Subunit Structure of the Fatty Acid Reductase Complex from *Photobacterium phosphoreum*†

Lee Wall and Edward A. Meighen*

Department of Biochemistry, McGill University, Montreal, Quebec H3G 1Y6, Canada

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ABSTRACT: The subunit structures of the fatty acid reductase complex from *Photobacterium phosphoreum* and its three component enzymes (designated acyl-CoA reductase, acyl-protein synthetase, and acyltransferase) have been investigated by cross-linking with bifunctional reagents, gel filtration, complementation experiments, and analysis of the polypeptide composition by quantitative protein staining. The acyl-CoA component eluted on gel filtration at a position [M_r 223 ($\pm$13) $\times$ 10^3] corresponding to a tetrameric structure. Protein bands corresponding to the dimer, trimer, and tetramer could be seen on sodium dodecyl sulfate–polyacrylamide gel electrophoresis after treatment of the enzyme with bifunctional cross-linking reagents. The dependence of the distribution of cross-linked species on the concentration and type of reagent indicated that acyl-CoA reductase has D_2 symmetry with the subunits arranged as a set of dimers. In contrast, both the acyl-protein synthetase and acyl-transferase enzymes behaved as monomeric species. The acyl-protein synthetase interacted with both the acyl-transferase and the acyl-CoA reductase whereas no interaction was found between the latter two enzymes. Four molecules of the acyl-protein synthetase monomer were required to completely complement the tetrameric acyl-CoA reductase, while a simple 1:1 heterodimer complex was formed between the acyl-transferase and acyl-protein synthetase subunits. Analysis of the polypeptide composition of the fatty acid reductase complex indicated that the complex is composed of four polypeptides each of the acyl-CoA reductase and acyl-protein synthetase enzymes and variable amounts (two to four polypeptides) of the acyl-transferase enzyme with the acyl-protein synthetase playing a central metabolic and structural role between the transferase and reductase subunits.

Fatty acid reductase from bioluminescent bacteria catalyzes the reduction of a long-chain fatty acid to aldehyde which then serves as the substrate for the light-emitting reaction catalyzed by luciferase (Ulitzur & Hastings, 1978, 1979; Meighen, 1979). During the growth-dependent development of bioluminescence and induction of luciferase in *Photobacterium phosphoreum*, three enzymes involved in fatty acid metabolism are specifically synthesized (Wall et al., 1984a): an acyltransferase, which produces fatty acids from fatty acyl precursors (Carey et al., 1984); an acyl-protein synthetase, which activates free fatty acid (+ATP) to form an acyl-protein intermediate (Rodriguez et al., 1983b); and a reductase, which reduces activated fatty acids (+NADPH), including acyl-CoA and the acylated synthetase, to the corresponding aldehydes (Rodriguez et al., 1983a,b). Both the synthetase and reductase enzymes are required for fatty acid reductase activity. Evidence that analogous enzymes involved in fatty acid reduction exist in other bioluminescent bacteria has also been obtained (Ulitzur & Hastings, 1980; Wall et al., 1984b; Engebrecht et al., 1983; Boylan et al., 1985).

Enzymes involved in fatty acid reduction to aldehyde and/or alcohol have also been detected in a variety of other organisms (Kolattukudy et al., 1981; Johnson & Gilberson, 1972; Snyder & Malone, 1970; Griffith et al., 1981). However, to date, purification of these systems has not yet been accomplished; hence, only limited information regarding their structure and regulation is available. Studies on *P. phosphoreum* fatty acid reductase may thus assist in providing a better overall understanding of the enzymes systems involved in fatty acid reduction.

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Previous results have indicated that the three *P. phosphoreum* proteins may exist as a complex since they can be copurified during the initial stages of purification before resolution by dye binding chromatography (Rodriguez et al., 1983b). Furthermore, the low level of acylation of the purified synthetase enzyme was stimulated severalfold by addition of either the reductase or the transferase proteins. Similarly, the synthetase enzyme not only stimulates the activity of the transferase but also alters its specificity (Carey et al., 1984). To gain an understanding of how interactions between these enzymes may regulate the specific steps in aldehyde production, the subunit structure and interactions of the transferase, synthetase, and reductase enzymes have been investigated.

Experimental Procedures

Materials. Cellex-D and sodium dodecyl sulfate (SDS)[1] were purchased from Bio-Rad. DEAE-Sepharose CL-6B, Blue Sepharose CL-6B, Sephacryl S-300, and molecular weight standards for gel filtration were from Pharmacia Fine Chemicals. Aminohexyl-Sepharose, acrylamide, *N*,*N*′-methylenebis(acrylamide), ATP, FMN, NADPH (type III), 2-mercaptoethanol, dithiothreitol, 1,5-difluoro-2,4-dinitrobenzene (DFDNB), bovine serum albumin (which was dried under phosphorus pentoxide before use), and molecular weight standards for SDS-polyacrylamide gel electrophoresis were all from Sigma. Bis(sulfosuccinimidyl) suberate (BS^3),

[1] Abbreviations: SDS, sodium dodecyl sulfate; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; PAGE, polyacrylamide gel electrophoresis; BS^3, bis(sulfosuccinimidyl) suberate; R_s, effective Stokes radius; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid.

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ethylene glycol bis(succinimidyl succinate), and dimethyl suberimidate were purchased from Pierce Chemical Co. Phosphate buffers were prepared by mixing the appropriate amounts of NaH_2PO_4 and K_2HPO_4. Fatty acids were stored at −20 °C as stock solutions in 2-propanol or ethanol.

[^{3}H]Tetradecanoic acid (31 Ci/mmol) was prepared by Amersham Corp. by reduction of *cis*-9-tetradecenoic acid with tritium gas in the presence of 5% palladium on carbon catalyst. [^{3}H]Tetradecanoyl-CoA was prepared from the radioactive acid as described previously (Rodriguez et al., 1983a).

