[Chem. Pharm. Bull. 32(6)2346—2352(1984)]

Peroxisomes in the Liver of Frog, Bombina orientalis

HIDENORI HAYASHI,*,[a] KENJI FUKUI,[a] FUMIE YAMASAKI,[a] and TETSUYA SUGA[b]

Faculty of Pharmaceutical Sciences, Josai University,[a] 1–1 Keyakidai, Sakado, Saitama 350–02, Japan and Tokyo College of Pharmacy,[b] 1432–1 Horinouchi, Hachiooji, Tokyo 192–03, Japan

(Received September 22, 1983)

The existence of peroxisomes was investigated in the liver of frog, Bombina orientalis, which characteristically cannot degrade cholesterol further than to C_{27} higher bile acids such as 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) or 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA). The activities of catalase, D-amino acid oxidase, urate oxidase, and the CN-insensitive fatty acyl–CoA β-oxidizing system (FAOS) were used as markers of peroxisomes. Catalase activity was present to almost the same extent in Bombina liver as in rat liver. D-Amino acid oxidase and urate oxidase were also present in the frog liver, but the activities were much lower than those in the rat liver. In a cell fractionation experiment, the highest specific activities of catalase, D-amino acid oxidase, urate oxidase and CN-insensitive FAOS were all found in the light mitochondrial fraction, and upon sucrose density gradient centrifugation, these activities were concentrated in the density fraction around 1.21—1.22. On the other hand, the densities of mitochondria and lysosomes were 1.19 and 1.20, respectively. From the above results, we concluded that Bombina liver contains peroxisomes.

Next, the characteristics of CN-sensitive (mitochondrial) or CN-insensitive (peroxisomal) FAOS and carnitine acyltransferase activities were compared in the partially purified peroxisomes and mitochondria using various chain length fatty acyl–CoAs (C_2—C_{20}). FAOS activities of the peroxisomes were effective for long-chain fatty acyl–CoAs (C_8—C_{16}), while the mitochondrial FAOS showed broad specificity for short- to long-chain fatty acyl–CoAs. Both the peroxisomes and the mitochondria showed high carnitine acyltransferase activities for acetyl–CoA and C_8 to C_{12} fatty acyl–CoAs. Administration of clofibrate for 7 d enhanced the activities of catalase and CN-insensitive FAOS, but the D-amino acid oxidase activity decreased and the urate oxidase activity was unchanged.

The results indicate that peroxisomes do exist in Bombina liver, but the particles may be involved primarily in the exhaustive degradation of general fatty acids, not in the synthesis of bile acids. When necessary, the mitochondria and peroxisomes may act cooperatively in order to obtain energy from various fatty acids.

Keywords——peroxisomes; Bombina; frog; fatty acid β-oxidation; catalase; clofibrate; mitochondria

In mammals, cholesterol is generally degraded to C_{24} bile acids such as cholic acid and chenodeoxycholic acid,[1] which are then excreted. It is known that the degradation of cholesterol to C_{24} bile acids passes through C_{27} higher bile acids such as 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) or 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA). THCA and DHCA are then subject to shortening of the side chains to yield cholic acid and chenodeoxycholic acid, respectively. The reaction involves the same β-oxidation system as that of general fatty acid β-oxidation.[2–4] The β-oxidation system had been considered to be located exclusively in liver mitochondria, but it has recently been reported that the system also exists in liver peroxisomes as well as mitochondria.[5–7] The characteristics of the peroxisomal system are significantly different in several respects from those of the mitochondrial system. Peroxisomal activities of the β-oxidation system are markedly enhanced by administration of

clofibrate,[5] acetylsalicylic acid,[8] LK-903,[9] or di(2-ethylhexyl)-phthalate,[10] which have a decreasing effect on serum cholesterol. We have reported that the biosynthesis of bile acids is stimulated by administration of clofibrate.[11] Pedersen and Gustafsson have recently reported that β-oxidation of rat liver peroxisomes participates in the degradation of cholesterol.[12] They suggested that peroxisomal β-oxidation activity for the reaction from THCA, the intermediate to cholic acid, is higher than that of the mitochondrial system. Further, Hagey and Krisans have also reported that peroxisomes take part in the cleavage of the cholesterol side chain into propionate.[13]

On the other hand, among the amphibia and reptiles, bile acids are excreted in the form of higher bile acids such as THCA and DHCA. Bombina orientalis,[14] Alligator mississippiensis,[15] and Trionyx[16] are examples. We are interested in studying the hepatic peroxisomes of such animals in order to elucidate the real significance of peroxisomes. This paper deals with the existence and possible significance of liver peroxisomes in frog, Bombina orientalis.

