THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1993 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 268, No. 4, Issue of February 5, pp. 2802-2808, 1993
Printed in U.S.A.

The Specific Subcellular Localization of Two Isoforms of Cytochrome b_5 Suggests Novel Targeting Pathways*

(Received for publication, August 31, 1992)

Antonello D'Arrigo‡§, Ernesto Manera¶, Renato Longhi¶, and Nica Borgese‡

From the ‡Consiglio Nazionale delle Recherche Center for Cytopharmacology and the Department of Medical Pharmacology, University of Milan, and the ¶Consiglio Nazionale delle Recherche Istituto della Chimica degli Ormoni, Milano, Italy

Two forms of cytochrome b_5 are present in rat tissues, with a sequence identity of ≈60% in the cytoplasmically exposed, tryptic fragments (Lederer, F., Ghrir, R., Guiard, B., Cortial, S., and Ito, A. (1983) *Eur. J. Biochem.* 132, 95–102). It has been suggested that the two isoforms have partially overlapping subcellular distributions, with each form localized to some extent on both endoplasmic reticulum and outer mitochondrial membranes (Ito, A. (1980) *J. Biochem. (Tokyo)* 87, 73–80). To investigate the degree of specificity of the localization of cytochrome b_5 isoforms, we studied their subcellular distributions with antipeptide antibodies, one specific for microsomal cytochrome b_5, one specific for outer membrane cytochrome *b*, and one against a sequence common to the two cytochromes. We first identified outer membrane Cyt *b* as a tightly bound, Triton X-114-extractable, 23-kDa polypeptide. We then analyzed biochemically characterized rat liver subcellular fractions by Western blotting and found that outer mitochondrial membrane cytochrome *b* was not present on endoplasmic reticulum membranes. Conversely, microsomal cytochrome b_5 was present on outer mitochondrial membranes in extremely low concentration, at a level <5% of that on endoplasmic reticulum membranes. Thus, the subcellular distribution of microsomal cytochrome b_5 is more restricted than previously thought, suggesting that novel posttranslational targeting mechanisms direct it to the endoplasmic reticulum.

Most integral membrane proteins which are found in compartments of the exocytic pathway are initially cotranslationally targeted to membranes of the rough endoplasmic reticulum (ER)[1] by the signal recognition particle-mediated mechanism (see Walter and Lingappa, 1986; Singer, 1990, for reviews). However, some ER enzymes, with large cytoplasmic domains and short membrane anchors, escape this pathway of membrane insertion. The posttranslational mechanisms responsible for the correct localization of this class of proteins represents an important aspect of ER biogenesis.

A well-known example of a microsomal protein which is synthesized on free polysomes and inserted into the membrane posttranslationally is offered by cytochrome (Cyt) b_5 (Rachubinski *et al.*, 1980). Cyt b_5 is a small heme-protein, which has been sequenced in many species (see Ozols, 1989), and which consists of a cytoplasmic domain of 90 amino acids and a C-terminal membrane anchor. There is disagreement as to whether the membrane anchor spans the entire phospholipid bilayer or is restricted to the cytoplasmic leaflet (Dailey and Strittmatter, 1981; Takagaki *et al.*, 1983; Ozols, 1989; Ladokhin *et al.*, 1991). It is widely believed that newly synthesized Cyt b_5 is not recruited into a specific targeting pathway, but that it can insert nonspecifically into any membrane, thanks to this C-terminal hydrophobic region. This belief stems from: (i) the capacity of Cyt b_5 to interact with preformed liposomes (Enoch *et al.*, 1979); (ii) its ability to insert into dog pancreas microsomes (MR) in an signal recognition particle-independent fashion (Anderson *et al.*, 1983); and (iii) the results of subcellular fractionation experiments, which indicated that Cyt b_5 has a fairly wide subcellular distribution. Cyt b_5 was found in Golgi (Ichikawa and Yamano, 1970; Fleischer *et al.*, 1971; Borgese and Meldolesi, 1980; Collot *et al.*, 1982) and in plasma membrane (Jarasch *et al.*, 1979; Bruder *et al.*, 1980) fractions, often at concentrations which could not be explained by MR contamination, and associated with structures morphologically and physicochemically distinguishable from ER elements. Nonetheless, the concentration of Cyt b_5 in these fractions was in general found to be low compared to that in the MR fraction, and morphological investigations showed a markedly heterogeneous distribution within the Golgi fraction (Collot *et al.*, 1982). Moreover, the presence of Cyt b_5 in the Golgi complex and on the plasma membrane could be explained by some escape of the cytochrome from the ER down the exocytotic pathway, rather than by its direct "opportunistic" insertion into any available phospholipid bilayer.

More telling with respect to the mechanism of insertion of Cyt b_5 would be the demonstration of its presence on a membrane biogenetically unrelated to the ER, such as the outer mitochondrial membrane (OMM). Indeed, Cyt b_5 has been detected spectrally in OMM fractions (Parsons *et al.*, 1968; Fukushima *et al.*, 1972), which also contain very high concentrations of the cytochrome's electron donor, NADH-Cyt-b_5 reductase (Borgese and Pietrini, 1986). Moreover, the presence of an antigen recognized by anti-Cyt b_5 antibodies (Abs) on OMM was demonstrated by immunoelectron microscopy (Fowler *et al.*, 1976). The interpretation of these data was, however, complicated by the finding that rat tissues contain an additional isoform of Cyt b_5, which cofractionates

* This work was supported in part by Consiglio Nazionale delle Recherche Grants Progetto finalizzato Ingegneria Genetica, Progetto finalizzato Biotecnologie e Biostrumentazione, and Progetto Speciale PS0408. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom reprint requests and correspondence should be addressed: Consiglio Nazionale delle Recherche Center for Cytopharmacology, via Vanvitelli 32, 20129 Milano, Italy. Tel.: 39-2-70146250; Fax: 39-2-7490574.

[1] The abbreviations used are: ER, endoplasmic reticulum; Abs, antibodies; Cyt, cytochrome; MAO, monoamino oxidase; MR, microsomes; MR b_5, microsomal cytochrome b_5; OM, outer mitochondrial; OM *b*, outer membrane cytochrome *b*; OMM, outer mitochondrial membranes; PL, phospholipid.

with OMM (Ito, 1980a, 1980b), and which probably has functions different from those of the "classical" Cyt b_5 (Ito *et al.*, 1981). We will refer to this cytochrome as OM b. A 92-amino acid tryptic fragment of the new isoform was purified and shown to have 60% sequence identity to the cytoplasmic domain of Cyt b_5 purified from microsomes (MR b_5; Lederer *et al.*, 1983). No information on nontrypsinized OM b in terms of size or type of association with the membrane was given in these studies. Since the two isoforms were spectrally very similar and would also be expected to cross-react with polyclonal Abs, these findings opened the interesting possibility that all the b_5 detected in OMM in previous studies might be attributable to OM b.