Cell Growth and Lysis. Photobacterium phosphorum (NCMB 844) was grown at 19–20 °C in 3% NaCl complete medium (Riendeau & Meighen, 1980). Twelve liters of culture were grown in a New Brunswick Magnaferm fermentor to maximum "in vivo" luminescence [$A_{660} \geq 4$, LU $\geq$ 4000; where 1 light unit (LU) equals 6×10^9 quanta/s based on the standard of Hastings and Weber (1963)]. Cells were isolated by centrifugation (15000*g*, 10 min) and then frozen at −20 °C. The cells were thawed and lysed by osmotic shock as previously described (Gunsalus-Miguel et al., 1972).

Purification of Enzymes from P. phosphoreum. The fatty acid reductase was partially purified from the extract by ion exchange on Cellex-D, ammonium sulfate precipitation, and gel filtration on Sephacryl S-300, then the individual components were separated by dye binding chromatography on Blue Sepharose, and the acyl-CoA reductase enzyme was further purified by chromatography on aminohexyl-Sepharose as previously described (Rodriguez et al., 1983a,b). Alternatively, the acyl-transferase enzyme was purified from a second peak of activity on Cellex-D in which it is not part of the fatty acid reductase complex (unpublished results).

In most cases, each of the fatty acid reductase components was further purified by gel filtration on Sephacryl S-300 to remove minor contaminants. For the acyl-CoA reductase and acyl-transferase enzymes, this was accomplished by precipitating the protein by dialysis against saturated ammonium sulfate, redissolving the precipitate in 50 mM phosphate (pH 7.0), 50 mM 2-mercaptoethanol, and 0.2 M NaCl, and running the gel filtration in the same buffer. The acyl-protein synthetase pool from Blue Sepharose was concentrated by dialysis against the same phosphate, 2-mercaptoethanol, NaCl buffer containing 10% glycerol (v/v), and 40–50% poly(ethylene glycol) (w/v), and the enzyme was then resolved on Sephacryl S-300 in the same buffer containing no poly(ethylene glycol). Enzyme preparations were stored at −20 °C in 50 mM phosphate (pH 7.0) containing 50 mM 2-mercaptoethanol (or 5 mM dithiothreitol), 0.2 M NaCl, and 15–30% (v/v) glycerol.

Protein Measurements. Protein assays were based on the biuret method (Layne, 1957), the method of Lowry as modified by Markwell et al. (1981), or the method of Bradford (1976), using bovine serum albumin as a standard. To estimate the response of the various proteins with the different dye staining methods, three to five samples of each of the purified fatty acid reductase proteins were dry-weighed as previously described (Bognar & Meighen, 1978), and the protein content in milligrams (± average deviation) of 1.68 (±0.02), 0.76 (±0.06), and 0.91 (±0.03) was determined by the biuret method for the acyl-CoA reductase, acyl-protein synthetase, and acyl-transferase proteins, respectively. These values were 100%, 95%, and 99% of the average dry weights for each of these proteins, respectively. The method of Lowry also agreed closely with the dry weights (105%, 96%, and 104%, respectively), while in agreement with previous reports (Markwell et al., 1981) the Bradford method underestimated the amounts of each protein (73%, 55%, and 65%, respectively) with bovine serum albumin as a standard. Protein concentrations were corrected by the appropriate factors for the individual polypeptides, and the average response was used for the partially purified complex. On the basis of these assays, specific extinction coefficients (0.1%, 1 cm) at 280 nm of 1.1, 1.0, and 1.1 for the reductase, synthetase, and transferase enzymes, respectively, were determined [in some cases background due to light scattering ($\leq$10%) was subtracted by extrapolation of values measured between 340 and 400 nm].

To determine the amounts of each protein in the fatty acid reductase complex, different amounts of each of the purified polypeptides were dissolved in 0.1 M Tris (pH 6.8) containing 25% glycerol, 1% SDS, 0.2 M 2-mercaptoethanol, and 0.05 M bromophenol blue and then separated by SDS–PAGE by the system of Laemmli (1970). Gels were stained for several hours with Coomassie brilliant blue (R) (Aldrich) in 25% 2-propanol and 10% acetic acid, followed by destaining in 10% 2-propanol and acetic acid. The dye staining intensity of each band was determined by scanning with an LKB 2202 Ultrascan laser densitometer. The relative staining intensities per unit weight of the transferase and synthetase proteins were about 13% lower than for the reductase protein. The amount of dye bound by each protein deviated slightly from linearity with up to 25 μg of protein applied on gels in the Laemmli system. The molar ratios of the polypeptides in the fatty acid reductase complex were determined from their staining intensities relative to the purified subunits and molecular weights of 54 000, 47 500, and 33 000 for the reductase, synthetase, and transferase components, respectively, which represent the average molecular weights on SDS–PAGE in the Laemmli (1970) and Weber and Osborn (1969) systems.

Analytical Gel Filtration. The molecular weights of the native enzymes were estimated by gel filtration on a Sephacryl S-300 column (85 × 1 cm) in 50 mM phosphate (pH 7.0) containing 0.2 M NaCl and 50 mM 2-mercaptoethanol. Protein samples (0.6 mL) were applied to the column and fractions (0.7 mL) collected at a flow rate of 3 mL per hour. Peak elution volumes (V_e) were determined from the A_{280} and/or enzyme activity in each fraction. The void volume (V_0) and the total column bed volume (V_t) were determined from the elution volumes of blue Dextran and [β-^{3}H]alanine, respectively, with the partition coefficient (σ) being defined as $(V_e - V_0)/(V_t - V_0)$. Native molecular weights and effective Stokes radii (R_s) were estimated by using plots of log molecular weight vs. σ or $(-\log \sigma)^{1/2}$ vs. R_s, respectively, which were linear for the range of standards used: thyroglobulin, M_r 669 000 (R_s = 85 Å); ferritin, M_r 440 000 (R_s = 61 Å); aldolase, M_r 158 000 (R_s = 48 Å); bovine serum albumin, M_r 67 000 (R_s = 35.5 Å); ovalbumin, M_r 43 000 (R_s = 30.5 Å); chymotrypsinogen A, M_r 25 000 (R_s = 21 Å); ribonuclease A, M_r 13 700 (R_s = 16.4 Å).

