termediate between these extremes (Albery & Knowles, 1976). With Pol I, most of the free energy change occurs in the rate-determining conformational change whereby the enzyme "selects" the correct dNTP. Thus, Pol I is similar to the ATPases in that both couple the favorable free energy of catalysis to a separate process—the ATPases caalyze coupled vectorial processes to do mechanical work (Jencks, 1980), while Pol I couples the favorable free energy change to a process that helps ensure the fidelity of DNA replication.

ACKNOWLEDGMENTS

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REFERENCES

Adelstein, R. S., & Eisenberg, E. (1980) *Annu. Rev. Biochem. 49*, 921.
Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5627.
Bambara, R. A., Uyemura, D., & Lehman, I. R. (1976) *J. Biol. Chem. 251*, 4090.
Barshop, B. A., Wrenn, R. F., & Frieden, C. (1983) *Anal. Biochem. 130*, 134.
Brody, R. S., & Frey, P. A. (1981) *Biochemistry 20*, 1245.
Bryant, F. R., Johnson, K. A., & Benkovic, S. J. (1983) *Biochemistry 22*, 3537.
Burgers, P. M. J., & Eckstein, F. (1979) *J. Biol. Chem. 254*, 6889.
Cleland, W. W. (1975) *Biochemistry 14*, 3220.
Eckstein, F., & Jovin, T. M. (1983) *Biochemistry 22*, 4546.
Ferrin, L. J., & Mildvan, A. S. (1986) *Biochemistry 25*, 5131.
Fierke, C. A., Johnson, K. A., & Benkovic, S. J. (1987) *Biochemistry 26*, 4085.
Gupta, A. F., & Benkovic, S. J. (1984) *Biochemistry 23*, 5874.
Jencks, W. P. (1983) *Adv. Enzymol. Related Areas Mol. Biol. 51*, 75.
Jovin, T. M., Englund, P. T., & Bertsch, L. L. (1969) *J. Biol. Chem. 244*, 2996.
Joyce, C. M., & Grindley, N. D. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1830.
Kornberg, A. (1980) *DNA Replication*, Freeman, San Francisco.
Lanzetta, P. A., Vlvarez, L. J., Reinach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 75.
McClure, W. R., & Jovin, T. M. (1975) *J. Biol. Chem. 250*, 4073.
Mizrahi, V., Henrie, R. N., Marlier, J. F., Johnson, K. A., & Benkovic, S. J. (1985) *Biochemistry 24*, 4010.
Mizrahi, V., Benkovic, P. A., & Benkovic, S. J. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 231.
Mizrahi, V., Benkovic, P., & Benkovic, S. J. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5769.
Ollis, D. L., Brick, P., Hamlin, R., Xuong, N. G., & Steitz, T. A. (1985) *Nature (London) 313*, 762.
Penefsky, H. S. (1985) *J. Biol. Chem. 260*, 13728.
Pickart, C. M., & Jencks, W. P. (1986) *J. Biol. Chem. 259*, 1629.
Raushel, F. M., & Garrard, L. J. (1984) Biochemistry *23*, 1791.
Setlow, P., Brutlag, D., & Kornberg, A. (1972) *J. Biol. Chem. 247*, 224.

N-Methyltransferase Function of the Multifunctional Enzyme Enniatin Synthetase†

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ABSTRACT: The *N*-methyltransferase function of the multifunctional enzyme enniatin synthetase was studied. Similar to other transmethylases, *S*-adenosyl-L-homocysteine (AdoHcy) and sinefungin were found to be potent inhibitors of the *S*-adenosylmethionine-dependent reaction. The K_m value was found to be 10 μM, and the K_i values for AdoHcy and sinefungin were 4 and 110 μM, respectively. Sinefungin acted as a competitive inhibitor with respect to *S*-adenosyl-L-methionine (AdoMet), whereas AdoHcy exhibited an inhibition pattern characteristic for a partial competitive inhibitor. This indicates that AdoHcy does not directly compete with AdoMet but binds to a discrete inhibitory site. In addition, AdoHcy inhibited the formation of the unmethylated depsipeptide formed in the absence of AdoMet. In contrast, sinefungin exhibited no influence on the synthesis of demethylenniatin. This finding confirms the assumption that two different binding sites for the inhibitors must be present. Like other methyltransferases, enniatin synthetase can be affinity labeled by UV irradiation of the protein in the presence of AdoMet labeled at the methyl group. The photoreaction was shown to be site specific, and a binding stoichiometry of one methyl group per enzyme molecule was observed. Limited proteolysis of the methyl-labeled enzyme yielded besides a number of unlabeled fragments only one radiolabeled fragment, size 25 kDa, obviously containing the binding site for AdoMet. Evidence was obtained that the binding site for valine, the substrate to be methylated, was not present on this fragment.

A peculiar property of many peptides from the secondary metabolism of fungi and actinomycetes is the occurrence of methylated peptide bonds in these molecules, as in the case of, e.g., cyclosporins, destruxins, enniatins, actinomycins, and quinoxaline antibiotics. These peptides or depsipeptides, which often contain unusual amino acids not present in proteins, are synthesized via nonribosomal pathways (Kleinkauf & von Döhren, 1981). The enzyme systems responsible for the formation of these metabolites obviously exhibit an *N*-methyltransferase function since in no case were N-methylated

