may mean the receptor exists in a relatively inaccessible area of the membrane. Nonetheless, this model should provide a useful basis for future studies.

References

Anderson, G. W., & McGregor, A. C. (1957) *J. Am. Chem. Soc. 79*, 6180–6183.

Aswanikumar, S., Corcoran, B. A., Schiffmann, E., Day, A. R., Freer, R. J., Showell, H. J., Becker, E. L., & Pert, C. B. (1977a) *Biochem. Biophys. Res. Commun. 74*, 810–817.

Aswanikumar, S., Corcoran, B., Schiffmann, E., Pert, C. B., Morell, J. L., & Gross, E. (1977b) in *Peptides* (Goodman, M., & Meienhofer, J., Eds.) pp 141–145, Wiley, New York.

Becker, E. L. (1979) *J. Reticuloendothel. Soc. 26 (Suppl.)*, 701–709.

Becker, E. L., Bleich, H. E., Day, A. R., Freer, R. J., Glasel, J. A., Latina, M., & Visintainer, J. (1979) *Biochemistry 18*, 4656–4668.

Day, A. R., Radding, J. A., Freer, R. J., Showell, H. J., Becker, E. L., Schiffmann, E., Corcoran, B., Aswanikumar, S., & Pert, C. B. (1977) *FEBS Lett. 77*, 291–294.

Day, A. R., Muthukumaraswamy, N., & Freer, R. J. (1980) *Peptides (N.Y.) 1*, 187–188.

Freer, R. J., Day, A. R., Radding, J. A., Schiffmann, E., Aswanikumar, S., Showell, H. J., & Becker, E. L. (1980) *Biochemistry 19*, 2404–2410.

Ho, P. P. K., Yourn, A. L., & Soughard, G. L. (1978) *Arthritis Rheum. 21*, 133–136.

Liao, C. S., & Freer, R. J. (1980) *Biochem. Biophys. Res. Commun. 93*, 566–571.

Lifson, S., & Sander, C. (1980) *J. Mol. Biol. 139*, 627–639.

Naccache, P. H., Volpi, M., Showell, H. J., Becker, E. L., & Sha'afi, R. I. (1979) *Science (Washington, D.C.) 203*, 463–465.

Neidel, J., Wilkinson, S., & Cuatrecasas, P. (1979) *J. Biol. Chem. 254*, 10700–10706.

Neidel, J., Kohane, I., Lackman, L., & Cuatrecasas, P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1000–1004.

Pike, M. C., Kredich, N. M., & Snyderman, R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2922–2926.

Pliska, V., & Fauchère, J. L. (1979) in *Peptides: Structure and Biological Function* (Gross, E., & Meinhofer, J., Eds.) pp 249–252, Pierce Chemical Co., Rockford, IL.

Schiffmann, E., Corcoran, B., & Wahl, S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1059–1062.

Schiffmann, E., Aswanikumar, S., Venkatasubramanian, K., Corcoran, B., Pert, C. B., Brown, J., Day, A. R., Freer, R. J., Showell, H. J., & Becker, E. L. (1980) *FEBS Lett. 117*, 1–7.

Sha'afi, R. I., Williams, K., Wacholtz, M. C., & Becker, E. L. (1978) *FEBS Lett. 91*, 305–309.

Showell, H. J., Freer, R. J., Zigmond, S. H., Schiffmann, E., Aswanikumar, S., Corcoran, B., & Becker, E. L. (1976) *J. Exp. Med. 143*, 1154–1169.

Snyderman, R., & Fudman, E. J. (1980) *J. Immunol. 124*, 2754–2757.

Tilak, M. A. (1970) *Tetrahedron Lett. 11*, 849–854.

Wilkinson, P. C. (1979) *Immunology 36*, 579–588.

Williams, L. J., Snyderman, R., Pike, M. C., & Lefkowitz, R. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1204–1208.

Accumulation of 6-Deoxyerythronolide B in a Normal Strain of *Streptomyces erythreus* and Hydroxylation at Carbon 6 of the Erythranolide Ring System by a Soluble Noninduced Cell-Free Enzyme System†

John W. Corcoran* and Auste M. Vygantas

ABSTRACT: Erythronolide B, a presumed intermediate in the biosynthesis of the erythromycins, has been shown to be formed from 6-deoxyerythronolide B by hydroxylation at C-6. The substrate, a metabolite of a blocked mutant of *Streptomyces erythreus* and postulated to be an intermediate in the biosynthesis of the erythromycins, is found also in wild-type cultures of *S. erythreus* CA340 either normally or in increased amount when an inhibitor of NADPH function is present. The hydroxylation of 6-deoxyerythronolide B is catalyzed by a stable and soluble cell-free enzyme preparation obtained from noninduced *S. erythreus* CA340, and the maximal specific activity of the hydroxylase system is found with the protein fraction precipitating between 50% and 90% of saturation with ammonium sulfate. The hydroxylase activity correlates well with the specific content of a cytochrome P-450 moiety present in the system and is inhibited by anaerobiosis and carbon monoxide.

The macrocyclic lactones (Figure 1) present in the known erythromycins A, B, C, and D each possess hydroxyl group functions that cannot be derived directly from the substrates believed used for their biosynthesis. The lactones are presumably formed from a "starter" molecule of activated propionate and six chain-extending units of activated 2-methylmalonate (Masamune et al., 1977; Corcoran, 1981). The mechanism proposed is that of a general fatty acid synthase (GFAS) in which chain growth does not depend on prior or "lock-step" removal of the β-oxo function introduced at each stage of elongation. The assembly of the "primative" and as yet unknown 14-member macrolide ring, with all substrate-derived oxo functions still present, may take place on a polyenzyme template since no partial structures or prelactonic intermediates have been detected. The simplest structure yet

† From the Department of Biochemistry, Northwestern University Medical School, Chicago, Illinois 60611. *Received March 26, 1981; revised manuscript received September 14, 1981.* This investigation was supported by grants from the U.S. Public Health Service (GM-22349 and AI-09158) and the National Science Foundation (PCM75-10096). This paper is dedicated to Professor David Shemin in honor of his 70th birthday.