Experimental

Materials——Bombina orientalis were purchased from Kobayashi Syoji Co. (Tokyo, Japan). Clofibrate was kindly provided by Sumitomo Kagaku Co. (Osaka, Japan). Carnitine was kindly donated by Taiho Yakuhin Co. (Tokushima, Japan). CoA, NAD^+, cytochrome c and acyl–CoAs were purchased from Sigma Chemicals Co. (St. Louis, U.S.A.). Other chemicals were obtained from Wako Pure Chemicals Co. (Tokyo, Japan).

Animals and Subcellular Fractionation——The frogs were purchased in April and kept at 25 °C. They were fed on maggots or crickets. The animals were anesthetized by cooling in ice-cold water and the livers were isolated. In the subcellular fractionation experiment, the livers collected from 30 animals were weighed and homogenized in ice-cold 0.25 M sucrose in a Potter-Elvehjem type Teflon homogenizer. Ten per cent (w/v) liver homogenate was prepared in 0.25 M sucrose and fractionated according to the method of De Duve *et al.*[17] Nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and supernatant (S) fractions were obtained by centrifugation at $600 \times g$ for 10 min, $300 \times g$ for 10 min, $12500 \times g$ for 20 min and $105000 \times g$ for 60 min, respectively. Centrifugation at $105000 \times g$ for 60 min was carried out in a Hitachi ultracentrifuge (model 70P) with an RP65 rotor.

Sucrose Density Gradient Centrifugation——The light mitochondrial fraction prepared from the frog liver homogenate was subjected to sucrose density gradient centrifugation. A linear sucrose density gradient (34 ml) from density 1.15 to 1.25 was prepared in 35 ml centrifuge tubes. Next, 1 ml of the light mitochondrial fraction corresponding to approximately 12 mg protein was layered on top of the gradient, and the tubes were centrifuged at 30000 rpm for 120 min (R_{min} $61000 \times g$, R_{max} $88000 \times g$, mean 13000 $rad^2/s \times 10^7$) in a Hitachi model 70P ultracentrifuge with a Hitachi RPV50T vertical rotor. In this centrifugation, a slow acceleration controller, which ensured slow and smooth acceleration up to 1000 rpm, was used. After centrifugation, the tubes were divided into 9 fractions of 4 ml each from the top of the gradient with an ISCO gradient fractionater (U.S.A.). Each fraction was appropriately diluted with ice-cold water, and centrifuged at $20000 \times g$ for 30 min. The respective precipitates were suspended in 0.25 M sucrose, and the enzyme activities in each fraction were determined.

Administration of Clofibrate——The frogs were given clofibrate orally *via* a microsyringe at a daily dose of about 10 mg for 7 d, then the light mitochondrial fraction was prepared, and the peroxisomal enzyme activities were determined.

Assays of Enzyme Activities and Protein Content——Catalase activity was determined according to the method of Leighton *et al.*[18] with a slight modification.[19] The activities of D-amino acid oxidase and urate oxidase were assayed according to the method of Hayashi *et al.*[20] Fatty acyl–CoA oxidizing system activity was estimated by the method of Lazarow and De Duve[5] with a slight modification.[9] Carnitine acyltransferase activity was determined by the method of Markwell *et al.*[21] Acid phosphatase activity was assayed according to the method of Hayashi *et al.*[19] Cytochrome c oxidase activity was estimated by the method of Wharton and Tzagoloff[22] with a slight modification.[9] Protein was determined according to Lowry *et al.*[23]

Results

Peroxisomal Enzyme Activities in Bombina Liver

Table I shows the activities of peroxisomal enzymes in liver homogenate of Bombina. The results suggest that D-amino acid oxidase and urate oxidase are present, although both

TABLE I. Activities of Peroxisomal Enzymes in Bombina Liver[a]

