Vol. 181, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1991 Pages 272-278

EXPRESSION OF BOVINE LUNG PROSTAGLANDIN F SYNTHASE IN *Escherichia coli* *

Kikuko Watanabe[1], Yutaka Fujii[2], Hiroaki Ohkubo[3#], Seiki Kuramitsu[4†], Hiroyuki Kagamiyama[4], Sigetada Nakanishi[3] and Osamu Hayaishi[1§]

[1]Department of Enzymes and Metabolism, Osaka Bioscience Institute, Suita 565, Japan

[2]Department of Chemistry, Fukui Medical School, Fukui 910-11, Japan

[3]Institute for Immunology, Kyoto University Faculty of Medicine, Kyoto 606, Japan

[4]Department of Medical Chemistry, Osaka Medical College, Takatsuki 569, Japan

Received October 14, 1991

Summary: The full-length bovine lung prostaglandin(PG) F synthase cDNA was constructed from partial cDNA clones and ligated into bacterial expression vector pUC8 to develop expression plasmid pUCPF1. This plasmid permitted the synthesis of bovine lung PGF synthase in *Escherichia coli*. The recombinant bacteria overproduced a 36-KDa protein that was recognized by anti-PGF synthase antibody, and the expressed protein was purified to apparent homogeneity. The expressed protein reduced not only carbonyl compounds including PGD_2 and phenanthrenequinone but also PGH_2; and the Km values for phenanthrenequinone, PGD_2, and PGH_2 of the expressed protein were 0.1, 100, and 8μM, respectively, which are the same as those of the bovine lung PGF synthase. The protein produced $PGF_{2\alpha}$ from PGH_2, and 9α,11β-PGF_2 from PGD_2 at different active sites. Moreover, the structure of the purified protein from *Eschericia coli* was essentially identical to that of the native enzyme in terms of C-terminal sequence, sulfhydryl groups, and CD spectra except that the nine amino acids provided by the lac Z' gene of the vector were fused to the N-terminus. These results indicate that the expressed protein is essentially identical to bovine lung PGF synthase. We confirmed that PGF synthase is a dual function enzyme catalyzing the reduction of PGH_2 and PGD_2 on a single enzyme and that it has one binding site for NADPH.

In 1981, we (1) and Wong (2) found an enzyme that catalyzed the reduction of prostaglandin(PG) D_2 to PGF_2 in rat lung and rabbit liver, respectively, and we purified the enzyme from bovine lung to apparent homogeneity (3). The purified enzyme showed a broad substrate specificity; and it reduced PGH_2 as well as PGD_2 and carbonyl compounds using NADPH as a cofactor. The enzyme catalyzes the reduction of PGH_2 to $PGF_{2\alpha}$ and that of PGD_2 to (5Z,13E)-(15S)-9α,11β,15-trihydroxyprosta-5,13-dien-

*This investigation was supported in part by research grants from the Ministry of Education, Science and Culture of Japan and the Japan Foundation for Applied Enzymology.

#Present address: Institute for Medical Genetics, Kumamoto University, Medical school, Kumamoto 860, Japan.

†Present address: Department of Biology, Faculty of Science, Osaka University Toyonaka 560, Japan.

§To whom correspondence should be addressed at Osaka Bioscience Institute, Furuedai, Suita, Osaka 565, Japan.

Abbreviations: PG, Prostaglandin; 9α,11β-PGF_2, (5Z,13E)-(15S)-9α,11β,15-trihydroxyprosta-5,13-dien-1-oic acid; *E. coli*, *Eschericia coli;* DTNB, 5,5-dithiobis (2-nitrobenzoic acid); SDS, sodium dodecyl sulfate; RP-HPLC, reverse phase-high performance liquid chromatography; CD, circular dichroism

0006-291X/91 $1.50

1-oic acid (9α,11β-PGF_2), which is a stereoisomer of $PGF_{2\alpha}$ (4), at different active sites on the same molecule. We named this enzyme PGF synthase (3). We isolated cloned cDNA sequences specific for PGF synthase from a cDNA library of bovine lung mRNA (5). Nucleotide sequence analysis of cloned cDNA insert revealed that PGF synthase consists of a 966-base pair open reading frame coding for a 322-amino acid polypeptide with a Mr of 36,517 excluding the initiation methionine.