Enzyme Assays. Fatty acid reductase and acyl-CoA reductase activities were measured by the luminescent coupled assay as previously described (Rodriguez et al., 1983a). The acyl-protein synthetase activity was determined from the incorporation of [^{3}H]tetradecanoic acid (6 Ci/mmol) into material insoluble in $CHCl_3/CH_3OH/CH_3CO_2H$ (Rodriguez et al., 1983b). Alternatively, the amount of acyl-protein synthetase enzyme was determined from the fatty acid reductase activity after complementation with excess acyl-CoA reductase. Acyl-transferase was measured by cleavage of [^{3}H]myristoyl-CoA (0.1 Ci/mmol) into hexane-soluble products as previously described (Carey et al., 1984).

Cross-Linking Experiments. Proteins were either dialyzed or diluted into the appropriate buffer before cross-linking. When two proteins were to be cross-linked with each other, they were mixed together at 4 °C for at least 2 h before addition of the bifunctional reagent. BS^3 was dissolved immediately before use in 50 mM phosphate (pH 7.0)/0.2 M NaCl at either 2 or 4 times the final concentration used in the cross-linking experiment, while DFDNB stocks were made in acetone at 50 times the final concentration used in the reactions. Final concentrations of protein, buffer constituents, and bifunctional reagents in the reaction mixtures are given in the figure legends.

The cross-linking reagents were added to the reaction mixture at room temperature, and the reaction was allowed to proceed for 2 h in all cases. Excess reagent was reacted by addition of $^1/_{20}$th volume of 0.2 M lysine. The protein was precipitated by addition of $^1/_{10}$th volume of 100% trichloroacetic acid (at 0 °C for 15 min) and centrifuged (15000*g*, 15 min), and the precipitate was washed with 200 μL of 1% trichloroacetic acid. Protein pellets were redissolved in 30 μL of SDS sample buffer containing 10 mM sodium phosphate (pH 7.0), 1% SDS, 0.2 M 2-mercaptoethanol, 25% glycerol, and 0.05% bromophenol blue and boiled for 10 min or incubated overnight at 37 °C.

Cross-linked proteins were separated on SDS–PAGE by the system of Weber and Osborn (1969) using slab gels containing 4.5% or 5% acrylamide. Although Laemmli gels can give better resolution, cross-linked proteins migrate anomalously on Laemmli gels. In some cases, gels containing 30 μg of cross-linked acyl-CoA reductase were scanned by laser densitometry to determine the amount of each cross-linked species. In the Weber and Osborn gel system, the dye staining intensity was found to be proportional to the amount of this protein with up to 30 μg applied to the gels (coefficient of correlation = 0.994); hence, the amount of protein in each band is expressed as a percent of the total dye staining intensity.

Results

The three fatty acid reductase enzymes, reductase (r), synthetase (s), and transferase (t), were determined, by their dye staining intensities on SDS–PAGE, to be present in the partially purified complex at molar ratios of 1.0:1.0:0.5, respectively. Although the molar ratio between these subunits remained relatively constant with different preparations of the fatty acid reductase, attempts to further purify the complex by gel filtration or chromatography on aminohexyl-Sepharose resulted in substantial losses of the transferase component. Recent results have also shown that part of the transferase enzyme is resolved from the fatty acid reductase complex during the initial stages of purification (Carey et al., 1984). These results indicate that the low molar ratio of the transferase component in the complex arises from partial dissociation. The molecular weight of 450 (±32) × 10^3 ($R_s \approx 68$ Å) determined for the fatty acid reductase complex by gel filtration on Sephacryl S-300 can therefore only be considered as a minimum estimate. However, this large size does indicate that the complex contains multiple copies of the reductase, synthetase, and/or transferase polypeptides.

Subunit Structure of Acyl-CoA Reductase. The molecular weight of the acyl-CoA reductase enzyme was determined by gel filtration on Sephacryl S-300 to be 223 (±13) × 10^3 ($R_s \approx 53$ Å). Assuming the enzyme is globular in shape, this result suggests the protein is a tetramer since the dissociated polypeptide chains migrate on SDS–PAGE at 58 kDa in Tris buffers (Laemmli system) or at 50 kDa in phosphate buffers (Weber and Osborn system).

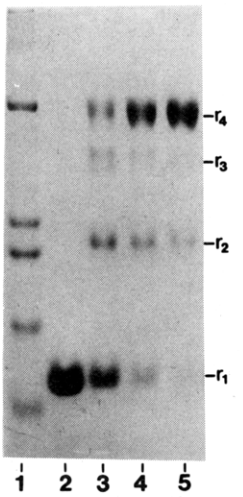

FIGURE 1: Cross-linking of acyl-CoA reductase with BS^3. Acyl-CoA reductase (0.1 mg/mL) in 50 mM phosphate (pH 7.0), 0.5 M NaCl, and 1 mM EDTA was reacted for 2 h with different concentrations of BS^3. The reaction was stopped and the protein (30 μg) analyzed by SDS–PAGE on Weber and Osborn gels containing 4.5% acrylamide as described under Experimental Procedures. Lane 2, 0 mM BS^3; lane 3, 0.5 mM BS^3; lane 4, 2 mM BS^3; lane 5, 8 mM BS^3 (r_1 = monomer, r_2 = dimer, r_3 = trimer, r_4 = tetramer). Lane 1, molecular weight standards: myosin, 205 000; β-galactosidase, 116 000; phosphorylase *b*, 97 400; bovine serum albumin, 67 000; ovalbumin, 45 000.