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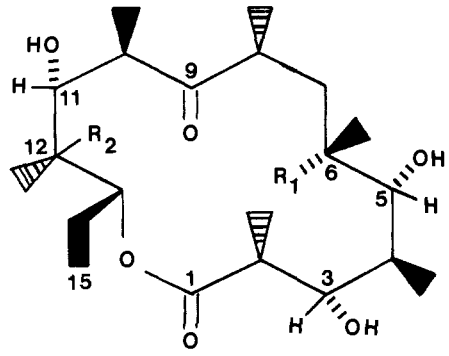

FIGURE 1: Structures of the erythronolides. 6-Deoxyerythronolide B (I, 6dEb; 3*L*,5*L*,11*L*-trihydroxyerythranolid-9-one), $R_1 = R_2 = H$. Erythronolide B (II, Eb; 3*L*,5*L*,6*D*,11*L*-tetrahydroxyerythranolid-9-one), R_1 = OH and R_2 = H. Erythranolide A (Ea; 3*L*,5*L*,6*D*,11*L*,12*D*-pentahydroxyerythranolid-9-one), $R_1 = R_2$ = OH. The semisystematic nomenclature used is one proposed by Corcoran (1981). It follows guidelines suggested by an IUPAC–IUB: Commission on Biochemical Nomenclature, in which substituents at carbon atoms of the macrolide ring are represented by dashed or open bonds if projecting to the rear of the plane defined by that carbon atom and the two adjacent ones and are solid if projecting to the front of that plane. Stereochemical designation of substituents as *D* or *L* is based on the Fischer convention as defined for substituted fatty acids where the carbon chain is oriented vertically with the carboxyl group uppermost. Substituents at an asymmetric center (H, OH, or alkyl) are *D* if projecting to the right and *L* if to the left. Erythr*a*nolide, the stereoparental structure, is the 14-member macrolide lactone that would be formed by the removal of all nonlactonic oxygen atoms from the aglycon present in erythromycins A and C.

known that is formed by this particular pathway is 6-deoxyerythronolide B (I, 6dEb), first identified by Martin & Rosenbrook (1967) as a product of a blocked mutant of *Streptomyces erythreus*. This lactone I possesses oxygen atoms only at carbon positions corresponding to carboxyl oxygen atoms in the presumed monomeric substrates, and the minimal chemical change required for the formation of I from the hypothetical first possible macrolide precursor includes reduction of the oxo groups at C-3, -5, -11, and -13 to secondary alcohol functions, lactonization (13→1), and complete elimination of the oxo group at C-7. It is not known which if any of these changes occur while the lipid chain is attached to the GFAS which makes it, but some evidence exists which suggests that oxygen group modifications may occur prior to release of the lactone from a synthase (Majer et al., 1976).

Erythronolide B (II, Eb) possesses one "extra" (not substrate-derived) hydroxyl oxygen atom at C-6, and its role in the biosynthesis of the erythromycins was shown by Hung et al. (1965). The demonstration that 6-deoxyerythronolide B (I) is also a biosynthetic intermediate (Martin & Rosenbrook, 1967) prompted the present study of the formation of 6dEb by nonmutant cultures of *S. erythreus* and the nature of the C-6 hydroxylase activity.

When a nonmutant and relatively active former production strain of *S. erythreus* (CA340, Abbott) was used, it was found that on the addition of an inhibitor of an NADPH-specific reductase step in sterol biosynthesis [GEB, or 3β-[2-(dimethylamino)ethoxy]androst-5-en-17-one], a significant accumulation of I occurred. With more sensitive methods of detection, it became evident also that lesser amounts of I are present in cultures lacking this inhibitor. The metabolism of I formed from radioactive propionate led to the accumulation of erythronolide B (II), observed both with the intact mycelium of *S. erythreus* and soluble cell-free extracts of the actinomycete. The hydroxylase activity in the crude extract is stable and possesses a significant amount of a cytochrome P-450 moiety. During modest purification of the hydroxylase activity, the specific content of the P-450 component rose in parallel with that of the hydroxylase activity, and it may be that the hydroxylase is a mixed-function oxydase dependent on the cytochrome. Preliminary findings reported here support this possibility.

Experimental Section

Materials

All solvents and chemicals used were of reagent grade and used without further purification unless mentioned otherwise. Ammonium sulfate (Sigma Grade I; purified) was recrystallized twice from glass-distilled water. Radiolabeled [2-^{14}C]propionate was obtained from New England Nuclear. Glucose 6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Calbiochemicals and NAD and NADP from P-L Biochemicals. Unisil (activated silicic acid) was obtained from Clarkson Chemical Co., Amberlite XAD-2 resin from Rohm and Haas, Sephadex LH-20 from Pharmacia, and silica gel G precoated glass plates for TLC (no fluorescence indicator) from Brinkmann. Reagents for preparing trimethylsilyl ether (Me_3Si) derivatives of 6-deoxyerythronolide B (I, 6dEb) and erythronolide B (Eb) were purchased from Pierce Chemicals.

Reference samples of 6dEb and Eb were gifts of Abbott Laboratories. The sample of AY-9944 was a gift of Dr. G. Schroepfer, Jr., of Rice University, and the GEB [3-β-[2-(dimethylamino)ethoxy]androst-5-en-17-one] was a generous gift of Dr. P. O'Connell of the Upjohn Co.

Streptomyces erythreus CA340. The culture of *S. erythreus* CA340 (Abbott) used in this study, and conditions for its maintainence, growth and the preparation of crude cell-free extracts have been described by Corcoran (1975). The ability of the culture to form erythronolides and erythromycins from propionate was assayed periodically by the method of Friedman et al. (1964). Biotransformation studies were carried out as described by these workers.