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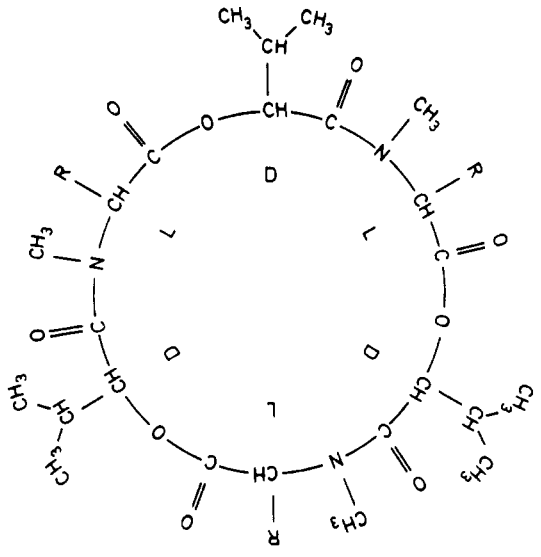

FIGURE 1: Enniatin A, R = $-CH(CH_3)CH_2CH_3$; enniatin B, R = $-CH(CH_3)_2$; enniatin C, R = $-CH_3CH(CH_3)_2$.

precursor amino acids incorporated into the peptide chains. However, a detailed study on the nature of this enzymatic activity of many peptide synthetases has not yet been reported. The present paper describes experiments on the characterization of the *N*-methyltransferase function of the multifunctional enzyme enniatin synthetase as a model system for other peptide and depsipeptide synthetases capable of N-methylation.

Enniatins are cyclic hexadepsipeptide antibiotics with ionophoretic properties produced by various strains of *Fusarium* (Plattner et al., 1948; Wipf et al., 1968). They are composed of alternating residues of D-2-hydroxyisovaleric acid (D-Hiv)[1] and an *N*-methyl L-amino acid, linked by amide and ester bonds (see Figure 1). Enniatin synthetase, the multifunctional enzyme responsible for the biosynthesis of these compounds from their primary precursors, has been isolated from *Fusarium oxysporum* and studied in our laboratory (Zocher et al., 1976, 1978, 1982, 1983). The protein consists of a single polypeptide chain with a molecular mass of about 250 kDa. The reaction sequence leading to the cyclic enniatin molecule can be divided into five partial reactions: (1) activation of D-Hiv and a branched-chain amino acid as adenylates; (2) transfer of adenylates to specific thiol groups to form thio esters; (3) N-methylation of the covalently bound amino acid residue with *S*-adenosylmethionine (AdoMet) as the donor of the methyl group; (4) peptide bond formation; (5) condensation of enzyme-bound dipeptidols and cyclization (enniatin formation).

The absence of AdoMet from the reaction mixture provokes synthesis of unmethylated enniatins at a reduced reaction rate (Zocher et al., 1982). Thus, enniatin synthetase can be considered as a depsipeptide synthetase capable of forming ester and amide bonds, joined with a methyltransferase. The question arises whether this methyltransferase function as an integral part of a multifunctional enzyme bears similarity to other AdoMet-dependent *N*-methylases occurring in the metabolism of eucaryotic cells. Therefore, kinetic and structural studies of the methyltransferase function of enniatin synthetase have been performed and are described in the present paper.

Materials and Methods

Materials. Commercial sources of reagents were as follows: adenosine, *S*-adenosyl-L-methionine, *S*-adenosyl-L-ethionine, *S*-adenosyl-L-homocysteine, 5′-deoxy-5′-(isobutylthio)-adenosine, 5′-deoxy-5′-(methylthio)adenosine, 5-methyltetrahydropteroylpentaglutamate, sinefungin, L-amino acid oxidase, and protease from *Staphylococcus aureus*, strain V8, were from Sigma Chemical Co., Deisenhofen, West Germany; acrylamide, methylenebis(acrylamide), and *N,N,N′,N′*-tetramethylethylenediamine were from Fluka, Buchs, Switzerland; *S*-[*methyl*-^{14}C]adenosyl-L-methionine (60 Ci/mol), *S*-[*methyl*-^{3}H]adenosyl-L-methionine (70 Ci/mmol), *S*-[*carboxy*-^{14}C]adenosyl-L-methionine (60 Ci/mol), [U-^{14}C]adenosine 5′-triphosphate (600 Ci/mol), [U-^{14}C]-L-valine (250 Ci/mol), and [2-^{14}C]iodoacetamide (60 Ci/mol) were purchased from Amersham Buchler, Braunschweig, West Germany. [U-^{14}C]-DL-Hydroxyisovaleric acid was prepared from [U-^{14}C]-L-valine by oxidation with L-amino acid oxidase and subsequent reduction of the keto acid with sodium borohydride. All other chemicals were of the purest available grades from standard commercial sources.

Preparation of Enzyme. Enniatin synthetase was isolated from *Fusarium oxysporum* strain ETH 1536/9 and purified as described (Zocher et al., 1982).

Analysis of Reaction Products by Thin-Layer Chromatography. Reaction products of enniatin synthetase were extracted with EtOAc and separated on reversed-phase (C-18) plates (Merck, Darmstadt, West Germany). Radioactive products were detected by autoradiography.

Kinetic Measurements. All measurements were performed in 50 mM potassium phosphate buffer (pH 7.2) containing 4 mM dithiothreitol (buffer A). For kinetic measurement of enniatin B formation, one of the four substrates (L-Val, D-Hiv, AdoMet, ATP) was varied, whereas the others were present in saturating concentrations [5 mM ATP, 10 mM $Mg(OAc)_2$, 1 mM D-Hiv, 1 mM L-valine, and 36 μM [*methyl*-^{14}C]AdoMet (0.25 μCi)]. The reaction was started by the addition of 3.2 μg of enniatin synthetase (0.1 μM); the total volume of the reaction mixture was 120 μL. Incubation was carried out at 25 °C and stopped after 8 min by addition of 2 mL each of water and EtOAc. Enniatin B was extracted into the organic layer and radioactivity determined by liquid scintillation counting (Bray, 1960).

