enzyme for the samples passed through the centrifuge columns was to measure the ratio of fluorescence of NBDA (excitation 480 nm, emission 530 nm) to that of the enzyme (excitation 280 nm, emission 330 nm). These ratios were converted to moles of NBDA per mole of fatty acid synthase by including an internal standard for which both the fluorescence and absorbance ratios were determined.

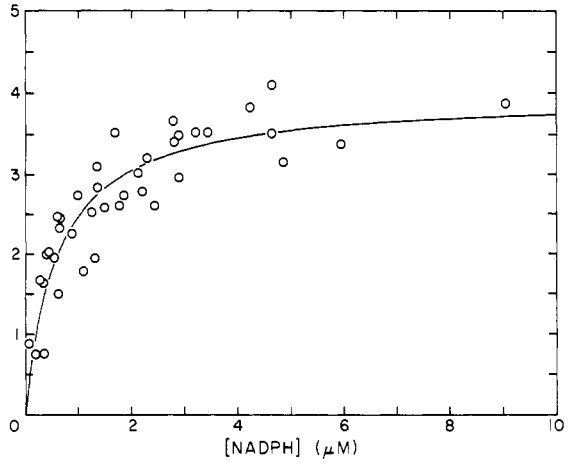

FIGURE 1: Plot of number of moles of NADPH bound per mole of fatty acid synthase, r, vs. concentration of free NADPH as determined by forced dialysis. The results of five separate experiments are shown. The enzyme concentration varied between 0.6 and 1.0 μM, and the experiments were carried out in 50 mM potassium phosphate, pH 7.0, and 1 mM EDTA at 23 °C. The line is the best fit to eq 3; n = 4.0 and K = 0.60 μM.

Resonance Energy Transfer Measurements. Enzyme with and without NBDA bound was titrated with NADPH in 50 mM potassium phosphate, pH 7.0, and 1 mM EDTA at 23 °C, and the changes in NADPH fluorescence were measured. By use of the dissociation constant and the number of binding sites determined in the NADPH-binding experiments, the fraction of enzyme-bound NADPH was determined. The efficiency of energy transfer is given by

$$E = 1 - F_{DA}/F_D \quad (2)$$

where F_{DA} is the fluorescence of NADPH when bound to NBDA-modified enzyme and F_D is the fluorescence of NADPH when bound to unmodified enzyme. The fluorescence of the bound species can be determined from the measured fluorescence of free NADPH and the calculated fraction of bound NADPH. The wavelength dependence of NADPH excitation and emission is not altered by the presence of NBDA so that the ratio of the fluorescence emission at 460 nm (345-nm excitation), F_{DA}/F_D, is proportional to the ratio of the quantum yields.

Results

Binding of NADPH by Fatty Acid Synthase. The results of five different forced dialysis experiments for which the binding of NADPH to the enzyme was determined are summarized in Figure 1. In this figure, r is the moles of bound NADPH per mole of enzyme. Because of the tightness of the binding and the small amounts of NADPH being manipulated, good data were difficult to obtain below $r \approx 2$. These results suggest that the binding can be described by the simple isotherm

$$r = n[L]/(K + [L]) \quad (3)$$

where [L] is the concentration of free ligand, K is the dissociation constant, and n is the number of binding sites per enzyme molecule. The results from the five experiments were fit to eq 3 by a nonlinear least-squares analysis. The curve in Figure 1 represents the best fit of the data; the values of n and K obtained were 4.0 and 0.60 μM. The scatter within each set of data for the five experiments is considerably less than in the summary in Figure 1, but essentially the same parameters were obtained if each data set was fit separately. The uncertainty in n is about ±10%, and that in K is about

