Reaven, E., Chen, Y.-D. I., Spicher, M., & Azhar, S. (1984) *J. Clin. Invest. 74*, 1384-1397.
Reaven, E., Chen, Y.-D. I., Spicher, M., Hwang, S.-F., Mondon, C. E., & Azhar, S. (1986) *J. Clin. Invest. 77*, 1971-1984.
Reaven, E., Boyles, J., Spicher, M., & Azhar, S. (1988) *Arteriosclerosis 8*, 298-309.
Reaven, E., Spicher, M., & Azhar, S. (1989) *J. Lipid Res. 30*, 1551-1560.
Reaven, E., Shi, X.-Y., & Azhar, S. (1990) *J. Biol. Chem. 265*, 19100-19111.
Rinninger, F., & Greten, H. (1990) *Biochim. Biophys. Acta 1043*, 318-326.
Rizzi, G. P., & Taylor, H. M. (1978) *J. Am. Oil Chem. Soc. 55*, 398-401.
Rosario, J., Sutherland, E., Zaccaro, L., & Simon, F. R. (1988) *Biochemistry 27*, 3939-3946.
Rouser, G., Nelson, G. J., Fleischer, S., & Simon, G. (1968) in *Biological Membranes* (Chapman, D., Ed.) pp 5-69, Academic Press, New York.
Seltzman, T. P., Finn, F. M., Widnell, C. C., & Hofmann, K. (1975) *J. Biol. Chem. 250*, 1193-1196.
Spooner, P. J. R., Hamilton, J. A., Gantz, D. L., & Small, D. M. (1986) *Biochim. Biophys. Acta 860*, 345-353.
Steck, T. L., & Wallach, D. F. H. (1970) *Methods Cancer Res. 5*, 93-153.
Stein, Y., & Stein, O. (1985) *Adv. Exp. Med. Biol. 183*, 37-46.
Vahouny, G. V., Borja, C. R., & Weersing, S. (1963) *Anal. Biochem. 6*, 555-559.
Valic, M. I., Gorrissen, H., Cushley, R. J., & Bloom, M. (1979) *Biochemistry 18*, 854-859.

Folate Binding Protein from Kidney Brush Border Membranes Contains Components Characteristic of a Glycoinositol Phospholipid Anchor†

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ABSTRACT: A number of cell surface proteins have been shown to be anchored to the plasma membrane by a covalently attached glycoinositol phospholipid (GPL) in amide linkage to the C-terminus of the mature protein. We applied several criteria to establish that folate binding protein (FBP) in brush border membranes of rat kidney contains a GPL anchor. Brush border membranes were isolated and labeled with [^{3}H]folate, and the complex of FBP and [^{3}H]folate was shown to be released to the supernatant by incubation with purified bacterial phosphatidylinositol-specific phospholipase C (PIPLC) but not by incubation with a purified bacterial phosphatidylcholine-specific phospholipase C. The FBP-[^{3}H]folate complex both in crude extracts and after FBP purification by ligand-directed affinity chromatography interacted with Triton X-114 micelles, and prior incubation with PIPLC prevented this detergent interaction. Individual residues characteristic of GPL anchors were found to be covalently associated with FBP following polyacrylamide gel electrophoresis in sodium dodecyl sulfate. These included glucosamine and ethanolamine, which were radiolabeled by reductive methylation and identified by chromatography on an amino acid analyzer, and inositol phosphate, which was inferred by Western blotting with an anti-CRD antisera. This antisera gave positive immunostaining only after FBP had been cleaved by PIPLC, a reliable diagnostic of a GPL anchor. The relationship between GPL-anchored FBP in biological membranes and soluble FBP in biological fluids also is discussed.

Integral membrane proteins generally maintain intimate contact with the hydrophobic phase of phospholipid bilayer membranes through hydrophobic amino acid residues in one or more transmembrane segments (Sabatini et al., 1982). These proteins require detergents for solubilization and bind detergents in extracts. However, it has become apparent that the membrane association of several intracellular proteins is mediated at least in part by covalently attached lipid groups. The attachments can involve acylation of protein residues by the fatty acids myristate or palmitate (Sefton & Buss, 1987) or the isoprenylation of cysteine residues by farnesyl or geranylgeranyl groups (Maltese, 1990). Recently, a new class of more than 50 extracellular membrane proteins has emerged that are anchored, not by a transmembrane peptide segment, but solely by a glycoinositol phospholipid (GPL) linked covalently to the protein C-terminus. Many features of proteins with GPL anchors are covered in excellent review articles by Ferguson and Williams (1988) and Cross (1990).

The most widely applied criterion for the identification of GPL-anchored proteins is their selective release from intact cells or plasma membrane preparations by highly purified

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bacterial phosphatidylinositol-specific phospholipase C (PIPLC)[1] [see Low (1987) and Rosenberry et al. (1989b)]. Among the proteins released by PIPLC is the folate binding protein (FBP) on the surface of the two cultured cell lines, the MA104 monkey kidney epithelial cell line (Lacey et al., 1989) and the KB human epidermoid carcinoma cell line (Luhrs & Slomiany, 1989). FBP mediates the high-affinity accumulation of 5-methyltetrahydrofolic acid in the cytoplasm of these cells, and it is of great interest because it appears to be the first GPL-anchored protein to be discovered whose function appears to require cellular internalization. Internalization could involve an endocytic pathway (Selhub et al., 1987; Hjelle et al., 1991). Since GPL anchors insert into the cell membrane outer bilayer only through their diacylglycerol or alkylacylglycerol moieties, the components that direct FBP internalization are unclear. Rothberg et al. (1990) have suggested that FBP physically moves in and out of the cell using a novel uncoated pit pathway that does not merge with the clathrin-coated pit endocytic machinery.

The kidney contains a high concentration of a high-affinity FBP which concentrates in the brush border membranes of proximal tubular cells (Selhub & Rosenberg, 1978; Selhub & Franklin, 1984). Studies in one of our laboratories have suggested that FBP in the kidney functions as a receptor for folates in the glomerular filtrate for subsequent folate transport across the tubular cell into the blood compartment (Selhub et al., 1987). The mechanism of this FBP-mediated folate transport also appears to involve an endocytic pathway (Hjelle et al., 1991). The present studies were undertaken to determine the mechanism of interaction of FBP with the luminal membrane of the kidney. In a preliminary report, we observed that PIPLC released FBP from kidney brush border membranes and that the released FBP no longer interacted with Triton X-114 detergent micelles (Lee et al., 1989). However, we consider these observations insufficient evidence of a GPL anchor on FBP because FBP could associate with membranes solely through another protein that is GPL anchored. To confirm a GPL anchor on FBP, we investigated the covalent attachment of known GPL anchor components to the FBP polypeptide. The complete structure for the GPL anchor on trypanosome variant surface glycoproteins (Ferguson et al., 1988) includes a core linear sequence of 6-*O*-(ethanolamine-PO_4)-α-Man*p*-(1→2)-α-Man*p*-(1→6)-α-Man*p*-(1→4)-α-GlcNH_2*p*-(1→6)-*myo*-inositol-1-PO_4. This core glycan sequence is completely conserved in the GPL anchor of rat Thy-1 (Homans et al., 1988), and information currently available on the GPL anchor of human erythrocyte AChE (Roberts et al., 1988b) is entirely consistent with it. Mammalian GPL anchors have an additional phosphoethanolamine moiety with a free amino group, and in the Thy-1 and AChE GPLs this group is in phosphodiester linkage to a hydroxyl group on the Man residue adjacent to GlcNH_2. We show here that kidney FBP contains glucosamine and ethanolamine components and an antigenic epitope that are characteristic of a GPL anchor.