To understand this enzyme at the molecular level, we examined the expression of the full-length cDNA encoding PGF synthase in *E. coli* using the expression vector pUC8. Here we report that *E. coli* harboring the complete sequence shows immuno-precipitable activity specific for PGF synthase and both the PGH_2 and PGD_2 reductase activities. Moreover, we compared the structure of the expressed protein with that of the native enzyme spectrophotometrically, and determined the binding number of PGF synthase for NADPH.

EXPERIMENTAL PROCEDURES

Construction of the Expression Plasmid The expression plamids were constructed from the cDNA recombinant clones pPF41 and pPF131 as described in Fig. 1 and used to transform the HB101 strain of *E. coli* as described previously (5). Clone pPF41 contained nucleotides-6~424, while clone pPF131 overlaps pPF41 and extends beyond the termination codon on the 3' end. pPF41 was digested with restriction enzymes EcoRI + MstII to generate an 85-base pair fragment, and clone pPF131, with restriction enzymes MstII +BamHI to generate a 1135-base pair one. These two fragments were joined together and then ligated into the expression vector pUC8. The resultant plasmids were used to transform *E. coli* HB101 and selected by ampicillin resistance. The lysates were prepared and analyzed by restriction enzyme digestion.

Purification of the Expressed PGF Synthase Cells were cultured in 1 liter of enriched medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) containing 50 μg/ml ampicillin overnight at 37°C after the preculture. The cells were collected by centrifugation at 3,000 x g for 10 min, and washed with Buffer A (10 mM potassium phosphate buffer, pH 7.0), and then disrupted by sonication in Buffer A. All the operations were performed at 0-4°C. The cell debris and unbroken cells were removed by centrifugation at 100,000 x g for 60 min. The supernatant was subjected to ammonium sulfate fractionation. The precipitate formed between 50 and 75% saturation was suspended in Buffer A and dialyzed in Buffer A. The dialyzed sample was applied to a column (2.4 x 170 cm) of Sephadex G-100, previously equilibrated with Buffer A. Elution was carried out with the same buffer at a flow rate of 7.5 ml/min. The active fraction was applied to Blue Sepharose column (2.4 x 12 cm). The overall purification was 5-fold with a yield of 53%. The enzyme assay was described previously (3).

Miscellaneous Procedures Circular dichroism (CD) spectrum was determined on a Jasco spectropolarimeter, Model J-600. Titration of sulfhydryl groups was carried out with 5,5-dithiobis(2-nitrobenzoic acid)(DTNB) essentially according to the procedure of Habeeb (6). The N- and C-terminal sequences were determined with an Applied Biosystems protein sequenator.

RESULTS

Expression of the PGF Synthase Gene The full-length bovine lung PGF synthase cDNA was constructed from restriction fragments of the two partial cDNA clones (pPF131 and pPF41) and inserted into the plasmid pUC8 by use of the strategy described under "EXPERIMENTAL PROCEDURES". Twelve of the plasmid DNA's examined showed the predicted restriction fragment pattern with Hind III (H)(225, 967, and 2,669 base pairs). The bacteria containing one of them, designated as pUCPF1, were chosen for further studies.

E.coli cells harboring pUCPF1 were tested for expression of chimeric bovine lung PGF synthase. Total cellular extracts were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Coomassie blue staining of the gel indicated that an approximately 36-KDa protein was produced in cells containing the expression plasmid (Fig. 2A). Western blot analysis revealed that the 36-KDa protein was recognized by PGF synthase-specific antibodies (Fig. 2B). No

