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Identification of protein-bound riboflavin in rat hepatocyte plasma membrane as a source of autofluorescence

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The presence of flavin compound(s) giving a yellowish-green autofluorescence in rat hepatocyte plasma membrane has recently been reported (Nokubo, M. et al. (1988) Biochim. Biophys. Acta 939, 441–448). The fluorophore can quantitatively be extracted with water at 80°C from isolated plasma membranes. Gel filtration of the extract eluted with water showed two peaks, the fluorescence of which closely resembled that of riboflavin. The major peak comigrated with proteins and the minor one displayed a position identical to authentic riboflavin. When the components of the major peak were rechromatographed after acetic acid treatment and eluted with 20 mM of acetic acid, the fluorescent compound separated from the proteins and eluted at the same position as riboflavin. In paper chromatography and HPLC, the behavior of the fluorescent compound (separated by acid treatment from the proteins) was identical to that of riboflavin. SDS gel filtration of subcellular fractions of rat liver revealed that riboflavin was the dominant flavin, whereas FAD and FMN were not detectable in the plasma membrane. Microsomes and mitochondria contain predominantly FAD and FMN, and only minor quantities of riboflavin. The presence of riboflavin in the plasma membrane is a novel finding, the functional significance of which is still unclear; however, a hypothesis can be forwarded on the basis of the ability of flavins to generate superoxide anion radicals during their autoxidation.

Introduction

A yellowish-green autofluorescence of the hepatocyte membrane proteins has been discovered during our previous studies on the lateral diffusion of proteins by means of the fluorescence recovery after photobleaching (FRAP) method in Fischer-344 rats [1]. This fluorescence was invisible in the fresh liver smears, however, it became evident after about 15–20 min exposure to atmospheric oxygen pressure. Since the intensity of it could considerably be increased by a mild (1 mM) hydrogen peroxide treatment, it has been designated as peroxide-induced autofluorescence (PIAF) [2,3]. The PIAF has also been encountered in Wistar rats [4] as well as in C57BL/6 mice [5]. It proved to be a useful endogenous fluorescent label for FRAP studies in various experimental approaches [6,7]. Apart from this practical usefulness, it seemed to be of interest to study in detail the molecular nature of PIAF. Therefore, specific studies have been undertaken to identify and characterize the compound being responsible for the formation of PIAF.

During the first step of these investigations, hepatocyte plasma membranes were isolated and their fluorescent properties were characterized. We observed a protein-bound fluorescence with an emission maximum at 525 nm when excited with blue laser light of 476.5 nm [8]. It has been established on the basis of the fluorescent excitation and emission spectra obtained that PIAF is due to an oxidized form of some flavin compound(s) [8]. We indicated as possible candidates riboflavin, FAD and FMN. These findings deserved further detailed interest for several reasons: (i) Although flavin compounds are abundant in cells and are distributed widely in subcellular fractions, the occurrence of flavins in the hepatocyte plasma membrane is a novel observation, therefore, their identification and understanding the cell-physiological significance of their particular localization may be of theoretical interest. (ii) The presence of FAD in leukocyte plasma membrane in

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Abbreviations: FRAP, fluorescence recovery after photobleaching; PIAF, peroxide-induced autofluorescence.

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form of a flavoprotein has recently been described and it was postulated that this flavoprotein acts as a superoxide anion radical generating enzyme [9,10]. This observation may further be interpreted in terms of free radical biochemistry.

The present paper demonstrates the experimental evidence that the protein-bound flavin present in the hepatocyte plasma membrane is riboflavin, and the hypothesis is forwarded that the presence of riboflavin in the plasma membrane, bound to proteins may represent a defense mechanism against OH˙ free radical induced cross-linking under conditions of oxidative stress.

Materials and Methods

Isolation of rat liver plasma membranes. Liver plasma membranes were prepared from male Fischer-344 rats (10–15 weeks old) by the method previously reported [8]. Characterization of subcellular fractions was performed by assaying marker enzymes such as 5′-nucleotidase [11], Mg^{2+}-ATPase, and Na^{+}/K^{+}-ATPase [12] for plasma membrane, succinate dehydrogenase [13] for mitochondria, and glucose-6-phosphatase [11] for microsomes.

Extraction of the fluorescence from rat liver plasma membrane. A suspension of rat liver plasma membrane preparation was heated at 80°C for 15 min, then centrifuged at 9000 × *g* for 20 min. The resultant supernatant was separated from the precipitates. The precipitates were suspended in water (half the volume of the original suspension). The hot-water extraction was repeated again. The supernatants were combined together and lyophilized.