Inhibitors when used were added to the growth medium prior to sterilization. With GEB the best yields of radiolabeled 6dEb resulted with 14 mg/L and when the GEB was present during both development of the vegetative inoculum and the subsequent growth with radioactive propionate.

Methods

Buffers. Two buffers were used. Buffer A [potassium phosphate, 0.5 M, dithiothreitol (DTT), 10^{-3} M, and ethylenediaminetetraacetic acid (EDTA), 10^{-3} M, pH 7.3] was used for all preparations of cell-free extracts and for assay of the erythranolide hydroxylase activity. Buffer B (buffer A minus the DTT) was used for washing the mycelium of *S. erythreus* prior to biotransformation studies or the making of cell-free extracts.

Protein Determinations. The biruet method with bovine serum albumin as standard was used.

Measurement of Radioactivity. Radioactivity was measured by using a Nuclear Chicago Mark I spectrometer. Samples were dissolved or suspended in the following mixture: naphthalene, 125 g; 2,5-diphenyloxazole, 7 g; 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene, 0.4 g; dissolved in *p*-dioxane, 1 L. Counting efficiencies and disintegration per minute (dpm) values were estimated by the channels ratio method using an external standard.

Thin-Layer Chromatography. Three solvent systems were used for the TLC analysis of 6dEb and Eb. These were system I (benzene–ethyl acetate–acetic acid, 1:9:0.01), system II [petroleum ether (bp 30–60 °C)–ethyl acetate, 70:39], and

system III (benzene–ethyl acetate, 4:1). Silica gel G precoated plates (no fluorescence indicator) were employed for both analytical and preparative TLC work. For assay of radioactive samples of 6dEb and Eb, the developed plates were marked at intervals of 1 cm from the origin, and each zone was scraped into a scintillation vial for determination of radioactivity. When the sample needed was recovered from the TLC plate, the recovered gel was extracted continuously with diethyl ether for 16 h by using a Soxhlet extractor.

Column Chromatography. Usually the erythronolide fraction from a culture of *S. erythreus* was recovered in crude form from the mycelium-free growth medium (after centrifugation at 1200*g* for 15 min) by the method of Martin et al. (1966). Further purification of the 6dEb and Eb was done by column chromatography using either Unisil or LH-20 adsorbant. With Unisil (2 × 15 cm), batch elution with petroleum ether (bp 30–60 °C)–benzene (1:1; 100 mL), benzene (100 mL), benzene–diethyl ether (3:2; 200 mL), diethyl ether (200 mL), ethyl acetate (200 mL), and methanol (100 mL) gave good initial separations, with the 6dEb eluting primarily in the diethyl ether fraction and the Eb in the ethyl acetate eluate. Rechromatography of the 6dEb either directly or after preparative TLC purification (system I) using a Unisil column (as above) with benzene (200 mL), diethyl ether (200 mL), ethyl acetate (200 mL), and methanol (100 mL) as eluants gave chromatographically pure 6dEb in the diethyl ether fraction. Column chromatography of the 6dEb using LH-20 lipophilic gel and a solvent system of chloroform–hexane (1:1) was an alternative to the Unisil chromatography and also yielded a single homogeneous peak of the lactone following prior Unisil or preparative TLC purification. As a useful alternative to the procedure described by Martin et al. (1966) for the preliminary recovery of the erythronolide fraction from culture filtrates, etc., the mycelium-free solution was passed over a column of XAD-2 macroreticular nonionic adsorbant resin which was then washed with water to free the adsorbed lipophilic substances from proteins, salts, small neutral molecules, etc. The total adsorbed erythronolide fraction (including erythromycins if present) was then eluted from the XAD-2 resin with ethanol. The residue obtained was suitable for further purification as described above. For the recovery of 6dEb and Eb from cell-free assay systems, the total assay mixture was passed over a small column (1 × 12 cm) of XAD-2 resin packed in water. The resin was washed with water (200 mL) and ethanol (95%; 80 mL). The water and first 5 mL of the ethanol were discarded. The remaining ethanol eluate contained all of the erythronolides plus other lipophilic substances. The residue was suitable for TLC analysis of the amounts of 6dEb and Eb present.

Gas–Liquid Chromatographic and Mass Spectral Analyses. Reference 6dEb and Eb were converted to their respective per(trimethylsilyl) derivatives by treatment with Tri-Sil TBT (Pierce Chemical Co.) reagent for 15 min at 25 °C. GLC analysis on a 6-ft OV-1 column (1.5%; 2-mm i.d.) using a Varian Model 2100 system (column temperature 215 °C; injector and detector temperatures 225 °C) showed the presence of a single diagnostic peak for each lactone which corresponded to the tris- and tetrakis(trimethylsilyl) derivatives, respectively, when analyzed by GLC–Mass spectrometry (courtesy of Professor Josef Fried, University of Chicago).

Radiolabeled 6-Deoxyerythronolide B. Incubation of *S. erythreus* with [^{14}C]propionate (1 or 2 labeled) or 2-methylmalonate ($^{14}CH_3$) in the presence (and absence) of GEB gave radioactive 6dEb which was purified and characterized as described above. The specific radioactivity of the 6dEb was assumed to be 7 times that of the monomeric substrate used (cf. Kaneda et al., 1962). The substrate used for the studies of the C-6 hydroxylase activity was derived from [2-^{14}C]-propionate, and its specific activity was calculated to be 5.6 × 10^{-3} μCi/μmol. Dilution of the 6dEb by endogenous propionate during its biosynthesis could make this value too high, but the error is probably less than 2-fold.