Materials and Methods

Materials. Several stocks of purified FBP were prepared. FBP with an intact GPL anchor was extracted from rat kidneys and partially purified by affinity chromatography on methotrexate resin (Selhub & Franklin, 1984). Alternatively, FBP was purified by affinity chromatography following its direct release from kidney brush border membranes by cleavage of its GPL anchor with PIPLC. Brush border membranes (1.4 g of protein) prepared from rat kidneys by the calcium precipitation method (Selhub & Rosenberg, 1978) were suspended in 100 mL of 10 mM Tris-HCl (pH 7.4) and incubated at 37 °C for 30 min with 100 milliunits of PIPLC. The incubation mixture was centrifuged (35000*g*, 30 min), and the supernatant fraction was subjected to affinity chromatography on methotrexate resin for the purification of released FBP (Selhub & Franklin, 1984). About 75% of the FBP binding activity in the brush border membranes was recovered in the purified fraction. Human and bovine erythrocyte acetylcholinesterase (AChE) were purified by affinity chromatography on acridinium resin (Roberts et al., 1987). Human AChE preparations contained no contaminant polypeptides, but bovine preparations contained slight amounts of a 96-kDa polypeptide and of spectrin. Phosphatidylinositol-specific phospholipase C (PIPLC) was purified from *Bacillus thuringiensis* by procedures based on those of Low et al. (1988). Some stocks (2500 units/mg of protein) were generous gifts of Dr. Martin Low, Columbia University College of Physicians and Surgeons. Phosphatidylcholine-specific phospholipase C (PCPLC) purified from *Bacillus cereus* was obtained from Sigma. Antibody to FBP was prepared as described previously (Selhub & Franklin, 1984). Anti-CRD antiserum, produced against trypanosome soluble VSG and affinity purified, was a kind gift of Dr. Paul Englund, Johns Hopkins University School of Medicine. Immunolabeling of electroblotted proteins utilized the Western blot AP system (Promega Corp.).

Preparation of Brush Border Membranes. Brush border membranes from rat kidneys were prepared using the calcium precipitation method as previously described (Selhub & Rosenberg, 1978). Membranes were suspended in 50 mM mannitol containing 10 mM Na/HEPES (pH 7.4) and freed of endogenous bound folates by washing with 0.1 M MES (pH 5.0) followed by centrifugation (20000*g*, 15 min) and discarding of the supernatant. This procedure was designed to ensure the integrity of membrane alkaline phosphatase activity, in contrast to a previous method (Selhub & Rosenberg, 1978) which used harsher acidification conditions and resulted in a complete loss of alkaline phosphatase activity. The acid-treated membranes were suspended in 0.3 M mannitol, 10 mM Na/HEPES (pH 7.4) at a final protein concentration of 4.4 mg/mL [as determined by the procedure of Lowry et al. (1951)].

Treatment of Brush Border Membranes with PIPLC. Acid-treated membranes (9 mg of protein) were incubated with [^{3}H]folic acid (90 pmol, 45 Ci/mmol; Amersham, Arlington Heights, IL) in 2 mL of 0.3 M mannitol, 10 mM Na/HEPES (pH 7.4). After repeated washing with the same mannitol–Na/HEPES buffer to remove unbound radiolabeled folic acid, the membranes were suspended in 2 mL of mannitol–Na/HEPES buffer. Aliquots of this suspension (250 μL) were incubated with 20 μL of PIPLC (100 milliunits) at 37 °C. Control incubations contained either 100 milliunits of PCPLC or no added phospholipase. At the indicated times, samples of the incubation mixtures were centrifuged (100000*g*, 60 min), and the supernatant and pellet fractions were analyzed for both [^{3}H]folic acid counts (by scintillation counting) and alkaline phosphatase activity (with *p*-nitrophenyl phosphate substrate; Marx et al., 1972). One unit of FBP corresponds to binding of 1 pmol of [^{3}H]folic acid.

Phase Separation with Triton X-114. The capacity of aqueous solutions of Triton X-114 to segregate into distinct

[1] Abbreviations: FBP, folate binding protein; PIPLC, phosphatidylinositol-specific phospholipase C; PCPLC, phosphatidylcholine-specific phospholipase C; AChE, acetylcholinesterase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

detergent-rich and detergent-poor phases above room temperature as originally described by Bordier (1981) was exploited to assess the detergent interactions of FBP before and after PIPLC treatment. Brush border membranes to which [^{3}H]folic acid had been bound (0.5 mL at 1 mg of protein/mL) were mixed with 0.05% Triton X-100 (0.05 mL) and incubated for 90 min at 30 °C with either PIPLC (50 milliunits), PCPLC (50 milliunits), or no added phospholipase. Aliquots (0.2 mL) were mixed with 4% Triton X-114 (0.3 mL) for 30 min at 4 °C and then warmed to 37 °C for 1 min before centrifugation at 600*g* for 3 min at 25 °C. Upper and lower phases were analyzed for ^{3}H counts and alkaline phosphatase activity. In a second set of similar experiments, FBP purified by affinity chromatography was partitioned in Triton X-114 before and after treatment with PIPLC.

Cross-Linking of FBP with [^{3}H]Folic Acid. [^{3}H]Folic acid (3.75 μCi) was mixed with *N*-hydroxysuccinimide (20 mg) and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (20 mg) in 0.5 mL of dimethyl sulfoxide (Henderson & Zevely, 1984). The reaction mixture was evaporated to dryness and the resulting *N*-hydroxysuccinimide ester of [^{3}H]folic acid was incubated with purified rat kidney FBP (180 units) in 150 mM Tris/HEPES (pH 7.4; 300-μL final volume) at 25 °C for 30 min. The mixture was passed through Bio-Gel P-30 (2-mL column) to separate bound from unbound radioactivity. Pooled fractions (0.9 mL) of ^{3}H-labeled FBP were adsorbed to phenyl-Sepharose (1.5-mL column). About 50% of the radioactivity was eluted with a 3-mL resin wash with 10 mM Tris/HEPES buffer (pH 7.4). The resin-bound radiolabeled protein was suspended in the same buffer (5 mL) and incubated with PIPLC (500 milliunits) overnight at 37 °C. Released ^{3}H-labeled FBP in the resin supernatant contained the remaining 50% of the radiolabel.