Gel filtration of the hot-water extract of rat liver plasma membrane. The lyophilized powder of the hot-water extract from rat liver plasma membrane was dissolved in a small volume of water, and centrifuged at 350 000 × *g* for 15 min to remove insoluble materials. The supernatant was applied to a column of TSK-gel HW-50 (1.5 × 26 cm) and eluted with water. The fractions (2.2 ml) were monitored for absorbance at 280 nm and for fluorescence at 525 nm (excited at 468 nm). Fractions showing fluorescence were collected and lyophilized. The residual powder was dissolved into a small volume of 20 mM acetic acid, and applied to a column of TSK-gel HW-50 equilibrated with 20 mM acetic acid. Fractions eluted with 20 mM acetic acid were monitored for absorbance at 280 nm, and for fluorescence at 525 nm.

Isolation of fluorophore after the gel filtration. After the gel filtration with 20 mM acetic acid, fractions with fluorescence were collected, lyophilized and submitted to paper chromatography (Whatman No. 1 chromatographic paper) developing with *n*-butanol/acetic acid/water (4 : 1 : 5, v/v, upper phase). After drying, the fluorescent zone on the filter paper was located by ultraviolet light, and cut out. The fluorescent material of the strip of filter paper was eluted with water.

Direct isolation of fluorophore from rat liver plasma membrane. Rat liver plasma membranes were partitioned between water and chloroform three times. The water fractions were combined, lyophilized and the fluorophore was isolated by paper chromatography as described above.

Identification of the fluorophore. The isolated fluorophore was identified by paper chromatography adopting two solvent systems; upper phase of *n*-butanol/acetic acid/water (4 : 1 : 5, v/v), and 4% sodium citrate. In addition, high-performance liquid chromatography (HPLC) was used for the identification. For this purpose, the following conditions were used: Bilepak-II column (JASCO, Tokyo, Japan) with a solvent system of methanol/acetonitrile/0.1 M sodium acetate buffer (pH 5.0) (1 : 1 : 8, v/v) at a flow rate of 1.0 ml/min. The fluorescence of the eluate was monitored at 525 nm emission with 468 nm excitation.

Comparison of elution patterns of SDS gel chromatography of subcellular fractions. Rat liver mitochondria and microsomes were prepared by the methods described in our previous paper [8]. SDS gel chromatography was performed with Toyopearl HW-50 column (15 × 700 mm) by flowing 1% SDS/20 mM Tris-HCl buffer (pH 7.4). Each subcellular fraction was solubilized with a small volume of the SDS-Tris buffer and applied to the column and eluted by the SDS-Tris buffer. Elution fluid was fractioned and fluorescence intensity and protein concentration of each fraction were measured. The fluorescence was monitored at the emission wavelength of 525 nm excited at 468 nm. The protein concentration was monitored by ultraviolet absorption at 280 nm.

Analytical instruments. Absorption and fluorescence spectra were measured by a Hitachi 124 spectrophotometer and a Hitachi 650-10S fluorescence spectrophotometer (Hitachi, Japan), respectively. For HPLC study, a Waters 6000A (Milford, Mass) instrument equipped with a fluorescence detector, Shimadzu RF-535 Fluorescence HPLC Monitor (Kyoto, Japan) and a JASCO Bilepak-II packed column (Tokyo, Japan) was used.

Chemicals. Riboflavin, FAD, AMP, ATP, glucose 6-phosphate, and *p*-iodonitrotetrazolium violet were purchased from Sigma Chemical Co. (St. Louis, MO), SDS and FMN from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and ouabain from E. Merck (Darmstadt, F.R.G.). TSK-gel HW-50 was obtained from Toyo Soda (Tokyo, Japan).

Results

Specific activities of 5′-nucleotidase, Mg^{2+}-ATPase and Na^{+}/K^{+}-ATPase, marker enzymes of liver plasma membrane, in the prepared rat liver plasma membranes

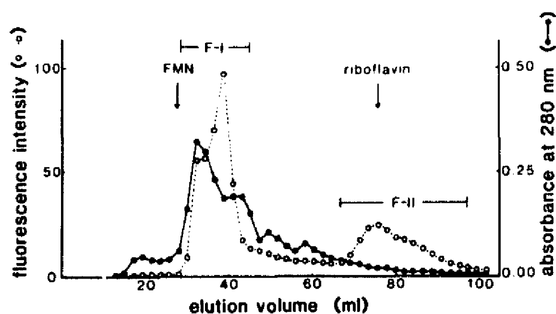

Fig. 1. TSK-gel HW-50 column chromatography of the hot-water extract from rat liver plasma membranes. The hot-water extract was concentrated by lyophilization and applied to a TSK-gel HW-50 column (1.5×26 cm) which was eluted with H_2O. The fluorescence of each fraction (2.2 ml) was monitored by emission at 525 nm with 468 nm excitation, and the protein concentration was determined as absorbance at 280 nm. The elution positions of riboflavin and FMN are shown. FAD was eluted at the position very close to that of FMN under the same chromatographic conditions. Blue Dextran 2000 was eluted at 20 ml of elution volume.