Cell-Free Enzyme Preparations. A vegetative culture of *S. erythreus* was washed once by centrifugation and resuspension in buffer B, followed by centrifugation for 10 min at 1200*g*. The mycelium was suspended in buffer A (three parts by volume) and the mixture passed through a French pressure cell at 15 000–20 000 psi. The exudate was centrifuged at 20000*g* for 30 min and the supernatant fraction again centrifuged at 165000*g* for 60 min. The resultant pellet of microsomes and mycelial debris was resuspended in buffer A and subjected to ultrasonic oscillation for a total of 1 min (4 × 15 s) with cooling in an ice bath. This product was used as the "particulate" enzyme fraction. The supernatant fraction from centrifugation at 165000*g* (soluble) was used as the "crude enzyme" for initial studies of the C-6 hydroxylase (15–20 mg of protein/mL) and for the preparation of fractions precipitated by ammonium sulfate. For the latter, the crude enzyme (100 mL) was treated with stirring at 0 °C with pulverized solid ammonium sulfate (23.3 g) and after adjustment of the pH to 7.1 (KOH) sitrred for an additional 20 min. The pellet collected after centrifugation at 20000*g* for 20 min was resuspended in a minimal amount of buffer A to give the fraction insoluble between 0% and 35% of saturation (15 mg of protein/mL). The supernatant fraction remaining was treated with additional solid ammonium sulfate (37.3 g/100 mL) under similar conditions to yield a precipitate (30–90% insoluble) and a supernatant fraction. The precipitate was suspended in a minimal amount of buffer A (13 mg of protein/mL) prior to assay. Another scheme for fractionation with ammonium sulfate used the same technique but with the following amounts of solid salt: 0–20%, 14 g; 20–50%, 18.9 g; 50–90%, 30.1 g (each amount per 100 mL of the supernatant fraction from the preceding step).

Assay of the Erythranolide C-6 Hydroxylase. Preliminary studies of the C-6 erythranolide hydroxylase were performed as follows. 6-[^{14}C]Deoxyerythronolide B (6dEb) (9000 dpm, 0.72 μmol) dissolved in a drop of ethanol was diluted with buffer A (0.2 mL) and the ethanol removed in a stream of dry nitrogen. The enzyme (2.0 mL) was added to the substrate, and then this solution was added to a solution of glucose 6-phosphate (15 mg), glucose-6-phosphate dehydrogenase (20 units), and $NADP^+$ (6 μmol) in buffer A (0.5 mL total volume) which had been preincubated for 10 min at 33 °C. The assay mixture (2.5 mL) was incubated for 60 min at 33 °C unless noted otherwise. In later studies of the correlation of hydroxylase activity and cytochrome P-450 content, carrier Eb (1 mg/assay) was added with the radiolabeled substrate (0.05 mL ethanol solution), and the evaporation of the ethanol was omitted. The NADPH-generating system was mixed with the enzyme–substrate solution without preincubation. Equivalent results were obtained, and recovery of the product Eb was greater.

Workup of the assay mixture by adsorption of substrate and product on XAD-2 resin, elution with ethanol, and analytical TLC fractionation was as described above. Graphical analysis of the radioactivity pattern observed on each TLC plate as compared with that of control assays from the same experiment was utilized for calculation of the amount of hydroxylated product formed. Standard reference samples of substrate and

product were included in each TLC analysis, but because of considerable dependency of the relative migration rate of 6dEb and Eb on the size of the sample applied, the main determinant used in the calculation was the location and shape of the radioactive peaks determined for each.

Abbreviations and Other Conventions. Standard biochemical abbreviations are used throughout for coenzymes and common components of buffers and other reagents. All procedures were carried out between 0 and 5 °C unless noted otherwise. Mixtures of solvents are stated as volume per volume, and concentrations of solutes are either given in terms of molarity or grams per 100 g of solution. Commonly accepted trivial names are used for the erythronolide lactones, but the hydroxylase described is named on a semisystematic basis proposed by Corcoran (1981) in which the fully reduced 14-member macrolide lactone related to both 6dEb (I) and Eb (II) is names *erythranolide* (cf. also legend to Figure 1).

Results and Discussion

Appearance of Erythonolides, Erythromycins, and Fatty Acids in the Normal Mycelium of Streptomyces erythreus CA340. Prior to a detailed study of the enzymes involved in the transformation of known erythromycin lactones (erythronolides) into the erythromycins, a brief study of the accumulation of these substances in the mycelium and culture medium of *S. erythreus* CA 340 was carried out. Earlier work (Masamune et al., 1977; Corcoran, 1981) had showed that the "apparent" specific activities of the lactone and fatty acid synthases of *S. erythreus* are quite time dependent, with a maximum between 6 and 12 h following inoculation of a vegetative growth medium. Erythromycin biosynthesis is similarly time dependent, with a peak of maximum specific activity that is only slightly delayed from that seen for the lactones. In this study, sterols, free fatty acids, erythronolides, and erythromycins were detected by TLC analysis of either ethyl acetate or methylene chloride extracts of mycelium or filtered culture broths. The sterols were found in trace amounts only in the mycelial fraction. Erythromycins were present after both 6 and 20 h of growth, but only in the growth medium. Fatty acids were detected after both time periods, but only in the mycelial fraction. Gas–liquid chromatographic (GLC) analysis of the methylated fatty acid fraction indicated a pattern of free acids like that previously reported by Hofheinz & Grisebach (1965). Erythronolides were detectable after 6 h of growth of the *S. erythreus* but were much less noticeable at 20 h.