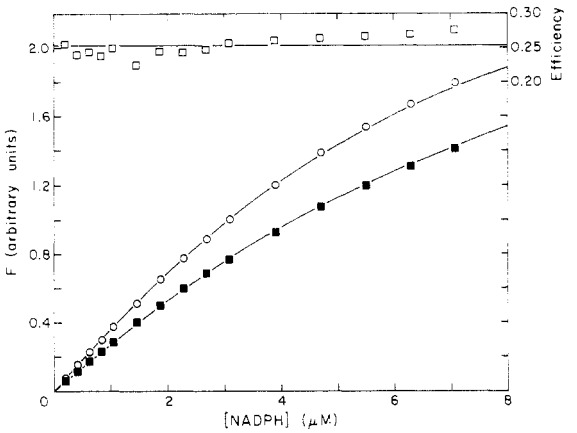

FIGURE 2: Fluorescence titrations of fatty acid synthase with NADPH in 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, and 1.04 μM enzyme at 23 °C. For the lower titration curve, the enzyme contained 2.5 NBDA/enzyme. The fluorescence, F, is plotted vs. the total concentration of NADPH, and the curves represent the best fits to eq 4 with $n = 4.0$. The efficiency of energy transfer throughout the titration is shown above the titration curves (□).

±25%. These experiments were done at room temperature, ~23 °C, in 50 mM potassium phosphate, pH 7.0, and 1 mM EDTA. The values of K and n were not significantly altered by the modification of the enzyme with tosyl fluoride or by the binding of 1 mol of NBDA/mol of enzyme.

A typical fluorescence titration curve of the enzyme with NADPH is shown in Figure 2 (upper curve). To calculate a binding isotherm from these data requires knowledge of the enhancement of the fluorescence of NADPH when bound to the enzyme. When the fluorescence enhancement is assumed to be the same for all bound ligands, the fluorescence, F, can be written as

$$F = f_0[L_f] + f_0R[L_b] = f_0[L_t] + f_0(R - 1)[L_b] \quad (4)$$

where f_0 is a constant determined by the quantum yield of the free ligand and the characteristics of the instrument, $[L_f]$ is the free ligand concentration, $[L_b]$ is the bound ligand concentration, $[L_t]$ is the total ligand concentration, and R is the ratio of the fluorescence of bound NADPH to that of free NADPH. If the binding is assumed to fit the hyperbolic isotherm of eq 3

$$2[L_b] = n[E_t] + [L_t] + K - [(n[E_t] + [L_t] + K)^2 - 4n[E_t][L_t]]^{1/2}$$

where $[E_t]$ is the total enzyme concentration. Equally good fits of the data could be obtained with $n = 3$ or $n = 4$; similar binding constants were obtained in both cases, but the enhancement ratio, R, was different. If n was assumed to be 4.0, as determined from the forced dialysis experiments, nonlinear least-squares analyses of six titrations gave values of $R = 3.6$ (±0.2) and $K = 0.70$ (±0.12) μM. The curves in Figure 2 have been calculated with these parameters. The fluorescence polarization of enzyme-bound NADPH was determined to be 0.42 under conditions identical with those for the titration experiments.

Reaction of NBDA-CoA with Fatty Acid Synthase. The reaction of NBDA-CoA with the enzyme is complicated by the fact that the enzyme catalyzes a slow hydrolysis of NBDA-CoA. The rate of binding of NBDA is sufficiently slow that the concentration of reactant changes during the course of the reaction. The amount of NBDA bound after a 2.25-h incubation of varying concentrations of NBDA-CoA with enzyme at 35 °C, in 0.1 M potassium phosphate, pH 7.5, and 1 mM EDTA, is shown in Figure 3. If the curve is interpreted as a simple binding isotherm, a dissociation constant of about 200 μM is obtained. However, this constant has no simple interpretation since the binding mechanism is complex, as indicated further below.

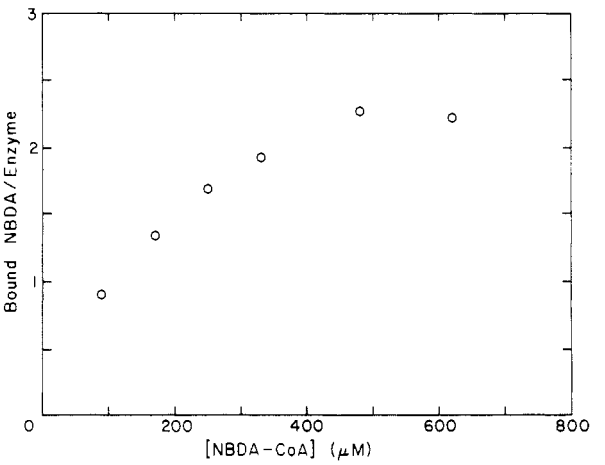

FIGURE 3: Plot of moles of NBDA bound per mole of fatty acid synthase vs. concentration of NBDA-CoA. The NBDA-CoA was incubated with 4.0 μM enzyme for 2.25 h at 35 °C in 100 mM potassium phosphate, pH 7.5, and 1 mM EDTA. After incubation, the solution was passed through a Sephadex G-50 centrifuge column, and the binding stoichiometry was determined by measurement of the absorbance at 475 and 280 nm as described under Materials and Methods.

