FORMYLATION OF ENZYME-BOUND VALINE AND STEPWISE ELONGATION OF INTERMEDIATE PEPTIDES OF GRAMICIDIN A BY A CELL-FREE ENZYME SYSTEM

Katsuhiko Akashi and Kiyoshi Kurahashi

Institute for Protein Research, Osaka University,
Yamada-kami, Suita, Osaka, 565, Japan

Received May 23,1977

SUMMARY

A partially purified enzyme fraction (Component I) of crude extracts of Bacillus brevis ATCC 8185 which produces gramicidin A activated L-valine and bound it as a thioester, and further formylated it in the presence of a formyl-tetrahydrofolate synthesizing system. The same fraction catalyzed the synthesis of enzyme-bound formylvalylglycine. The addition of Component II, which was also partially purified, together with the constituent amino acids of gramicidin A brought about the synthesis of enzyme-bound formylvalylglycylalanine and formyl-valylglycylalanylleucine (or a longer intermediate).

Gramicidin A is a linear pentadecapeptide with an antibiotic activity. It is produced by Bacillus brevis ATCC 8185 which produces another antibiotic peptide tyrocidine. The structure of gramicidin A was elucidated to be N-formyl-Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Trp-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-ethanolamine by Sarges and Witkop (1). Bauer et al. obtained a cell-free system which synthesized a pentadecapeptide bound to an enzyme protein by a thioester bond with the sequence of gramicidin A and suggested that the formylation of the N-terminal valine took place after completion of the peptide chain (2). Akers et al. further reported the synthesis of the N-terminal heptapeptide lacking a formylgroup by a partially resolved enzyme system (3).

On the contrary we reported previously that an enzyme fraction (Component I) partially purified from crude extracts of B. brevis ATCC 8185 could activate and bind L-valine as a thioester and that the formylation of this enzyme-bound L-valine took place in the presence of a formyl-THFA synthesizing system (4). We also demonstrated the formation of enzyme-bound formylvalylglycine. In this paper

Abbreviations: THFA, tetrahydrofolic acid; DTT, dithiothreitol; BSA, bovine serum albumin.

ISSN 0006-291X

we present evidence that Component I itself catalyzes the transformylation reaction to enzyme-bound L-valine and that stepwise elongation of the peptide chain takes place starting from formylvaline.

MATERIALS AND METHODS.

Enzyme Preparation. Cultivation of Bacillus brevis and preparation of crude extracts were carried out as described by Fujikawa et al. (5). The cell-free extracts (130 ml) obtained from 30 g wet weight of cells were brought to 45 % saturation with solid $(NH_4)_2SO_4$. Thirty minutes after all $(NH_4)_2SO_4$ had dissolved, the suspension was centrifuged (10,000 x g, 15 min). The precipitates were dissolved in 12 ml of potassium phosphate buffer (pH 7.1, 0.1 M) containing 1 mM DTT and 2 mM $MgCl_2$ (Buffer A) and dialyzed against 2 l of the same buffer for 15 hours. Fifteen milliliters of the dialyzed sample was applied to a Sephadex G 200 superfine column (5 x 100 cm) which was equilibrated with Buffer A. The column was eluted with Buffer A and 4 ml fractions were collected. Fractions which had ATP-PP_i exchange activity dependent on all the five constituent amino acids were pooled and applied to a DEAE-cellulose column (2 x 32 cm) equilibrated with Buffer A containing 10 % glycerol. The column was eluted with a linear gradient of 800 ml potassium phosphate buffer (pH 7.1) from 0.1 to 0.4 M which contained 1 mM DTT, 2 mM $MgCl_2$ and 10 % glycerol, and 5 ml fractions were collected. The elution patterns of ATP-PP_i exchange activity dependent on glycine, L-valine, L-alanine and L-leucine are shown in Fig. 1. Fractions of Peak I which activated L-valine and glycine and fractions of Peak III which activated L-alanine, L-leucine and L-valine were combined, separately, and condensed by DiaFlo membrane PM 10. These two fractions were termed Component I and II, respectively, and used in the following experiments.

Sucrose Density Gradient Centrifugation. Two tenths milliliter of Component I mixed with bovine liver catalase [EC 1.11.1.6], bovine serum albumin (BSA) and cytochrome C as standards was layered on 11.6 ml of a 5-20 % sucrose density gradient in 50 mM potassium phosphate buffer, pH 7.1, containing 2 mM $MgCl_2$ and 1 mM DTT, and centrifuged for 18 hours at 38,000 rpm in a SW 41 rotor in a Beckman ultracentrifuge. Three tenths milliliter fractions were collected with an ISCO Model 640 density gradient fractionator.