Radiomethylation of FBP and AChE. FBP (500 pmol) that had been cleaved with PIPLC and purified by affinity chromatography on methotrexate resin was incubated with 10 mM [^{14}C]HCHO (ICN, 40 mCi/mmol), 50 mM $NaCNBH_3$, 25 mM sodium phosphate (pH 7.0) in 80-μL total volume for 30 min at 37 °C (Haas & Rosenberry, 1985; Haas et al., 1986). Water (420 μL) was added, and the sample was dialyzed against 10 mM sodium phosphate (pH 7.0) for 48 h with three changes of the outside dialysis buffer. Of the total radioactivity in the incubation mixture, 2–3% remained inside the dialysis bag in apparent association with FBP (2000–4000 cpm/pmol of FBP). A purified fraction (3.2 mL) of human erythrocyte AChE (18 nmol) in 50 mM NaCl, 0.1% Triton X-100, 5 mM decamethonium bromide, 5 mM sodium phosphate (pH 7) was concentrated 8-fold and incubated with [^{14}C]HCHO and unlabeled HCHO (1:9 molar ratio, 10 mM), 50 mM $NaCNBH_3$, 25 mM sodium phosphate (pH 7.0) in a 660-μL total volume for 30 min at 37 °C and dialyzed extensively.

Analytical Procedures. SDS–PAGE with gels at the indicated percent total acrylamide was conducted as in Rosenberry et al. (1989a) except that NaCl was added to 0.2 M in all sample onputs and all lanes were loaded to minimize lane width distortion in gels subjected to fluorography. Electroblotting of SDS–PAGE gel bands onto either Immobilon-P or Immobilon-P^{SQ} (Millipore) followed procedures in Burnette (1981). Blot recoveries of radiomethylated FBP and AChE were less than 10% relative to gel onputs when conventional Immobilon-P was used over a wide range of electroblotting times. Blot recoveries of AChE improved to 20–25% with Immobilon-P^{SQ}, but recoveries of FBP remained in the 3–5% range and the bulk of the radioactivity was found in the electroblot transfer buffer. Analysis of the labeled amine components of radiomethylated proteins was conducted on a Beckman 119CL amino acid analyzer following hydrolysis of dried labeled samples in 6 N HCl at 115 °C for 16 h as described previously (Haas & Rosenberry, 1985; Haas et al., 1986). Label recovery in analyzer fractions generally was >90% of that in the hydrolysate onput. Quantitation of the radiomethylated amines was based on an observed specific radioactivity of 69 cpm/pmol of methyl groups from the [^{14}C]HCHO stock (Haas & Rosenberry, 1985). Quantities of AChE in picomoles were calculated from amino acid recoveries (measured by ninhydrin staining) and the following residue numbers per subunit of human erythrocyte AChE [determined by Soreq et al. (1990)]:[2] alanine (53), valine (52), leucine (56), phenylalanine (28), arginine (39). AChE picomole estimates by this method were 0.89 times those used in the previous approximation (Haas & Rosenberry, 1985). Total radiomethylated glucosamine was estimated from the radioactivity only in its earlier hydrolysis peak (X_1; total = X_1/0.69; Haas et al., 1986). The N-terminal amino acid of FBP was identified by coelution with the corresponding [^{3}H]HCHO-radiomethylated amino acid standard (Haas & Rosenberry, 1985).

Detection of the CRD Epitope by Western Blotting. Appropriate criteria (Rosenberry et al., 1989b) were applied to confirm that putative GPL anchors on the protein samples were intact: partially purified rat kidney FBP was shown to partition into the Triton X-114 detergent phase (see Results) and purified bovine erythrocyte AChE was observed to bind (>98%) to phenyl-Sepharose. Sample aliquots (1–7 μg of protein in 10–30 μL of 0.1% Triton X-100 and either 10 mM Tris-HCl or 20 mM sodium phosphate, pH 7) were incubated with or without PIPLC (10 μg/mL) for 1 h at 37 °C, subjected to SDS–PAGE on 10% gels, and immunoblotted by a procedure based on Burnette (1981) as outlined in Rosenberry et al. (1989a). The initial blot was blocked with 5% defatted milk in 150 mM NaCl, 10 mM Tris (pH 8.0) for 30 min prior to incubation with the primary anti-CRD antiserum at a 1:500 dilution in the same buffer plus 0.05% Tween 20 for 1 h at 25 °C. Incubation in alkaline phosphatase-conjugated second antibody (1:7500 dilution) was for 1 h.

Results

Treatment of Brush Border Membranes with Phospholipases. Rat kidney brush border membranes were isolated and washed with a mild acid solution to preserve the enzymatic activity of endogenous alkaline phosphatase, a protein known to contain a GPL anchor (Low et al., 1987; Low, 1987). To investigate the susceptibility of membrane-bound FBP to release by PIPLC, the high-affinity ligand [^{3}H]folic acid was employed. This ligand has an equilibrium dissociation constant of 140 pM with FBP (Selhub & Franklin, 1984) and should show little dissociation from the FBP–[^{3}H]folic acid complex during the course of a release experiment. As shown in Figure 1A,B, incubation of the membranes with PIPLC resulted in a rapid release of up to 95% of FBP-bound [^{3}H]folic acid from the membranes to the supernatant. The radioactivity released by the action of PIPLC eluted in the void volume of a Bio-Gel P-30 column, and about 90% of the released radioactivity was precipitated by antibody to purified FBP (data not shown). These observations confirmed that the released [^{3}H]folic acid remained complexed to FBP. Parallel measurements from the

[2] The C-terminal sequence of human erythrocyte AChE was a personal communication from Dr. P. Taylor, University of California at San Diego.