In the present study, we have used noncross-reactive antipeptide Abs to investigate the subcellular distribution of the two Cyt b_5 isoforms and to identify and characterize the holoform of OM b. The results indicate that the two proteins have nonoverlapping distributions, and suggest the existence of novel posttranslational targeting mechanisms, which could result in the restricted localization of each Cyt b_5 isoform within the cell.

MATERIALS AND METHODS

Subcellular Fractionation—Microsomal (MR), mitochondrial, and OMM fractions were prepared from perfused livers of male Sprague-Dawley rats, as previously described (Borgese and Pietrini, 1986). Briefly, the mitochondrial and MR (rough + smooth) fractions were obtained by differential centrifugation, whereas OMM was purified from hypotonically shocked mitochondria by isopycnic flotation. In all experiments, volumes of samples at each step were registered and aliquots were kept, so that recoveries of constituents with respect to the total homogenate could be calculated.

Biochemical Assays—Protein, rotenone-insensitive NADH-Cyt-*c* reductase, NADPH-Cyt-*c* reductase, and monoamine oxidase (MAO), were assayed as previously described (Borgese and Pietrini, 1986). Cyt b_5 was determined by the difference spectrum between the oxidized and the reduced form, using Na^+ dithionite at 2 mg/ml as reductant. The concentration of Cyt b_5 was calculated using a mM^{-1} extinction coefficient of $163/OD_{429-409}$ (Garfinkel, 1958).

Production and Purification of Antipeptide Antibodies—Based on the published sequences of the tryptic fragments of rat liver MR b_5 and OM b (Ozols and Heinemann, 1982; Lederer *et al.*, 1983) and on predictions of immunogenicity made with the Surfaceplot program obtained from the Alberta Peptide Institute (Synthetic Peptides Incorporated, Edmonton, Alberta, Canada), three peptides were synthesized by the solid-phase method (Merrifield, 1986) with a Beckman 990/3 synthesizer, using *tert*-butyloxycarbonly/benzyl or fluorenylmethoxycarbonyl/*tert*-butyl-based protocols (Stewart and Young, 1984). Resins and amino acid derivatives were from Novabiochem (Laufelfingen, Switzerland). Peptides were cleaved from the polymeric support by standard procedures (Stewart and Young, 1984).

Two peptides, "Pep 1" (DGQGSDPAVTY[GGC-amide]) and "Pep 2," (ac-AEQSDKDVKY[GGC-amide]) corresponding to the divergent N-terminal regions of the tryptic fragments of OM b and MR b_5, respectively (Fig. 1), were synthesized with the aim of obtaining monospecific Abs against each Cyt b_5 isoform. An additional peptide, called "Pep 3" ([C]GADATENFEAVGHS-amide), was synthesized with the aim of obtaining Abs against both isoforms. Pep 3 synthesis was based on a conserved 14-residue stretch, with only two conservative substitutions between the two isoforms (Fig. 1). All the peptides contained an extra sequence, (*S*)-(acetamidomethyl)cysteine (Veber *et al.*, 1972), at the N or C terminus to enable conjugation to a carrier protein. For Pep 1 and Pep 2, the protected Cys was incorporated at the C terminus, preceded by 2 glycines used as spacer amino acids. For Pep 3 it was at the N terminus.

The peptides were purified to apparent homogeneity by ion exchange chromatography and semipreparative reversed-phase high-performance liquid chromatography, converted to the CysSH-containing peptides (Veber *et al.*, 1972), and conjugated to soybean trypsin inhibitor or ovalbumin activated with the heterobifunctional reagent sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate from Pierce, according to the method of Liu *et al.* (1979). Conjugates were separated from unbound peptide by gel filtration. The molar ratio of peptide to carrier was determined by amino acid analysis (Briand *et al.*, 1979), and found to be in the range of 5–10:1 for the Pep 1- and Pep 2-soybean trypsin inhibitor conjugates, and ≈3:1 for the Pep 3-ovalbumin conjugate.

Three-month-old white male rabbits were immunized with 350–500 μg of peptide-carrier conjugates emulsified in complete Freund's adjuvant by multiple subcutaneous injections on the back. The injections were repeated an additional two times, at 2-week intervals. After a further 2 weeks the rabbits were bled, and the anti-Cyt b_5 antibodies were purified by affinity chromatography as previously described (Borgese and Meldolesi, 1980), using the immunogenic peptides immobilized on cyanogen bromide-activated Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Treatment of Mitochondria with Na_2CO_3, Urea, or Triton X-114—0.5-ml aliquots of a mitochondrial fraction, containing 2.5 mg of protein, were incubated in 0.1 M Na_2CO_3, pH 11.2 (Fujiki *et al.*, 1982), or in 4 M urea. Extracted material was separated from the insoluble residue by ultracentrifugation (150,000 × *g* for 90 min in a TFT.65.13 rotor (Kontron Instruments, Zürich, Switzerland)). The supernatants were concentrated by trichloroacetic acid precipitation and resuspension in 0.25 M sucrose, while the pellets were directly resuspended in the same solution. Equal aliquots of the pellets and supernatants were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

A 40-μl aliquot of a mitochondrial fraction, containing 200 μg of protein, was treated with Triton X-114, as described by Bordier (1981). Equal aliquots of detergent and aqueous phase were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

Trypsinization of the Mitochondrial Fraction—An aliquot of a mitochondrial fraction (16 mg of protein) was first resuspended in 10 mM Tris-HCl, pH 7.5, at 4 mg of protein/ml, recovered by centrifugation at 170,000 × *g* for 1 h, and then treated with 0.1 M Na_2CO_3 (see above). The washed cell fraction was resuspended in 50 mM sodium phosphate buffer, pH 7.5, at a concentration of 2 mg of protein/ml and incubated with 0.02 mg of trypsin (Boehringer, Ingelheim, Germany) per mg of protein in the samples for 9 h at 4 °C. Proteolysis was stopped by addition of 1 mM phenylmethylsulfonyl fluoride, and the released material was recovered after centrifugation of the samples for 1 h at 170,000 × *g*.