increased 28-, 8-, and 23-fold compared with their respective values in the homogenates. On the other hand, specific activities of glucose-6-phosphatase, a marker enzyme of microsomes, and succinate dehydrogenase, a marker enzyme of mitochondria, were 0.35 and 0.10 in the prepared plasma membrane relative to their respective values in the total homogenates. These data demonstrate that the plasma membrane preparations were sufficiently pure, and contaminations of other subcellular fractions were minimal.

The recovery of fluorescing material from the rat liver plasma membranes by repeated hot-water extraction was practically 100%. The extracts were concentrated by lyophilization and applied to gel filtration chromatography. Fig. 1 shows the gel filtration pattern of the hot-water extracts. When the column was eluted with water, two fluorescent peaks appeared at the elution volume of about 40 ml (F-I) and 75 ml (F-II). F-I was eluted with proteins and F-II was eluted at the same position as riboflavin. When F-I was collected, lyophilized and applied to a column of HW-50 gel which was equilibrated with 20 mM acetic acid, the fluorescence suggesting the separation from proteins was eluted at the position of riboflavin as examined under the same conditions (Fig. 2). This fluorescing material was combined to F-II, concentrated and subjected to a paper chromatographic purification. When the paper chromatography was developed by a solvent system of *n*-butanol/acetic acid/water (4:1:5, v/v, upper phase), only one fluorescent band was observed at R_f 0.32 which corresponded to that of riboflavin. The behavior of the fluorescent compound, isolated by paper chromatography, was compared to that of authentic flavin compounds in paper chromatography and HPLC. R_f values in paper chromatography of the isolated fluorescing material, riboflavin, FMN and FAD are shown in Table I. R_f value of the fluorescing

TABLE I

R_f values on paper chromatography of fluorescence of rat liver plasma membrane and authentic flavin compounds

Solvent systems: I, *n*-butanol/acetic acid/water (4:1:5, v/v, upper phase); II, 4% sodium citrate. Paper: Whatman chromatographic paper No. 1.

Compounds	Solvent system	
	I	II
FAD	0.08	0.38
FMN	0.14	0.53
Riboflavin	0.34	0.32
Membrane fluorescence	0.34	0.32

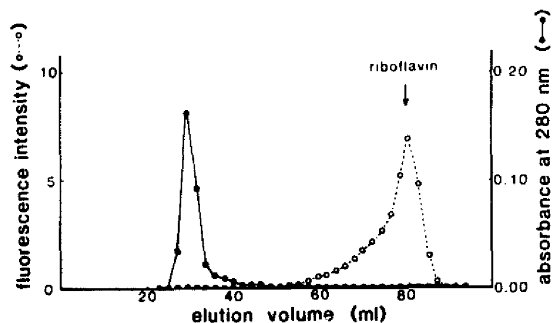

Fig. 2. TSK-gel HW-50 column chromatography of F-1 fraction eluting with 20 mM acetic acid. F-I fraction in Fig. 1 was collected and applied to a TSK-gel HW-50 column (1.5×26 cm) equilibrated with 20 mM acetic acid. The column was eluted with the same solution. Fluorescence and protein concentration were monitored as described in the legend to Fig. 1.

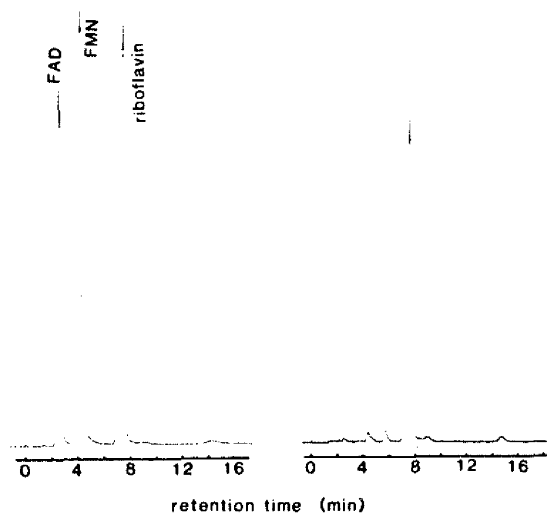

Fig. 3. HPLC pattern of authentic flavins and isolated fluorescence of rat liver plasma membrane. The left panel reveals a separation pattern of riboflavin, FMN and FAD. The right panel reveals a chromatogram of the isolated fluorescence from rat liver plasma membranes. HPLC conditions are described in the text.

compound from rat liver plasma membrane was close to that of riboflavin. Fig. 3 shows HPLC pattern of the authentic flavin compounds and the isolated membrane fluorescing material. The retention time of the membrane fluorescing compound was found to be identical to that of riboflavin.