Assay for the Synthesis of Formylated Valine and Intermediate Peptides Bound to Enzyme Proteins. The reaction mixture (0.13 ml) contained tritiated amino acids (New England Nuclear) together with unlabeled amino acids as indicated in each legend, 20 mM $MgCl_2$, 3 mM ATP, 7 mM DTT, 1.5 mM phosphoenolpyruvate, 0.5 µg of inorganic pyrophosphatase [EC 3.6.1.1] (Boehringer), 1 µg of pyruvate kinase [EC 2.7.1.40] (Boehringer), 40 mM potassium phosphate, pH 7.0, and an appropriate amount of Component I alone or together with Component II. The pH was adjusted to 6.5 by the addition of 0.3 M CH_3COONa buffer, pH 3.7. After 5 min incubation at 37°, twenty microliters of a formyl-THFA synthesizing system consisting of HCOONa (final 1 mM), NH_4Cl (final 4 mM), THFA (final 1mM, Sigma) and formyl-THFA synthetase [EC 6.3.4.3] [100 µg, purified from pigeon liver acetone powder through the ASI stage according to the method of Jaenicke and Brode (6)] was added to the above reaction mixture. Incubation was further carried out at 37° for 15 min. The reaction was stopped by the addition of 1 ml of cold trichloroacetic acid (10 %). The procedures for liberation of enzyme-bound amino acids, formylated valine and peptides by alkali treatment were described previously (4). Formylated products liberated from the enzymes were separated by passage through Dowex 50 column free of amino acids. The validity of this method was shown in the previous paper (4).

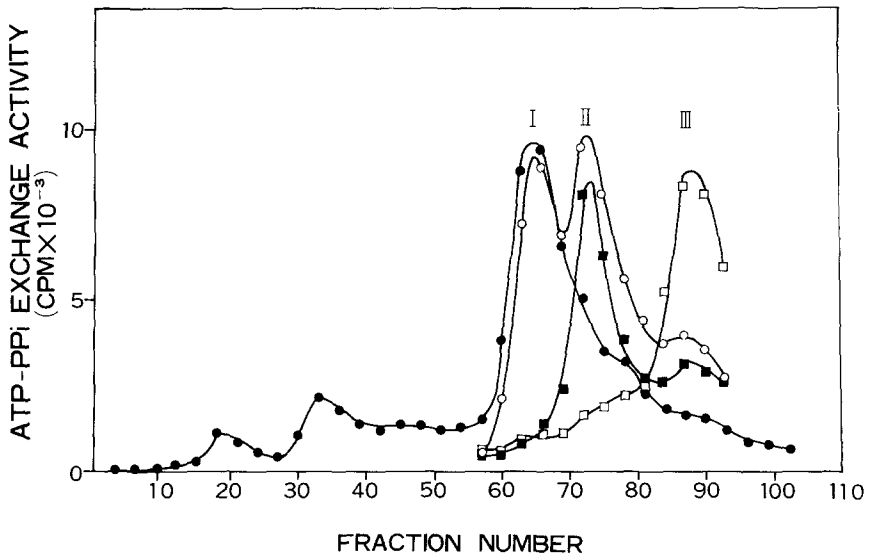

Figure 1. ATP-PP_i Exchange Activity Dependent on Constituent Amino Acids of Gramicidin A in DEAE-cellulose Fractions. The experimental conditions were described in MATERIALS AND METHODS. The ATP-PP_i exchange reaction dependent on the amino acid as indicated was performed as described previously except that 50 mM concentration of each amino acid was used (5). ○, L-valine; ●, glycine; □, L-alanine; ■, L-leucine.

RESULTS

Separation of Gramicidin A Synthesizing Enzymes from the Heavy Enzyme of Tyrocidine Synthetase. Since the heavy enzyme of tyrocidine synthetase contains L-valine and L-leucine dependent ATP-PP_i exchange activity and is capable of binding these two amino acids as thioesters (7, 8), it is essential to remove this enzyme from Component I and Component II of gramicidin A synthetase which activate and bind L-valine and L-leucine (possibly as D-leucine), respectively. Fig. 1 shows that the contaminating heavy enzyme (Peak II) of tyrocidine synthetase was separated from Component I (Peak I) and II (Peak III) by DEAE-cellulose column chromatography.