For the measurement of demethylenniatin B formation, AdoMet was omitted, and [^{14}C]-L-valine was used as the radiolabel; incubation was extended to 30 min. For the determination of the kinetic parameters, 5 μCi of labeled valine was used, and the total concentration of valine was 800 μM. For the demonstration of the inhibition of demethylenniatin B synthesis by AdoHcy with respect to ATP and D-Hiv, 0.5 μCi of [^{14}C]valine (17 μM) was used; that means in this experiment there was no substrate saturation with respect to valine. The reaction rates of enniatin B and demethylenniatin B formation were linear under these conditions for 15 and 60 min, respectively.

ATP–Pyrophosphate Exchange. Valine- or D-Hiv-dependent ATP–pyrophosphate exchange was carried out as described by Gevers et al. (1969).

Determination of Covalent Enzyme–Substrate Complexes. This was carried out by incubation of enniatin synthetase (0.1 μM) in the presence of ATP (5 mM), $Mg(OAc)_2$ (10 mM), and either 0.5 μCi of labeled valine or 1 μCi of labeled DL-Hiv. The reaction was stopped after 10 min by addition of 1 mL of 7% trichloroacetic acid and the protein filtered off with membrane filters ME 25 (Schleicher & Schüll, Dassel, West Germany). Retained radioactivity was determined after washing and drying of the filters by liquid scintillation counting.

[1] Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; D-Hiv, D-2-hydroxyisovaleric acid; EtOAc, ethyl acetate; $NaDodSO_4$, sodium dodecyl sulfate.

Affinity Labeling of Enniatin Synthetase. To 7.9 μg (31.6 pmol) of enniatin synthetase was added [*methyl*-^{14}C]AdoMet (up to 0.25 μCi), [*methyl*-^{3}H]AdoMet (5 μCi), [*carboxy*-^{14}C]AdoMet (0.25 μCi), or [U-^{14}C]ATP (0.5 μCi) in a total volume of 30 μL. For the studies of the effect of inhibitors on the affinity labeling, the concentrations were as follows: 4–600 μM adenosine (control experiment), 150–600 μM sinefungin, and 4–100 μM AdoHcy.

The mixtures were irradiated at 4 °C for 10 min with short-wave UV light (254 nm) from a 44-W Hg lamp from a distance of 10 cm. The reaction was stopped by the addition of 100 μL of 7% trichloroacetic acid, and the protein was filtered off with membrane filters (see above). After washing and drying of the filters, radioactivity was determined by liquid scintillation counting.

Modification of Enniatin Synthetase with Iodoacetamide. To 12 μg (48 pmol) of enniatin synthetase in buffer A was added 0.5 μCi of [2-^{14}C]iodoacetamide to a final volume of 35 μL. After 10 min at 25 °C, the sample was subjected either to proteolytic digestion or to polyacrylamide gel electrophoresis directly.

Proteolytic Digestion of Enniatin Synthetase. To 12 μg (48 pmol) of enniatin synthetase in buffer A was added 1 μg of protease V8 to a final volume of 30 μL; incubation was for 10 min. The reaction was stopped by the addition of 10 μL of electrophoresis sample buffer (3% $NaDodSO_4$, 30% 2-mercaptoethanol).

Polyacrylamide Gel Electrophoresis. This was performed according to Laemmli (1970); gels contained 15% acrylamide. Fluorography of radiolabeled proteolytic digests was performed with Amplify from Amersham Buchler, Braunschweig, West Germany, according to the instructions of the manufacturer.

Protein Determinations. The $E^{280}_{1\%}$ value of enniatin synthetase was determined to be 8.66. Therefore, the protein concentration in solutions of the enzyme purified to over 98% homogeneity could be measured photometrically.

Results

Kinetic Properties of the Methyltransferase Function. In a previous paper (Zocher et al., 1982), it was shown that in the presence of AdoMet the rate of depsipeptide formation is about 10-fold higher than when the methyl donor is absent. In order to get knowledge about the reason for this effect of AdoMet on the biosynthetic process, we performed kinetic measurements of enniatin B and demethylenniatin B synthesis. One substrate was varied, while the other substrates were kept at saturating concentrations; the kinetic parameters were obtained from double-reciprocal plots. The rate constant of product formation, k_{cat},[2] for enniatin B synthesis was found to be 0.13 s^{-1}, while that for demethylenniatin B was only 0.019 s^{-1}. On the other hand, the K_m values for ATP, valine, and D-Hiv (350, 80, and 5 μM, respectively) did not differ significantly when the synthesis of methylated or unmethylated product was measured. Thus AdoMet affects the overall reaction rates but not the affinities of the substrates to the enzyme. The K_m value for AdoMet itself was found to be 10 μM.

A characteristic property of almost all *N*-methyltransferases that have been studied so far is their sensitivity to inhibition by the reaction product *S*-adenosylhomocysteine (AdoHcy); in addition, sinefungin, another compound structurally related to AdoMet (see Figure 2), is known to act as a competitive inhibitor of a variety of N-methylating enzymes (Cantoni et al., 1979). Therefore, we tested the effect of AdoHcy and sinefungin on the product formation by enniatin synthetase. As can be seen in Figure 3, sinefungin inhibited N-methylation, but its presence even in excessive amounts still allowed synthesis of demethylenniatin B (lane D). At lower concentrations of sinefungin, besides enniatin B and demethylenniatin B two further bands appeared (2 and 3, lane C). These bands were identified as monomethyl- and dimethylenniatin B, respectively, by their content of valine and *N*-methylvaline after acid hydrolysis.

In contrast to the findings with sinefungin, AdoHcy blocked not only formation of enniatin B but also that of the unmethylated product (see Figure 3, lanes E and F). A 50% inhibition of demethylenniatin B synthesis was observed at a concentration of about 30 μM AdoHcy.