After being cross-linked with the bifunctional reagent bis(sulfosuccinimidyl) suberate (BS^3) at a concentration of 8 mM, over 90% of the reductase protein was found to migrate on Weber and Osborn SDS slab gels at a molecular weight (205 × 10^3) corresponding to a cross-linked tetramer (r_4) when compared to the monomer (r_1) which migrated at 50 kDa (Figure 1). At lower concentrations of the reagent, a prominent band corresponding to the dimer (r_2, 104 kDa) and also a doublet representing the cross-linked trimer (r_3, 150–165 kDa) are seen. The existence of multiple bands for a cross-linked species has been observed by other workers and may reflect the inability of some of the cross-linked structures to completely unfold due to intrapeptide linkages (Hucho et al., 1975). Similar cross-linking patterns were also obtained with the bifunctional reagents dimethyl suberate and ethylene glycol bis(succinimidyl succinate) and were independent of protein concentrations from 0.01 to 1.0 mg/mL.

Hucho et al. (1975) have demonstrated that the probability of forming different cross-linked species upon reaction of a polymeric protein with a bifunctional reagent can be related to the subunit geometry of the native enzyme, as well as its reactivity toward the given reagent. On the basis of the theoretical probabilities presented by these investigators, the expected distributions of dimer, trimer, and tetramer, as a function of the percent monomer remaining un-cross-linked, were derived for model tetrameric structures. In this manner, complete experimental patterns, for a given protein and reagent, can be compared directly to the calculated distributions.

In Figure 2a, the lines show the theoretical distribution calculated for a square planar tetramer model, with only two sites of contact between subunits, if both sites were to react equivalently with the given reagent. It should be realized, however, that a tetramer with tetrahedral geometry, and three contact faces between subunits, could also give rise to this same pattern if only two of the sites were accessible to the reagent, or if one site did not contain groups in close enough proximity to be cross-linked. In Figure 2b, the lines represent the theoretical patterns expected if three contact sites in a tetrahedron

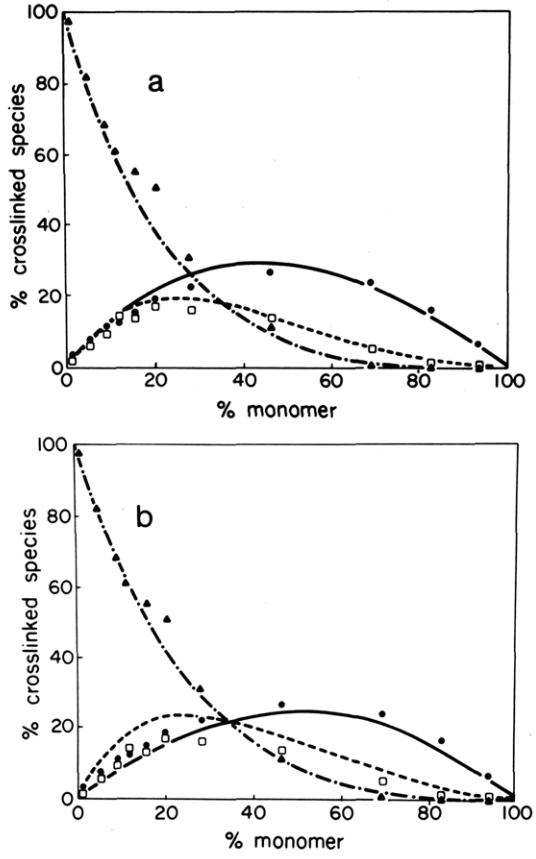

FIGURE 2: Distribution of cross-linked species of acyl-CoA reductase as a function of the percentage of monomer. (a) Acyl-CoA reductase (0.25 mg/mL) was treated with BS^3 at various reagent concentrations between 0.025 and 4 mM. The amount of each cross-linked species was determined by the dye staining intensity and is expressed as a percent of the total dye staining intensity as described under Experimental Procedures. The amounts of dimer (●), trimer (□), and tetramer (▲) are plotted as a function of the percent monomer remaining un-cross-linked. The lines represent the theoretical values for cross-linked dimer (—), trimer (---), and tetramer (–·–) expected for a square planar model with two sites of contact between subunits that are cross-linked with the same probability. (b) Theoretical distribution of cross-linked species for a tetrahedral arrangement of subunits with three equivalently reactive sites of contact between subunits. The distributions for the model structures were calculated on the basis of the probability equations described by Hucho et al. (1975).