Enzyme	Specific activity	
	Bombina	Rat[b]
Catalase[c]	46.0±15.3	55.05±3.27
D-Amino acid oxidase[d]	1.52±0.42	8.43±1.52
Urate oxidase[d]	4.46±1.35	12.29±0.96
Protein (mg/g liver)	171.4±16.5	196.9±4.9

a) Means±S.D. of 30 animals.
b) Data from H. Hayashi *et al.* [*Biochem. Pharm.*, **30**, 1817 (1981)].
c) U/mg protein. *d*) mU/mg protein.

activities are much lower than those of rat liver. The specific activities of catalase and the protein content, however, were almost the same as those of rat liver. The specific activities of rat liver enzymes reported from our laboratory are also listed in Table I for comparison.[9]

Subcellular Fractionation

Figure 1 shows the results of cell fractionation of the liver of Bombina according to the method of De Duve *et al.*[17] The activities of the fatty acyl–CoA oxidizing system, as well as catalase, D-amino acid oxidase and urate oxidase, were found to be highest in the light mitochondrial fraction. The activity of cytochrome c oxidase (a mitochondrial marker) appeared in the heavy mitochondrial fraction and the activity of acid phosphatase in the light mitochondrial fraction.

Sucrose Density Gradient Centrifugation

The results of the sucrose density gradient centrifugation of the light mitochondrial fraction are shown in Fig. 2. The patterns of catalase, D-amino acid oxidase, urate oxidase and the fatty acyl–CoA oxidizing system were similar to one another, but different from those of cytochrome c oxidase and acid phosphatase. The mean densities of the hepatic organella were calculated from the distribution patterns of the appropriate enzyme activities and are shown in Table II. The density of peroxisomes was found to be 1.21—1.22, that of mitochondria was 1.19, and that of lysosomes was 1.20.

Substrate Specificity of the Fatty Acyl–CoA Oxidizing System and Carnitine Acyltransferase

Substrate specificity patterns of the fatty acyl–CoA oxidizing system in partially purified peroxisomes and mitochondria prepared by sucrose density gradient centrifugation are shown in Fig. 3. The determination of the system activity was carried out by the method of Lazarow and De Duve,[5] except that 1 mM KCN was omitted in the assay of the mitochondrial system. The peroxisomes had the highest activity towards long-chain acyl–CoAs (C_8—C_{16}), while the mitochondria had activity towards a broad range of acyl–CoAs from short- to long-chain. These results on the peroxisomal enzymes of Bombina are quite similar to those obtained in the case of rat liver.[6]

Figure 4 shows substrate specificity patterns of carnitine acyltransferase of liver peroxisomes and mitochondria of Bombina. Peroxisomes as well as mitochondria had the highest activity towards acetyl–CoA and medium-chain acyl–CoAs (C_8—C_{12}).

Effect of Clofibrate on Peroxisomal Enzymes

Clofibrate was orally administered to Bombina for 7 d, then the light mitochondrial fraction of the liver was prepared. Table III shows the changes of liver peroxisomal enzyme activities induced by clofibrate. The activities of catalase and the fatty acyl–CoA oxidizing system were increased significantly by the administration (1.43- and 2.34-fold, respectively), as

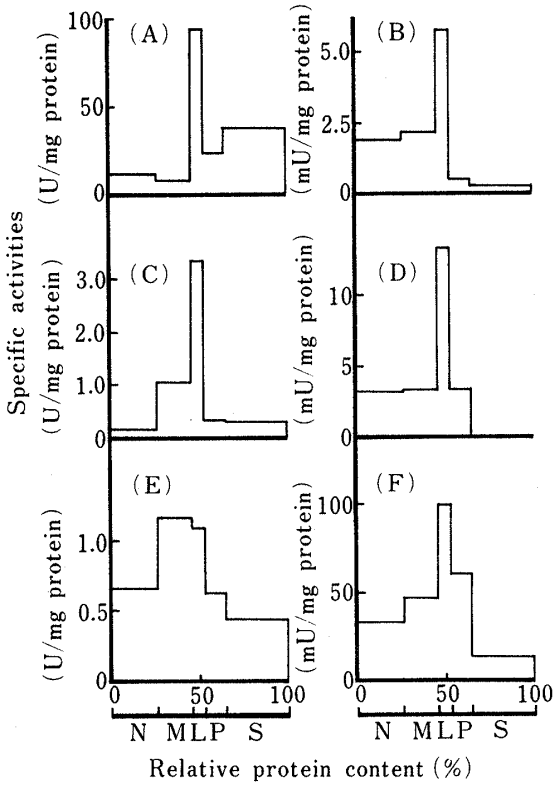

Fig. 1. Subcellular Distributions of Peroxisomal Enzyme Activities of Bombina Liver

Homogenates were fractionated according to the method of De Duve *et al.*[17] The ordinates represent specific activities. The abscissas indicate relative cumulative protein content; N, M, L, P, and S represent nuclear, heavy mitochondrial, light mitochondrial, microsomal and supernatant fractions, respectively.