Kinetic measurements of demethylenniatin B synthesis revealed that AdoHcy acts as a noncompetitive inhibitor with respect to all of the necessary substrates, i.e., ATP, valine, and D-Hiv (data not shown). This prompted us to measure the

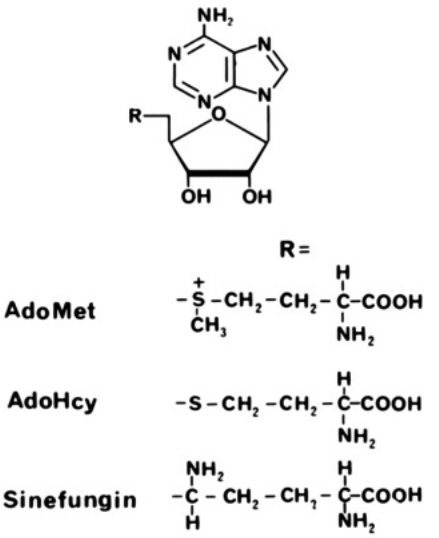

FIGURE 2: Structures of *S*-adenosyl-L-methionine (AdoMet), *S*-adenosyl-L-homocysteine (AdoHcy), and sinefungin.

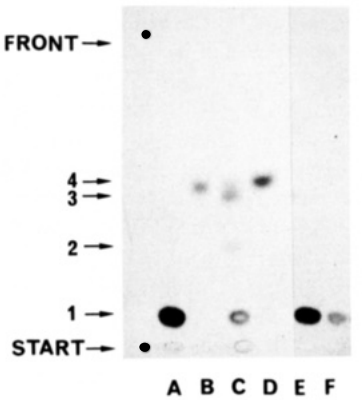

FIGURE 3: Influence of AdoHcy and sinefungin on product formation of enniatin synthetase. The enzyme was incubated with all necessary substrates for enniatin synthesis with [^{14}C]valine as the radiolabel (5 μCi) as described under Materials and Methods. The AdoMet concentration was 10 μM. Products formed in the presence and absence of AdoHcy and sinefungin were separated by reversed-phase TLC on RP18. (Lane A) enniatin B (marker); (lane B) demethylenniatin B (marker); (lane C) plus 510 μM sinefungin; (lane D) plus 6.7 mM sinefungin; (lane E) plus 16 μM AdoHcy; (lane F) plus 160 μM AdoHcy. Exposure of the chromatogram to X-ray film was for 2 days.

[2] The symbols used for kinetic quantities are as follows: e_0, initial concentration of free enzyme; k_{cat} ($=v_{max}/e_0$), rate constant of product formation; K_i, inhibition constant; K_m, Michaelis constant; K_s, dissociation constant of enzyme–substrate complex; v, rate of reaction; v_{max}, rate of reaction at infinite concentration of substrate.

influence of AdoHcy on the partial reactions of the multifunctional enzyme. However, neither were the rates of valyl or D-hydroxyisovaleryl adenylate synthesis, as studied by means of the valine- or D-Hiv-dependent ATP–pyrophosphate exchange reaction, reduced, nor was the formation of valine or D-2-hydroxyisovaleric acid thio ester inhibited. Therefore, AdoHcy must exhibit an influence on the elongation of cyclization reactions of depsipeptide synthesis.

When the synthesis of the methylated depsipeptide, enniatin B, was studied, AdoHcy also acted as a noncompetitive inhibitor with respect to ATP, valine, and D-Hiv (data not shown). However, kinetic measurements with AdoMet as the varied substrate revealed another pattern of inhibition; the double-reciprocal plots in the presence of different concentrations of AdoHcy show a family of lines intersecting at a common $1/v_{max}$ value (see Figure 4A). The secondary plot of the slopes of these lines versus the inhibitor concentration (Figure 4B) yields a hyperbola, which is indicative of a so-called partial competitive inhibition (Segel, 1975).

In this type of inhibition, the substrate (S) and the inhibitor (I) bind to the enzyme (E) at different sites to yield ES, EI, and ESI complexes; the substrate binds to the free enzyme with greater affinity (dissociation constant K_s) than to the EI complex (αK_s, where $\alpha > 1$); both ES and ESI complexes yield product with equal facility. To determine the dissociation constant of the inhibitor K_i and the parameter α, one has to plot the reciprocal of the slopes of the primary Lineweaver–Burk plots versus the reciprocal of the inhibitor concentration. The resulting line intersects with the ordinate at $v_{max}/e_0K_s(\alpha - 1)$ and with the abscissa at $-1/\alpha K_i$.

As can be seen in Figure 4C, a straight line is obtained by replotting the data of Figure 4A. With $v_{max}/e_0 = 0.13\ s^{-1}$ and $K_m = 10\ \mu M$ derived from the primary plot of the uninhibited synthesis of enniatin B and by approximating $K_s \approx K_m$, $K_i = 4\ \mu M$ and $\alpha = 5$ are calculated.

In contrast to AdoHcy, sinefungin shows a pattern of pure competitive inhibition as indicated by the linear secondary plot (Figure 5); $K_i = 110\ \mu M$ for sinefungin was determined from the intersection point of the line with the abscissa.

In addition to AdoHcy and sinefungin, some other compounds known to inhibit specific transmethylation reactions were tested; 5′-deoxy-5′-(isobutylthio)adenosine, 5′-deoxy-5′-(methylthio)adenosine, and 5-methyltetrahydropteroylpentaglutamate did not have any effect on the formation of the unmethylated or the methylated depsipeptide.

