Functional properties in health and disease. *Biochem Soc Trans* **18**: 87–89, 1990.
18. Christiansen EN, Gray TJB and Lake BG, Effect of very long chain fatty acids on peroxisomal β-oxidation in primary rat hepatocyte cultures. *Lipid* **12**: 929–932, 1985.
19. Hertz R, Bar-Tana J, Sujatta M, Pill J, Schmidt FH and Fahimi HD, The induction of liver peroxisomal proliferation by β-β′-methyl-substituted hexadecanedioic acid (MEDICA 16). *Biochem Pharmacol* **37**: 3571–3577, 1988.
20. Eacho PI and Foxworthy PS, Inhibition of hepatic fatty acid oxidation by benzafibrate and benzafibroyl CoA. *Biochem Biophys Res Commun* **157**: 1148–1152, 1988.

Biochemical Pharmacology, Vol. 42, No. 1, pp. 188–190, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Role of glutathione *S*-transferases in heme transport

(*Received* 6 *November* 1990; *accepted* 28 *January* 1991)

Newly synthesized heme must cross the inner and outer membranes of mitochondria and then diffuse through cytoplasm preceding incorporation into apocytochromes within the endoplasmic reticulum. Senjo *et al.* [1] reported that the reconstitution of apo-b_5* by mitochondrial heme is increased in the presence of GST Yb_2Yb_2 (see [2] for nomenclature). The latter result suggests a dual role for the GSTs, i.e. enhancing efflux of heme from mitochondria and then shuttling it to the apocytochromes. To define whether these transport functions are limited to GST Yb_2Yb_2, we examined the effect of homogenous GSTs on mitochondrial heme efflux and subsequent reconstitution of apo-b_5.

Methods

Materials. All fine chemicals were purchased from Sigma. Heme was prepared fresh daily.

Preparation of rat liver mitochondria and cytosol. Rat livers were perfused *in situ* with ice-cold 0.25 M sucrose and then excised, minced and resuspended in 5 vol. of a 0.25 M sucrose, 10 mM Hepes (pH 7.0) buffer for preparation of cytosol [3] and mitochondria. The mitochondria were prepared according to standard techniques and were washed five times with an EDTA-containing buffer [1]. Mitochondrial coupling was determined on each preparation with succinate, and only preparations with a respiration control ratio of >4.0 were used.

Enzymatic and protein assays and preparation of enzymes. GST activity was determined spectrophotometrically [4]. Protein was determined as described previously with bovine serum albumin as the standard [5]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed as described by Maizel [6]. The various GST isozymes were purified using a GSH affinity column and chromatofocusing [3]. GSTs also were purified according to Senjo *et al.* [1].

Preparation of apocytochrome b_5. Detergent-solubilized holocytochrome b_5 was prepared from rabbit liver microsomes [7]. Apo-b_5 was prepared by acid/acetone extraction of the holocytochrome [7, 8].

Measurement of heme transfer. Our experimental approach was similar to that of Senjo and colleagues [1] in which the reconstitution of apo-b_5 is used to quantitate the movement of heme from mitochondria into the aqueous phase. Apo-b_5 (0.02 to 0.04 mg) was mixed in 1.0 mL of buffer (10 mM Tris, pH 7.4) with the putative transfer protein and/or mitochondria (2 to 3.5 mg). The mixture was incubated for 10 min at 37°. The mitochondria were removed by centrifugation in a microfuge, and 0.5 mL of the supernatant was used for measurement in a spectrophotometer of the difference spectra (424–409 nm) of the dithionite reduced minus oxidized cytochrome b_5 [1]. Heme transfer activity is defined as nanomoles of cytochrome b_5 reconstituted per milligram of transfer protein. In experiments without mitochondria, the reconstitution of the apocytochrome also was measured at 410 nm and the concentration of the holocytochrome calculated using an absorption coefficient of 117 mM^{-1} cm^{-1} [9].