Formylation of Component I-Bound L-Valine. In the previous paper (4) we reported that the addition of an ammonium sulfate fraction of crude extracts of B. brevis together with a formyl-THFA synthesizing system to Component I in the presence of L-valine and glycine brought about the synthesis of Component I-bound formyl-

TABLE I. Formylation of Component I-Bound L-Valine

Incubation time (min)	Radioactivity: Before Dowex 50 column (cpm)	Radioactivity: Passed through Dowex 50 column (cpm)
0	2,350	50
5	2,490	430

The reaction conditions for the binding of L-[^{3}H]valine (12.5 Ci/mmole, 12 μM) to Component I were described in MATERIALS AND METHODS, except that sucrose (final 2 %) and 100 μg of BSA were added to 0.1 ml of the reaction mixture. After 20 min incubation at 37° the reaction mixture was passed through Sephadex G 50 fine column (0.5 x 5 cm) equilibrated with 20 mM potassium phosphate buffer (pH 6.0) containing 5 % sucrose, 1 mM DTT and 2 mM $MgCl_2$. The effluent containing Component I-bound [^{3}H]valine (4,300 cpm) was incubated for the indicated time with the formyl-THFA synthesizing system in the final volume of 0.15 ml. The procedures for isolation of formylvaline were described in MATERIALS AND METHODS. The amount of radioactivity recovered as an acid-stable form was about a half of that put into the reaction mixture, indicating that a half of Component I-bound valine was acid-labile valyl adenylate as shown by Gevers et al. (9).

valine and formylvalylglycine. In order to confirm that Component I-bound L-valine was formylated but not free valine, the Component I-L-valine complex was isolated by Sephadex G 50 gel filtration and incubated with the formyl-THFA synthesizing system. As shown in Table I, the amount of radioactivity which passed through a Dowex 50 column after incubation increased 9-fold that of the 0-time control, indicating that a part of Component I-bound L-valine was formylated. Since the formylation of Component I-bound valine took place without formyl-THFA synthetase when formyl-THFA was used (data not shown), the above results suggest that Component I itself possesses transformylase activity.

Sucrose Density Gradient Centrifugation of Component I. Fig. 2a shows the profile of ATP-PP_i exchange activities of Component I dependent on L-valine and glycine in sucrose density gradient centrifugation. Both activities are in parallel throughout the fractions of Peak II suggesting that they reside in one protein moiety. The nature of the valine activating activity at Peak II was not further studied. The sedimentation coefficient for Component I was estimated to be 9.0 in comparisons with the S values of catalase, BSA and cytochrome C. The approxi-

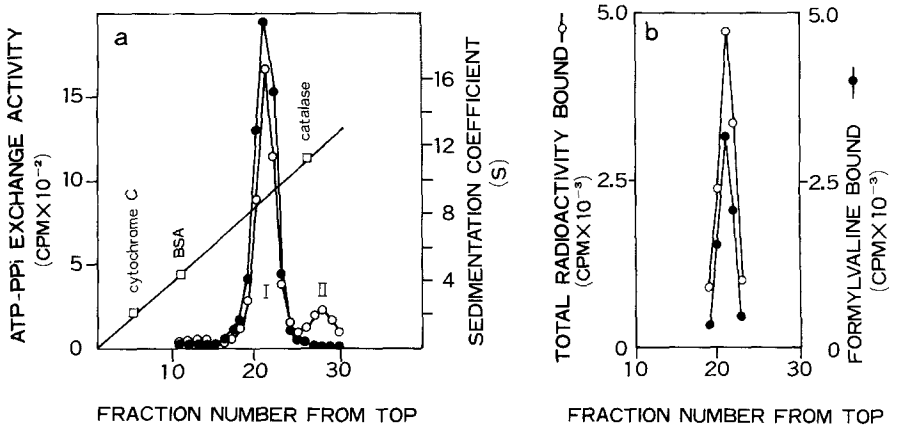

Figure 2. Sucrose Density Gradient Centrifugation of Component I. a. ATP-PP_i exchange activity dependent on L-valine and glycine. O, L-valine; ●, glycine. b. Total radioactivity and formyl[^{3}H]valine bound to Component I. The procedures are described in MATERIALS AND METHODS. [^{3}H]valine (12.5 Ci/mmole, 12 μM) was used. The positions of cytochrome C (2.1 S), BSA (4.3 S) and catalase (11.3 S) were determined by their absorbancy at 280 nm. The ATP-PP_i exchange activity was determined as described in the legend to Fig. 1.