Photoaffinity Labeling of Enniatin Synthetase. A method for a site-specific affinity labeling of methyltransferases has been introduced by Yu (1983) and by Hurst et al. (1984). Upon irradiation of protein *O*-carboxymethyltransferase and phenylethanolamine *N*-methyltransferase with short-wave UV light in the presence of [*methyl*-^{3}H]AdoMet, they observed covalent radioactive labeling of the proteins. Enniatin synthetase, too, was radiolabeled when irradiated in the presence of AdoMet ^{14}C- or ^{3}H-labeled at the methyl group; using AdoMet that was labeled at the carboxylate or the nucleoside moieties did not lead to radiolabeling of the protein. Also, irradiation in presence of [U-^{14}C]ATP did not have any effect (not shown). It has to be concluded that it is the methyl group of AdoMet that is specifically transferred to the protein during UV irradiation.

In order to determine the number of methyl groups that can be transferred from AdoMet to enniatin synthetase, we irradiated the protein in presence of different concentrations of its methyl-labeled substrate and determined covalently bound radioactivity after acid precipitation of the protein. As can be seen in Figure 6, the enzyme was labeled with the [^{14}C]-methyl group in a molar ratio of about 1:1. This stoichiometry did not change when irradiation was prolonged.

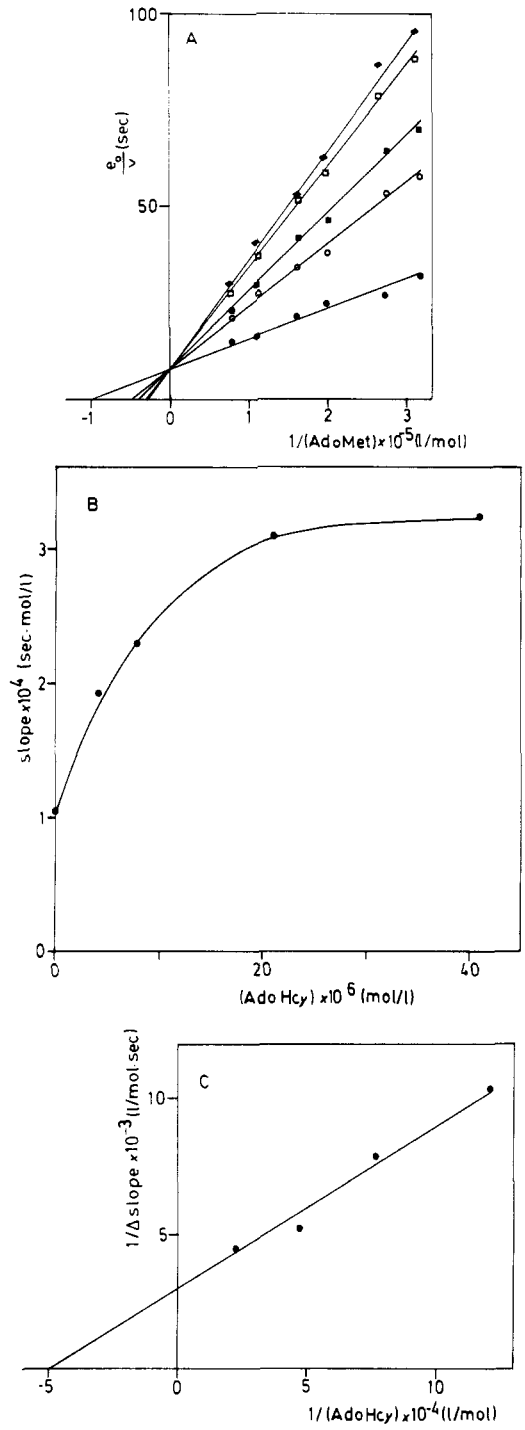

FIGURE 4: (A) Effect of AdoHcy on the synthesis of enniatin B by enniatin synthetase. AdoMet was used as the radiolabel and was varied from 3 to 12.5 μM. The concentrations of AdoHcy used were 0 (●), 4.2 (○), 8.3 (■), 20.8 (□), and 41.6 (◆) μM. The assay was performed as described under Materials and Methods. (B) Secondary plot of the slopes of (A) versus the concentration of AdoHcy. (C) Plot of $1/\Delta$slope versus the reciprocal of the concentration of AdoHcy. By use of Δslope, the abscissa of (B) is raised so that the curve becomes a hyperbola that starts at the origin.

To demonstrate the effect of adenine nucleoside inhibitors of the methyltransferases on the rate of AdoMet photoaffinity labeling, one has to consider that the inhibitors have the same UV absorption spectrum as AdoMet; they might reduce labeling because they act as filters as has been pointed out by Hurst et al. (1984). To overcome this problem, we performed