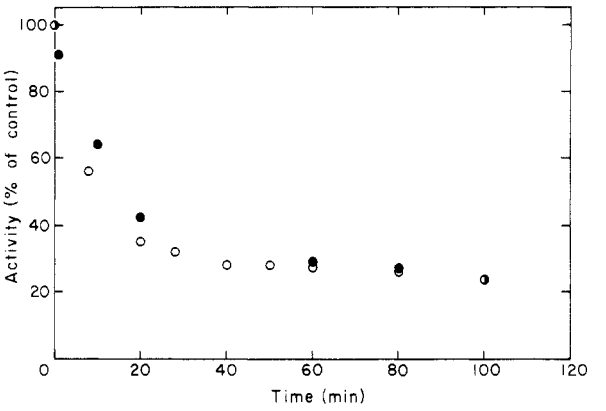

FIGURE 4: Plot of enzyme activity vs. time of incubation of 150 μM NBDA-CoA with 2 μM enzyme at 35 °C in 100 mM potassium phosphate, pH 7.5, and 1 mM EDTA. Aliquots were removed for assay at the times indicated. The aliquots were either assayed directly (○) or passed through a Sephadex G-50 centrifuge column prior to assay (●).

When NBDA binds to the enzyme, overall fatty acid synthesizing activity is lost, as shown in Figure 4. The enzyme was incubated with 150 μM NBDA-CoA in 50 mM potassium phosphate, pH 7.5, and 1 mM EDTA at 35 °C, and aliquots were removed for assay at various times. The loss of activity was the same if the aliquot was first passed through a Sephadex G-50 centrifuge column to remove excess NBDA-CoA prior to the assay. However, if the free NBDA-CoA was removed in this way and the enzyme with NBDA bound was incubated at 35 °C in 50 mM potassium phosphate, pH 7.5, and 1 mM EDTA for 15 min, part of the activity could be removed. This partial recovery varied from about 95% (for 60% active enzyme) after a short (less than 15-min) incubation with NBDA-CoA to about 37% (for 27% active enzyme) after an 80-min incubation. If enzyme with NBDA bound (1.2–1.7 mol/mol of enzyme) was treated with neutralized hydroxylamine (0.45 M, room temperature, 30 min), approximately 75% of the activity was restored, as compared with control enzyme similarly treated.

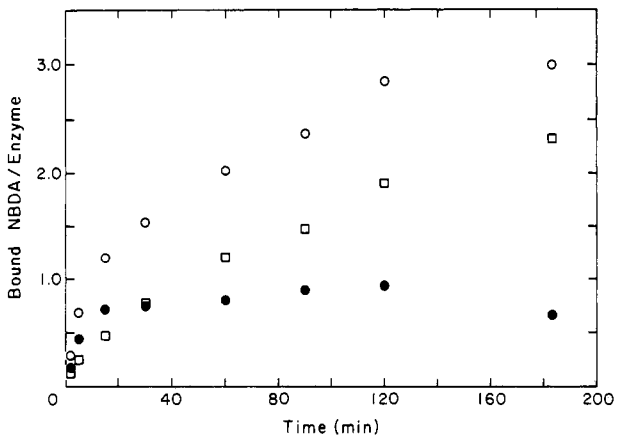

FIGURE 5: Plot of moles of NBDA bound per mole of enzyme vs. time of incubation of 450 μM NBDA-CoA with 6.5 μM enzyme in 100 mM potassium phosphate, pH 7.5, and 1 mM EDTA at 35 °C. Aliquots were removed at the times indicated and passed through a centrifuge column (○). The amount of NBDA bound was determined by measuring the ratio of NBDA fluorescence (480-nm excitation, 530-nm emission) to protein fluorescence (280-nm excitation, 330-nm emission). The aliquot was allowed to stand at 35 °C in the same buffer for 15 min. It then was passed through a second centrifuge column, and the amount of bound NBDA was determined (□). The difference between the two measurements also is plotted (●).