Hydroxylapatite Chromatography—The supernatant obtained from the trypsinized mitochondrial fraction (5 ml, corresponding to 16 mg of protein of nontreated sample) was applied to a 6.7 × 1.4-cm hydroxylapatite column (Biogel HTP from Bio-Rad) equilibrated in 5 mM potassium phosphate buffer, pH 6.7. Tryptic fragments of Cyt b_5 isoforms were eluted with a linear potassium phosphate gradient (100 ml), from 5 to 100 mM (Ito, 1980a). Fractions (1.6 ml each) were assayed spectrally for Cyt b_5, and equal aliquots were analyzed by radioimmunoblotting, after precipitation with trichloroacetic acid in the presence of 50 μg of bovine serum albumin as carrier.

Electrophoretic Techniques—Sodium dodeyl sulfate-polyacrylamide gel electrophoresis of β-mercaptoethanol reduced samples was carried out according to Maizel (1971). 1.5-mm thick, 12.5% gels were either of standard or of "mini" size (Miniprotean II Slab Cell, Bio-Rad).

Electroblotting onto nitrocellulose (Schleicher and Schuell, Dassel, Germany; type BA 85) and radioimmunostaining of blots with affinity-purified Abs and ^{125}I-protein A (Amersham, United Kingdom) was carried out as previously described (Borgese and Pietrini, 1986). Abs were used at concentrations between 5 and 30 μg/ml.

For quantitation of Cyt b_5 isoforms in subcellular fractions, radioimmunostained blots were exposed to x-ray film without intensifying screens, and the autoradiographs were scanned with an LKB Ultroscan XL Laser Densitometer. For each cell fraction, multiple samples, with differing protein content, were analyzed, and the linearity of the response with respect to sample load was always verified.

RESULTS

Production of Anti-MR b_5 and Anti-OM b Abs with Synthetic Peptides as Immunogens—Fig. 1 shows the comparison of the amino acid sequences of the tryptic fragments of the two Cyt b_5 isoforms and the peptides which were used as immunogens in this study. Pep 1 and Pep 2, corresponding to the N-terminal divergent regions of OM b and MR b_5, respectively, were used to generate antibodies specific for each isoform. Pep 3, corresponding to a conserved sequence, was chosen with the aim of generating a third antiserum reactive with

```
1  acAEQSDKDVK YYTLEEIEKH KDSKSTW-VI LHHKVYDLTK FLEEHPGGEE   48
       ||  |  || |||  |       || ||  |  ||| |  || |||||||
1   DGQGSDPAVT YYRLEEVAKR NTAEETWMVI -HGRVYDITR FLSEHPGGEE   49
    **********

49  VLREQAGGDA TENFEDVGHS TDARELSKTY IIGELHPDDR SK           90
    || |||| || ||||||||  ||||  | |  ||  || |
50  VLLEQAGADA TESFEDVGHS PDAREMLKQY YIGDVHPNDL KPK          92
```

FIG. 1. **Alignment of the amino acid sequences of the tryptic fragments of MR b_5 and OM b, showing peptides used as immunogens in this study.** The upper sequence is MR b_5 (Ozols and Heineman, 1982); the lower sequence is OM b (Lederer *et al.*, 1983). *Vertical lines* indicate amino acid identities. The sequence *underlined* with *asterisks* corresponds to Pep 1; the sequence *overlined* with *dashed lines* corresponds to Pep 2; the two nearly identical sequences *overscored* and *underscored* with the *continuous line* were used for Pep 3 synthesis; at the divergent positions, the residues indicated by the *dots* are the ones that were incorporated into the peptide.

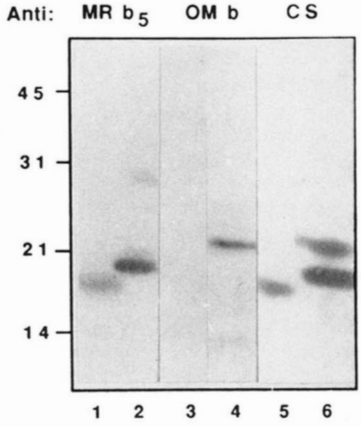

FIG. 2. **Specificity of antipeptide Abs tested by radioimmunoblotting.** *lanes 1, 3,* and *5* contained ≈0.1 μg of tryptic peptide of MR b_5, purified according to Omura and Takesue (1970); *lanes 2, 4,* and *6* contained rat liver total homogenate (50 μg of protein in *lanes 2* and *6*; 100 μg protein in *lane 4*). Antibodies used were: *lanes 1* and *2*, anti-MR b_5; *lanes 3* and *4*, anti-OM b; *lanes 5* and *6*, anti-common sequence (*CS*). Numbers on the left indicate M_rs and positions of Biorad mol weight standards.

both isoforms. We will refer to the Abs generated against Pep 1, 2, and 3, as anti-OM b, anti-MR b_5, and anticommon sequence, respectively.

The affinity-purified Abs were first tested in radioimmunoblotting against a total rat liver homogenate. As can be seen from Fig. 2, *lane 2*, anti-MR b_5 Abs recognized in the homogenate a polypeptide with an apparent M_r of 19,000. This M_r is somewhat different from the expected one of 15,000, based on the primary structure of Cyt b_5 (Ozols and Heinemann, 1982). To verify whether the 19-kDa band was due to MR b_5, we also assayed the reactivity of the Abs against the purified tryptic fragment of Cyt b_5, prepared as described by Omura and Takesue (1970). As shown in *lane 1* of Fig. 2, the purified protein was recognized by the anti-MR b_5 Abs; however, it also migrated anomalously on the gel, with an apparent M_r of ≈17,500 instead of the expected 11,000.

The anti-OM b Abs recognized a polypeptide with apparent M_r of ≈23,000 (Fig. 2, *lane 4*), but were incapable of binding to the purified tryptic fragment of MR b_5 (*lane 3*). Both the 19- and 23-kDa bands were recognized by the anti-common sequence Abs (*lane 6*), which also bound to the purified tryptic fragment (*lane 5*). These results indicated that the anti-MR b_5 Abs did indeed recognize MR b_5 on radioimmunoblots, and also suggested that the 23-kDa band, recognized by anti-OM b and anti-common sequence Abs, corresponded to OM isoform of Cyt b_5 (OM b).

The specificity of the Abs was also verified in a competition experiment, in which anti-MR b_5 and anti-OM b were used in radioimmunoblotting in the presence of each of the three peptides used as immunogens (Fig. 3). Each of the two Abs was prevented from binding to its cognate blotted antigen only by the peptide which had been used as immunogen (*lane 1* of the upper panel and *lane 2* of the lower panel). The lanes exposed to other peptide-Ab combinations were indistinguishable from the lane incubated with Abs in the absence of competing peptide (*lane 0*).