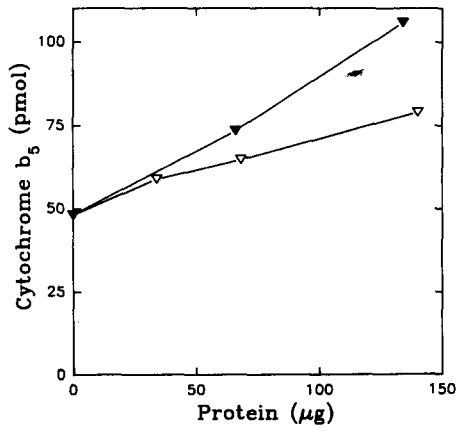

Fig. 1. Reconstitution of apocytochrome b_5 with heme in the presence of GSTs. The transfer proteins [mixture of purified GST (▽) or GST Yb_2Yb_2 (▼) were mixed with 100 pmol heme. Apocytochrome b_5 (1 nmol) was added and the mixture was incubated at 37° for 10 min. Cytochrome b_5 content was measured at 410 nm as described in Methods. Each point is the average of two determinations.

* Abbreviations: apo-b_5, apocytochrome b_5; and GST, glutathione *S*-transferase.

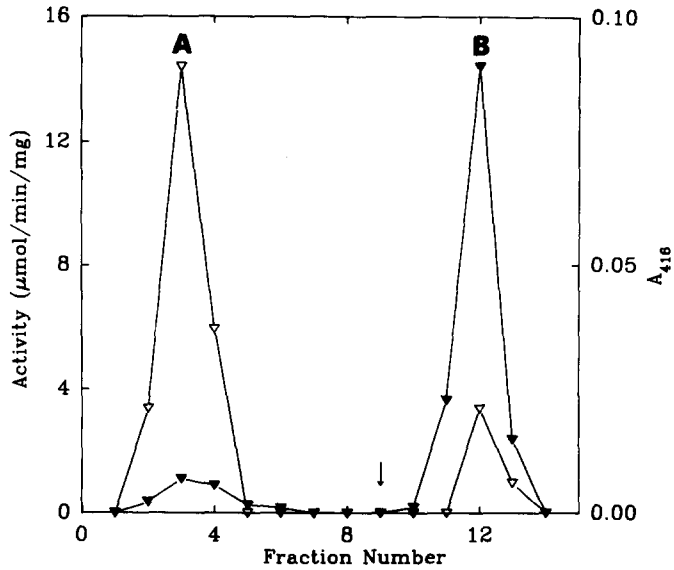

Fig. 2. GSH affinity column of CM-52 purified GSTs. The material obtained from the 0.5 M NaCl wash of a CM-52 column was applied to a GSH-affinity column. The column was eluted with 0.9 M NaCl (fractions 1–8) and 100 mM GSH (fractions 9–15, arrow). Enzymatic activity (▼) and A_{416} (▽) were determined on each fraction. Fractions 2–4 (peak A) and 11–13 (peak B) were pooled for analysis.

Results and Discussion

When crude rat liver cytosol was added to a mixture of apo-b_5 and mitochondria, holocytochrome b_5 was formed. However, when affinity purified GSTs (mixture or YaYa or Yb_2Yb_2) were substituted for cytosol, essentially no heme transfer activity could be identified. To determine the effect of purified GSTs on reconstitution of apo-b_5, increasing amounts of the affinity purified mixture of GSTs or the Yb_2Yb_2 isozyme were mixed with 100 pmol heme. Reconstitution of cytochrome b_5 was determined at 410 nm following the addition of 1 nmol apo-b_5. In the absence of GST only about 50% of the added heme bound to the apocytochrome, whereas in the presence of a high concentration of GST fully 100% (100 pmol) of the added heme bound to the heme binding site of the apocytochrome (Fig. 1).