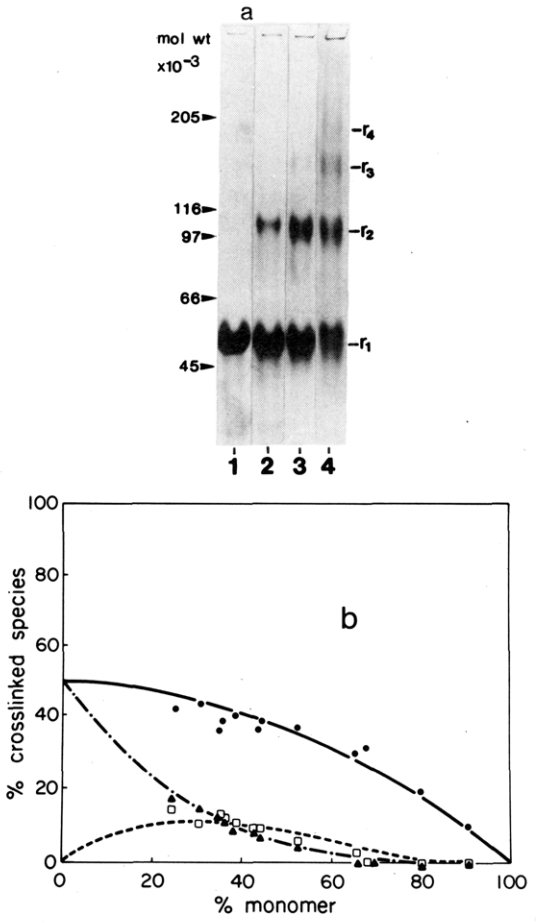

FIGURE 3: Cross-linking of acyl-CoA reductase with DFDNB. (a) Acyl-CoA reductase (0.06 mg/mL) was reacted with 0 (lane 1), 1 (lane 2), 2 (lane 3), or 4 mM (lane 4) DFDNB in 50 mM PO_4, pH 7.0, 0.5 M NaCl, and 1 mM EDTA, and the protein (30 μg) was separated on 4.5% polyacrylamide Weber and Osborn slab gels and stained with Coomassie blue as described under Experimental Procedures. (b) The amounts of each cross-linked species obtained with varying concentrations of DFDNB between 0.5 and 6 mM were quantitated by the dye staining intensities and plotted as a function of the percent monomer remaining un-cross-linked (symbols as in Figure 2). The lines represent the theoretical values expected for a tetramer in a square planar configuration with D_2 symmetry, where the bifunctional reagent cross-links one contact site between subunits with 3.5 times more probability than the second site.

subunit arrangement reacted with equal probability with a bifunctional reagent.

The percentage of each cross-linked species (dimer, trimer, and tetramer), as a function of the percent free monomer, obtained on reaction of acyl-CoA reductase with varying concentrations of BS^3 closely parallels that expected if the reagent were reacting at only two subunit contact faces with equal probability (Figure 2a) but deviates significantly from that expected if the reagent were reacting equivalently at three contact sites (Figure 2b). In the latter case, the amount of cross-linked trimer would be expected to exceed the level of dimer at high levels of cross-linking, a result which is not observed experimentally. Although these results do indicate that only two contact sites in the reductase tetramer react with BS^3, they do not distinguish between a square and a tetrahedral model.

To further investigate the reactivity of the subunit contact sites in the acyl-CoA reductase tetramer, cross-linking with a shorter bifunctional reagent, DFDNB, was investigated. After reaction with DFDNB, bands corresponding to the dimer (r_2), trimer (r_3), and tetramer (r_4) as well as the monomer of the acyl-CoA reductase can be seen on SDS–PAGE (Figure 3a). However, unlike BS^3, the most prominent band observed with DFDNB is the dimer, with only low amounts of the trimer and tetramer being produced. The distribution of the cross-linked species, with this reagent (Figure 3b), closely followed that expected for a square structure with D_2 symmetry where cross-linking at one contact site between subunits occurs 3–4 times more rapidly then the second site and therefore results in preferential formation of the dimer. This same pattern would also arise for a tetrahedral subunit arrangement where one site is completely unreactive and there is a differential rate of cross-linking at the other two subunit contact faces. If the reductase enzyme, after treatment with DFDNB, was reacted with BS^3, it could be fully cross-linked to the tetrameric species (as in Figure 1), indicating that DFDNB does not perturb the structure. These results suggest that the reductase tetramer is composed of a pair of dimers either in a square planar or in a tetrahedral arrangement.

Subunit Structure of the Acyl-Protein Synthetase and Acyl-Transferase Enzymes. The acyl-protein synthetase protein eluted on gel filtration at a molecular weight of 66 (±5) $\times 10^3$ ($R_s \approx 35$ Å) compared to 45 $\times 10^3$ and 50 $\times 10^3$ for

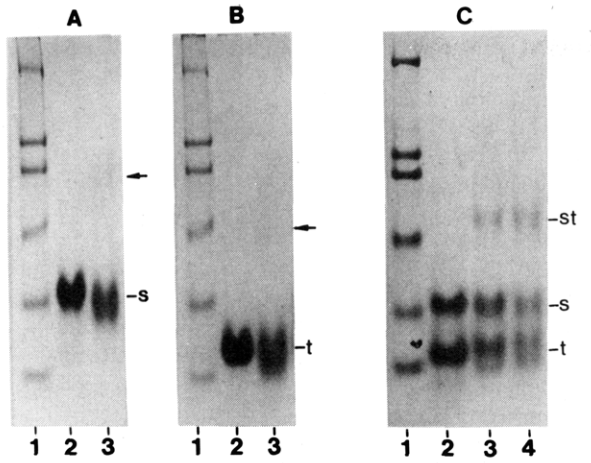

FIGURE 4: Cross-linking of the acyl-protein synthetase and acyl-transferase enzymes. Acyl-protein synthetase, acyl-transferase, or a mixture of the two enzymes (0.15 mg/mL) in 50 mM phosphate (pH 7.0), 0.2 M NaCl, 0.5 mM dithiothreitol, and 15% glycerol was reacted with different concentrations of BS^3. After 2 h, the protein was precipitated and then run on 5% Weber and Osborn slab gels as described under Experimental Procedures. (a) Acyl-protein synthetase: lane 2, 0 mM BS^3; lane 3, 4 mM BS^3 (30 μg of protein per well). (b) Acyl-transferase: lane 2, 0 mM BS^3; lane 3, 4 mM BS^3 (30 μg of protein per well). (c) Mixtures of the two enzymes: lane 2, 0 mM BS^3; lane 3, 1 mM BS^3; lane 4, 4 mM BS^3 (40 μg of total protein per well). Symbols: s, acyl-protein synthetase monomer; t, acyl-transferase monomer; st, heterodimer. Arrows depict the positions expected for cross-linked homodimers of the acyl-protein synthetase or acyl-transferase enzymes. Lane 1, molecular weight standards: myosin, 205 000; β-galactosidase, 116 000; phosphorylase *b*, 97 400; bovine serum albumin, 67 000; ovalbumin, 45 000; carbonic anhydrase, 29 000.