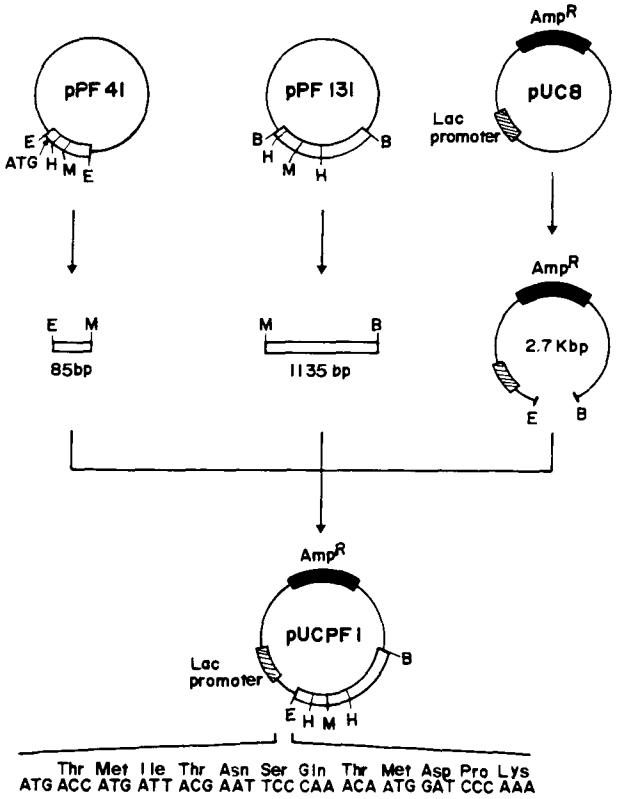

Figure 1. Construction of plasmid pUCPF 1 for the synthesis of bovine lung PGF synthase in *E. coli*. Plasmid pPF41 contains the 5' region of the bovine lung cDNA. Due to the presence of a MstII site (M), it was digested with MstII + EcoRI (E), and the fragments were isolated. Plasmid pPF131, which contains the 3' region of the cDNA, was digested with MstII +BamHI (B), and the fragments were isolated. Unique cohesive ends of these fragments and those of the vector pUC8 permit unidirectional ligation to result in the formation of pUCPF 1.

protein from the control *E. coli* cells alone or from *E. coli* containing the expression vector pUC8 interacted with these antibodies.

Purification and Properties of the PGF Synthase from *E. coli* The expressed protein was purified to apparent homogeneity as described under "EXPERIMENTAL PROCEDURES". The N-terminal

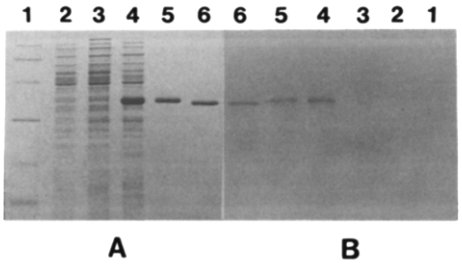

Figure 2. SDS-polyacrylamide gel analysis (A) and Western blot analysis (B) of bovine lung PGF synthase in *E. coli*. Samples were loaded on a 12.5% SDS-polyacrylamide gels. Lane 1, molecular weight markers: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000) carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), lactalbumin (14,400); Lane 2, crude extract of *E. coli* HB101; Lane 3, crude extract of *E. coli* HB101 containing the expression vector pUC8; Lane 4, crude extract of *E. coli* HB101 harboring pUCPF 1; Lane 5, purified *E. coli*-derived protein; Lane 6, purified bovine lung PGF synthase. For immunoblots, samples were subjected to SDS-PAGE, transferred to a Zeta-probe blotting membrane (Bio-Rad), and immunostained. Preparation of antisera against PGF synthase purified from bovine lung was reported earlier (3).

TABLE I
Substrate specificity of the expressed protein

Substrate	Concentration (mM)	Expressed Protein		Lung PGF synthase	
		Relative Activity (%)	Km (mM)	Relative Activity (%)	Km (mM)
Phenylglyoxal	1	124	83	128	80
4-Nitroacetophenone	0.5	97	20	115	13
Menadione	0.25	204	13	111	-a/
9,10-Phenanthrenequinone	0.01	77	0.13	100	0.7
4-Nitrobenzaldehyde	0.5	100	8.3	100	125
PGD_2	1	70	100	34	120
PGH_2	0.08	7	8	15	10
PGE2	1	6	-	2	-
Testosterone	0.05	4	-	1	-
NADPH			10		10

a/ not determined.
The reaction mixture contained 0.1M potassium phosphate buffer, pH 6.5, 80μM NADPH, substrate, and the expressed protein (1.5 to 6 μg) in the total volume of 0.7ml. The initial velocity of decrease in the absorbance at 340nm of NADPH was followed at 37°C. In the case of PGH_2 9,11-endoperoxide reductase, the enzyme activity was expressed as the amount of reaction product, $[^{14}C]PGF_{2\alpha}$, under the same assay conditions as those for other substrates.The 4-nitrobenzaldehyde reductase activity (115 nmol/min/mg of protein) represents 100% activity.

sequence was $^{-6}$Thr-$^{-5}$Met-$^{-4}$Ile-$^{-3}$Thr-$^{-2}$Asn-$^{-1}$Ser-^{0}Gln-^{1}Thr-^{2}Met-^{3}Asp-^{4}Pro-^{5}Lys. The first six amino acis, from $^{-6}$Thr to $^{-1}$Ser were derived from the β-galactosidase gene of pUC8; and the next three, ^{0}Gln-^{1}Thr-^{2}Met, were derived from the noncoding region and the initiation methionine of the cDNA for PGF synthase. These results suggest that the expressed vector pUCPF1 is initiated from the initiation codon of pUC8 and that 9 amino acids are fused to the N-terminus of the native protein. On the other hand, the C-terminal sequence of the expressed protein was Gly-Ile-Gly-His-Pro-Glu-Tyr-Pro-Phe-Ser-Glu-Glu-Tyr, identical to that of lung PGF synthase. Due to the additional amino acids at the N-terminus of the native enzyme, the Mr of the expressed enzyme is larger than that of lung PGF synthase, as shown in Fig.2.