(A) catalase; (B) D-amino acid oxidase; (C) CN-insensitive FAOS; (D) urate oxidase; (E) cytochrome c oxidase; (F) acid phosphatase.

Data are means of 6 experiments.

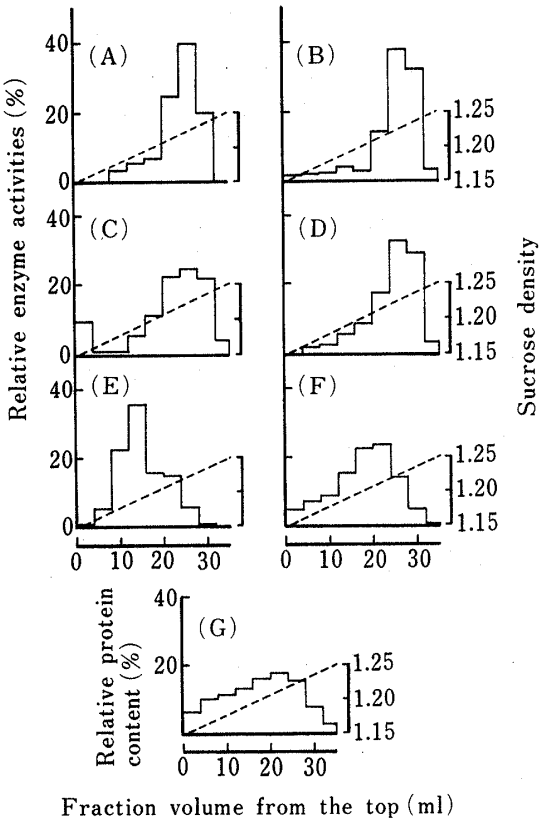

Fig. 2. Sucrose Density Gradient Centrifugation Patterns of the Light Mitochondrial Fraction of Bombina Liver

The procedure is described in Experimental.

(A) catalase; (B) D-amino acid oxidase; (C) CN-insensitive FAOS; (D) urate oxidase; (E) cytochrome c oxidase; (F) acid phosphatase; (G) protein.

Data are means of 5 experiments.

TABLE II. The Densities of the Hepatic Organella of Bombina[a)]

Organella	Enzyme	Density
Peroxisomes	Catalase	1.218
	D-Amino acid oxidase	1.219
	Urate oxidase	1.220
	CN-Insensitive FAOS	1.211
Mitochondria	Cytochrome c oxidase	1.192
Lysosomes	Acid phosphatase	1.199

a) Mean density values were calculated from the data in Fig. 2.

was the case in rat liver.[5] However, D-amino acid oxidase activity was decreased, while the activities of urate oxidase, cytochrome c oxidase and acid phosphatase were unchanged. The protein content of the liver increased significantly (1.58-fold).

Discussion

Peroxisomes are widely distributed in various tissues, but their true physiological

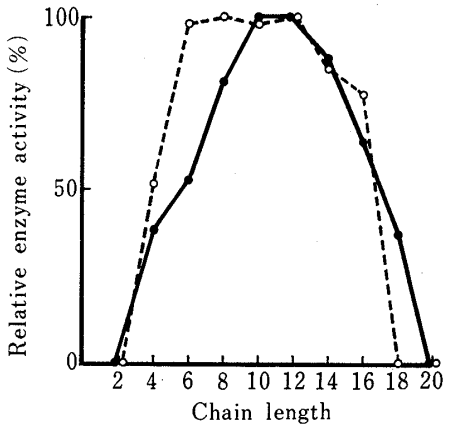

Fig. 3. Substrate Specificity Patterns of the Fatty Acyl–CoA Oxidizing System of Partially Purified Peroxisomes and Mitochondria

Fractions No. 4 and 7 of Fig. 2 were used as partially purified mitochondrial and peroxisomal preparations, respectively. Various fatty acyl–CoAs from C_2 to C_{20} chain length were used as substrates of this system. The ordinate represents relative enzyme activities with respect to the maximum activity (C_{12} fatty acyl–CoA for both mitochondria and peroxisomes).