mate molecular weight of Component I is calculated to be 180,000 according to the method of Martin and Ames (10). Fig. 2b shows the total radioactivity (valine plus formylvaline) and the radioactivity of formylvaline bound to Component I in each fraction of Peak I of sucrose density gradient centrifugation after incubation with L-[^{3}H]valine and the formyl-THFA synthesizing system. The profile of the total radioactivity and that of the radioactivity of formylvaline formed are similar and the ratios of the two throughout the central three fractions are constant, 0.65 That the ratios in the adjacent tubes, No. 19 and No. 23 are lower, about 0.4, may be resulted from dissociation of the transformylase subunit from Component I caused by dilution in these two fractions. In fact, when the center fraction, Fraction No. 21, was assayed at the same protein concentration as in Fraction No. 23, the ratio of enzyme-bound total valine to formylvaline formed was reduced from 0.65 to 0.4. Thus, the efficiency of the formylation of Component I-bound valine throughout the Component I fractions in sucrose density gradient centrifugation would be constant, 0.65, confirming that Component I itself is capable of formylating valine bound to itself as was shown in the previous section.

TABLE II. Stepwise Elongation of Formylated Intermediate Peptides.

Amino acids added	Radioactivity passed through Dowex 50 column (cpm)
[^{3}H]Val	3,050
[^{3}H]Val, Gly	3,750
[^{3}H]Val, Gly, Ala	4,900
[^{3}H]Val, Gly, Ala, Leu	4,800

Experimental procedures were as described in MATERIALS AND METHODS, except that Tris buffer, 125 mM, pH 7.4 was used. L-[^{3}H]valine (5.0 Ci/mmole, 12 μM) and unlabeled amino acids (20 μM) were used.

Elongation of Formylvaline to Intermediate Peptides of Gramicidin A. Component I catalyzes the synthesis of enzyme-bound formylvaline and formylvalylglycine as reported previously (4). The addition of Component II which activates L-alanine, L-leucine and L-valine together with these amino acids to this system revealed the stepwise elongation of the formylated peptide chain. Table II shows that the amount of formylated L-[^{3}H]valine associated with Component I and II increased with the stepwise addition of the constituent amino acids according to the sequence of gramicidin A when both Component I and II were present.

Figs. 3b-3g show elongation products revealed by TLC, when a labeled amino acid was added together with other unlabeled constituent amino acids to the reaction mixture. The detailed conditions are described in the legend. Spot I and II in Fig. 3a are formylvaline and formylvalylglycine as were proven in the previous paper (4). When labeled alanine was added together with unlabeled valine and glycine, Spot III appeared as shown in Fig. 3e, indicating that Spot III must be formylvalylglycylalanine. When labeled leucine together with unlabeled valine, glycine and alanine was used, Spot IV appeared as shown in Fig. 3f, suggesting that Spot IV is formylvalylglycylalanylleucine (or a longer intermediate). Spot V and VI in the same figure may be the further elongated intermediate peptides containing leucine of gramicidin A, but their identities are not known at present. It should be noted in Fig. 3e that there is also a faint spot corresponding to Spot IV without the addition of L-leucine to the reaction mixture