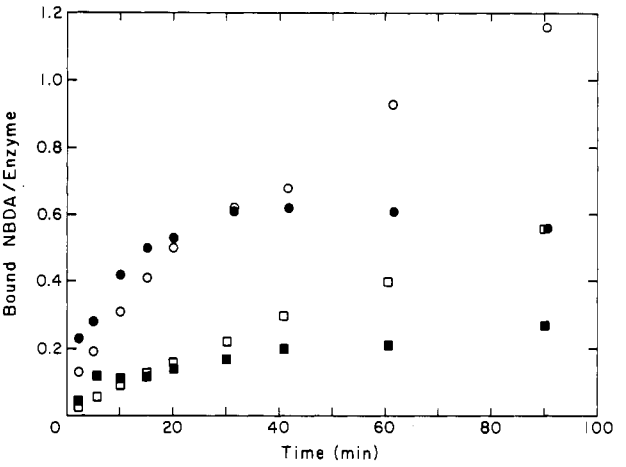

FIGURE 6: Plot of moles of NBDA bound per mole of fatty acid synthase vs. time of incubation of 120 μM NBDA-CoA with 2 μM enzyme with (□, ■) or without (○, ●) 200 μM acetyl-CoA at 35 °C in 100 mM potassium phosphate, pH 7.5, and 1 mM EDTA. The procedure used was the same as described in the legend to Figure 5. Open symbols represent the amount of NBDA bound after two passes through centrifuge columns. Filled symbols represent the amount removed by the second centrifuge column.

If the enzyme–NBDA adduct, isolated from free NBDA-CoA by a centrifuge column, is incubated at 35 °C for 15 min in 100 mM potassium phosphate, pH 7.5, and 1 mM EDTA and then passed through a second centrifuge column, a significant portion of the NBDA is lost. Third and fourth columns, however, remove very little more NBDA. Typical results of an experiment showing the amount of NBDA bound to the enzyme after one and two centrifuge columns are shown in Figure 5. The difference between these two curves also is shown. In this experiment, 450 μM NBDA-CoA and 6.5 μM enzyme were incubated at 35 °C in 100 mM potassium phosphate, pH 7.5. The amount of enzyme-bound NBDA was determined fluorometrically. Similar results were obtained over a wide range of NBDA-CoA concentrations (20–450 μM). In all cases, the less tightly bound ligand, i.e., that removed by the second column, is the predominant species at short times and plateaus within 45 min. The amount of more tightly bound ligand rises even at relatively long times (2–3 h). Both types of binding can be inhibited equally well by acetyl-CoA (200 μM) as shown in Figure 6. In other experiments, both malonyl-CoA and NADPH (25 and 30 μM) were shown to inhibit total binding although not as well as acetyl-CoA (25 μM). When both acetyl-CoA (125 μM) and malonyl-CoA (125 μM) were added together, the effect was the same as that with acetyl-CoA alone. Both acetyl-CoA and malonyl-CoA are hydrolyzed by the enzyme; therefore, the concentration of these substrates diminishes during the reaction. These results indicate that both types of NBDA binding are specific but are sufficiently complex that we are unable to derive a detailed binding mechanism. The reaction is so slow that the limiting stoichiometry of the tight binding process is uncertain because of enzyme denaturation. In addition, because of the thioesterase activity of the complex, this limiting stoichiometry does not represent the maximum stoichiometry. [For the simplest mechanism, the observed stoichiometry will be reduced by $k_b/(k_b + k_h)$, where k_b is the effective rate constant for binding and k_h is the rate constant for hydrolysis.] The maximum amount of ligand bound per enzyme was about 3.0 mol/mol.