the dissociated monomer on SDS–PAGE in the systems of Laemmli and of Weber and Osborn, respectively. The acyl-transferase had a subunit molecular weight of (32–34) $\times 10^3$ on SDS–PAGE and migrated on Sephacryl S-300 at a position of 41 ($\pm$3) $\times 10^3$ ($R_s \approx 28$ Å). Upon treatment of either enzyme with relatively high concentrations of BS^3 (4 mM), the protein remains un-cross-linked, migrating at the position of the monomer on SDS–PAGE (Figure 4). Although in some preparations low amounts of protein can be detected at the positions expected for cross-linked dimers, these bands could also be detected in samples run on nonreducing gels without treatment with cross-linking reagents. Similar cross-linking results were obtained with other bifunctional reagents, showing that both the acyl-protein synthetase and acyl-transferase enzymes are monomers. The differences between molecular weight estimates by gel filtration and SDS–PAGE may be due to asymmetric shapes for these enzymes and/or anomalous behavior on SDS–PAGE.

Previous results have shown that the acylation of the synthetase enzyme can be stimulated by the acyl-tranferase enzyme (Rodriguez et al., 1983b). Moreover, the activity of the acyl-transferase can be increased more than 2-fold upon addition of the acyl-protein synthetase enzyme, and its primary specificity then involves acyl transfer to water rather than to thiol groups (Carey et al., 1984). Figure 5 demonstrates that upon mixing of the two enzymes, maximum stimulation of both the acyl-protein synthetase and acyl-transferase activities is reached at a molar ratio of 1:1 between the two proteins. In similar experiments, no further stimulation of either activity was seen on increasing the molar ratios up to 4:1. Upon treatment of mixtures of these two enzymes with BS^3, a new cross-linked protein band can be detected on SDS–PAGE (Figure 4). This band (76 $\times 10^3$ kDa) migrates between the positions expected for homodimers of the acyl-transferase and

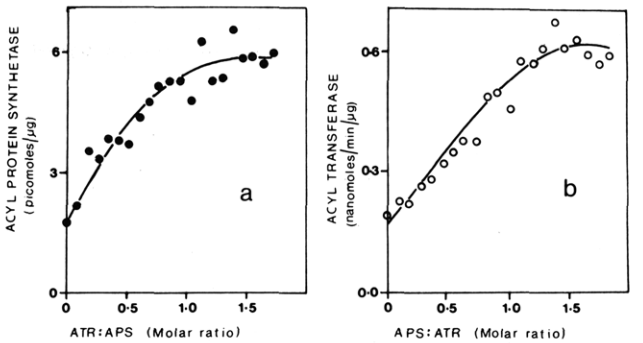

FIGURE 5: Titration of acyl-protein synthetase and acyl-transferase enzymes. (a) A constant amount of the acyl-protein synthetase enzyme (APS) (3.7 μg) was assayed in the presence of varying amounts of the acyl-transferase enzyme (ATR) in a total volume of 100 μL containing 50 mM phosphate (pH 7.0), 0.1 M NaCl, 35 mM 2-mercaptoethanol, 15% glycerol (v/v), 5 mM ATP, and 10 μM [^{3}H]myristic acid (6 Ci/mmol). The activity is expressed as picomoles of fatty acid incoporated per microgram of acyl-protein synthetase enzyme. Assays were performed in triplicate, and background, in the absence of enzyme, has been subtracted. (b) A constant amount of acyl-transferase enzyme (0.65 μg) was assayed for 1 min, in duplicate, in the presence of increasing amounts of the acyl-protein synthetase enzyme in a total volume of 100 μL containing 0.55 M phosphate (pH 7.0), 0.1 M NaCl, 50 mM 2-mercaptoethanol, 15% glycerol (v/v), and 10 μM [^{3}H]myristoyl-CoA (0.1 Ci/mmol). Acyl-transferase activity is expressed in nanomoles of acyl-CoA cleaved per minute per microgram of acyl-transferase enzyme.

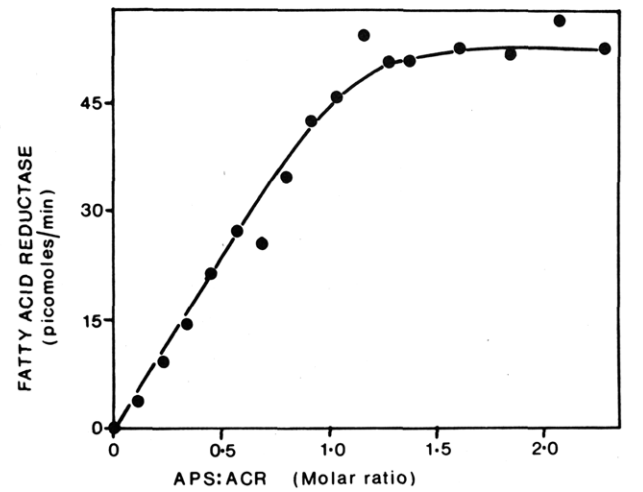

FIGURE 6: Titration of fatty acid reductase activity. A constant amount of the acyl-CoA reductase (ACR) (2.5 μg) in 1 mL of 50 mM phosphate (pH 7.0) containing 20 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 100 μM NADPH, 1 mM ATP, and 5 μM tetradecanoic acid was assayed, in duplicate, for fatty acid reductase activity in the presence of increasing amounts of the acyl-protein synthetase enzyme (APS) as described under Experimental Procedures. The molar ratios for the reductase and synthetase enzymes are based on their subunit molecular weights of 54 000 and 47 500, respectively.

acyl-protein synthetase (arrows, Figure 4) and shows that a heterodimer can form between the two enzymes. Since additional aggregates were not seen, either at higher concentrations of cross-linking reagents or protein or with either protein at a 2-fold excess, the heterodimer (st) appears to be the limiting complex between the two enzymes, demonstrating that they form a direct 1:1 protein complex.