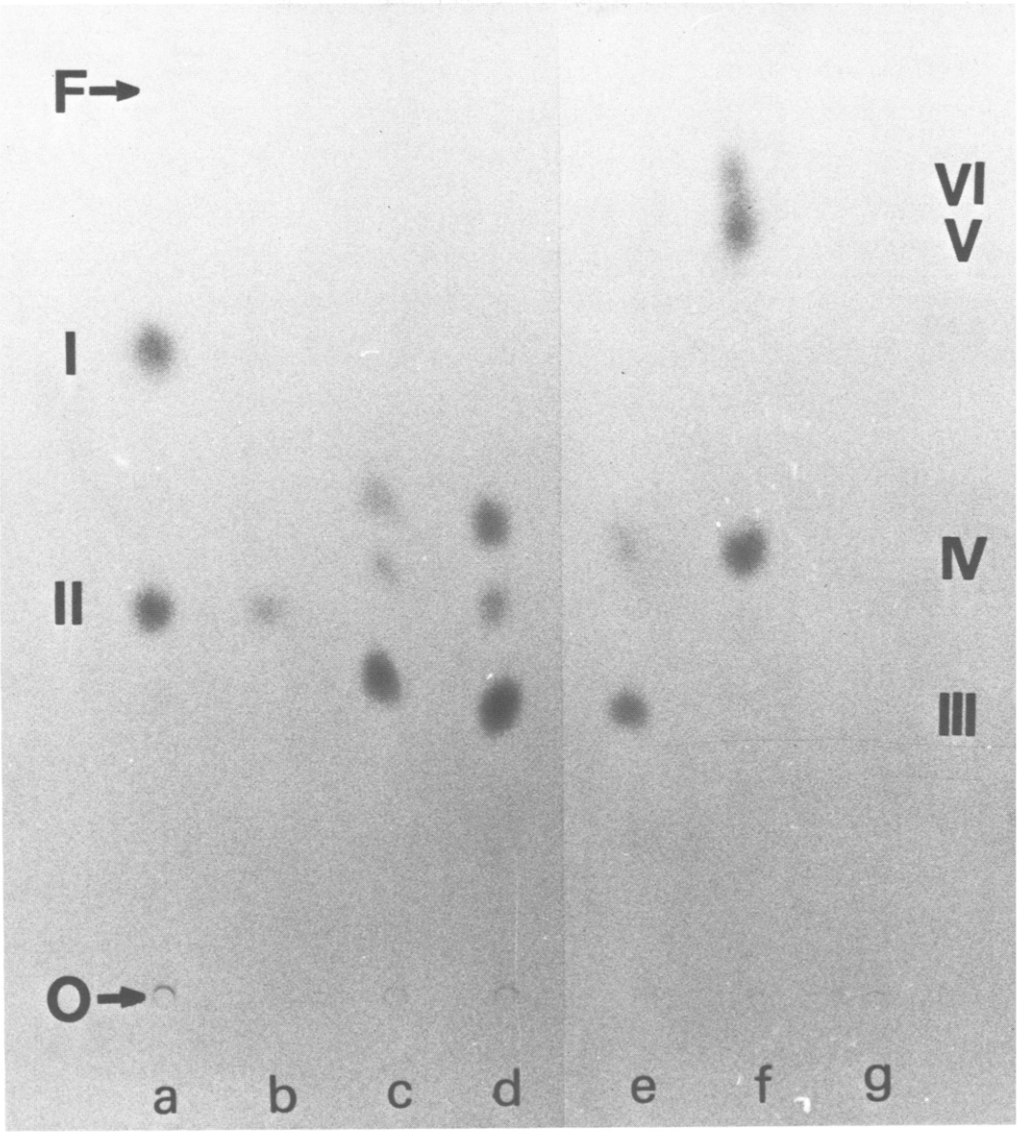

Figure 3. Incorporation of Labeled Amino Acids to Formylated Intermediate Peptides. Experimental procedures were the same as those in the legend to TABLE II. Labeled amino acids (5.0 Ci/mmole, 12 μM) and unlabeled amino acids (20 μM) were used. The procedures for TLC and autoradiography were described previously (4). O, Origin; F, Front. Roman numerals indicate radioactive spots. The additions to each reaction mixture are as follows.

	Enzymes added		Amino acids added
a.	Component I		[^{3}H]Val, [^{3}H]Gly
b.	Component I		Val, [^{3}H]Gly
c.	Component I,	Component II	Val, [^{3}H]Gly, Ala
d.	Component I,	Component II	Val, [^{3}H]Gly, Ala, Leu
e.	Component I,	Component II	Val, Gly, [^{3}H]Ala
f.	Component I,	Component II	Val, Gly, Ala, [^{3}H]Leu
g.		Component II	Val, Gly, [^{3}H]Ala

suggesting that there is endogenous leucine in the enzyme preparations, which brings about the product of Spot IV. This fact may explain the result in Table II that the addition of unlabeled leucine did not increase the amount of labeled formylated valine associated with the enzyme above the level of the addition of unlabeled amino acids upto L-alanine. Fig. 3g shows that the omission of Component I from the same reaction mixture as in Fig. 3e abolishes the synthesis of formylvalylglycylalanine, indicating that Component I is essential for the peptide chain initiation. Fig. 3b, c and d show the results of the incubation of labeled glycine together with unlabeled valine, unlabeled valine and alanine, and unlabeled valine, alanine and leucine, respectively. In Fig. 3b, only Spot II corresponding to formylvalylglycine is found. In Fig. 3c, Spot II, III and IV are observed, formylvalylglycylalanine corresponding to Spot III being distinct. Further addition of leucine intensified the radioactivity of Spot IV (Fig. 3d). These results are compatible with the results presented in Table II that elongation of the peptide chain takes place in a stepwise fashion.