The expressed protein purified from *E.coli* had the same broad substrate specificity as lung PGF synthase, and the enzymatic properties were essentially identical (Table I). The Km value for 4-nitrobenzaldehyde was about one-fifteenth that of the lung enzyme. This difference may be caused by the additional 9 amino acids at the N-terminus of the expressed protein.

The reaction products from $[^{14}C]PGH_2$ and $[^{3}H]PGD_2$ were identified as $[^{14}C]PGF_{2\alpha}$ and $[^{3}H]9\alpha,11\beta$-PGF_2, respectively, by reverse phase-high performance liquid chromatography (RP-HPLC) (data not shown) done according to the method of Liston and Roberts (7). Moreover, in an attempt to obtain the information on the active sites of the expressed protein, we performed kinetic analyses using PGH_2, PGD_2, and phenanthrenequinone. Phenanthrenequinone did not inhibit the conversion of PGH2 to $PGF_{2\alpha}$ (Fig.3A), but inhibited that of PGD_2 to $9\alpha,11\beta$-PGF_2 competitively (Fig. 3B). These results suggest that PGD_2 and phenanthrenequinone are reduced at the same active site and that PGH_2 is reduced at a different active site.

These results taken together with the data regarding the substrate specificity, the identification of the reaction products from PGH_2 and PGD_2, and the kinetic analysis indicated that the expressed protein was essentially equal to bovine lung PGF synthase.

The CD spectra of both proteins displayed a maximum at 192 nm and double minima at 210 nm and 225 nm, and were very similar in shape and magnitude (data not shown). For these proteins, the calculated secondary structure of PGF synthase is on the average 35% α-helix. In the 250-350nm wavelength region, the CD spectra of both proteins were also similar in shape and magnitude.

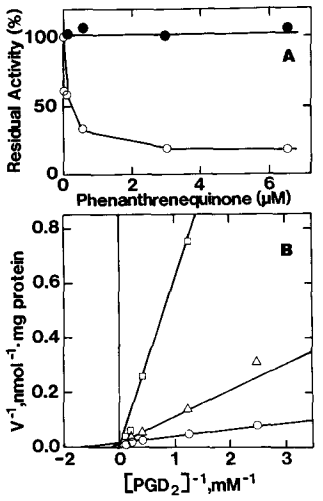

Figure 3. Effect of phenanthrenequinone on the PGD_2 11-ketoreductase and PGH_2 reductase activities of the expressed protein. The upper figure (A) shows PGD_2 11-ketoreductase (○) and the PGH_2 reductase (●) activities determined as described under "EXPERIMENTAL PROCEDURES" with the addition of increasing amounts of phenanthrenequinone. The lower figure (B) shows Lineweaver-Burk plots for the competitive inhibition of the PGD_2 11-ketoreductase activity by phenanthrenequinone. The PGD_2 11-ketoreductase activity was assay as described under "EXPERIMENTAL PROCEDURES" in the presence of phenanethrenequinone in the following concentrations: none(○); 3 μM(△); 6.5 μM (□).

Furthermore, the number of SH groups of the expressed protein and the native enzyme was determined by titration with DTNB. The number of free SH groups of both proteins was both approximately 7 mol/mol of protein. This value quantitatively agrees with the number of cysteines dictated by the cDNA for PGF synthase (5). This result suggests that the SH groups of the expressed proteins are all free, indicating the identity to those of the native protein. The structural analyses suggest that the backbone conformation of the polypeptides of both proteins is identical in terms of CD spectra and the titration of SH groups. Therefore, the structure and the properties of the expressed protein were essentially identical to those of lung PGF synthase.

The Binding Number of PGF Synthase for NADPH Using the expressed protein, we determined the binding number of PGF synthase for NADPH. Formation of a binary complex between NADPH and PGF synthase caused a red shift of the absorption maximum of the cofactor from 340 to 353 nm (Fig. 4A). The difference spectrum of free and protein-bound NADPH showed a minimum at 330 nm, a maximum at 378 nm (Fig. 4B). From the results of spectral titration of PGF synthase (Fig. 5), the binding ratio was estimated to be 0.81 mol of cofactor/mol of protein.