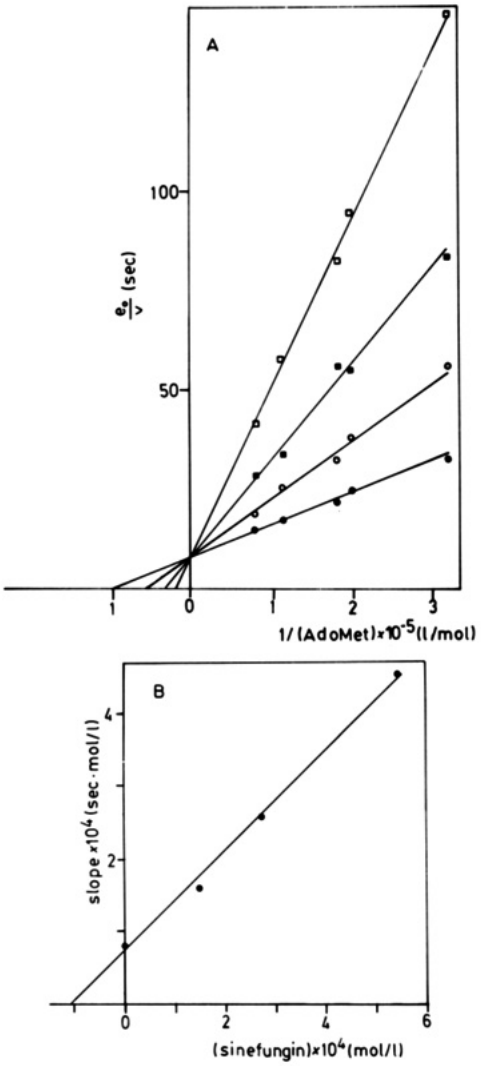

FIGURE 5: (A) Effect of sinefungin on the synthesis of enniatin B by enniatin synthetase. AdoMet was used as the radiolabel and was varied from 3 to 12.5 μM. The concentrations of sinefungin used were 0 (●), 135 (○), 271 (■), and 542 (□) μM. The assay was performed as described under Materials and Methods. (B) Secondary plot of the slopes in (A) versus the concentration of sinefungin.

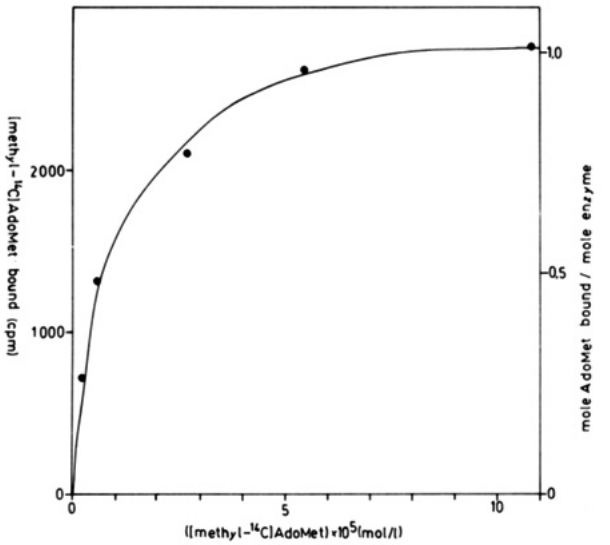

FIGURE 6: Photoaffinity labeling of enniatin synthetase. A total of 31.6 nmol of enniatin synthetase was irradiated with UV light in the presence of [*methyl*-^{14}C]AdoMet, and acid-stable counts were determined, as described under Materials and Methods.

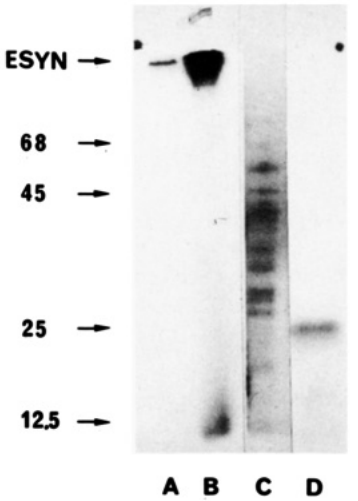

FIGURE 7: Fluorogram of a polyacrylamide–NaDodSO$_4$ gel of labeled enniatin synthetase. The enzyme was labeled with (lane A) [^{14}C]-iodoacetamide, (lane B) [*methyl*-^{3}H]AdoMet, (lane C) [^{14}C]iodoacetamide (subsequent digestion with protease V8), and (lane D) [*methyl*-^{3}H]AdoMet (subsequent digestion with protease V8). Exposure to X-ray film was for 2 days at −80 °C. Numbers at the side indicate molecular mass in kilodaltons.

control experiments using adenosine, which does not have any measurable affinity to the enzyme, at the same concentrations as the inhibitors; in fact, a linear reduction of AdoMet labeling with rising adenosine concentrations was observed (not shown). With these blank values, the effect of sinefungin and AdoHcy on photoaffinity labeling was studied.

With sinefungin a dose-dependent reduction of labeling was observed when it was added to the incubation mixture. This is expected for a competitive inhibitor. The concentration range (150–600 μM) affecting the binding of labeled AdoMet to the enzyme was the same as that necessary for blocking of the methyltransferase function (data not shown).

AdoHcy diminished labeling with AdoMet but even at high concentrations (100 μM) was not able to totally prevent the photoreaction, as did sinefungin; in contrast, the enzyme-bound radioactivity reached a reduced but constant level of 40% of the uninhibited control under the conditions described under Materials and Methods. This behavior is expected for a partial competitive inhibitor, which reduces the affinity of the substrate to the enzyme but even at infinite high inhibitor concentrations allows formation of an ESI complex.

Proteolytic Digestion of Photoaffinity-Labeled Enniatin Synthetase. Enniatin synthetase was photoaffinity labeled with [*methyl*-^{3}H]AdoMet and subsequently subjected to limited proteolytic digestion with protease V8 from *Staphylococcus aureus*. As can be seen in Figure 7, lane D, only one labeled fragment with M_r 25 000 is observed after 10 min of incubation. A kinetic study of its formation showed that labeled fragments with higher molecular masses are only transient precursors of the 25-kDa band, which only disappears slowly during prolonged incubation with the protease (not shown).

It can be assumed that the fragment contains all or part of the methyltransferase function of the enzyme. Consequently, the question arose whether this fragment contains the binding site for the other substrate of the methyltransferase, i.e., valine, too. Attempts to isolate a radioactively labeled fragment after covalent loading of the enzyme with [^{14}C]valine were unsuccessful due to the instability of the thio ester under the alkaline conditions required during electrophoresis. As an alternative, we used iodoacetamide, which has been shown to act as a selective inhibitor of the valine binding site of enniatin synthetase by its reaction with a catalytic thiol group (Zocher

et al., 1982). Figure 7, lane C, shows that a variety of fragments of the V8 digest were labeled, when [^{14}C]iodoacetamide was used. However, in the position of the AdoMet-labeled fragment no radioactive band appeared, indicating the absence of the catalytic thiol group from the 25-kDa fragment.