Effect of Inhibitors of NADPH Function. In the hope that inhibitors of NADPH function could cause accumulation of intermediates in the biogenesis of the erythronolides, *S. erythreus* was grown in the presence of AY-9944 and 3-β-[2-(dimethylamino)ethoxy]androst-5-en-17-one (GEB). Both inhibit sterol biosynthesis, with GEB being a specific agent against the Δ^{24}-reductase step. Analysis of the fermentation products with AY-9944 showed no changes from a control culture, but after exposure to GEB, three new components were detected in extracts of the culture medium. Analysis (TLC) of the fraction extractable with ethyl acetate showed one product with mobility similar to the known lactone 6-deoxyerythronolide B (6dEb, I) and two other substances of higher polarity. The latter have not been identified. The product similar to 6dEb was purified by chromatography of the residue on a silicic acid column using a batch elution method (petroleum ether to methanol). The 6dEb-like product eluted with the diethyl ether while erythronolide B (Eb, II) was found in the ethyl acetate fraction. Numerous fermentations were performed aimed at optimizing the yield of the product as well as its identification. Radiolabeled propionate and 2-methylmalonate were efficiently incorporated into both Eb and the 6dEb-like product, and the addition of the GEB greatly increased (3–4-fold) not only the total amount of radioactivity incorporated from these precursors but also the relative amount of the 6dEb-like product. The best results followed addition of the GEB to both the inoculum culture and the subsequent fermentation with radioactive substrate. In cultures lacking the GEB the main product was Eb (II), while with GEB a significant amount of the 6dEb-like product was found (in a typical experiment, 140000 cpm out of 800000 cpm found in the original ethyl acetate extract). The proof of identity of the isolated product with reference 6dEb (courtesy of Dr. Jerry R. Martin, Abbott Laboratories) rests both on direct and circumstantial data. Cochromatography of the isolated product with authentic 6dEb on TLC analysis (systems I and II) and on column chromatography using either silicic acid (Unisil) or Sephadex LH-20 gel suggested strongly that the product from the *S. erythreus* after addition of GEB was 6dEb (I). Treatment of the product with Tri-Sil TBT and GLC analysis of the single per(trimethylsilyl) derivative formed showed that the recovered product behaved exactly as did authentic 6dEb. A comparable study of Eb (II) was done, and GLC–mass spectral analysis (courtesy of Professor Josef Fried, University of Chicago) of these Me_3Si derivatives showed that they corresponded to the $(Me_3Si)_3$ derivative of 6dEb and the $(Me_3Si)_4$ derivative of Eb, respectively. Alternate support for the structural assignment comes from the observation that the radiolabeled product (6dEb-like) was efficiently transformed by the intact mycelium of *S. erythreus* into Eb and also that a cell-free enzyme system derived from this organism utilizes both the radiolabeled product and authentic 6dEb as substrates for hydroxylation at C-6, leading to Eb (II).

Transformation of Radiolabeled 6dEb into Eb by Intact S. erythreus CA340. Following preliminary indications that slightly impure ^{14}C-labeled 6dEb (I) was transformed into Eb (II) by the intact mycelium of *S. erythreus*, a sample of the substrate was carefully purified by a combination of silicic acid and XAD-2 column chromatography and TLC preparative fractionation. The purified substrate (Figure 2A) showed a single peak on TLC analysis and was used for further studies. Incubation of the ^{14}C-labeled 6dEb with intact *S. erythreus* in the presence of nonradioactive Eb led to the formation of a significant amount of radioactive Eb (Figure 2B) under conditions where [^{14}C]propionate was incorporated well into both 6dEb and Eb in a control experiment. The proof that the radioactive product is Eb rests on cocrystallization of the radioactivity with added Eb and on chromatographic behavior of the product and derivatives on TLC and GLC–mass spectral analysis (not shown). The biotransformation experiments suggested that *S. erythreus* possesses a C-6 erythranolide hydroxylase activity (below) and support the proposal of Martin & Rosenbrook (1967) that 6dEb is a direct precursor of Eb. The control experiment not only verified that the *S. erythreus* possessed the complete pathway for the biosynthesis of Eb but also provided the first evidence for the formation of 6dEb in "normal" cultures of *S. erythreus*. In the control experiment (no GEB), significant amounts of radioactive 6dEb accumulated together with the expected radiolabeled Eb.

Hydroxylation of 6-Deoxyerythronolide B by a Cell-Free System from S. erythreus CA340. A culture of *S. erythreus* (15-h growth) was ruptured by using a French pressure cell, and the exudate was divided by differential centrifugation into 165000*g* (supernatant) and pellet (microsomes, insoluble)

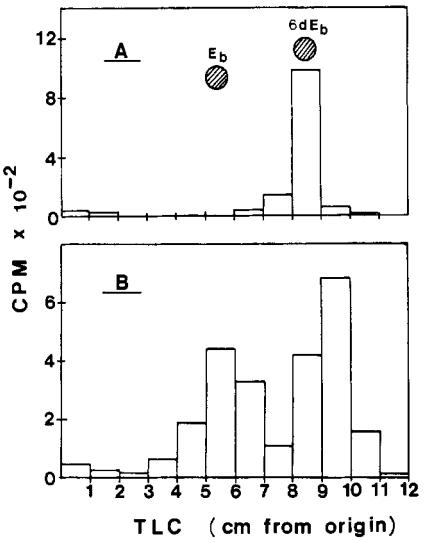

FIGURE 2: (A) Thin-layer chromatogram of 6-deoxyerythronolide B (I, 6dEb) recovered from incubation of radiolabeled [2-^{14}C]-propionate with intact mycelium of *S. erythreus* CA340. [2-^{14}C]-Propionate (13 μmol, 0.27 μCi/μmol) and nonradioactive erythronolide B (II, Eb) were incubated with a suspension of *S. erythreus* [6 mL of a 25% suspension (Corcoran, 1975)] diluted with 3 mL of medium M1-102 in a 50-mL Erlenmeyer flask for 2.5 h at 33 °C with shaking. Workup was as described under Methods, and the specific radioactivity of the purified 6dEb was calculated be approximately 5.6 × 10^{-3} μCi/μmol; (B) Thin-layer chromatogram of radioactive 6-deoxy-erythronolide B (6dEb, I) after incubation with the intact mycelium of *S. erythreus* CA340. 6-Deoxyerythronolide B (16380 dpm, ~1.3 μmol) and nonradioactive Eb (II) (2 mg) were incubated with a suspension of washed *S. erythreus* as described in part A.