One mM KCN was omitted from the incubation mixture when mitochondrial activities were determined.

●—●, peroxisomes; ○---○, mitochondria.

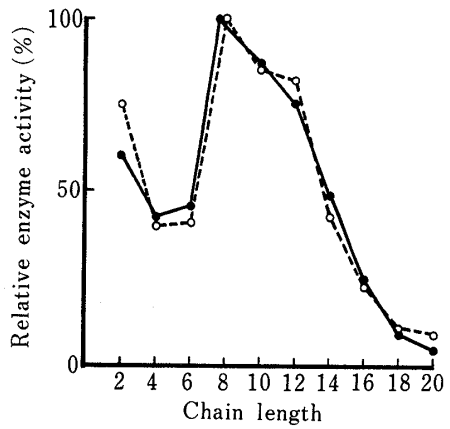

Fig. 4. Substrate Specificity of Carnitine Acyltransferase of the Partially Purified Peroxisomes and Mitochondria

Enzyme preparations and substrates were the same as in Fig. 3. The ordinate represents relative activities with respect to the maximum activity (C_8 fatty acyl–CoA for both mitochondria and peroxisomes).

●—●, peroxisomes; ○---○, mitochondria.

TABLE III. Effect of Clofibrate on the Activities of Peroxisomal Enzymes in Bombina Liver[a]

Enzyme	Control	Clofibrate	Clofibrate / Control
Catalase[b]	111.37±26.11	159.24±26.39	1.43[d]
D-Amino acid oxidase[c]	5.69±1.09	4.62±0.61	0.81[e]
Urate oxidase[c]	11.07±3.73	13.58±3.14	1.23
CN-Insensitive FAOS[b]	3.89±1.08	9.09±2.15	2.34[d]
Cytochrome c oxidase[b]	1.10±0.33	1.18±0.34	1.07
Acid phosphatase[c]	94.98±17.58	82.92±15.70	0.87
Protein (mg/g liver)	12.63±2.42	19.47±2.58	1.58[d]

a) The frogs were orally given clofibrate (*ca.* 1.67 mg/g body weight) daily for 7 d. Enzyme preparations were the light mitochondrial fractions from liver homogenate. Data are means±S.D. of 12 animals.
b) U/mg protein. *c*) mU/mg protein.
d) and *e*) represent statistically significant changes induced by clofibrate *d*) $p<0.01$; *e*) $p<0.05$).

significance is not yet clear. Studies of peroxisomal functions have revealed that the fatty acid β-oxidation system of peroxisomes is different from that of mitochondria, and the peroxisomal β-oxidation system of the rat liver was suggested to participate in the biosynthesis of bile acids from cholesterol.[12,13] In this experiment, peroxisomes of the liver of Bombina orientalis were studied, because the animals excrete excess cholesterol as higher bile acids, such as THCA and DHCA,[14] which are intermediates in the formation of C_{24} bile acids.[1] Peroxisomes were found in Bombina liver, and contained the activities of catalase, D-amino acid oxidase, urate oxidase and the cyanide-insensitive fatty acid β-oxidation system.