Interaction between Acyl-CoA Reductase and Acyl-Protein Synthetase. Reconstitution of fatty acid reductase activity requires only the acyl-protein synthetase and acyl-CoA reductase enzymes (Rodriguez et al., 1983b). Although the acyl-transferase can cause stimulation in activity, its primary function appears to be in supplying free fatty acids from activated precursors (Carey et al., 1984). Figure 6 demonstrates

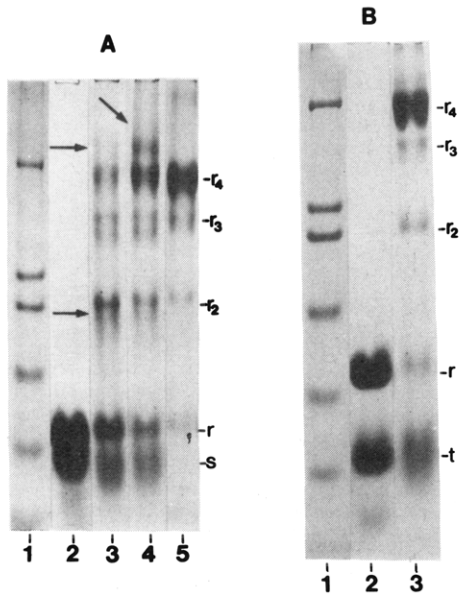

FIGURE 7: Cross-linking of the acyl-protein synthetase and acyl-CoA reductase enzymes. (a) A mixture of the acyl-protein synthetase (s) and acyl-CoA reductase (r) enzymes at 0.2 mg/mL in 50 mM phosphate (pH 7.0), 0.2 M NaCl, 1 mM dithiothreitol, and 10% glycerol (v/v) was treated with 0 (lane 2), 4 (lane 3), or 8 mM (lane 4) BS^3 and the protein separated on 4.5% Weber and Osborn gels as described under Experimental Procedures. Lane 5 is acyl-CoA reductase treated with 4 mM BS^3 under the same conditions. Additional bands arising from cross-linking between two different subunits are indicated by arrows. (b) Mixtures of the acyl-transferase (t) and acyl-CoA reductase (r) enzymes at 1 mg/mL in the same buffer were treated with 0 (lane 2) or 8 mM (lane 3) BS^3 and separated on a 5% polyacrylamide gel. Lanes 1 are molecular weight standards as in Figure 5.

that when a constant amount of acyl-CoA reductase is titrated with increasing acyl-protein synthetase, maximum fatty acid reductase activity is reached when the polypeptides of the two enzymes are at a molar ratio of 1:1. A similar result was obtained on complementing a constant amount of acyl-protein synthetase with increasing amounts of acyl-CoA reductase (Rodriguez et al., 1983b). This finding is in agreement with the 1:1 molar ratio of the two enzymes in the partially purified complex and indicates that the tetrameric acyl-CoA reductase enzyme is capable of binding four acyl-protein synthetase monomers.

Cross-linking between the acyl-CoA reductase and acyl-protein synthetase enzymes is shown in Figure 7. A band can be seen in lane 3, which migrates just below the cross-linked dimer of the acyl-CoA reductase, at 95 kDa (arrow) and may represent a heterodimer between the two enzymes. At least two new cross-linked protein bands can be detected above the position corresponding to the cross-linked tetramer of the acyl-CoA reductase (r_4) which are not seen for the acyl-CoA reductase cross-linked alone. These bands may represent the tetramer of acyl-CoA reductase cross-linked to one and two monomers of the acyl-protein synthetase. Mixed heterotrimers and heterotetramers between the two enzymes were also expected; however, these species would not be easily resolved from the trimer and tetramers of the reductase and would only be detected as small differences in the protein staining in these regions. These results provide further evidence of a direct protein–protein interaction between the reductase and the synthetase polypeptides in the complex.

In contrast to the acyl-protein synthetase, the acyl-transferase enzyme does not appear to interact with the acyl-CoA reductase enzyme (Figure 7) even at very high protein and reagent concentrations. Cross-linked bands between the two enzymes cannot be detected, and the majority of the acyl-transferase enzyme remains as a monomer while the acyl-CoA reductase enzyme can be cross-linked fully to the tetrameric species.

Discussion

Cross-linking of acyl-CoA reductase with bifunctional reagents and comparison of the molecular weights of the native and dissociated enzymes clearly demonstrated that this enzyme is a tetramer. The preferential formation of a dimeric species on cross-linking the enzyme with DFDNB demonstrated that the reductase has different sets of subunit contact sites and is therefore composed of a pair of dimers, with D_2 symmetry. Assuming only closed subunit contacts, two simple models can be used to describe a tetramer with D_2 symmetry (Cornish-Bowden & Koshland, 1970; Klotz et al., 1970): a square planar structure with two, nonequivalent isologous contact sites, or a tetrahedral arrangement with three isologous contacts between subunits. In contrast, a square planar model with heterologous contact sites does not have D_2 symmetry as all subunit interactions are identical. The results presented here show that the acyl-CoA reductase has isologous contact sites in a tetrameric structure in which only two subunit contact domains could be cross-linked. These results cannot differentiate between a square subunit arrangement or a tetrahedral structure in which one of the three subunit contacts cannot be cross-linked.