DISCUSSION

PGF synthase has at least two active sites on a monomeric protein (3), and catalyzes the reduction of PGH_2 to $PGF_{2\alpha}$ and that of PGD_2 to $9\alpha,11\beta$-PGF_2 at the different active sites (3, 4). This enzyme belongs to the group of aldo-keto reductases in terms of cofactor requirement, molecular weight, and substrate specificity; and its amino acid sequence shows high similarity with those of aldo-keto reductases (5, 8). We reported (9) that, like PGF synthase, human liver aldehyde reductase also reduces PGH_2 as well as aldehyde compounds at different active sites. These facts indicate that the structures of the active sites of these enzymes are similar.

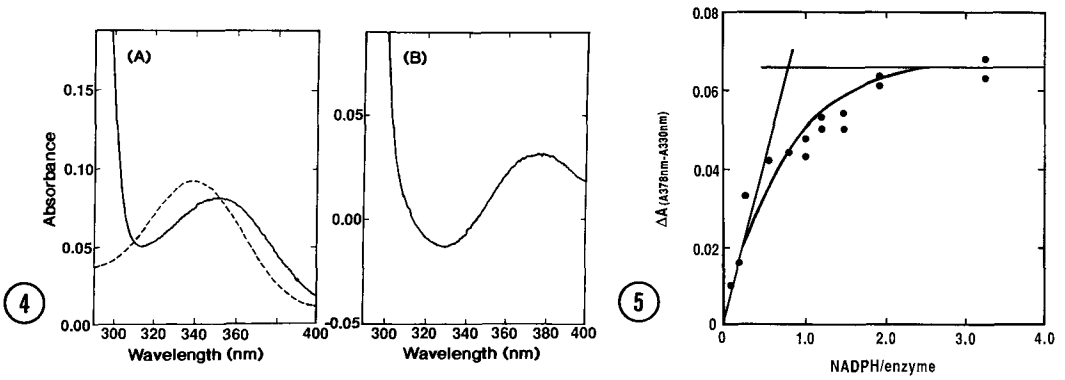

Figure 4. Absorption spectra of free and expressed protein-bound NADPH. The spectra (A) of 14 μM NADPH in the absence (– – –) and in the presence (——) of 21 μM the expressed protein, were recorded at room temperature in 25 mM sodium phosphate buffer, pH 7.2. Difference spectrum (B) between the expressed protein-bound and free NADPH was obtained.

Figure 5. Spectral titration of the expressed protein by NADPH. Concentration of the expressed protein was 21 μM. ΔA was obtained from difference spectra (Fig.4B) generated with various concentrations of NADPH.

To understand PGF synthase at the molecular level, we expressed the full-length bovine lung PGF synthase cDNA in *E. coli* HB101 using the expression vector pUC8 containing the lac Z' gene promoter, and compared the properties of the expressed protein with those of the native enzyme. The PGF synthase cDNA was inserted into the polylinker region of pUC8 and resulted in the production of PGF synthase with 9 additional amino acids at the natural N-terminus. However, the CD spectra of both proteins were similar in shape and magniude. From the results of the CD spectra, the α-helical content of both proteins was about 35%. This value coincides with that predicted from the primary structure of bovine lung PGF synthase by the method of Chou and Fasman (10). That method also showed about 35% β-sheet, and the hypothetical model of the secondary structure proposes a reciprocal repeat of α-helix and β-sheet. The α/β structure is specific for the nicotinamide-nucleotide-linked dehydrogenases (eg., lactate dehydrogenase, malate dehydrogenase, etc) (11). The primary structure of PGF synthase is not similar to that of lactate dehydrogenase (5), but the secondary structure of NADPH-linked PGF synthase may be similar to that of NADH-linked enzymes. Therefore, PGF synthase has the nucleotide linking fold like NADH-linked enzymes. Recently, Schade *et al.* reported that bovine lens aldose reductase also has a β-α-β secondary structure (12). Therefore, the group of aldo-keto reductases, which have high similarity in amino acid sequence to PGF synthase and aldose reductase, may also have the α/β structure.