The 23-kDa Antigen Is an Integral Membrane Protein—To characterize the nature of the interaction of the presumptive OM b (23-kDa antigen) with membranes, we treated a mitochondrial fraction with three agents commonly used to discriminate between integral and peripheral membrane proteins. As shown in Fig. 4, *lanes 1–4*, both 0.1 M Na_2CO_3 and 4 M urea failed to extract any 23-kDa antigen from the mitochondrial membranes. Moreover, when the mitochondrial

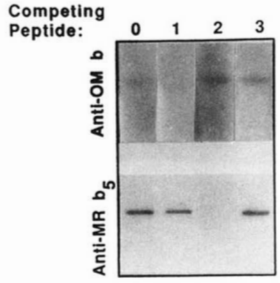

FIG. 3. **Inhibition of Ab-antigen interaction by competing peptides.** Anti-OM b (*upper lanes*) or anti-MR b_5 (*lower lanes*) were used for radioimmunostaining of blots in the absence or in the presence of competing peptides. *Upper lanes* contained 20 μg of protein of a partially purified OMM fraction; *lower lanes* contained 20 μg of protein of total liver homogenate. Radioimmunostaining of *lane 0* was carried out in the absence of competing peptide; radioimmunostaining of *lanes 1–3* was carried out in the presence of 500 μM Pep 1, Pep 2, and Pep 3, respectively (see Fig. 1).

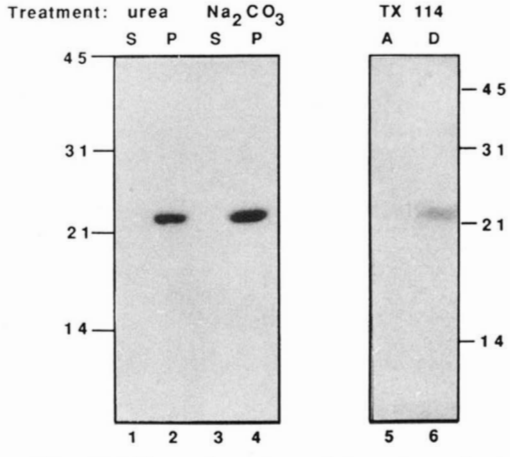

FIG. 4. **The 23-kDa antigen is an integral membrane protein.** *Lanes 1–4*, a mitochondrial fraction was incubated in 4 M urea (*lanes 1* and *2*) or 0.1 M Na_2CO_3, pH 11.2 (*lanes 3* and *4*) and then centrifuged to obtain pellet (*P*) (*lanes 2* and *4*), and supernatant (*S*) (*lanes 1* and *3*) fractions. Samples were analyzed by radioimmunostaining with anti-OM b Abs. In each lane, the equivalent of 100 μg of protein of initial mitochondrial fraction were loaded. *Lanes 5–6*, a mitochondrial fraction was extracted with Triton X-114 and separated into aqueous (*A*) (*lane 5*) and detergent (*D*) (*lane 6*) phases. Samples were analyzed by radioimmunostaining with anti-OM b Abs. The lanes contain the equivalent of 20 μg of protein of initial mitochondrial fraction. Numbers on the left and on the right of the two panels indicate M_r values and positions of Bio-Rad molecular weight standards.

fraction was treated with Triton X-114, all the 23-kDa antigen was recovered in the detergent phase (Fig. 4, *lanes 5* and *6*). Thus, by all three criteria the 23-kDa antigen could be classified as an integral membrane protein.

Identification of the 23-kDa Antigen as OM b—Our next goal was to demonstrate that the 23-kDa antigen corresponded to the previously unidentified intact form of OM b. Previous work characterized only a Cyt b_5-like fragment released from mitochondria by tryptic digestion (Ito, 1980a; Lederer *et al.*, 1983). We took advantage of the observation of Ito (1980a) that the tryptic fragments of MR b and OM b can be separated by hydroxylapatite chromatography, to investigate whether the antigen recognized by anti-OM b Abs had Cyt b_5-like spectral properties.

A mitochondrial fraction (which contains also contaminating microsomes; see Table I) was prewashed sequentially with a hypotonic solution and with 0.1 M Na_2CO_3 and then treated with trypsin and centrifuged; the supernatant (released proteins) and pellet were analyzed by radioimmunoblotting with anti-MR b_5 (Fig. 5*A*, *lanes 1–2*) and anti-OM b Abs (Fig. 5*A*, *lanes 3–4*). Trypsinization resulted in the release of the apparent M_r 17,500 fragment of MR b_5 into the supernatant (Fig. 5*A*, *lane 1*, *upward pointing arrowhead*), while no MR b_5 remained in the pellet (*lane 2*). An additional weak band in the supernatant with similar migration to the nonproteolyzed form of MR b_5 (*horizontal arrow* on the left of Fig. 5*A*) was also detected. The release of this polypeptide by trypsin had not been observed previously. We observed that the release of this polypeptide occurred only if the membranes had been pretreated with hypotonic solution and Na_2CO_3.

Analysis of the supernatant and pellet of the trypsinized mitochondrial fraction with anti-OM b Abs showed that the 23-kDa antigen generated a 21- and a 14-kDa polypeptide. The 14-kDa fragment was released into the supernatant (Fig. 5*A*, *lane 3*), while the 21-kDa polypeptide was retained in the pellet (*lane 4*). Prolonging the time of digestion did not increase the yield of the 14-kDa soluble fragment (not shown).

The proteins released by trypsin were loaded onto a hydroxylapatite column, and the fractions were spectrally assayed for Cyt b_5 (Fig. 5*B*). Spectrally detectable Cyt b_5 was eluted in two peaks, the first one at an elution volume expected for OM b, and the second one in the fractions expected for MR b_5 (Ito, 1980a). When the fractions were analyzed by radioimmunoblotting (Fig. 5*C*), it was found that the first peak contained the 14-kDa tryptic fragment recognized by anti-OM b Abs and the second peak contained the 17.5–19-kDa doublet recognized by anti-MR b_5 Abs. The excellent correlation between the position of elution of spectrally detectable Cyt b_5 and antigen indicated that the 23-kDa polypeptide indeed corresponds to OM b.

TABLE I

Recovery of protein and marker enzymes in rat liver subcellular fractions

Percentage relative to the total homogenate. Values are averages ± half-range of two experiments. Absolute values for constituents in the total homogenate (per g of liver) were: protein, 162.2 ± 6.2 mg; NADH-Cyt-*c* reductase, 32.1 ± 4.1 μmol/min; NADPH-Cyt-*c* reductase, 3.0 ± 0.3 μmol/min; MAO, 128.6 ± 7.4 μmol/min. Recoveries of constituents in the sum of all fractions (including those not shown in the table) were: protein, 87.8 ± 2.8%; NADH-Cyt-*c* reductase, 112.5 + 12.5%; NADPH-Cyt-*c* reductase, 129 ± 13%; MAO, 98.2 ± 16%.