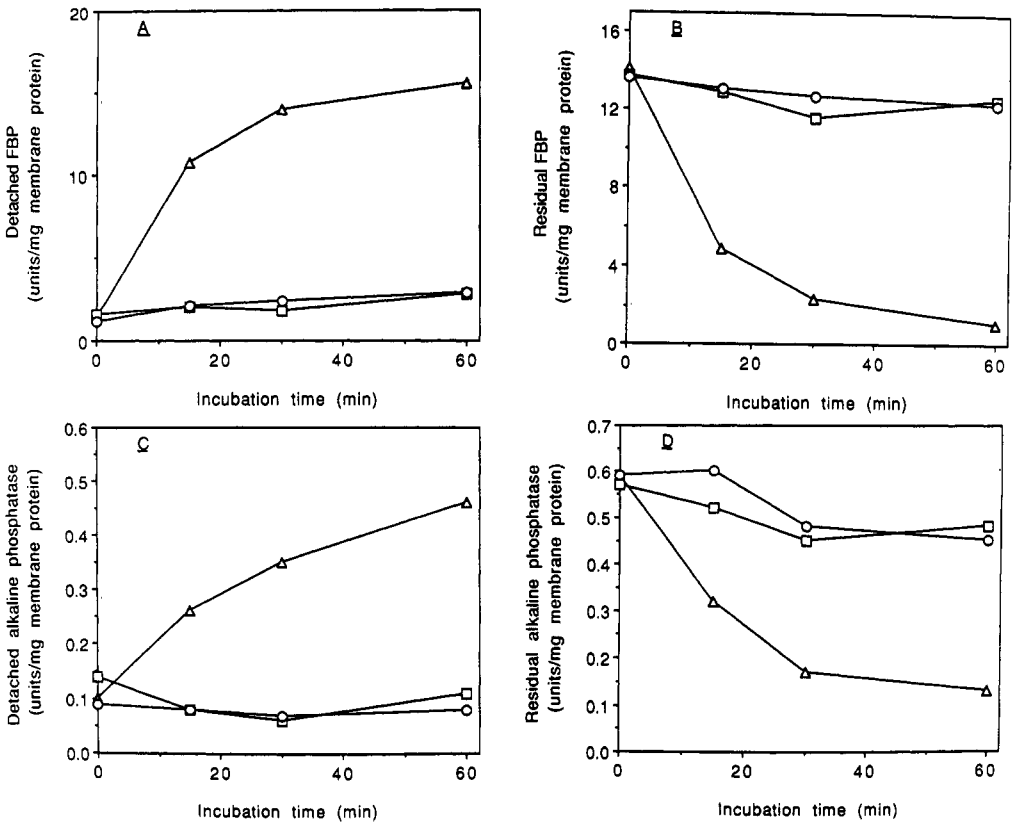

FIGURE 1: Selective release of proteins from brush border membranes with purified bacterial PIPLC. Brush border membranes were mixed with [^{3}H]folic acid to label FBP and incubated with PIPLC (△), PCPLC (□), or no added phospholipase (○) as outlined in Materials and Methods. Radiolabeled FBP was measured in the incubation supernatant (panel A) and the sedimented membranes (panel B). As a positive control, alkaline phosphatase activity was also measured in the supernatant (panel C) and membrane (panel D) fractions.

Table I: Incubation with PIPLC Prevents the Interaction of FBP-Bound [^{3}H]Folic Acid with Triton X-114 Micelles[a]

phospho-lipase	FBP in detergent phase (units)		FBP in aqueous phase (units)	
	0 min	90 min	0 min	90 min
PIPLC	8.5 ± 0.3	2.6 ± 0.1	0.76 ± 0.13	5.2 ± 0.1
PCPLC	8.7 ± 0.1	8.6 ± 0.1	0.62 ± 0.02	0.61 ± 0.01
none	8.7 ± 0.1	8.4 ± 0.1	0.62 ± 0.01	0.57 ± 0.04

[a] Brush border membranes to which [^{3}H]folic acid was bound were extracted with Triton X-100, incubated with phospholipases for 90 min, and partitioned with Triton X-114 as outlined in Materials and Methods. Units of FBP in each phase were calculated from the radioactivity.

incubation mixtures showed that PIPLC released more than 80% of the alkaline phosphatase activity (Figure 1C,D). Incubation of these membranes with bacterial phosphatidylcholine-specific phospholipase C (PCPLC) or with no phospholipase gave no appreciable release of FBP-bound [^{3}H]folic acid or alkaline phosphatase activity beyond the initial background level.

Phase Separation with Triton X-114. Proteins with intact GPL anchors partition almost exclusively into the detergent phase during Triton X-114 extraction, and cleavage by PIPLC nearly abolishes any detergent interactions [Bordier, 1981; see Rosenberry et al. (1989b)]. Brush border membranes to which [^{3}H]folic acid had been bound were solubilized with Triton X-100 and incubated with PIPLC, PCPLC, or no phospholipase. Aliquots were then mixed with Triton X-114 and partitioned into detergent and aqueous phases. As shown in Table I, the FBP–[^{3}H]folic acid complex in crude membrane extracts responds to PIPLC digestion like a typical GPL-anchored protein. Nearly 92% of the initial detergent-binding complex was converted to a form that partitioned into the aqueous phase by PIPLC treatment. In contrast, no conversion was observed in the control or PCPLC incubations. FBP purified by affinity chromatography retained its interaction with Triton X-114 micelles and also was converted to a form that partitioned into the aqueous phase on incubation with PIPLC (Figure 2).

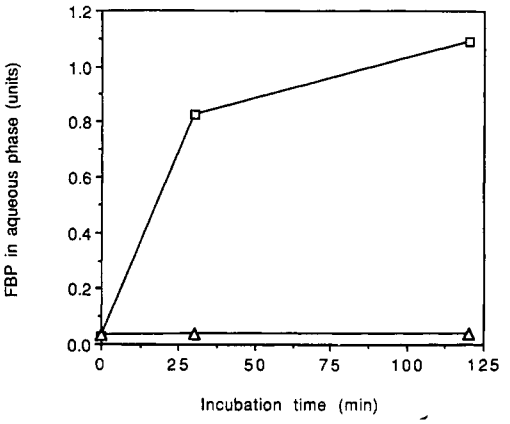

FIGURE 2: PIPLC digestion of purified FBP prevents FBP association with Triton X-114 micelles. FBP from brush border membranes was purified by affinity chromatography and equilibrated with [^{3}H]folic acid. Unbound radioactivity was removed by chromatography on Bio-Gel P-30. FBP-[^{3}H]folic acid complex was incubated with 50 milliunits of PIPLC (□) or without PIPLC (△) for the indicated period of time and subjected to Triton X-114 partitioning as outlined in Materials and Methods. The amount of FBP–[^{3}H]folic acid complex in the aqueous phase was calculated from the radioactivity.