From the data shown in Fig. 1, peroxisomes in Bombina liver are assumed to be approximately the same size as those in rat liver. The density of the peroxisomes calculated from the results of density gradient centrifugation was 1.21—1.22, which is slightly less than that of rat liver peroxisomes. Thus, it became apparent that even Bombina liver, which cannot oxidize THCA and DHCA to cholic acid and chenodeoxycholic acid, respectively, contains peroxisomes and some fatty acid β-oxidizing system. Further, the activities of catalase and the fatty acyl–CoA oxidizing system in the peroxisomes were significantly increased by the administration of clofibrate, as was the case in rat liver peroxisomes. The activity of D-amino acid oxidase, however, was decreased, and the urate oxidase activity was unchanged. The activities of cytochrome c oxidase (mitochondrial marker enzyme) and of acid phosphatase (lysosomal enzyme) were also unaffected by the administration. These results suggest that the characteristics of Bombina liver peroxisomes may be similar in many respects to those of rat liver peroxisomes. Even though the peroxisomes of Bombina liver are not associated with biosynthesis of bile acids, the peroxisomes may have roles in the metabolism of general fatty acids. It was reported that rat liver peroxisomes have specificity for long-chain acyl–CoA, while mitochondria have specificity for short-chain acyl–CoA.[6] Therefore, it can be presumed that hepatic peroxisomes of the rat may degrade long-chain fatty acids into fatty acids of suitable length, which are then further metabolized in mitochondria. In the case of Bombina, the hepatic peroxisomes can oxidize long-chain fatty acids, while the fatty acid oxidizing system of the mitochondria has broad specificity for fatty acids from short- to long-chain. Carnitine acyltransferases of the peroxisomes act on medium-chain fatty acids in almost the same way as those of mitochondria. The above results suggest that mitochondria in the liver of Bombina are the predominant or exclusive providers of energy from fatty acids in the normal or static states, but in abnormal states, (for example, excessive uptake of higher fatty acids,[24] fasting,[25] diabetes[26] and others), peroxisomes may act co-operatively with mitochondria.

References and Notes

1) H. Danielsson, "The Bile Acids," Vol. 2, ed. by P. P. Nair and D. Kritchevsky, Plenum Press, New York, 1973, pp. 1—32.
2) H. M. Suld, E. Staple, and S. Gurin, *J. Biol. Chem.*, **237**, 338 (1962).
3) T. Masui and E. Staple, *Biochim. Biophys. Acta*, **104**, 305 (1965).
4) T. Masui and E. Staple, *J. Biol. Chem.*, **241**, 3889 (1966).
5) P. B. Lazarow and C. De Duve, *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 2043 (1976).
6) P. B. Lazarow, *J. Biol. Chem.*, **253**, 1522 (1978).
7) H. Hayashi, S. Hino, and F. Yamasaki, *Eur. J. Biochem.*, **120**, 47 (1981).
8) H. Ishii and T. Suga, *Biochem. Pharmacol.*, **28**, 2829 (1979).
9) H. Hayashi, S. Hino, F. Yamasaki, T. Watanabe, and T. Suga, *Biochem. Pharmacol.*, **30**, 1817 (1981).
10) T. Osumi and T. Hashimoto, *J. Biochem.* (Tokyo), **83**, 1361 (1977).
11) H. Hayashi, K. Fukuyama, and F. Yamasaki, *Chem. Pharm. Bull.*, **31**, 653 (1983).
12) J. I. Pedersen and J. Gustafsson, *FEBS Lett.*, **121**, 345 (1980).
13) L. R. Hagey and S. K. Krisans, *Biochem. Biophys. Res. Commun.*, **107**, 834 (1982).
14) T. Kuramoto, H. Kikuchi, H. Sanemori, and T. Hoshita, *Chem. Pharm. Bull.*, **21**, 952 (1973).
15) G. A. D. Halsewood, *Biochem. J.*, **52**, 583 (1952).
16) K. Amimoto, T. Hoshita, and T. Kazuno, *J. Biochem.* (Tokyo), **57**, 565 (1965).
17) C. De Duve, B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans, *Biochem. J.*, **60**, 604 (1955).
18) F. Leighton, B. Polle, H. Beaufay, P. Baudhuin, J. W. Coffey, S. Fowler, and C. De Duve, *J. Cell Biol.*, **37**, 482 (1968).
19) H. Hayashi and T. Suga, *J. Biochem.* (Tokyo), **84**, 513 (1978).
20) H. Hayashi, T. Suga, and S. Niinobe, *Biochim. Biophys. Acta*, **252**, 58 (1971).
21) M. A. K. Markwell, E. J. McGroarty, L. L. Bieber, and N. E. Tolbert, *J. Biol. Chem.*, **248**, 3426 (1973).
22) D. C. Wharton and A. Tzagoloff, *Methods in Enzymol.*, **10**, 245 (1973).
23) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

24) H. Ishii, N. Fukumori, S. Horie, and T. Suga, *Biochim. Biophys. Acta*, **617**, 1 (1980).
25) H. Ishii, S. Horie, and T. Suga, *J. Biochem.* (Tokyo), **87**, 1855 (1980).
26) S. Horie, H. Ishii, and T. Suga, *J. Biochem.* (Tokyo), **90**, 1691 (1981).