In order that a better estimate of the stoichiometry of NBDA binding could be obtained, the hydrolysis of NBDA-CoA by fatty acid synthase was inhibited by modification of the enzyme with tosyl fluoride. This modification has been shown to inhibit selectively the thioesterase activity (Shrinivasan & Kumar, 1976; Kumar, 1975; Lin & Smith, 1978). When enzyme modified with tosyl fluoride is incubated with NBDA-CoA (0.1 M potassium phosphate, 1 mM EDTA, pH 7.5, 35 °C, 350 μM NBDA-CoA, 4 μM enzyme), the initial rate of binding is somewhat less than that for unmodified enzyme. The final amount of NBDA bound, however, is 3.6 mol/mol of enzyme (4.75 h incubation). If a second addition of NBDA-CoA (200 μM) is made after a 3.25-h incubation, the final level of bound NBDA reached after an additional 1.25 h is 4.1 mol/mol of enzyme.

The nature of the enzyme–ligand bond was examined by treating the adduct (in 100 mM potassium phosphate, pH 7.5) with neutralized hydroxylamine (0.55 M, pH 6.8, 23 °C). This should cleave a thioester but not an oxygen ester linkage. Hydroxylamine has other effects as well, however. Overall enzyme activity decreases after this treatment, and the fluorescence of NBDA is destroyed. Because of this latter effect, [^{14}C]NBDA was used to monitor the loss of covalently bound ligand. At various times, aliquots were removed from the mixture of NBDA-CoA and enzyme and added either to perchloric acid or to hydroxylamine. Those added to hydroxylamine were then incubated for 20 min, and two aliquots were removed and added to perchloric acid to determine the amount of enzyme-bound radioactivity. The results of a typical experiment are shown in Figure 7. In this experiment (160 μM [^{14}C]NBDA-CoA, 2.7 μM enzyme, 23 °C), the amount of bound NBDA that is insensitive to hydroxylamine increases to a maximum of about 1 mol/mol of enzyme for extremely long (overnight) incubations. Similar results were obtained with tosyl fluoride modified enzyme. These results are qualitative because of the other effects of hydroxylamine and because of the enzyme denaturation that occurs after long incubations. However, most of the NBDA is apparently bound as a thioester; a small amount may be bound as an oxygen ester. The maximum number of specific binding sites for NBDA on the enzyme appears to be about 4. At pH 7.0, the rate of modification of the enzyme by NBDA-CoA was slower than at pH 7.5, and the hydroxylamine sensitivity of the final adduct was similar. At pH 8, the rate of binding and the final

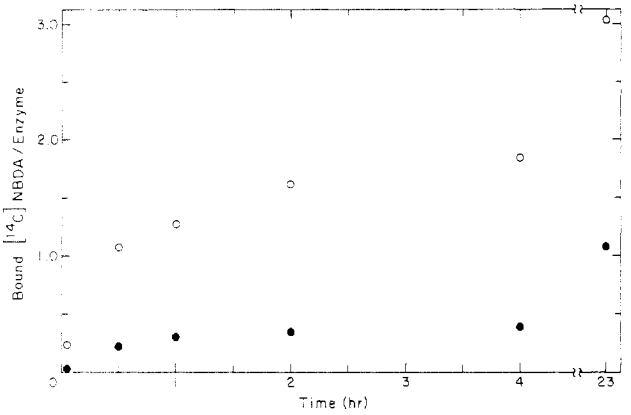

FIGURE 7: Effect of hydroxylamine on enzyme-bound NBDA. The moles of [^{14}C]NBDA bound to enzyme is plotted vs. the time of incubation of 160 μM [^{14}C]NBDA-CoA with 2.7 μM enzyme in 50 mM potassium phosphate, pH 7.5, and 1 mM EDTA at 23 °C. Aliquots were quenched in perchloric acid (O) or added to neutralized hydroxylamine (0.55 M final concentration) for 20 min before quenching in perchloric acid (●). All points are averages of duplicate experiments.

stoichiometry were significantly increased; in addition, less of the NBDA was released by hydroxylamine. The results at pH 8 suggest a loss in the specificity of labeling and an increased transfer of NBDA to oxygen. Under the conditions of the energy transfer experiments, the fluorescence emission polarization of NBDA bound tightly to the enzyme (2 mol/mol of enzyme) was found to be 0.35 (480-nm excitation, 530-nm emission).