Detection of Glycoinositol Phospholipid Anchor Components by Radiomethylation. Experiments in Figures 1 and 2 and Table I suggest that FBP is associated with kidney brush border membranes through a GPL anchor, but they do not

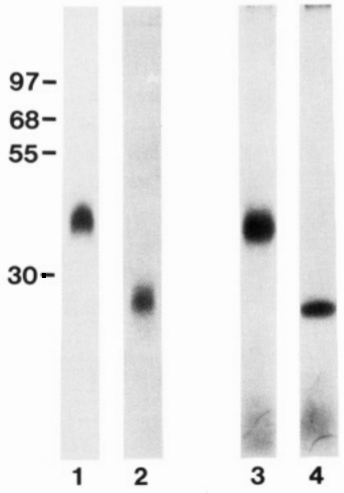

FIGURE 3: Affinity labeling of FBP with [^{3}H]folic acid and assessment of purity of radiomethylated FBP by SDS–PAGE. Purified FBP was either covalently cross-linked to [^{3}H]folic acid with NHS–EDC (lanes 1 and 2) or radiomethylated (lanes 3 and 4), incubated with (lanes 2 and 4) or without (lanes 1 and 3) *N*-glycanase, and analyzed by fluorography in 10% SDS–PAGE gels as outlined in Materials and Methods. Molecular masses of standards (in kilodaltons) run in parallel are indicated on the left. Sample loads: lanes 1 and 2, 10000 ^{3}H cpm; lanes 3 and 4, 30000 ^{14}C cpm. Fluorographic exposures were 5–20 days.

directly show the attachment of such an anchor to FBP. In particular, they do not exclude the possibility that FBP is associated with the membrane only through interaction with another GPL-anchored protein. To confirm the presence of a GPL anchor on FBP, direct chemical identification of GPL anchor components was pursued. Perhaps the most sensitive procedure for the detection of these components in proteins isolated from tissue is the reductive radiomethylation of free amine groups in the glucosamine and ethanolamine residues that are present in all mammalian GPL anchors that have been characterized to date. The radiomethylated amines are stable to acid hydrolysis and can be fractionated with great sensitivity on an automated amino acid analyzer. This procedure was first used to identify and quantitate the glucosamine and the ethanolamine with a free amino group in the GPL anchor of human erythrocyte AChE (Haas et al., 1986) and radiomethylated AChE was included as a positive control in the current analysis.

To obtain selective radiolabeling, FBP was released from brush border membranes with PIPLC and purified by affinity chromatography on methotrexate resin. Purified samples then were either radiomethylated or affinity-labeled with [^{3}H]folic acid by a covalent cross-linking procedure. Aliquots of both radiolabeled samples were then digested with *N*-glycanase to remove N-linked oligosaccharides. In Figure 3, the radiolabeled samples both treated and untreated with *N*-glycanase were subjected to SDS–PAGE and fluorography. Lanes 1 and 2 show that FBP affinity-labeled with [^{3}H]folic acid moves as a 40-kDa polypeptide prior to *N*-glycanase digestion and as a 24-kDa polypeptide after *N*-glycanase treatment relative to protein standards. These values are in reasonable agreement with values of 38 and 28 kDa reported for placental FBP before and after *N*-glycanase treatment (Ratnam et al., 1989). The radiomethylated FBP samples in lanes 3 and 4 migrate as bands with mobilities virtually identical to those in the corresponding lanes 1 and 2 and thus show no evidence of significant polypeptide contaminants.

Analyses of the labeled amine components in radiomethylated FBP are shown in Figure 4. Acid hydrolysis and chromatography of the dialyzed radiomethylated FBP sample

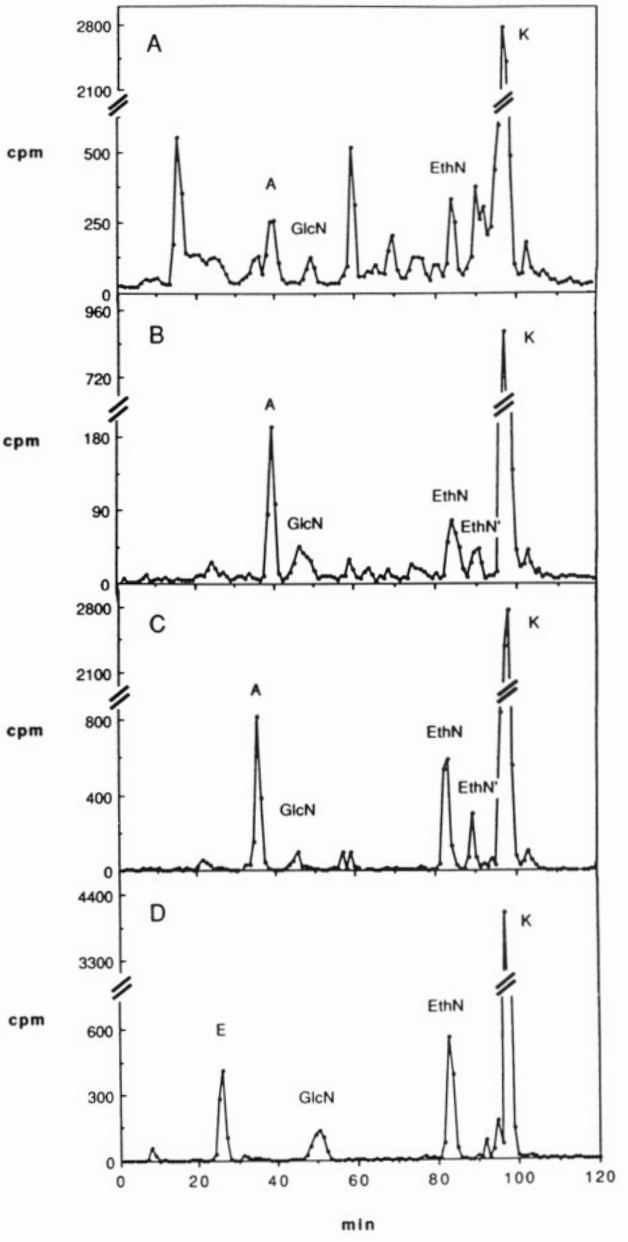

FIGURE 4: Identification of glucosamine and ethanolamine in FBP by radiomethylation and amino acid analysis. FBP and human erythrocyte AChE were radiomethylated with [^{14}C]HCHO as outlined in Materials and Methods. Acid hydrolysates prepared from the following samples were fractionated on an amino acid analyzer, and radiolabeled components were identified by their elution times relative to standards (Haas et al., 1986). Panel A, FBP after radiomethylation and dialysis only; panel B, the dried gel slice containing the silver-stained FBP band in Figure 3, lane 3; panel C, the FBP band from an SDS–PAGE gel similar to that in Figure 3, lane 3, electroblotted onto Immobilon-P; panel D, the AChE band from an SDS–PAGE gel electroblotted on Immobilon-P^{sq}. Peaks labeled E, A, GlcN, EthN, and K refer to N-dimethylated glutamate, alanine, glucosamine, ethanolamine, and N$^{\epsilon}$-dimethylated lysine, respectively; EthN′ refers to N-monomethylated ethanolamine. Recoveries of total label applied to SDS–PAGE gels in hydrolysates of either the gel slice or the electroblot were variable: in B, 20%; in C, 3%; and in D, 20%. Amino acid recoveries of alanine, valine, leucine, phenylalanine, and arginine from the electroblot in panel D (measured by ninhydrin staining) were in good agreement with the composition predicted from the sequence of human erythrocyte AChE (Soreq et al., 1990), and procedures outlined in Materials and Methods gave the following estimates of radiomethylated amines per AChE subunit: E, 0.8; GlcN, 0.7; EthN, 1.0; K, 5.8. These estimates are in reasonable agreement with those made previously from radiomethylated AChE samples in solution (Haas et al., 1986).