The expressed protein crossreacted with the anti-PGF synthase antibody and had the same enzymatic properties as the lung PGF synthase in terms of Km values for several substrates and NADPH, the reaction products from PGH_2 and PGD_2, and the kinetic analysis. On the other hand, the Vmax values for several substrates and the Km value for 4-nitrobenzaldehyde of the expressed protein were different from those of the lung enzyme. While the backbone conformation of the expressed protein was identical with that of the native enzyme as judged from the CD spectrum and SH groups of the expressed protein were identical to those of the lung enzyme, it is possible that some alteration in the local conformation of the expressed protein has not yet been detected. Although a few cases in which a β-galactosidase fusion protein exhibited activity were reported (13-18), the comparison of the local conformation between the native enzyme and the expressed protein had not yet been reported. The slight difference between the expressed protein and lung PGF synthase noted above may be caused by the

additional amino acids. However, the properties of the expressed protein in *E.coli* are essentially identical to those of lung PGF synthase. Therefore, we confirmed in this paper that PGF synthase is a dual function enzyme catalyzing the reduction of PGH_2 and that of PGD_2 at different acive sites on a single enzyme and that it has one binding site for NADPH.

ACKNOWLEDGMENTS: We are grateful to Dr. Y. Kawata and Prof. K. Hamaguchi of Department of Biology, Faculty of Science, Osaka University for spectrophotometrical analyses. We also thank Dr. Y. Urade of the CIBA-GEIGY (Japan) Limited for the preparation of antibody, and Drs. S. Ito and H. Hayashi for useful discussion, and Dr. L. D. Frye for critical reading of this manuscript. We are indebted to N. Eguchi and M. Miyata for their skillful technical assistant.

REFERENCES

1. Watanabe, K., Shimizu, T., and Hayaishi, O. (1981) Biochem. Int. **2**, 603-610
2. Wong, P.Y.K. (1981) Biochem. Biophys. Acta **659**, 169-178
3. Watanabe, K., Yoshida, R., Shimizu, T., and Hayaishi, O. (1985) J. Biol. Chem. **260**, 7035-7041
4. Watanabe, K., Iguchi, Y., Iguchi, S., Arai, Y., Hayaishi, O., and Roberts, L.J., II (1986) Proc. Natl. Acad. Sci. USA **83**, 1583-1587
5. Watanabe, K., Fujii, Y., Nakayama, K., Ohkubo, H., Kuramitsu, S., Kagamiyama, H., Nakanishi, S., and Hayaishi, O. (1988) Proc. Natl. Acad. Sci. USA **85**, 11-15
6. Habeeb, A.F.S.A. (1972) Methods Enzymol. **25**, 457-464
7. Liston, T.E., and Roberts, L.J., II (1985) Proc. Natl. Acad. Sci. USA **82**, 6030-6034
8. Carper, D. A., Wistow, G., Nishimura, C., Graham, C., Watanabe, K., Fujii, Y., Hayashi, H., and Hayaishi, O. (1990) Exp. Eye Res. **49**, 377-388
9. Hayashi, H., Fujii, Y., Watanabe, K., Urade, Y., and Hayaishi, O. (1989) J. Biol.Chem. **264**, 1036-1040
10. Chou, P.Y., and Fasman, G.D. (1978) Annu. Rev. Biochem. **47**, 251-276
11. Eventoff,W., Rossmann, M.G., Taylor, S.S., Torff, H.,J., Meyer, H., Keil, W., and Kiltz, H.,H. (1977) Proc. Natl. Acad. Sci. USA **74**, 2677-2681
12. Schade, S. Z., Early, S. L., Williams T. R., Kezdy, F. J., Heinrikson, R. L., Grimshaw, C.E., and Doughty, C. C. (1990) J. Biol. Chem. **265**, 3628-3635
13. Johnson, A.D., and Herskowitz, I. (1985) Cell **42**, 237-247
14. Kanfman, D.L., McGinnis, J.F., Krieger, N.R., and Tobin, A.J. (1986) Science **232**, 1138-1140
15. Mutzel, R., Lacombe, M.L., Simon, M.N., de Gunzburg, J., and Vernon, M. (1987) Proc. Natl. Acad. Sci. USA **84**, 6-10
16. Eberwine, J.H., Barchas, J.D., Hewlett, W.A., and Evans, C.J. (1987) Proc. Natl. Acad. Sci. USA **84**, 1449-1453
17. Saraswat, L.D., Filutowicz, M., and Taylor, S.S. (1986) J. Biol.Chem. **261**, 11091-11096
18. Kunapuli, S.P., Prasad, G.L., and Kumar, A. (1987) J. Biol. Chem.**262**, 7672-7675