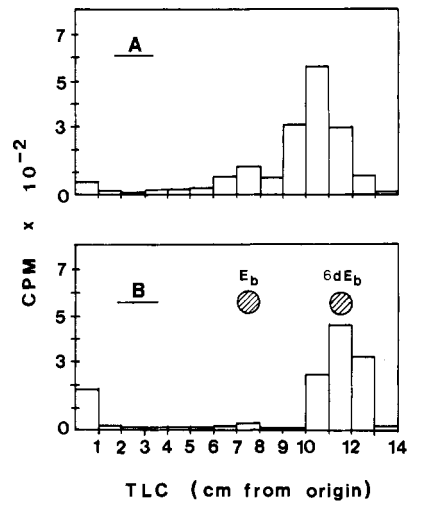

FIGURE 3: Thin-layer chromatographic analysis of radiolabeled 6dEb (I) after incubation with soluble (system A) and particulate (system B) enzyme fractions from *S. erythreus* CA340. System A utilized a 165000*g* supernatant enzyme fraction (16 mg of protein/mL) and system B a sonicated pellet collected at 165000*g* (8 mg of protein/mL). The incubation (75 min) of the 6dEb (1.2 μmol, 15 000 dpm) and the workup of the assay were as under Methods.

fractions. These were assayed under aerobic conditions with an NADPH-generating system and radiolabeled 6dEb (I) as substrate. No product (Eb, II) was added. When the lipophilic products of the incubation were fractionated by the TLC method (Figure 3), it was seen that hydroxylase activity existed in the 165000*g* fraction (panel A) but that no significant hydroxylation was promoted by the microsomal pellet fraction (panel B). The soluble C-6 erythranolide hydroxylase activity was reasonably stable. After storage for 2 weeks in the frozen state, the thawed enzyme was able to hydroxylate nonradioactive 6dEb (direct visualization after spraying with an anisaldehyde reagent; not shown), and after 5 weeks the thawed hydroxylase was about one-fifth as active as the fresh enzyme.

Table I: Comparison of C-6 Hydroxylase Activity and Cytochrome P-448 Content of Soluble Enzyme System from *Streptomyces erythreus*[a]

protein fraction	hydroxylase act. [mmol of 6dEb utilized min^{-1} L^{-1} (mg of protein)$^{-1}$]	cytochrome P-448 content [nmol (mg of protein)$^{-1}$ × 10^2]
crude	4.7	4.2
165000*g* supernatant	3.4	4.5
$(NH_4)_2SO_4$ ppt; 0–20%	1.8	0
$(NH_4)_2SO_4$ ppt; 20–50%	1.2	2.0
$(NH_4)_2SO_4$ ppt; 50–90%	9.7	10.6

[a] Substrate 6dEb (I) (1.63 μmol, 2.63 × 10^4 dpm) incubated with enzyme protein (crude, 14.3 mg; 165000*g* supernatant, 12.5 mg; ammonium sulfate precipitates: 0–20%, 4.5 mg; 20–50%, 12.3 mg; 50–90%, 8.8 mg) for 60 min at 35 °C. Assay procedure and workup as described under Methods. Cytochrome P-450 content of fraction measured by method of Omurs & Sato (1964).

Preliminary Characterization of the C-6 Hydroxylase. The time course of hydroxylation of 6dEb by a crude 165000*g* supernatant fraction of *S. erythreus* was determined with a frozen and thawed enzyme preparation 1-week old. The rate of the reaction was nearly linear for 60 min but fell off markedly by 120 min. An assay time of 60 min was used for characterization of the system, and it was found that about 12 mg of crude protein per mL of assay mixture gave a nearly maximal rate. Treatment of the crude C-6 hydroxylase with ammonium sulfate gave three fractions, insoluble between 0% and 20%, 20% and 50%, and 50% and 90% of saturation, respectively. The maximum specific activity was found in the last (50–90%) fraction (cf. Table I).

Characterization of the C-6 Hydroxylase Activity in the Protein Insoluble between 50% and 90% of Saturation with Ammonium Sulfate. A more active C-6 hydroxylase preparation obtained by fractionation of the 165000*g* supernatant solution with ammonium sulfate was used for further characterization of the reaction. This enzyme was able to promote conversion of nonradioactive 6dEb into Eb, as shown by the mobility of the product Eb on TLC analysis as well as by GLC and mass spectral properties of the Eb after recovery from the TLC plate. The time course of the hydroxylation reaction is shown as Figure 4, and although similar to that seen with the crude hydroxylase, the specific activity is higher, and the reaction still continues at 120 min. The dependency of the hydroxylation on the amount of protein added is shown as Figure 5, and the rate of the reaction is proportional to the amount of enzyme added to at least 13 mg of protein per mL of assay mixture.

Dependency of the C-6 Hydroxylase on a Cytochrome P-450 Moiety. The possibility that a cytochrome P-450 heme protein is required for the C-6 hydroxylase was investigated. The active hydroxylase fraction was first treated with dithionite followed by carbon monoxide gas, and a difference absorption spectrum was measured. A peak with a maximum at 448-nm was observed when the 165000*g* enzyme solution was so examined (Figure 6). Little of this 448-nm peak was seen with the microsomal pellet fraction. A large peak at 430 nm was

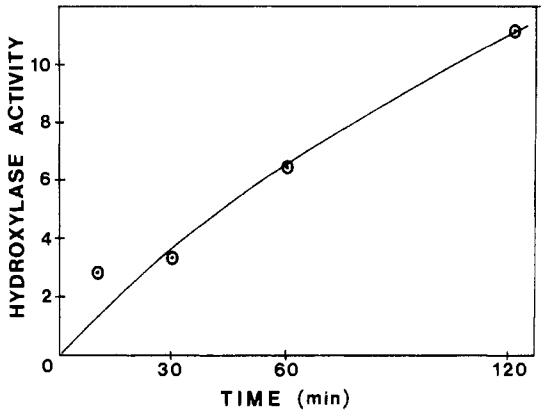

FIGURE 4: Time course of the cell-free C-6 hydroxylation of 6-deoxyerythronolide B (I). Substrate 6dEb (2.4 μmol, 30000 dpm) was incubated with enzyme (8.8 mg of a freshly prepared dialyzed 50–90% ammonium sulfate precipitated protein) for varying times at 33 °C. Assay conditions and workup as described under Methods. Hydroxylase activity expressed as erythronolide B formed per assay (nmol × 10^1).