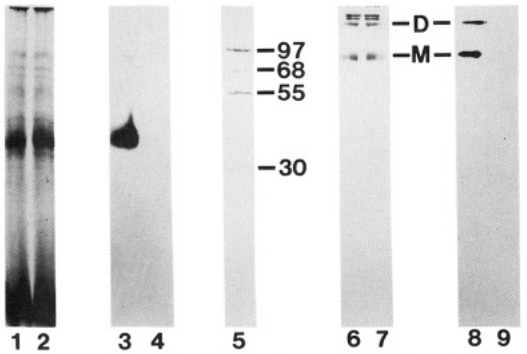

FIGURE 5: Generation of the CRD epitope on FBP by cleavage with PIPLC. Samples of FBP partially purified from detergent extracts of rat intestinal brush border membranes were treated with or without PIPLC (10 μg/mL) and analyzed on SDS–PAGE Western blots with anti-CRD antisera as outlined in Materials and Methods. Purified bovine erythrocyte AChE treated in parallel was analyzed as a positive control. Sample loads: lanes 1 and 2, 10 μg of FBP; lanes 3 and 4, 5 μg of FBP; lane 5, standard proteins (1 μg of each, with the molecular masses indicated); lanes 6 and 7, 1.5 μg of AChE; lanes 8 and 9, 0.5 μg of AChE. Lanes 1, 2, 5, 6, and 7 were stained with Coomassie brilliant blue R; lanes 3, 4, 8, and 9 were immunoblotted with an alkaline phosphatase detection system. Samples in lanes 1, 3, 6, and 8 were treated with PIPLC. The AChE monomer (M) and dimer (D) are indicated.

gave a number of minor labeled peaks in the amino acid analyzer elution profile (Figure 4A). Most of these minor peaks appear to represent residual contaminant radioactivity that can be removed by SDS–PAGE, as they were absent in acid hydrolysates of either the SDS–PAGE gel slice containing the GPL-anchored protein (Figure 4B) or the gel band electroblotted onto nitrocellulose (data not shown) or Immobilon-P (Figure 4C). The simplified profiles in panels B and C of Figure 4 permitted clear identification of glucosamine and ethanolamine in the FBP band by comparison to the corresponding profile previously established for human erythrocyte AChE (Figure 4D). In addition, a radiolabeled peak at 37–40 min (A) in the FBP hydrolysate profiles in Figure 4A–C was absent in the corresponding profile for AChE (Figure 4D). This peak coeluted with ^{3}H-labeled N-dimethylated alanine (data not shown) and thus indicated that the N-terminal amino acid of FBP is alanine.

Detection of the CRD Epitope after Cleavage of FBP with PIPLC. Strong evidence in support of a glycoinositol phospholipid anchor on a membrane protein is the emergence of the CRD epitope on the protein after incubation with PIPLC. The term CRD epitope arose from a determinant that is present on all soluble variants of trypanosome VSGs, even those with no amino acid sequence homology (Cardoso de Almeida & Turner, 1983). The only structure common to the membrane-bound forms of these VSGs is their GPL anchor, and the glycan moiety of this anchor represents the only site of immunological cross-reaction between the variants. The epitope is contained entirely within the glycan and includes the 1,2-cyclic inositol phosphate produced by PIPLC cleavage, and it is also exposed on mammalian proteins with GPL anchors following PIPLC treatment (Zamze et al., 1988). To investigate the generation of a CRD epitope on FBP, a sample of the partially purified rat kidney FBP preparation in Figure 2 and a positive control, purified bovine erythrocyte AChE, were incubated with PIPLC. As shown in Figure 5, PIPLC treatment produced the CRD epitope both on a prominent 40-kDa band in the FBP sample and on bands corresponding to monomeric and dimeric AChE. The coincidence of the molecular masses of the [^{3}H]FBP band in Figure 3 (lane 1) and the CRD-positive band in Figure 5 (lane 3) provides strong evidence that the CRD epitope is generated on FBP.

Discussion

We have confirmed that FBP is detached from the brush border membranes of rat kidney by purified bacterial PIPLC in a manner that paralleled the release of GPL-anchored alkaline phosphatase from the same membranes. Detachment could not be obtained by purified bacterial PCPLC. These observations suggest that FBP also is anchored by a GPL. Incubation with PIPLC prevents the interaction of purified FBP with Triton X-114 micelles. This observation supports the suggestion that a GPL is an integral part of the FBP structure and indicates that FBP, like other GPL-anchored proteins, is devoid of any hydrophobic amino acid sequence that can insert into the lipid bilayer of cell membranes. The lack of such a sequence renders it unlikely that kidney FBP can function as a membrane "carrier" protein in mediating folate recovery from glomerular filtrates, as recently implied by Bhandari et al. (1988). Conclusive evidence of a GPL anchor on rat kidney FBP was obtained by the identification of two residues with free amino groups that are characteristic of mammalian GPL anchors, glucosamine, and ethanolamine, and by the unmasking of a latent antigenic epitope on FBP called the CRD by digestion with PIPLC. This epitope includes the inositol 1,2-cyclic phosphate produced by PIPLC cleavage of the GPL inositol phospholipid. Our information complements the recent report that PIPLC releases an alkylacylglycerol from FBP in KB cells (Luhrs & Slomiany, 1989). Alkylacylglycerols also appear to be characteristic components of mammalian GPL anchors (Walter et al., 1990).

The detection of glucosamine and ethanolamine in FBP involved analysis of radiomethylated FBP, and the quality of the analysis was dependent upon the extent to which radiolabeled contaminants were removed prior to acid hydrolysis of the radiomethylated sample. In the initial studies of human erythrocyte AChE, the analysis was improved by repurification of the radiomethylated AChE by ligand-directed affinity chromatography. The data in Figure 4B,C show that contaminant radioactivity also can be removed by SDS–PAGE and hydrolysis of either the gel slice or the electroblotted protein band. In Figure 4D, estimates of the numbers of radiomethylated amine residues in each human erythrocyte AChE subunit were obtained by acid hydrolysis and amino acid analysis of an electroblotted band transferred from an SDS–PAGE gel to Immobilon-P^{sq}. The electroblotting efficiency of FBP was too low to estimate the amount of FBP protein in the blot hydrolysate from the amino acid composition (see Materials and Methods), and thus absolute quantitation of the numbers of radiomethylated amines per FBP polypeptide by procedures applied to AChE in Figure 4D was not possible. Such quantitation also is compromised if radiomethylation does not completely saturate the protein amine groups as the dimethyl derivatives, and the presence of considerable amounts of monomethylated ethanolamine in the FBP hydrolysates in Figure 4A–C suggests that this also is a concern. Nevertheless, the amount of radiolabeled ethanolamine relative to the radiolabeled N-terminal amino acid is nearly identical for radiomethylated FBP and AChE, and the relative amount of radiolabeled glucosamine in FBP is within 2–3-fold of that in AChE. This comparison provides clear evidence of GPL anchor components in the isolated FBP polypeptide.