	MR	Mitochondria	OMM
Protein	11.5 ± 0.4	7.0 ± 0.9	0.06 ± 0.02
NADH-Cyt *c* reductase	39.4 ± 0.6	7.3 ± 1.2	1.1 ± 0.0
NADPH-Cyt *c* reductase	65.3 ± 1.7	1.55 ± 0.05	0.04 ± 0.0
MAO	8.6 ± 2.1	21.0 ± 4.3	2.1 ± 0.1

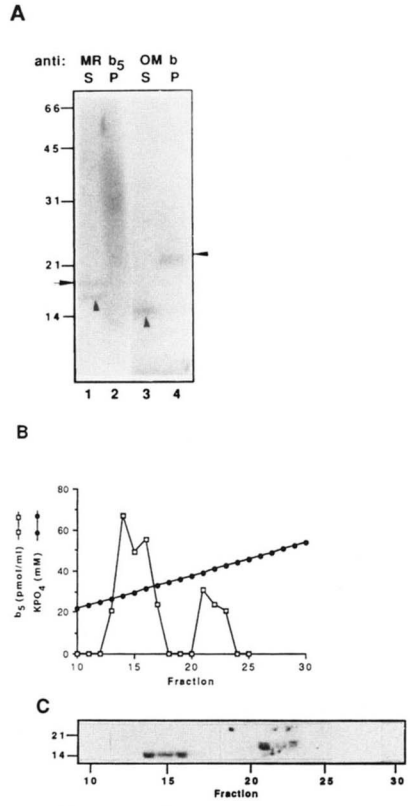

FIG. 5. **Copurification of tryptic fragments of 23-kDa and 19-kDa antigens with spectrally determined Cyt b_5.** *A*, radioimmunoblotting analysis with anti-MR b_5 (*lanes 1–2*) and anti-OM b (*lanes 3–4*) of products generated by trypsin from a mitochondrial fraction. After treatment with trypsin (see "Materials and Methods"), equivalent aliquots of supernatant (*S*) (*lanes 1* and *3*) and pellet (*P*) (*lanes 2* and *4*) fractions, deriving from 50 μg of untreated mitochondria, were analyzed. *Upward-pointing arrowheads* in *lanes 1* and *3* indicate the 17.5- and 14-kDa bands generated from trypsinization of MR b_5 and OM b, respectively. *Horizontal arrows* on the left and right of the panel indicate the positions of undigested MR b_5 (19-kDa) and 23-kDa antigen, respectively. Numbers on the left indicate M_r values and positions of Bio-Rad molecular weight standards. *B*, separation of the tryptic fragments of the two Cyt b_5 isoforms by hydroxylapatite chromatography. See "Materials and Methods" for details. *C*, radioimmunoblotting analysis of fractions eluted from hydroxylapatite column. One-half of each fraction was precipitated with trichloroacetic acid and analyzed by radioimmunoblotting. The blot was first radioimmunostained with anti-OM b, which recognized the 14-kDa band visible in fractions 13–17. It was then reincubated with anti-MR b_5 Abs, which revealed the polypeptide doublet in fractions 21–24. Numbers on the left indicate M_r values and positions of Bio-Rad molecular weight standards.

Subcellular Distribution of MR b_5 and OM b, as Determined in Cell Fractionation Experiments—The antipeptide Abs were used to probe blots of biochemically characterized liver MR, mitochondrial, and OMM fractions.

The recoveries of protein and marker enzymes in the three fractions are shown in Table I. The enzymes chosen for assay were: NADPH-Cyt-*c* reductase, as an ER marker; MAO as an OMM marker; and rotenone-insensitive NADH-Cyt-*c* reductase, an enzyme found on both ER and OMM membranes. The distributions of these enzyme markers were found to be in good agreement with those found previously (Borgese and Pietrini, 1986). Autoradiographs of representative immunoblots obtained with the three Abs are shown in Fig. 6. The anti-MR b_5 probe (Fig. 6*A*) revealed that the corresponding antigen was concentrated in the MR fraction (*e.g.* compare signal obtained with 10 μg of MR protein in *lane 6* with that obtained from the same amount of total homogenate protein

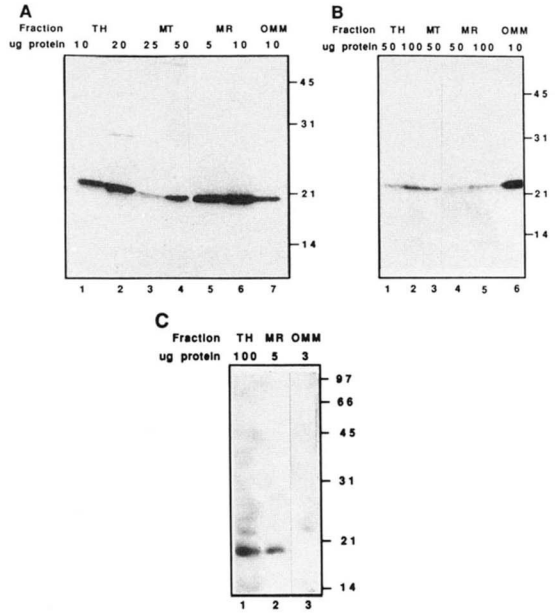

FIG. 6. **Radioimmunoblotting analysis of rat liver subcellular fractions with anti-MR b_5, anti-OM b, and anti-common sequence Abs.** *A*, blot radioimmunostained with anti-MR b_5. Lanes contained: *1* and *2*, total homogenate, 10 and 20 μg of protein respectively; *3* and *4*, mitochondrial fraction, 25 and 50 μg of protein, respectively; *5* and *6*, MR fraction, 5 and 10 μg of protein, respectively; *7*, OMM fraction, 10 μg of protein. *B*, blot radioimmunostained with anti-OM b. Lanes contained: *1* and *2*, total homogenate, 50 and 100 μg of protein, respectively; *3*, mitochondrial fraction, 50 μg of protein; *4* and *5*, MR fraction, 50 and 100 μg of protein, respectively; *6*, OMM fraction, 10 μg of protein. *C*, blot radioimmunostained with anti-common sequence. Lanes contained: *1*, total homogenate, 100 μg of protein; *2*, MR fraction, 5 μg of protein; *3*, OMM fraction, 3 μg of protein. Numbers on the right of the panels indicate positions and M_r values of Bio-Rad molecular weight standards.

in *lane 1*). Some MR-b_5 was detected also in the OMM fraction (Fig. 6*A*, *lane 7*). Conversely, probing with anti-OM b (Fig. 6*B*) showed that the corresponding antigen was highly concentrated in the OMM fraction (*e.g.* compare signal from 10 μg of protein OMM in *lane 6* with that obtained from 50 and 100 μg of total homogenate protein in *lanes 1* and *2*). Hardly any OM b could be detected in the MR fraction (*lanes 4* and *5*). The different subcellular distribution of the 2 Cyt b_5 isoforms was strikingly apparent when anti-common sequence Abs were used as probe (Fig. 6*C*). The two polypeptides revealed in the total homogenate (Fig. 6*C*, *lane 1*) were clearly separated into the MR (MR b_5, *lane 2*) and the OMM (OM b, *lane 3*) fractions.