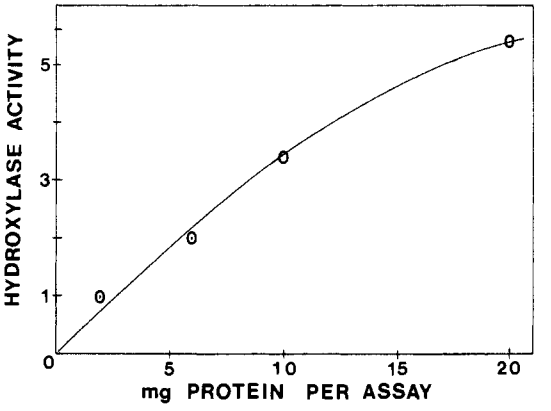

FIGURE 5: Hydroxylation at C-6 of 6-deoxyerythronolide B (I, 6dEb) as a function of enzyme concentration. Substrate 6dEb (1.63 μmol, 2.63 × 10^4 dpm) was incubated at 33 °C for 60 min with varying amounts of enzyme protein (a thawed 1-week-old dialyzed 50–90% ammonium sulfate fraction stored at −20 °C). Assay conditions and workup as described under Methods. Hydroxylase activity is expressed as product (Eb) formed per 60 min (nmol × 10^1).

also seen, and this absorption seemed to increase in aged preparations at the expense of the 448-nm peak. When the specific content of the cytochrome P-448 moiety was measured, a good correlation between the hydroxylase activity and the P-448 content was seen (Table I). Both were highest in the 50–90% ammonium sulfate fraction. In addition to this strong suggestion of a dependency on the cytochrome P-448 moiety, studies with this enzyme source revealed that both anaerobiosis and replacement of the oxygen in the gas phase with carbon monoxide produced significant decreases in hydroxylase activity (90% and 50%, respectively). It was not possible with this enzyme preparation to demonstrate an unequivocal dependency of the reaction on the NADPH-generating system, but the relatively crude nature of the hydroxylase makes this failure of questionable significance [more recent studies of J. W. Corcoran and T. Nishikiori (unpublished results) have fully confirmed a dependency of the hydroxylase system both on the cytochrome P-448 moiety and on NADPH].

Conclusions

An intermediate in the biosynthesis of the erythromycins, 6-deoxyerythronolide B (6dEb, I), described before only as a product of a blocked mutant of *S. erythreus*, has been found to accumulate in the culture medium of a normal strain of *S.*

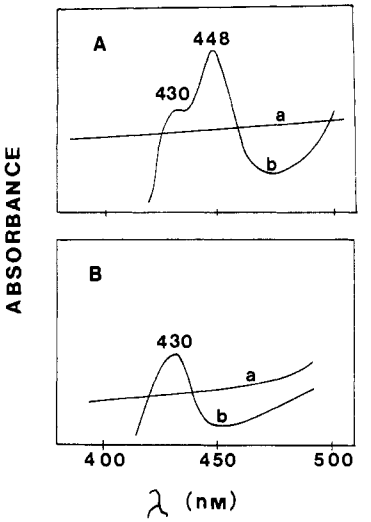

FIGURE 6: Spectral analysis of the erythranolide C-6 hydroxylase and the carbon monoxide adduct of its reduction product with dithionite. Panel A is a 165000*g* supernatant fraction (12.5 mg of protein), and panel B depicts a sonicated 165000*g* pellet fraction (5–10 mg of protein). Curve a in each panel is the untreated enzyme while curve b shows the same solutions after treatment with solid sodium dithionite followed by equilibration with carbon monoxide gas (Omura & Sato, 1964). The important absorptions (in nm and *A* units) are (A) A_{430} = 0.074, A_{448} = 0.095, and A_{480} = −0.014 and (B) A_{430} = 0.4.

erythreus. This lactone I is the most complex erythronolide known which still possesses only carbon and oxygen atoms derived from the activated propionate and 2-methylmalonate units used for its biosynthesis. Transformation of 6dEb into Eb, the next lactone in the biosynthetic pathway, involves the introduction of an "extra" oxygen atom at C-6 of the erythranolide ring system. The intact mycelium of *S. erythreus* as well as a partially purified enzyme system derived from it transforms 6dEb (I) into Eb (II).

The C-6 erythranolide hydroxylase is present in the soluble fraction from *S. erythreus*, and it introduces a hydroxyl group stereospecifically with retention of configuration at C-6 of the erythranolide ring system. The enzyme activity appears to involve a cytochrome P-448 component, and since it is both stable and soluble, it could prove to be a useful model system for the study of the mechanism of activation of molecular oxygen and insertion of the activated species into a sterically hindered, structurally rigid, tertiary center possessing well-defined stereochemistry. Thus, the enzyme system from *S. erythreus* CA340 may be of considerable interest beyond its role in the biosynthesis of the erythromycins. It will be interesting to compare this C-6 hydroxylase with the few other soluble hydroxylases already well studied. Among these are the one from *Pseudomonas putida*, active with camphor and camphor derivatives (Tyson et al., 1972), another from *Candida tropicalis*, active as an alkane and alkanoic acid ω-hydroxylase (Peterson et al., 1966), and one from *P. oleovorans*, also capable of hydroxylating the ω position of alkanes and alkanoic acids (Ruettinger et al., 1974). Of these other hydroxylase systems, the first two require a cytochrome P-450 moiety, and the last functions with a non-heme iron electron carrier.

Work is in progress to determine the detailed nature of the C-6 hydroxylase of *S. erythreus*. It is of further interest to question the apparent lack of a requirement for an added "inducer" of the hydroxylase activity. Is the present system really different from the others cited, each of which requires the addition of substrate to stimulate production of hydroxylase activity, or rather is the development of the C–6 hydroxylase the result of autocatalytic induction by the endogenous pro-

duction of substrate 6dEb during development of the mycelium? The C-6 erythranolide hydroxylase may play an important regulatory role in the biosynthesis of the erythromycins, and continued study of this enzyme system may thus be important for a variety of reasons.