DISCUSSION

The results presented above show that the initiation of gramicidin A synthesis takes place by the formylation of the enzyme-bound N-terminal L-valine residue This reaction is very unique in that an enzyme protein activates and binds an amino acid, and that it in turn serves as an acceptor for the transformylation reaction catalyzed by a transformylase which is also present as a complex with the substrate, an enzyme-bound amino acid.

Once Component I-bound valine is formylated, apparently it is transferred to glycine which is also bound to Component I as a thioester. It is not known at present that the transfer of formylvaline to glycine involves phosphopantetheine arm as in the case of gramicidin S, tyrocidine and bacitracin synthesis (11-14). When Component II with thioesterified alanine and leucine is present, the formyldipeptide is transferred to alanine as in the case of tyrocidine synthesis, and further to leucine (15). It seems that the biosynthetic mechanism of gramicidin A

is very similar to those of gramicidin S, tyrocidines and bacitracins. However, in order to elucidate the detailed mechanism of gramicidin A biosynthesis, it is necessary to obtain the enzyme fractions which elongate further the peptide chain and terminate it by adding ethanolamine at the C-terminal of the peptide.

In the work presented we used only L-form of amino acids as the constituent amino acids of gramicidin A. However, the antibiotic peptide contains two D-valine residues and all four leucine residues of it have the D configuration. Component II, in fact, activates both L- and D-leucine. We obtained a fraction which activated L-valine and D-valine. This situation is similar to that of gramicidin S synthesis, in that both L- and D-phenylalanine are activated by the light enzyme of gramicidin S synthetase (16, 17). We have also isolated another fraction which activates mainly leucine and tryptophan. We are now testing these fractions for further elongation of the intermediate peptides formed by Component I and Component II.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. H. Takeda of Banyu Pharmaceutical Co., Ltd. for his help in maintaining the strains used in this work.

REFERENCES

1. Sarges, R., and Witkop, B. (1964) J. Am. Chem. Soc. 86, 1862-1863
2. Bauer, K., Roskoski, R., Jr., Kleinkauf, H., and Lipmann, F. (1972) Biochemistry 11, 3266-3271
3. Akers, H., Lee, S. G., and Lipmann, F. (1976) Federation Proc. 35, 1533
4. Akashi, K., Kubota, K., and Kurahashi, K. (1977) J. Biochem. 81, 269-272
5. Fujikawa, K., Suzuki, T., and Kurahashi, K. (1968) Biochim. Biophys. Acta 161, 232-246
6. Jaenicke, L., and Brode, E. (1961) Biochem. Z. 334, 108-132
7. Kurahashi, K. (1974) Ann. Rev. Biochem. 43, 445-459
8. Lee, S. G., Roskoski, R., Jr., Bauer, K., and Lipmann, F. (1973) Biochemistry 12, 398-405
9. Gevers, W., Kleinkauf, H., and Lipmann, F. (1969) Proc. Nat. Acad. Sci. U. S. A. 63, 1335-1342
10. Martin, R. G., and Ames, B. N. (1961) J. Biol. Chem. 236, 1372-1379
11. Gilhuus-Moe, C. C., Kristensen, T., Bredesen, J. E., Zimmer, T. L., and Laland, S. G. (1970) FEBS Lett. 7, 287-290
12. Kleinkauf, H., Gevers, W., Roskoski, R., Jr., and Lipmann, F. (1970) Biochem. Biophys. Res. Commun. 41, 1218-1222
13. Ishihara, H., Endo, T., Abe, S., and Shimura, K. (1975) FEBS Lett. 50, 43-46
14. Frøyshov, ø., and Laland, S. G. (1974) European J. Biochem. 46, 235-242
15. Roskoski, R., Jr., Kleinkauf, H., Gevers, W., and Lipmann, F. (1970) Biochemistry 9, 4846-4851
16. Yamada, M., and Kurahashi, K. (1969) J. Biochem. 66, 529-540
17. Takahashi, H., Sato, E., and Kurahashi, K. (1971) J. Biochem. 69, 973-976