To determine whether the OM b found in the MR fraction, and, vice versa, the MR b_5 found in the OMM fraction were due to cross-contamination between the fractions or to real overlap in the distributions of the two isoforms, we quantitated the results of immunoblots and compared the relative concentrations of MR b_5 and OM b in the fractions with the values for marker enzymes (Table II). As can be seen in lines 1 and 2 of the table, the distribution of OM b closely paralleled that of the OMM marker enzyme, MAO. OM b actually appeared to be somewhat *more* enriched in the OMM fraction than MAO itself (compare lines 1 and 2, column 3). Contamination of the MR fraction by OMM was on the order of 2%, if expressed on a protein basis (% MAO concentration in MR compared to MAO concentration in OMM fraction). This value is close to the percentage of OM b concentration in the MR fraction compared to its concentration in the OMM fraction (1.55: 55 × 100 = 2.9%). Thus, the very low amounts

TABLE II

Comparison between distributions of marker enzymes and MR b_5 and OM b antigens among rat liver subcellular fractions

Values given are enrichments (enzyme activity or peak area of scanned autoradiographs of radioimmunoblots/mg protein in cell fractions divided by the value of the total homogenate) ± half-range of two cell fractionation experiments.

	MR	Mitochondria	OMM
MAO[a]	0.77 ± 0.15	3.2 ± 0.9	38.7 ± 9.8
OM b[b]	1.55 ± 0.25	3.0 ± 1.3	55 ± 8.0
NADPH-Cyt-*c* reductase[a]	5.7 ± 0.0	0.23 ± 0.3	0.78 ± 0.21
MR b_5[b]	5.4 ± 0.1	0.18 ± 0.0	1.26 ± 0.3

[a] Enzyme activity.

[b] 23- or 19-kDa bands from Western blots with anti-OM b or anti-MR b_5 Abs.

of OM b in the MR fraction are due entirely, or almost entirely, to contaminating OMM, and not to OM b bound to ER membranes.

The comparison of the distribution of MR b_5 and the ER marker enzyme, NADPH-Cyt-*c* reductase, can be seen in lines 3 and 4 of Table II. The two distributions were strikingly similar, with both enriched ≈5.5-fold in the MR fraction. Note that rotenone-insensitive NADH-Cyt-*c* reductase activity (due to the enzyme NADH-Cyt-b_5 reductase), which is present on both ER and OMM membranes, was enriched only ≈3.5-fold in the MR fraction (see Table I), as expected. Contamination of the OMM fraction by MR was on the order of 14% (% NADPH-Cyt-*c* reductase concentration in OMM fraction compared to its concentration in the MR fraction: line 3, columns 1 and 3). Again, this value is close to the percentage of MR b_5 concentration in the OMM fraction, compared to its concentration in the MR fraction (1.26: 5.4 × 100 = 23%; columns 1 and 3 of line 4). Thus, over half of the MR b_5 (14 out of 23%) found in the OMM fraction is due to microsomal contamination, however, a certain amount may be due to MR b_5 bound to OMM. The concentration of this endogenous MR b_5 on OMM would in any case be quite low compared to ER membranes (≈10% on a protein basis).

The data of Table II address the question of the relative concentrations of the two Cyt b_5 isoforms on ER and OM membranes. We were also interested in determining the absolute concentrations of the two isoforms in these two compartments. To do this, we first assayed Cyt b_5 in the two fractions by difference spectroscopy, a method which does not distinguish between the two isoforms (Ito, 1980a). The concentration of spectrally measurable b_5 was found to be considerably higher in MR than in OMM (Table III, line 1). In line 2, cytochrome concentrations have been expressed on a phospholipid (PL) basis, by using our previously published data on the PL to protein ratio in the MR and OMM fractions (Borgese and Pietrini, 1986). PL content is a better measure of membrane surface area than protein, and is higher in the OMM fraction, which contains purified membranes, than in the MR fraction, whose membrane vesicles carry lumenal proteins as well as bound ribosomes. Assuming that all of the spectrally measurable Cyt b_5 in MR is due to MR b_5 and that all of the MR b_5 and OM b assayed by radioimmunoblotting are derived from spectrally measurable cytochromes, it is possible to use the data obtained by radioimmunoblotting (Table II) to calculate the relative contributions of MR b_5 and OM b to the spectrally measurable b_5 endogenous to OMM, as explained in Table III. The results of this analysis, shown in the last three lines of Table III, indicate that MR b_5 is 8-fold more concentrated in ER membranes than is OM b in OMM and that approximately one-fifth of the spectrally measurable cytochrome b_5 bound to OMM may be attributed

TABLE III

Distribution of the two cytochrome b_5 isoforms on MR and OM membranes

	MR	OMM
nmol of b_5/mg protein[a]	0.71 ± 0.075	0.39 ± 0.075
nmol of b_5/mg PL[b]	1.85	0.4
nmol of b_5/mg PL (corrected)[c]	1.85	0.31
Portion of b_5 attributable to MR b_5[d]	1.85	0.07
Portion of b_5 attributable to OM b[e]		0.24

[a] Cyt b_5 was measured spectrally. Values represent the averages ± half-range of the two fractionation experiments of Table I.

[b] Obtained by dividing the values of line 1 by the PL to protein ratios of the MR and OMM fractions (0.383 and 0.97 for MR and OMM respectively; data from Borgese and Pietrini, 1986).

[c] The contamination of MR by OMM is neglected (Table II). The contamination of OMM by MR expressed on a PL basis is 14% (Table I) × 0.383 (PL/protein of MR) divided by 0.97 (PL/protein of OMM fraction) = 5.5%. Therefore, 1.85 × 0.055 = 0.1 nmol/mg PL in the OMM fraction is due to contaminating MR. The remaining 0.30 nmol are endogenous to OMM, which contribute 94.5% of the PL to the fraction (neglecting other sources of contamination).