FBP is found in both membrane-bound and soluble forms, and the relationship between these forms remains to be clarified. It is possible that the soluble forms are derived from GPL-anchored FBP by proteolytic or phospholipase cleavage. Selective proteolysis near the GPL anchor has been observed

for purified human erythrocyte AChE (Haas et al., 1986) and alkaline phosphatase (Jemmerson & Low, 1987), but no case of proteolytic or phospholipase release of a GPL-anchored protein from the cell surface under physiological conditions has yet been clearly documented. Alternatively, soluble FBP could reflect a distinct translation product, as illustrated by the Qa-2 antigen in splenocytes. Mitogenic stimulation of these cells with concanavalin A results in the appearance of a soluble, secreted form of the Qa-2 antigen that partially replaces a GPL-anchored cell surface Qa-2 of approximately the same size. The C-terminus of GPL-anchored Qa-2 is encoded by exon 5, while in the secreted Qa-2 form exon 4 is spliced to exon 6 to give a hydrophilic C-terminus (Ulker et al., 1990). The distinction between expression of soluble Qa-2 from an alternatively spliced mRNA and release of GPL-anchored Qa-2 by a putative endogenous PIPLC or protease can be difficult to make (Robinson, 1987).

Previous reports do not resolve the relationship between GPL-anchored and soluble FBP. Prior to the identification of its GPL anchor, FBP was suggested to interact with membranes through several fatty acids covalently linked to FBP through amide and ester bonds (Luhrs et al., 1987) or through a peptide segment that could be removed by proteolytic cleavage (Antony & Verma, 1989; Antony et al., 1989). A more recent report suggests that GPL-anchored FBP is released from KB cell membrane fractions by an endogenous metalloprotease (Elwood et al., 1991). Structural analysis of the C-terminus of soluble FBP could be useful in deducing its relationship to GPL-anchored FBP. Probing for the CRD epitope, for example, addresses the possibility of cleavage of GPL-anchored FBP by an endogenous phospholipase C. Our preliminary analyses fail to find evidence of a CRD epitope on milk FBP.[3] Isolation of a C-terminal peptide fragment would permit analysis for GPL anchor components and/or identification of the C-terminal amino acid. The C-terminal sequence of FBP deduced from cDNA clones in KB or Caco-2 (a human carcinoma cell line) cDNA libraries is consistent with a hydrophobic segment that can serve as a GPL-attachment signal sequence (Lacey et al., 1989; Sadasivan & Rothenberg, 1989). A second FBP cDNA clone obtained from a human placental cDNA library shows 68% sequence homology with the first FBP sequence and appears to be a product of a distinct gene (Ratnam et al., 1989). This cDNA also predicts a hydrophobic C-terminal segment that could direct attachment of a GPL anchor. Of perhaps greatest interest is a comparison of these two sequences to that obtained for bovine milk FBP (Svendsen et al., 1984). The milk sequence shows near identity with that deduced from the KB FBP cDNA clone except at its C-terminus, where the last 12 amino acids diverge sharply in a much less hydrophobic segment (Ratnam et al., 1989). This sequence divergence is precisely what would be predicted for an alternative mRNA splicing mechanism like that noted above for Qa-2. Further directed searching for FBP sequence divergence in cDNA or genomic libraries could help to resolve the relationship between GPL-anchored and soluble FBPs.

References

Antony, A. C., & Verma, R. S. (1989) *Biochim. Biophys. Acta 789*, 62–68.

Antony, A. C., Verma, R. S., Unune, A. R., & LaRosa, J. A. (1989) *J. Biol. Chem. 264*, 1911–1914.

Bhandari, S. D., Joshi, S. K., & McMartin, K. E. (1988) *Biochim. Biophys. Acta 937*, 211–218.

Bordier, C. (1981) *J. Biol. Chem. 256*, 1604–1607.

Burnette, W. N. (1981) *Anal. Biochem. 112*, 195–203.

Cardoso de Almeida, M. L., & Turner, M. J. (1983) *Nature (London) 302*, 349–352.

Cross, G. A. M. (1990) *Annu. Rev. Cell Biol. 6*, 1–39.

Elwood, P. C., Deutsch, J. C., & Kolhouse, J. F. (1991) *J. Biol. Chem. 266*, 2346–2353.

Ferguson, M. A. J., & Williams, A. F. (1988) *Annu. Rev. Biochem. 57*, 285–320.

Ferguson, M. A. J., Homans, S. W., Dwek, R. A., & Rademacher, T. W. (1988) *Science 239*, 753–759.

Haas, R., & Rosenberry, T. L. (1985) *Anal. Biochem. 148*, 154–162.

Haas, R., Brandt, P. T., Knight, J., & Rosenberry, T. L. (1986) *Biochemistry 25*, 3098–3105.

Henderson, G. B., & Zevely, E. M. (1984) *J. Biol. Chem. 259*, 4558–4562.

Hjelle, J. T., Christensen, E. I., Carone, F. A., & Selhub, J. (1991) *Am. J. Physiol. 260 (Cell Physiol. 29)*, C338–C346.

Homans, S. W., Ferguson, M. A. J., Dwek, R. A., Rademacher, T. W., Anand, R., & Williams, A. F. (1988) *Nature (London) 333*, 269–272.

Jemmerson, R., & Low, M. G. (1987) *Biochemistry 26*, 5703–5709.

Lacey, S. W., Sanders, J. M., Rothberg, K. G., Anderson, R. G. W., & Kamen, B. A. (1989) *J. Clin. Invest. 84*, 715–720.

Lee, H.-C., Selhub, J., & Rosenberry, T. (1989) *FASEB J. 3*, A1058.

Low, M. G. (1987) *Biochem. J. 244*, 1–13.

Low, M. G., & Saltiel, A. R. (1988) *Science 239*, 268–275.

Low, M. G., & Zilversmit, D. B. (1980) *Biochem. J. 19*, 3913–3918.

Low, M. G., Futerman, A. H., Ackermann, K. E., Sherman, W. R., & Silman, I. (1987) *Biochem. J. 241*, 615–619.

Low, M. G., Stiernberg, J., Waneck, G. L., Flavell, R. A., & Kincade, P. W. (1988) *J. Immunol. Methods 113*, 101–111.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Luhrs, C. A., & Slomiany, B. L. (1989) *J. Biol. Chem. 264*, 21446–21449.