Resonance Energy Transfer. A fluorescence titration of fatty acid synthase (with 2.5 mol of bound NBDA/mol of enzyme) with NADPH is included in Figure 2 (lower curve). Because the loosely bound NBDA is readily lost after the removal of free NBDA-CoA, these experiments were done with enzyme from which the loosely bound NBDA had been removed, as described previously. The binding of up to 3.4 mol of NBDA/mol of enzyme was accomplished by using enzyme modified with tosyl fluoride. In this case, the two classes of NBDA-binding sites are not observed because the thioesterase activity of the enzyme is inhibited. Also shown in Figure 2 is the efficiency of energy transfer between enzyme-bound NADPH and NBDA calculated with eq 2 for each point on the curve. The efficiency of energy transfer does not change during the course of the titration, supporting the conclusion that the NADPH-binding sites are essentially equivalent. The average efficiency of energy transfer for three different NBDA–enzyme adducts is presented in Table I. The energy transfer efficiency for equivalent donors and m equivalent energy acceptors present at a stoichiometry of r (with a maximum stoichiometry of m) is given by (Matsumoto & Hammes, 1975)

$$E = i\sum_{i=1}^{m} f_i \frac{i(R_0/R)^6}{1 + i(R_0/R)^6} \qquad (5)$$

with

$$f_i = \frac{m!}{i!(m-i)!}\left(\frac{r}{m}\right)^i\left(1 - \frac{r}{m}\right)^{m-i}$$

Here R is the distance from each donor to the ith acceptor and

$$R_0 = (9.79 \times 10^3)(Q_D J \kappa^2 n^{-4})^{1/6}$$

where n is the refractive index of the medium, κ^2 is a dipole–dipole orientation factor, Q_D is the quantum yield of the donor

Table I: Energy Transfer Parameters for Enzyme-Bound NADPH and NBDA

bound NBDA/ enzyme (mol/mol)	energy transfer efficiency	R_4[a] (Å)	R_2[b] (Å)	R_1[c] (Å)
1.1	0.13	41	36	31
2.5	0.25	42	37	32
3.4[d]	0.31	42	37	33

[a] Calculated with eq 5 on the assumption that four NBDA are equidistant from each NADPH. [b] Calculated with eq 5 on the assumption that two NBDA are equidistant from each NADPH. [c] Calculated with eq 5 on the assumption that only one NBDA is close to each NADPH. [d] The enzyme was modified with tosyl fluoride.

in the absence of the acceptor, and J is the overlap integral of the donor fluorescence emission and the acceptor absorption spectrum. The overlap integral is

$$J = \frac{\int F(\lambda)\epsilon(\lambda)\lambda^4\, d\lambda}{\int F(\lambda)\, d\lambda}$$

where F is the corrected emission of the donor, ϵ is the molar extinction coefficient of the acceptor, and λ is the wavelength in centimeters. If the energy donors and acceptors rotate rapidly relative to the fluorescence lifetime, $\kappa^2 = {}^2/_3$. With $\kappa^2 = {}^2/_3$, $n = 1.33$, and the measured values of Q_D (0.076) and the overlap integral, the calculated value of R_0 is 30 Å. The distance between the donors and acceptors has been calculated with eq 5 for three cases: (1) four acceptors (NBDA) are equidistant from each donor (R_4); (2) two acceptors are equidistant from each donor, and the other two are much more distant (R_2); and (3) only one acceptor is sufficiently close to each donor for significant energy transfer to occur (R_1). The values of R_4, R_2, and R_1 corresponding to these cases are included in Table I.

The fluorescence polarizations of both NADPH and NBDA when bound to the enzyme are high, indicating that both have little rotational freedom. These measured polarizations can be used to set limits on κ^2, the orientation factor (Dale et al., 1979). From the contour plots of Dale et al. (1979), the lower limit on κ^2 is 0.10 and the upper limit is 3.3. This leads to a *maximum* uncertainty in the calculated values of the distances (R_1, R_2, and R_4) of about ±30%.