Discussion

The kinetic properties of the *N*-methyltransferase function of the multienzyme enniatin synthetase were studied. The K_m value for AdoMet was found to be 10 μM and lies in the range of K_m values reported for other *N*-methyltransferases (Cantoni et al., 1979). The double-reciprocal plot of kinetic measurements with AdoMet as the varied substrate is linear over the whole range of concentrations used (3–50 μM); thus, there is no sign of a positive cooperativity of AdoMet binding as observed in the case of glycine *N*-methyltransferase from rat liver (Ogawa & Fujioka, 1982).

The K_m values for the other substrates of the enzyme are identical for both enniatin and demethylenniatin synthesis. Obviously, AdoMet does not affect the affinity of the enzyme to ATP, valine, or D-Hiv. However, the maximal velocity of the reaction, as described by the k_{cat} value, is about 6 times higher for enniatin synthesis than for the formation of its unmethylated congener. The N-methylated enzyme-bound amino acid residue seems to be more reactive than the unmethylated one, leading to an increase of the rate of peptide bond formation; this might be due either to the higher basicity or to a favorable orientation of the secondary amino group.

The methyltransferase function of enniatin synthetase is inhibited by AdoHcy. This is not unexpected, since a regulatory role of this compound has been postulated for many methyltransferases (Cantoni et al., 1979), e.g., protein (arginine) methyltransferase (Lee et al., 1977), guanidinoacetate methyltransferase (Im et al., 1979), and indolethylamine methyltransferase (Lin et al., 1973), where AdoHcy is acting as a competitive inhibitor. This means that AdoHcy is able to occupy the same binding site as the substrate AdoMet. In the case of enniatin synthetase, in the presence of AdoHcy a pattern of inhibition is observed, which has been described as partial competitive or hyperbolic competitive inhibition (Segel, 1975). This pattern can only be explained if one assumes that there is a discrete inhibitory site to which the reaction product AdoHcy is able to bind. When the site is occupied, the affinity of the enzyme to AdoMet is lowered by a factor $\alpha = 5$, while the v_{max} of the reaction is not diminished.

In contrast, sinefungin acts as a pure competitive inhibitor interacting with the AdoMet binding site. This is not surprising, since at pH 7.2 sinefungin carries a positive charge at its δ-NH_2 group, which is situated at about the same location as the charge of the sulfur in the AdoMet molecule. AdoHcy does not possess a positive charge in this position. This may be the discriminating factor for the binding to the two sites.

The study of the effect of inhibitors on the photoaffinity labeling of enniatin synthetase with AdoMet confirmed the kinetic data: sinefungin is able to totally prevent the labeling, as expected for a competitive inhibitor, while AdoHcy only lowers the affinity to AdoMet and still allows binding even at high inhibitor concentrations.

Other examples for a separate binding site of AdoHcy on a methyltransferase have not yet been published; however, extended kinetic studies might reveal partial competitive inhibition patterns for other enzymes, too.

AdoHcy also inhibits the formation of demethylenniatin B. The kinetic analysis gave with all substrates a noncompetitive inhibition pattern. Since the activation reaction of the hydroxy amino acids is not affected by AdoHcy, the inhibitor seems to block the elongation or cyclization reactions leading to the cyclodepsipeptide enniatin. This shows that in the case of enniatin synthetase the end product of the methylase reaction, AdoHcy, not only influences the methyltransferase function itself but also exhibits an inhibitory effect on other reactions of the multifunctional enzyme. Obviously, the active sites for depsipeptide formation and for methylation are not independent from each other.

Like other methyltransferases, enniatin synthetase can be affinity labeled in a photoreaction using AdoMet. The mechanism of this reaction as well as the acceptor group on the protein is not known; however, from our data it becomes clear that it is a site-specific, stoichiometric reaction, in which the methyl group of AdoMet is transferred to the protein. A ratio of one transferred methyl group per enzyme molecule was measured here. This indicates that there is only one binding site for AdoMet on the enzyme, while the enniatin molecule consists of three methylated dipeptidols. This equimolar stoichiometry has also been determined for valine and D-Hiv (data will be published elsewhere).

Limited proteolytic digestion of the enzyme after UV irradiation in the presence of [*methyl*-^{3}H]AdoMet yielded only one radioactively labeled fragment, which was relatively stable against further degradation. It has to be concluded that this fragment represents all or at least part of a methyltransferase domain of enniatin synthetase. The size of the 25-kDa fragment is in agreement with reported molecular masses for *N*-methyltransferases (or their monomers), which lie in the range of 30 kDa [e.g., Irace et al. (1982), Im et al. (1979), and Ogawa and Fujioka (1982)]. The fragment seems not to contain the binding site for valine, the substrate to be methylated. However, epitope mapping studies using monoclonal antibodies interacting with both the methyltransferase site and the valyl binding site of enniatin synthetase have shown that the sites must be located close to each other (Billich et al., 1987).

It has recently been demonstrated that the biosynthesis of other N-methylated peptides from fungi, like cyclosporin A (Zocher et al., 1986) and beauvericin (Peeters et al., 1983), and from actinomycetes (Keller, 1987) proceeds via a similar mechanism; i.e., the methylation occurs on the stage of the thioesterified amino acid. Therefore, this pathway seems to be of general importance in the synthesis of peptides and depsipeptides. Work is in progress to investigate if the findings described here hold true for the other enzyme systems, too.

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References

Billich, A., Zocher, R., Kleinkauf, H., Braun, D. G., Lavanchy, D., & Hochkeppel, H. (1987) *Biol. Chem. Hoppe-Seyler 368*, 521.