Luhrs, C., Pitiranggon, P., Costa, M. D., Rothenberg, S. P., Slomiany, B. L., Brink, L., Tours, G. I., & Stein, S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6546–6549.

Maltese, W. A. (1990) *FASEB J. 4*, 3319–3328.

Marx, S. J., Fedak, S. A., & Surbach, G. D. (1972) *J. Biol. Chem. 247*, 6913–6918.

Ratnam, M., Marquardt, H., Duhring, J. L., & Freisheim, J. H. (1989) *Biochemistry 28*, 8249–8254.

Roberts, W. L., Kim, B. H., & Rosenberry, T. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7817–7821.

Roberts, W. L., Myher, J. J., Kuksis, A., Low, M. G., & Rosenberry, T. L. (1988a) *J. Biol. Chem. 263*, 18766–18775.

Roberts, W. L., Santikarn, S., Reinhold, V. N., & Rosenberry, T. L. (1988b) *J. Biol. Chem. 263*, 18776–18784.

Robinson, P. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 527–531.

Rosenberry, T. L., Krall, J. A., Dever, T. E., Haas, R., Louvard, D., & Merrick, W. C. (1989a) *J. Biol. Chem. 264*, 7096–7099.

Rosenberry, T. L., Toutant, J.-P., Haas, R., & Roberts, W. L. (1989b) *Methods Cell Biol. 32*, 231–255.

[3] J. A. Krall and T. L. Rosenberry, unpublished observations.

Rothberg, K. G., Ying, Y., Kolhouse, J. F., Kamen, B. A., & Anderson, R. G. W. (1990) *J. Cell Biol. 110*, 637-649.
Sabatini, D. D., Kreibich, G., Morimoto, T., & Adesnik, M. (1982) *J. Cell Biol. 92*, 1-22.
Sadasivan, E., & Rothenberg, S. P. (1989) *J. Biol. Chem. 264*, 5806-5811.
Sefton, B. M., & Buss, J. E. (1987) *J. Cell Biol. 104*, 1449-1453.
Selhub, J., & Franklin, W. A. (1984) *J. Biol. Chem. 259*, 6601-6606.
Selhub, J., & Rosenberg, I. H. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3090-3093.
Selhub, J., Nakamura, S., & Carone, F. A. (1987) *Am. J. Physiol. 252*, F757-F760.
Soreq, H., Ben-Aziz, R., Prody, C. A., Seidman, S., Gnatt, A., Neville, L., Lieman-Hurwitz, J., Lev-Lehman, E., Ginzberg, D., Lapidot-Lifson, Y., & Zakut, H. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9688-9692.
Svendsen, I. D., Hansen, S. I., Holm, J., & Lyngbye, J. (1984) *Carlsberg Res. Commun. 49*, 123-131.
Ulker, N., Lewis, K. D., Hood, L. E., & Stroynowski, I. (1990) *EMBO J. 9*, 3839-3847.
Walter, E. I., Roberts, W. L., Rosenberry, T. L., Ratnoff, W. D., & Medof, M. E. (1990) *J. Immunol. 144*, 1030-1036.
Zamze, S. E., Ferguson, M. A. J., Collins, R., Dwek, R. A., & Rademacher, T. W. (1988) *Eur. J. Biochem. 176*, 527-534.

Ca^{2+}/Calmodulin-Dependent NO Synthase Type I: A Biopteroflavoprotein with Ca^{2+}/Calmodulin-Independent Diaphorase and Reductase Activities†

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ABSTRACT: NO synthase (NOS; EC 1.14.23) catalyzes the conversion of L-arginine into L-citrulline and a guanylyl cyclase-activating factor (GAF) that is chemically identical with nitric oxide or a nitric oxide-releasing compound (NO). Similar to the other isozymes of NOS that have been characterized to date, the soluble and Ca^{2+}/calmodulin-regulated type I from rat cerebellum (homodimer of 160-kDa subunits) is dependent on NADPH for catalytic activity. The enzyme also possesses NADPH diaphorase activity in the presence of the electron acceptor nitroblue tetrazolium (NBT). We investigated the requirements of NOS and its content of the proposed additional cofactors tetrahydrobiopterin (H_4biopterin) and flavins, further characterized the NADPH diaphorase activity, and quantified the NADPH binding site(s). Purified NOS type I Ca^{2+}/calmodulin-independently bound the [^{32}P]2′,3′-dialdehyde analogue of NADPH (dNADPH), which, at near K_m concentrations during 3-min incubations was utilized as a substrate and at higher concentrations or after prolonged incubations and cross-linking inhibited NOS activity. The NADPH diaphorase activity was Ca^{2+}/calmodulin-independent, required higher NADPH concentrations than NOS activity, and was affected by dNADPH to a lesser degree. Divalent cations interfered with the diaphorase assay. Per dimer, native NOS contained about 1 mol each of H_4biopterin, FAD, and FMN, classifying it as a biopteroflavoprotein, and incorporated 1 mol of dNADPH. No dihydrobiopterin (H_2biopterin), biopterin, or riboflavin was detected. These findings suggest that NOS may share cofactors between two identical subunits via high-affinity binding sites. They also explain why different preparations of NOS have different requirements for exogenous flavins and H_4biopterin for maximal catalytic activity. Furthermore, H_4biopterin alone reduced NBT, and purified dihydropteridine reductase was found to have diaphorase activity. The absence of enzyme-bound biopterins other than H_4biopterin, the reported lack of effect of methotrexate on enzyme activity, and the potent and noncompetitive inhibition of NOS activity by NBT suggest also that NOS type I may have a novel methotrexate-insensitive quinoid-H_2biopterin reductase activity.

In various mammalian cells and tissues a terminal guanidino nitrogen of L-arginine (Iyengar et al., 1987; Schmidt et al., 1988) is oxidized to yield a factor with physicochemical and pharmacological properties similar to nitric oxide (NO;[1] Ignarro et al., 1987; Palmer et al., 1987; Forray et al., 1990; Greenberg et al., 1990; Mordvintsev et al., 1990; Myers et al., 1990). Both in the NO-producing cell and in specific NO-

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[1] Abbreviations: biopterin, 6-(L-*erythro*-1′,2′-dihydroxypropyl)pterin; dNADPH, 2′,3′-dialdehyde NADPH; GAF, guanylyl cyclase-activating factor; H_2biopterin, (6*R*)-7,8-dihydrobiopterin; H_4biopterin, (6*R*)-5,6,7,8-tetrahydrobiopterin; IBMX, 3-isobutyl-1-methylxanthine; Me_2SO, dimethyl sulfoxide; NBT(F), nitroblue tetrazolium (formazan); NO, nitric oxide or nitric oxide-releasing compound; NOS, NO synthase; q-H_2biopterin, (6*R*)-6,7-dihydrobiopterin; SDS, sodium dodecyl sulfate.

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