References

Corcoran, J. W. (1975) *Methods Enzymol. 43*, 487.
Corcoran, J. W. (1981) in *Antibiotics IV. Biosynthesis* (Corcoran, J. W., Ed.) pp 132–174, Springer-Verlag, Berlin, Heidelberg, and New York.
Friedman S. M., Kaneda, T., & Corcoran, J. W. (1964) *J. Biol. Chem. 239*, 2386.
Hofheinz, W., & Grisebach, H. (1965) *Z. Naturforsch. B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol. 20B*, 43.
Hung, P. P., Marks, C. L., & Tardrew, P. L. (1965) *J. Biol. Chem. 240*, 1322.
Kaneda, T., Butte, J. C., Taubman, S. B., & Corcoran, J. W. (1962) *J. Biol. Chem. 237*, 322.
Majer, J., McAlpine, J. B., Egan, R. S., & Corcoran, J. W. (1976) *J. Antibiot. 29*, 769.
Martin, J. R., & Rosenbrook, W. (1967) *Biochemistry 6*, 435.
Martin, J. R., Perun, T. J., & Girolami, R. (1966) *Biochemistry 5*, 2852.
Masamune, S., Bates, G. S., & Corcoran, J. W. (1977) *Angew. Chem. Int. Ed. Engl. 16*, 585.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370.
Peterson, J. A., Basu, D., & Coon, M. J. (1966) *J. Biol. Chem. 241*, 5162.
Ruettinger, R. T., Olson, S. T., Boyer, R. F., & Coon, M. J. (1974) *Biochem. Biophys. Res. Commun. 57*, 1011.
Tyson, C. A., Lipscomb, J. D., & Gunsalus, I. C. (1972) *J. Biol. Chem. 247*, 5777.
Vygantas, A. M., & Corcoran, J. W. (1974) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33*, 1233.

Functional Groups at the Catalytic Site of BF_1 Adenosinetriphosphatase from *Escherichia coli*†

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ABSTRACT: The rates of inactivation of BF_1 adenosinetriphosphatase (BF_1-ATPase) from *Escherichia coli* by 7-chloro-4-nitro-2,1,3-benzoxadiazole, 1-fluoro-2,4-dinitrobenzene, 2,4,6-trinitrobenzenesulfonate, phenylglyoxal, and *N,N'*-dicyclohexylcarbodiimide have been measured in the presence and absence of various concentrations of inorganic phosphate, ADP, ATP, or magnesium ion. Dissociation equilibrium constants and rate constants for the labeling reactions have been deduced from a quantitative treatment of the kinetic data. The results suggest that the essential Tyr, Lys, Arg, and Glu or Asp residues are probably located at the catalytic site of BF_1-ATPase and that in addition to steric interference, the effect of charge interaction should also be considered in interpreting the kinetic data on the protection of BF_1-ATPase by substrate molecules against inactivation by the above labeling reagents. Examination of the relative values of the rate constants for the labeling reactions in the presence and absence of inorganic phosphate, ADP, ATP, or magnesium ion, respectively, and the effect of NBD label on the rates of labeling of the essential guanidinium, amino, and carboxyl groups suggest that the arrangement of these four functional groups at the catalytic site of BF_1 may be similar to that previously proposed for MF_1-ATPase from bovine heart; namely, the essential amino group and the unusually reactive phenol group are probably located near the bound inorganic phosphate or the γ-phosphate group of the bound ATP, the essential guanidinium group is probably located nearer to the α- or β-phosphate group than to the γ-phosphate group of the bound ATP or the bound inorganic phosphate, and the essential carboxylate group is probably complexed with a magnesium ion which it shares with the bound inorganic phosphate.

Escherichia coli BF_1-ATPase[1] has the subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Bragg & Hou, 1975; Yoshida et al., 1979) or $\alpha_2\beta_2\gamma_2\delta_2\epsilon_2$ (Vogel & Steinhart, 1976). The isolated enzyme has 3 mol of tightly bound nucleotides, 2 mol of ATP, and 1 mol of ADP per mol of enzyme (Maeda et al., 1976). The tight nucleotide sites seem to be on the α subunits, since the isolated α subunit contains a single tight nucleotide binding site with a K_d value of 0.1 μM for ATP and 0.9 μM for ADP, whereas the β, γ, δ, and ϵ subunits do not bind ATP or ADP in the ligand concentration range from 0.1 to 2 μM (Dunn and Futai, 1980). One of the tight nucleotides may be more rapidly exchangeable with external nucleotides with a K_d value of 1 μM for ADP (Lunardi et al., 1981). *E. coli* BF_1-ATPase has three loose nucleotide binding sites with a K_m of 200–400 μM for ATP hydrolysis (Futai et al., 1974), the K_i for ADP inhibition in hydrolysis is ~80 μM, and K_d for ADP at the

† From the Bioenergetics Laboratory, State University of New York, Buffalo, New York 14214. *Received July 20, 1981.* This work was supported in part by research grants from the National Institute of General Medical Sciences (GM 19990) and the National Science Foundation (PCM 7715002).

[1] Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EEDQ, 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; FDNB, 1-fluoro-2,4-dinitrobenzene; FSBA, 5'-[(*p*-fluorosulfonyl)benzoyl]adenosine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; NAP_4-ADP, 3'-*O*-[4-[*N*-(4-azido-2-nitrophenyl)amino]butyryl]adenosine 5'-diphosphate; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole (also named 4-chloro-7-nitrobenzofurazan); PEP, phosphoenolpyruvate; PG, phenylglyoxal; TNBS, 2,4,6-trinitrobenzenesulfonate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; BF_1, *Escherichia coli* coupling factor; MF_1, beef heart mitochondrial coupling factor; Mops, 3-(*N*-morpholino)propanesulfonic acid.

0006-2960/82/0421-0269$01.25/0