Isolation of the membrane fluorescing fraction was carried out without using gel-filtration to avoid the possible artificial production of riboflavin during its process. An aqueous phase by a direct partitioning of rat liver plasma membrane between water and chloroform was concentrated by lyophilization and subjected to paper chromatography. The chromatogram showed a single fluorescence spot at the position corresponding to that of riboflavin.

As flavins are abundant in most subcellular fractions, the possibility that the riboflavin in the plasma membrane derived as a contaminant from other subcellular fractions had to be further examined. Patterns of 1% SDS gel chromatography of the subcellular fractions are shown in Fig. 4. The chromatogram of the plasma membrane revealed two fluorescent peaks as shown in the bottom panel of Fig. 4. The first peak at an elution volume of 45 ml was one-fifth the second one in relative amount, as judged from the area under the fluorescence peak. The larger peak was eluted at an elution volume of about 120 ml, corresponding to the elution position of riboflavin under the same chromatographic conditions. No fluorescent peak was observed at the position of either FMN or FAD. These flavins were not clearly separated from each other under such chromatographic conditions. On the other hand, microsomes and mitochondria displayed different patterns, as compared to the plasma membrane: a main fluorescence peak was observed at the position of FMN or FAD, and the relative fluorescence intensity at the position of riboflavin was only 40–50% of the main peak.

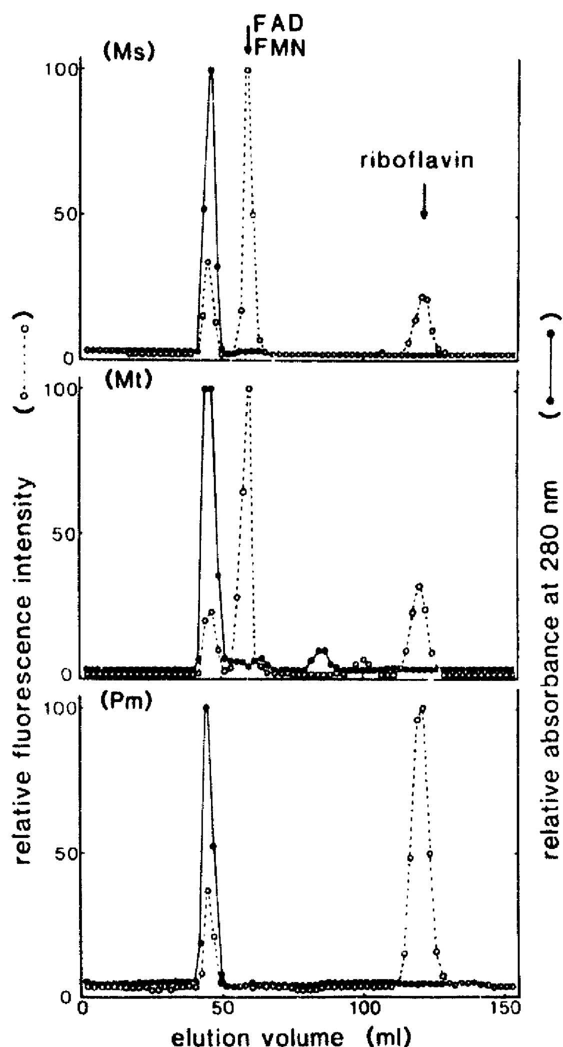

Fig. 4. SDS gel filtration pattern of subcellular fractions of rat liver on TSK-gel HW-50 column. Each subcellular fraction was solubilized in 1% SDS/20 mM Tris-HCl buffer (pH 7.4) and applied to a column of TSK-gel HW-50 (1.5 × 70 cm) equilibrated with the same buffer. Each fraction (2.2 ml) was monitored for fluorescence and protein concentration as described in the legend to Fig. 1. Values are expressed as per cent of the maximal value.