[d] The ratio of *endogenous* MR b_5 in the OMM fraction to MR b_5 in the MR fraction on a protein basis is 0.09 (Table II), which, expressed on a PL basis is 0.035. Therefore, the concentration of endogenous MR b_5 in OMM is

$$\frac{1.85 \times 0.035}{0.945} = 0.068 \text{ nmol/mg PL.}$$

[e] Considered to be the difference between the values of lines 3 and 4.

to MR b_5. The concentration of MR b_5 bound to OMM relative to its concentration in MR, expressed on a PL basis, amounts to 4% (line 4: 0.07 *vs.* 1.85 nmol/mg PL).

DISCUSSION

The discovery of a second Cyt b_5 isoform, OM b (Ito, 1980a), which generates a tryptic fragment with 60% sequence identity to the previously characterized cytoplasmic domain of the MR form of Cyt b_5 (Lederer *et al.*, 1983), cast doubt on the conclusion of early studies concerning the presence of Cyt b_5 on OMM. Indeed, the two isoforms have similar spectra, and would also be expected to cross-react with polyclonal Abs. Therefore, OM b could have been mistaken for MR b both in biochemical and immunological studies.

Because of its implications for membrane biogenesis, we decided to investigate the subcellular distribution of the two Cyt b_5 isoforms, using monospecific, antipeptide Abs. We first used the anti-OM b Abs to identify OM b as a 23-kDa polypeptide and to characterize the nature of its association with membranes. The identification of the 23-kDa antigen as OM b was confirmed: (i) by the fact that it was recognized by another anti-peptide Ab (anti-common sequence) directed against a different part of the reported sequence of OM b (Lederer *et al.*, 1983); (ii) by the observation that it generated a tryptic fragment which coeluted with spectrally measurable Cyt b_5 from a hydroxylapatite column; and (iii) by the finding that it was highly enriched in the OMM fraction. In this location, OM b behaved like an integral membrane protein according to all accepted criteria.

To investigate the subcellular distributions of the two Cyt b_5 isoforms, we used the antipeptide Abs to obtain quantitative data on biochemically characterized rat liver subcellular fractions. We also attempted to confirm the results obtained in fractionation experiments by immunofluorescence on tissue culture cells. Unfortunately, we could not obtain a significant signal over background with any of our Abs. However, since MR b_5 is known to be bound to biological membranes in a tight, nonexchangeable form (Enoch *et al.*, 1979; Poengsen and Ullrich, 1980) and since the data reported in this study showed that also OM b is a tightly bound integral membrane protein, the two proteins are not expected to undergo redistribution artifacts during cell fractionation. Two main points emerged from our analysis: (i) the specificity in the localization of the two Cyt b_5 isoforms is very high. The density of MR b_5 on the ER membrane is ≈25 times higher than its density on the "wrong" membrane, *i.e.* on OMM. Vice versa, we could detect essentially no OM b on ER membranes; (ii) notwithstanding the specificity in the localization of the two forms, a small fraction of Cyt b_5 on OMM could be attributed to MR b_5 (≈one-fifth of the total). This is comprehensible if we consider that OM b is a much less abundant protein than MR b_5 in rat liver cells. From a combination of the data of Tables III and I, it can be calculated that there are ≈20 nmol of MR b_5 and only ≈1 nmol of OM b per g of liver. Given the much higher concentration of MR b_5 on ER membranes than of OM b on OMM, a very small degree of "mislocalization" of MR b_5 to OMM results in it contributing significantly to the spectrally measurable Cyt b_5 found in the latter membranes.

The conclusions of the present study are in contrast with those reached by Ito (1980b). This author used polyclonal Abs, thought to be monospecific for the tryptic fragments of MR b_5 and OM b, to inhibit rotenone-insensitive NADH-Cyt-*c* reductase activity in mitochondrial and MR fractions from various rat tissues, including liver. Since each Ab partially inhibited the activity in both fractions, it was concluded that there was substantial overlap in the localization of the two isoforms. However, analysis of the data reveals that the sum of the inhibitions of the two Abs in each cell fraction was well over 100%, indicating that in these inhibition assays the antigens were cross-reactive.

At steady state, the rate of delivery of a protein to a subcellular compartment is balanced by its rate of removal, and its concentration within the compartment is determined by the kinetics of these two processes. Our results on the specific subcellular localization of the two Cyt b_5 isoforms could therefore be explained either by specific targeting mechanisms, or by an accelerated removal of each Cyt b_5 isoform from the wrong compartment. We believe that targeting mechanisms are more likely to be involved, for the reasons discussed below.

OMM proteins are known to be delivered to their target membrane via a receptor-mediated mechanism (Soellner *et al.*, 1989, 1990). The proteins analyzed so far do not have cleavable mitochondrial localization signals (Steger *et al.*, 1990), however, for their insertion, they do use a receptor (MOM 19) involved also in the import of matrix-directed proteins, which carry positively charged cleavable presequences (Soellner *et al.*, 1989, 1990). Given the apparently exclusive localization of OM b to OMM, it is likely that it uses the same targeting mechanisms as other OMM proteins. The verification of this hypothesis awaits the cloning and *in vitro* expression of OM b.

Possible pathways of delivery of MR b_5 to the ER are more difficult to fit into a known framework. This protein is synthesized on free polysomes (Rachubinski *et al.*, 1980), without a presequence, and inserts posttranslationally into membranes via an signal recognition particle-independent pathway (Benazko *et al.*, 1982; Anderson *et al.*, 1983). Based also on the demonstration of its interaction with preformed liposomes (Enoch *et al.*, 1979), it was widely believed that MR b_5 can opportunistically insert into any PL bilayer. Its C-terminal hydrophobic sequence was called an "insertion sequence" to distinguish it from signal sequences involved in targeting (Benazko *et al.*, 1982). However, the MR b_5 attached to

preformed liposomes is not bound in the same way as it is to MR membranes. Cyt b_5 bound to preformed liposomes is in a "loose" conformation, in that it is transferable to other liposomes, and is susceptible to digestion with carboxypeptidase Y, characteristics which are absent from the Cyt b_5 endogenous to MR or incorporated into liposomes via detergent dialysis (Enoch *et al.*, 1979; Poensgen and Ullrich, 1980). Even if the preformed liposomes are constructed with lipids extracted from MR, they support only the loose type of binding. Thus, lipids alone are not sufficient to permit tight, physiological binding of MR b_5 to the bilayer (Enoch *et al.*, 1979; Poensgen and Ullrich, 1980). In the light of the distinction between loose and tight binding, also, the significance of early studies, showing the interaction of Cyt b_5 with a variety of biological membranes (Enomoto and Sato, 1977; Remacle, 1978; Remacle, 1980), is doubtful.