Bray, G. (1960) *Anal. Biochem. 1*, 279.

Cantoni, G. L., Richards, H. H., & Chiang, P. K. (1979) in *Transmethylation* (Usdin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 155–164, North-Holland, Amsterdam.

Gevers, W., Kleinkauf, H., & Lipmann, F. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 1335.

Hurst, J. H., Billingsley, M. L., & Lovenberg, W. (1984) *Biochem. Biophys. Res. Commun. 122*, 499.

Im, Y. S., Chiang, P. K., & Cantoni, G. L. (1979) *J. Biol. Chem. 254*, 11047.

Irace, G., Colonna, G., Camardella, M., Pietra, G. D., & Porta, R. (1982) *Biochemistry 21*, 1464.
Keller, U. (1987) *J. Biol. Chem. 262*, 5852.
Kleinkauf, H., & von Döhren, H. (1981) *Curr. Top. Microbiol. Immunol. 91*, 129.
Laemmli, U. (1970) *Nature (London) 227*, 680.
Lee, H. W., Kim, S., & Paik, W. K. (1977) *Biochemistry 16*, 78.
Lin, R. H., Narasimhachari, N., & Himwich, H. E. (1973) *Biochem. Biophys. Res. Commun. 54*, 751.
Ogawa, H., & Fujioka, M. (1982) *J. Biol. Chem. 257*, 3447.
Peeters, H., Zocher, R., Madry, N., Oelrichs, P. B., Kleinkauf, H., & Kraepelin, G. (1983) *J. Antibiot. 36*, 1762.
Plattner, P. A., Nager, U., & Boller, A. (1948) *Helv. Chim. Acta 31*, 594.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 161–166, 182–185, Wiley, New York.
Wipf, H. K., Pioda, L. A. R., Stefanac, Z., & Simon, W. (1968) *Helv. Chim. Acta 51*, 377.
Yu, P. H. (1983) *Biochim. Biophys. Acta 742*, 517.
Zocher, R., & Kleinkauf, H. (1978) *Biochem. Biophys. Res. Commun. 81*, 1162.
Zocher, R., Salnikow, J., & Kleinkauf, H. (1976) *FEBS Lett. 71*, 13.
Zocher, R., Keller, U., & Kleinkauf, H. (1982) *Biochemistry 21*, 43.
Zocher, R., Keller, U., & Kleinkauf, H. (1983) *Biochem. Biophys. Res. Commun. 110*, 292.
Zocher, R., Nihira, T., Paul, E., Madry, N., Peeters, H., Kleinkauf, H., & Keller, U. (1986) *Biochemistry 25*, 550.

Regulation of Carboxylester Lipase Adsorption to Surfaces. 1. Chemical Specificity†

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ABSTRACT: The chemical specificity of the adsorption of porcine pancreatic carboxyl ester lipase to pure lipid surfaces was examined. Adsorption of native and catalytically inactivated enzyme was measured at the argon–buffer interface by using lipid films near the point of collapse. Protein adsorbed readily to films of triolein, 1,3-diolein, methyl oleate, oleonitrile, oleyl alcohol, and 13,16-docosadienoic acid. However, recovery of enzyme activity was variable. These differences and the changes in surface pressure accompanying adsorption indicated the occurrence of enzyme denaturation at the interface. Denaturation was controlled largely by surface free energy but showed some chemical specificity at high surface pressures. Adsorption of protein to the lipids was comparable when measured under either equilibrium or initial rate conditions. Together with surface pressure changes that accompany adsorption, the data indicate a relative lack of specificity for the enzyme–surface interaction. Adsorption to 13,16-docosadienoic acid and 1,3-diolein obeyed the Langmuir adsorption isotherm. Dissociation constants ranged from 10 to 50 nM, depending on enzyme form, ionic strength, and pH. With both lipids, a monolayer of enzyme was adsorbed at saturation. In contrast to these results, adsorption of enzyme activity and protein to films of 1-palmitoyl-2-oleoylphosphatidylcholine was ≤5% of that observed with the other lipids under all conditions. Comparison of rate constants for adsorption to 13,16-docosadienoic and 1,3-diolein as a function of subphase pH indicated a marked dependence on the ionization state of the fatty acid. Overall, the data suggest that the presence of zwitterionic and anionic lipids may regulate the interaction of the enzyme with substrate-containing surfaces in vivo.

Lipolytic enzymes are a class of esterases that exhibit a high activity toward water-insoluble lipids. In contrast to their substrates, many lipases and phospholipases are well-behaved, water-soluble proteins. Thus, for catalysis to occur it is necessary that enzyme and substrate partition to a common phase. As recently reviewed (Brockman, 1984), the site of lipolysis is the interphase that exists between bulk lipid and water phases. Previous studies with lipases and other proteins have shown that both the rate and extent of protein adsorption from aqueous phases to interphases, as well as denaturation at interfaces, are in some way regulated by the physical properties of the surface phase. Taken together, those physical properties of interphases such as charge, lipid composition, and lipid-packing density have been termed the "quality" of the interface (Verger, 1980).

Although it is reasonable to postulate that changes in surface quality accompany changes in lipid packing or composition, it has been difficult to quantitatively apply the concept to understanding the regulation of protein adsorption to lipid–water interfaces. In large part, this difficulty has arisen from lack of an adequate model to describe the structure of such interfaces. Recently, however, it has been shown that monomolecular lipid films at the point of collapse to a bulk or bilayer phase display regular behavior with respect to the area of each lipid species as well as interfacial tension (Smaby & Brockman, 1984). Analysis of this behavior has yielded an equation of state that describes the surface in terms of lipid–water "building blocks". In one of these studies, the properties of a series of lipids containing exclusively oleoyl or oleyl

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