Discussion

The binding of NADPH by fatty acid synthase has been studied with enzyme from various sources: pigeon and rat liver (Dugan & Porter, 1970), goose uropygial gland (Poulose et al., 1980), and chicken liver (Srinivasan & Kumar, 1976; Hsu & Wagner, 1970). The results obtained have not been in agreement as to the number of binding sites for NADPH on the enzyme. Fatty acid synthases from all of these sources are apparently very similar so that a similar binding stoichiometry for NADPH would be anticipated. However, Hsu & Wagner (1970) reported four sites per enzyme for the chicken liver enzyme; Dugan & Porter (1970) reported three sites for the pigeon liver enzyme and four sites for the rat liver enzyme; Srinivasan & Kumar (1976) reported three sites for the chicken liver enzyme; and Poulose et al. (1980) reported four sites for the goose enzyme. All of these studies used the fluorescence enhancement of NADPH bound to the enzyme to estimate the stoichiometry of binding, although Poulose et al. (1980) developed a new method to analyze their data. The results reported here clearly indicate the difficulty inherent

in using spectroscopic methods to determine binding stoichiometry. An unambiguous interpretation of the fluorescence data was possible only after the stoichiometry of binding was determined through direct binding measurements. Our results indicate four independent binding sites for NADPH are present on the enzyme with an intrinsic dissociation constant of about 0.6 μM.

The nature of the acylation sites on fatty acid synthase has been the subject of many studies (Jacob et al., 1968; Chesterton et al., 1968; Phillips et al., 1970a,b). All of these have analyzed an acidic peptic digest of the acid-precipitated protein after the binding of radioactive acetate, malonate, or palmitate. As a result of such studies, three acyl-binding sites have been identified: (1) 4′-phosphopantetheine (–SH); (2) cysteine (–SH); and (3) threonine or serine (–OH). The relationship of these sites to those actually functioning on the enzyme during catalysis has not been elucidated. However, the hydroxyl site has been proposed as the site where acyl groups first bind before being transferred to a sulfhydryl (Phillips et al., 1970a,b). This transfer of acyl groups from serine to sulfhydryl seems unlikely since the oxygen ester normally is the more thermodynamically stable of the two (Jencks, 1969). Of course, the occurrence of a special enzyme environment that alters the normal order of stability cannot be excluded. The most direct evidence for a hydroxyl loading site has been the observation that modification of the enzyme with iodoacetamide (which presumably reacts with sulfhydryl groups) can almost eliminate the recovery of peptides having acyl groups bound as thioesters while slightly enhancing recovery of those peptides having oxygen esters bound (Phillips et al., 1970a,b; Nixon et al., 1970). Concomitantly the ability of the enzyme to transfer acetate or malonate from CoA to 4′-phosphopantetheine is essentially unaffected (Phillips et al., 1970b). This evidence is subject to other interpretations, and in the absence of evidence that acyl groups are loaded directly to a hydroxyl site, an alternative mechanism is that sulfhydryls are the loading sites, as was originally proposed (Jacob et al., 1968). The hydroxyl sites, if not the result of sulfur to oxygen transfers that are not catalytically significant, are more likely to be the thioesterase sites, which bind the active serine reagents tosyl fluoride and diisopropyl fluorophosphate (Kumar, 1975; Lin & Smith, 1978). They also may be related to the hydroxylamine-insensitive sites implicated in malonyl-CoA inhibition of the enzyme (Kumar & Srinivasan, 1981). Our results indicate that the binding to the hydroxylamine-insensitive sites (presumably hydroxyl sites) is most likely subsequent to binding to the thiol sites. Obviously, more work is needed to elucidate this aspect of the mechanism.

The binding of NBDA to the enzyme is a complex process. When the thioesterase is inhibited by the binding of tosyl groups, a maximum of four NBDA appears to bind to the enzyme. At least three of these can be removed by hydroxylamine treatment and, therefore, are presumably bound at sulfhydryl sites. Two classes of hydroxylamine-sensitive binding sites exist. One class seems to be in a relatively rapid steady state, being hydrolyzed and then regenerated from medium NBDA-CoA. Under our conditions, a level of about one site of this class per enzyme molecule is found. This establishes the minimum number of such sites. The other class is continuously generated more slowly and does not appear to be readily hydrolyzed. A simple interpretation of these results is that the first class represents the loading site for NBDA, from which it can be transferred to the second site. The loading site, therefore, would be readily available to the thioesterase. However, an alternative possibility is that binding proceeds independently at the two sites. The finding of two classes of sulfhydryl sites is consistent with previous results showing that acetate can bind to both the 4′-phosphopantetheine and the cysteine, while malonate binds only to the former (Chesterton et al., 1968). This result also explains why acetyl-CoA is a more effective inhibitor of NBDA binding than malonyl-CoA. Our results do not, of course, identify which class of sites is 4′-phosphopantetheine.