Although at present we do not know anything about the putative targeting mechanism which restricts the intracellular localization of MR b_5, it is possible that such a mechanism is common to other posttranslationally inserted ER proteins. Recently, the prototype "nontransmembrane" tyrosine phosphatase, PTP-1B, has been reported to be localized to the ER, and the information for this localization has been found to reside in a hydrophobic C-terminal region (Frangioni *et al.*, 1992). It is tempting to speculate that PTP-1B and MR b_5 share a common, previously undescribed, targeting mechanism. Appropriate systems for the *in vitro* and *in vivo* expression of the cloned Cyt b_5 isoforms and of mutant forms thereof will hopefully rapidly generate the data required to describe and understand these novel posttranslational targeting pathways.

Acknowledgments—We are grateful to Drs. S. Bonatti, F. Clementi, and F. Navone for critically reading the manuscript; to Dr. A. Zanini for helpful discussions; and to P. Tinelli for technical assistance with the preparation of the illustrations.

REFERENCES

Anderson, D. J., Mostov, K. E., and Blobel, G. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 7249–7253
Benazko, P., Brehn, S., Pfeil, W., and Rapoport, T.A. (1982) *Eur. J. Biochem.* **123,** 121–126
Bordier, C. (1981) *J. Biol. Chem.* **256,** 1604–1607
Borgese, N., and Meldolesi, J. (1980) *J. Cell. Biol.* **85,** 501–515
Borgese, N., and Pietrini, G. (1986) *Biochem J.* **239,** 393–403
Briand, J. P., Mutter, S., and Van Regenmortel, M. H. V. (1985) *J. Immunol. Methods* **78,** 59–69
Bruder, G., Bretscher, A., Franke, W. W., and Jarasch, E.-D. (1980) *Biochim. Biophys. Acta* **600,** 739–755
Collot, M., Kalff, M., and Remacle, J. (1982) *Eur. J. Cell Biol.* **29,** 34–42
Dailey, H. A., and Strittmatter, P. (1981) *J. Biol. Chem.* **256,** 3951–3955
Enoch, H., Fleming, J., and Strittmatter, P. (1979) *J. Biol. Chem.* **254,** 6483–6488
Enomoto, K., and Sato, R. (1977) *Biochim. Biophys. Acta* **466,** 136–147
Fleischer, S., Fleischer, B., Azzi, A., and Chance, B. (1971) *Biochim. Biophys. Acta* **225,** 194–200
Fowler, S., Remacle, J., Trouet, A., Beaufay, H., Berthet, J., Wibo, M., and Hauser, P. (1976) *J. Cell Biol.* **71,** 535–550
Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A., and Neel, B. G. (1992) *Cell* **68,** 545–560
Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarow, P. B. (1982) *J. Cell Biol.* **93,** 97–102
Fukushima, L., Ito, A., Omura, T., and Sato, R. (1972) *J. Biochem. (Tokyo)* **71,** 447–461
Garfinkel, D. (1958) *Arch. Biochem. Biophys.* **77,** 493–509
Ichikawa, Y., and Yamano, T. (1970) *Biochem. Biophys. Res. Commun.* **40,** 297–305
Ito, A. (1980a) *J. Biochem. (Tokyo)* **87,** 63–71
Ito, A. (1980b) *J. Biochem. (Tokyo)* **87,** 73–80
Ito, A., Hayashi, S., and Yoshida, T. (1981) *Biochem. Biopys. Res. Commun.* **101,** 591–598
Jarasch, E.-D., Kartenbeck, J., Bruder, G., Fink, A., Morre', D. J., and Franke, W. W. (1979) *J. Cell Biol.* **80,** 37–52
Ladokhin, A. S., Wang, L., Steggles, A. W., and Holloway, P. W. (1991) *Biochemistry* **30,** 10200–10206
Lederer, F., Ghrir, R., Guiard, B., Cortial, S., and Ito, A. (1983) *Eur. J. Biochem.* **132,** 95–102
Liu, F.-T., Zinnecker, M., Hamaoka, T., and Katz, D. H. (1979) *Biochemistry* **18,** 690–697
Maizel, J. V. (1971) *Methods Virol.* **5,** 179–246
Merrifield, R. B. (1986) *Science* **232,** 341–347
Omura, T., and Takesue, S. (1970) *J. Biochem. Tokyo* **67,** 249–257
Ozols, J. (1989) *Biochim. Biophys. Acta* **997,** 121–130
Ozols, J., and Heinemann, F. (1982) *Biochim. Biophys. Acta* **704,** 163–173
Parsons, D. F., Williams, G. R., Thompson, W., Wilson, D., and Chance, B. (1968) in *Mitochondrial Structure and Compartmentation* (Quagliariello, E., Papa, S., Slater, E. C., and Tager, J. M., eds) pp. 29–70, Adriatica Editrice, Bari, Italy
Poensgen, J., and Ullrich, B. (1980) *Biochim. Biophys. Acta* **596,** 248–263
Rachubinski, R. A., Verma, D. P. S., and Bergeron, J. J. M. (1980) *J. Cell Biol.* **84,** 705–716
Remacle, J. (1978) *J. Cell Biol.* **79,** 291–313
Remacle, J. (1980) *Biochim. Biophys. Acta* **597,** 564–576
Singer, S. J. (1990) *Annu. Rev. Cell Biol.* **6,** 247–296
Soellner, T., Griffiths, G., Pfaller, R., Pfanner, N., and Neupert W. (1989) *Cell* **59,** 1061–1070
Soellner, T., Pfaller, R., Griffiths, G., Pfanner, N., and Neupert, W. (1990) *Cell* **62,** 107–115
Steger, H. F., Soellner, T., Kiebler, M., Dietmeier, K. A., Pfaller, R., Trülzsch, K. S., Tropschug, M., Neupert, W., and Pfanner, N. (1990) *J. Cell Biol.* **111,** 2353–2363
Stewart, J. M., and Young, J. D. (1984) *Solid Phase Peptide Synthesis*, Ed. 2, Pierce Chemical Co., Rockford, IL
Takagaki, Y., Radhakrishnan, R., Wirtz, K. W. A., and Khorana, H. G. (1983) *J. Biol. Chem.* **258,** 9136–9142
Veber, D. F., Milkowsky, J. D., Varga, S. L., Denkewalter, R. G., and Hirschmann, R. (1972) *J. Am. Chem. Soc.* **94,** 5456–5461
Walter, P., and Lingappa, V. R. (1986) *Annu. Rev. Cell Biol.* **2,** 499–516