Hucho et al. (1975) suggested that the selectivity of bifunctional reagents for binding domains between subunits would increase with increasing distance between the two functional groups on the cross-linking reagent. However, by comparing the experimental distribution of cross-linked species for the reductase tetramer with theoretical distributions (Figures 2 and 3), we have found that DFDNB, with its short bridging length (3 Å), could distinguish between two different contact sites. In contrast, a distinction between subunit contacts could not be made on using a cross-linking reagent, BS^3, with a much longer span (11 Å), although both reagents react with amino groups and appear to react at only two sites of subunit contact.

Evidence for a 1:1 molar ratio between the acyl-CoA reductase and acyl-protein synthetase polypeptides in the fatty acid reductase complex was provided by their molar ratio in the partially purified complex, as well as by the reconstitution of maximum fatty acid reductase activity (Figure 6). However, the degree of cross-linking between the two different subunits was relatively limited, with only a few cross-linked species being observed (Figure 7). This result may be due to several factors, including preferential cross-linking of the acyl-CoA reductase tetramer in the complex, an inadequate number of lysyl residues in proper proximity for cross-linking to the synthetase component, or possible dissociation of the complex under these conditions. For example, blocking of lysyl residues appears to be detrimental to the interaction between the two enzymes since acyl-protein synthetase could not be cross-linked to the acyl-CoA reductase if the reductase was first reacted with BS^3 (data not given).

Low levels of the acyl-transferase enzyme in the isolated fatty acid reductase appear to be due to dissociation during purification. Previous results have shown that a large amount of the transferase (~50%) is separated from the complex by ion exchange on Cellex-D in the first step of purification (Carey et al., 1984). Reconstitution of the acyl-transferase and acyl-protein synthetase enzymes demonstrated, however, that maximum stimulation of both enzymes occurred when the two proteins were at equal molar amounts (Figure 5).

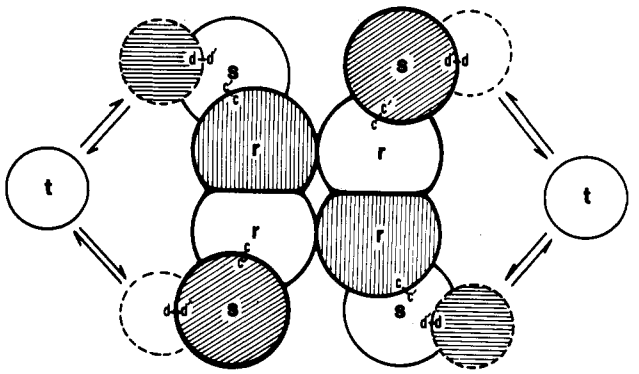

FIGURE 8: Representation of the structure of the fatty acid reductase complex. r, acyl-CoA reductase subunits; s, acyl-protein synthetase subunits; t, acyl-transferase subunits. Binding sites between r and s are denoted c-c′ while binding sites between s and t are denoted d′-d. For further details, see text.

Cross-linking mixtures of the two enzymes also confirmed that they interact to form a 1:1 heterodimer (Figure 4). On the other hand, no interaction between the transferase and reductase proteins could be demonstrated.

A working model depicting the possible interactions between the subunits in the fatty acid reductase complex is presented in Figure 8. It should be noted that the fatty acid reductase structure, which from gel filtration data appears to be globular in nature, has been expanded into two dimensions so that subunit contacts could be clearly depicted. For example, the reductase subunits may be in a tetrahedral and not a planar structure, and the synthetase subunits may be asymmetric as the monomer runs on gel filtration at 67 kDa (R_s = 35 Å) compared to 47.5 kDa on SDS–PAGE. The four subunits of the acyl-CoA reductase, with at least two sets of isologous subunit contacts, form the central core of the complex. Four monomers of the acyl-protein synthetase (s) bind to the reductase tetramer in a symmetrical fashion through at least one contact site between subunits (denoted c-c′). Acyl-transferase subunits (t) bind to the acyl-protein synthetase subunits through a contact site, d-d′, so that four heterodimers (st) would be expected to be bound to the central reductase tetramer (r_4) in the fully associated fatty acid reductase complex. However, since a complete complex, in terms of the acyl-transferase, has not been isolated, the t subunits have been denoted in a partially dissociated state. On the basis of the subunit molecular weights on SDS–PAGE, such a complex [$r_4(st)_4$] would have an expected molecular weight of (510–570) × 10^3.

The formation of the complex between these three enzymes may play an important regulatory role in diverting fatty acids from the normal metabolic pathways into the bioluminescent system. The transferase enzyme preferentially converts acyl precursors into free fatty acids only when complexed with the synthetase. Similarly, although the level of acylation of the synthetase can be stimulated by either the transferase or the reductase subunits, the turnover of the acyl group on the synthetase and its conversion to aldehyde cannot occur unless the reductase is present. In this manner, the formation of the complex between the three enzymes would regulate the metabolic flow of fatty acids into the luminescent system with the acyl-protein synthetase playing a central role between the acyl-transferase and reductase subunits. Further experiments will be necessary to determine if the fatty acyl precursors are channeled by the fatty acid reductase complex directly to luciferase and to investigate how the protein interactions regulate the activities of the transferase and synthetase subunits.

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Registry No. Fatty acid reductase, 75718-33-1; acyl-CoA reductase, 50936-56-6; acyl-protein synthetase, 82657-98-5; fatty acyl-CoA acyltransferase, 51484-53-8.

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