The resonance energy transfer results suggest that the acyl groups and the bound NADPH are not very close. Unfortunately, the high emission polarization values found for the energy acceptor and donor create a rather large uncertainty in the measured distance. However, the 4′-phosphopantetheine is about 20 Å long, and the NBDA may not be bound precisely at the acyl site so that the 4′-phosphopantetheine could easily span the distance between the acyl-binding site and the NADPH-binding site. Two classes of NADPH-binding sites have been suggested (Poulose et al., 1980), one with a reactive lysine nearby and one without, that are separately implicated in the enoyl reductase activity and in the β-ketoacyl reductase activity. Other evidence has indicated that the β-ketoacyl moiety is bound primarily to the 4′-phosphopantetheine rather than to other acyl-binding sites (Nixon et al., 1970). Thus, the two reduction steps may take place near different sulfhydryl sites, each near its related NADPH-binding sites and relatively far from other NADPH-binding sites. The shortest calculated distance in Table I assumes one NBDA is close to each NADPH. The actual distance for the closest NADPH–NBDA pair could be still shorter if only a fraction of the bound NADPH is close enough to NBDA for energy transfer to occur. Experiments are under way to define better these spatial relationships.

The question of whether the two subunits of fatty acid synthase are identical has been difficult to resolve. In the yeast system, genetic evidence convincingly suggests two different subunits (Schweizer et al., 1971; Tauro et al., 1974). However, recent work with avian enzymes (Arslanian et al., 1976; Stoops & Wakil, 1981; Poulose et al., 1980) has suggested that the two subunits are identical, each having two sulfhydryl sites for acyl groups, one thioesterase site, and two NADPH-binding sites for each of the two reductase activities. Our results are consistent with this structure: four binding sites per enzyme molecule are found for NADPH and four for acyl groups; moreover, the sites for acyl groups appear to be divided into two classes of two sites each.

Added in Proof

Recent experiments suggest that NBDA binds initially to a hydroxyl site on the protein and is removed by passage through the first centrifuge column.

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Direct Transfer of One-Carbon Units in the Transformylations of de Novo Purine Biosynthesis†

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ABSTRACT: It is shown that the transfer of formyl units in the de novo purine biosynthetic pathway as catalyzed by glycinamide ribonucleotide (GAR) transformylase and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase probably proceeds through a direct displacement mechanism involving only formyl donor (10-CHO-H_4folate) and formyl acceptor (GAR or AICAR). The inability to observe enzyme-catalyzed solvent oxygen incorporation or uncoupling by hydroxylamine of 1:1 stoichiometry between formylated acceptor [formylglycinamide ribonucleotide or 5-(formylamino)imidazole-4-carboxamide ribonucleotide] and deformylated donor implies the absence of an amidine intermediate and suggests that either a formylated enzyme-bound intermediate is not formed or such an intermediate is not accessible to hydroxylamine.

AICAR[1] transformylase and GAR transformylase are the two reduced folate requiring transformylases in de novo purine biosynthesis. They catalyze the formylation of AICAR and GAR, respectively, by using 10-formyl-H_4folate as the cofactor (Hartman & Buchanan, 1959; Smith et al., 1981a) to produce FAICAR and FGAR.

The mechanism of formyl transfer may involve the nucleophilic attack by the amino group of the acceptor (GAR or AICAR) to lead to products in one step—direct transfer. Alternatively, a nucleophilic function on the enzyme could attack the formyl donor and result in a covalently bound one-carbon unit at the formate level of oxidation—indirect transfer. In the latter case one may imagine two routes through either (1) a formyl enzyme or (2) an amidine composed of H_4folate and the transformylase that was formed by dehydration of a putative tetrahedral intermediate. Attack

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[1] Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; GAR, glycinamide ribonucleotide; FAICAR, 5-(formylamino)imidazole-4-carboxamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; TFase, transformylase; H_4folate, tetrahydrofolate; EDTA, ethylenediaminetetraacetic acid.

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