Discussion

The results of the present study suggest that the fluorescence of the plasma membrane is due to riboflavin. Our previous experiments have demonstrated the existence of autofluorescence in rat liver plasma membrane [8]. This autofluorescence exhibited the characteristics common to various flavins in terms of fluorescence spectra under oxidizing and reducing conditions, and reaction to photolysis in alkaline medium. In the present experiments, the fluorescence was quantitatively extracted with hot water from rat hepatocyte plasma membranes. When the extract was applied to a gel filtration with water, most of the fluorescence comigrated with protein (Fig. 1, F-I). This elution position was relatively close to but clearly different from that of FMN or FAD. A minor amount of fluorescence appeared at the position of free riboflavin. The fluorescent compound of this fraction was identified to be riboflavin by paper chromatography and HPLC. When F-I fraction was applied to gel filtration under acidic condition, the fluorescence appeared at the position of F-II. These findings suggest that the fluorescence of F-I fraction can be easily converted to that of F-II fraction. It is well known that FMN is hydrolysed into riboflavin under acidic conditions [14]. But is it unlikely that the fluorescence of F-I fraction was due to FMN, because F-I appeared at an elution position different from that of FMN on gel filtration with water. Furthermore, no FMN could be detected in the fluorescent compound(s) directly isolated from plasma membranes. Furthermore,

SDS gel chromatography of plasma membrane did not show any free FMN. One can conclude that the fluorescence of F-I fraction is consistent with that of a riboflavin-protein complex.

Riboflavin-binding proteins were found in hen's egg white [15], yolk [16] and plasma [17], in plasma of pregnant cows [18], rats [19] and humans [20]. Egg yolk riboflavin-binding protein is synthesized in the liver and transported via blood to the oocyte [21]. Riboflavin-binding protein in egg white was reported to be heat stable [15]. It maintained its riboflavin-binding activity after heat treatment at 100°C for 15 min. No information is available as to whether these riboflavin-binding proteins exist or not in the plasma membrane of rat liver. It is suspected, however, that these riboflavin-binding proteins documented in previous reports may be different from our riboflavin-protein complex of the plasma membrane, because the binding of riboflavin to the apo-riboflavin-binding protein quenched the fluorescence of riboflavin [15], whereas in our experiments the riboflavin-protein complex maintained its fluorescence being similar to riboflavin.

Several authors reported that the uptake of riboflavin by the intestinal epithelium [22,23] and hepatocytes [24] is a saturable process. Aw et al. [24] suggested the existence of a specific carrier protein for riboflavin in rat hepatocyte plasma membrane. This assumed carrier protein may be one of the candidates for the proteins binding riboflavin in the plasma membrane of hepatocytes as shown in the present study. However, it should be stressed that the presence of riboflavin was observed in a wide range of protein fractions [8], therefore, the riboflavin-binding can hardly be attributed to a single class of proteins.

There exists a theoretical possibility that the riboflavin-protein complex is at least in part an artifact produced during hot-water extraction, i.e., the riboflavin may be attached to the membrane during the preparation procedure. Nevertheless, indirect evidence listed below shows that this is not the case. (i) The PIAF is present in liver smears always in the upper most layer where the plasma membrane was identified by electron microscopy [2,25]. (ii) The plasma membrane preparations maintained their fluorescence even after several washings at room temperature, i.e., riboflavin cannot be just a loosely bound component of membrane. (iii) The lipid fraction of the plasma membrane never contained any significant part of the PIAF [8].

On the basis of our experimental data we can conclude that the formation of PIAF in the hepatocyte plasma membrane is consistent with the oxidation of riboflavin bound to a wide range of proteins. This represents a convenient tool for FRAP measurement of the average lateral diffusion of membrane proteins [1-7], since the membrane integrity is not perturbed by the introduction of any external label which may cause cross-linking and other artifacts, too. Apart from this pragmatic point of view, the question arises whether the presence of riboflavin in the plasma membrane has a deeper physiological significance or not. It seems to us that the following hypothesis can be forwarded as regards its functional role in the cell membrane.

It is well known that the autoxidation of reduced flavins is accompanied by a monovalent reduction of oxygen, i.e., it produces superoxide anion radicals [26]. Apparently most of the riboflavin is in the reduced form in vivo, and becomes oxidized only when exposed to higher oxygen partial pressures. Considering that superoxide radicals can quench very efficiently $OH^{\cdot}$ free radicals [27], one can assume that the presence of reduced riboflavin in the plasma membrane may represent a sort of defense against $OH^{\cdot}$ free radical attacks taking place obviously to an increasing extent under any oxidative stress situation. Such a defense mechanism may be of great importance, if we consider that the range of oxygen consumption under physiological conditions, i.e., in mammals may increase from the necessary minimum value to about 5-10-fold or more, even though only temporarily, in cases of excessive physical load.

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