The above results plus the finding that crude cytosol reconstituted apo-b_5 in the absence of mitochondria (data not shown) suggested that the method of purification may influence the results of the transfer assay due to the presence of variable amounts of heme bound to GSTs. The GSTs, therefore, were purified according to the method of Senjo *et al.* [1]. Heme transfer activity (0.98 nmol/mg) and the GSTs were recovered together in the 0.5 M NaCl wash of a CM-52 column (3 cm × 10 cm). The fraction also appeared to contain a significant amount of protein-bound heme based on an absorbance at 416 nm. GSH is known to affect the affinity of GSTs for nonsubstrate ligands [10, 11]. Therefore, the CM-52 salt wash fraction was applied to a GSH affinity column. Heme (A_{416}) (peak A, Fig. 2) was recovered in the salt wash of the affinity column, whereas GST activity was recovered in the GSH wash (peak B, Fig. 2). Peak A contained heme transfer activity (1.26 nmol/mg), whereas there was minimal activity in peak B (0.1 nmol/mg).

The results of this study are consistent with the recent report of Liem *et al.* [12], i.e. the GSTs did not appear to increase the rate of heme efflux from mitochondria. Whether other proteins such as fatty acid binding protein [12, 13] or diffusion from the mitochondria [14, 15] account for heme efflux is currently unclear. However, our finding that apo-b_5 was fully reconstituted by exogenous heme only in the presence of GSTs implies that GSTs and perhaps other heme binding cytosolic proteins increase the aqueous phase concentration of heme available for binding to specific sites and, therefore, have an important role in the reconstitution of apocytochromes. This view is consistent with earlier suggestions on the role of GSTs in the transport of nonpolar molecules [16] and the finding that cytosolic proteins are necessary for reconstitution of cytochrome P_{450} [17].

In conclusion, the GSTs may play an important role in the synthesis of heme proteins, not by increasing the rate of efflux of heme from mitochondria but instead by increasing the availability of heme to apocytochromes in the endoplasmic reticulum.

Acknowledgements—The work was supported in part by a grant from the N.I.H. (GM31555).

THOMAS D. BOYER*†‡
ERIC OLSEN†

**Division of Digestive Diseases*
Emory University School of Medicine
Atlanta, GA 30322; and
† Liver Studies Unit
Veterans Administration Medical Center
San Francisco
CA 94121, U.S.A.

‡ Correspondence: Dr. Thomas D. Boyer, Emory University School of Medicine, P.O. Drawer AL, Atlanta, GA 30322.

REFERENCES

1. Senjo M, Ishibashi T and Imai Y, Purification and characterization of cytosolic liver protein facilitating heme transport into apocytochrome b_5 from mitochondria. Evidence for identifying the heme transfer protein as belonging to a group of glutathione *S*-transferases. *J Biol Chem* **260**: 9191–9196, 1985.
2. Jakoby WB, Ketterer B and Mannervik B, Glutathione transferases: Nomenclature. *Biochem Pharmacol* **33**: 2539–2540, 1984.
3. Boyer TD, Kenney WC and Zakim D, Structural, functional and hybridization studies of the glutathione *S*-transferases of rat liver. *Biochem Pharmacol* **32**: 1843–1850, 1983.
4. Habig WH, Pabst MJ and Jakoby WB, Glutathione *S*-transferases: The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139, 1974.
5. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
6. Maizel JV Jr, Polyacrymamide gel electrophoresis of viral proteins. *Methods Virol* **5**: 179–246, 1971.
7. Konopka K and Waskell L, Modification of trypsin-solubilized cytochrome b_5, apocytochrome b_5, and liposome-bound cytochrome b_5 by diethylpyrocarbonate. *Arch Biochem Biophys* **261**: 55–63, 1988.
8. Cinti DL and Ozols J, Binding of homogeneous cytochrome b_5 to rat liver microsomes. Effect of *N*-demethylation reactions. *Biochim Biophys Acta* **410**: 32–44, 1975.
9. Rogers MJ and Strittmatter P, Evidence for random distribution and translational movement of cytochrome b_5 in endoplasmic reticulum. *J Biol Chem* **249**: 895–900, 1974.
10. Takikawa H, Sugiyama Y and Kaplowitz N, Binding of bile acids by glutathione *S*-transferases from rat liver. *J Lipid Res* **27**: 955–966, 1986.
11. Caccuri AM, Aceto A, Piemonte F, Di Ilio C, Rosato N and Federici G, Interaction of hemin with placental glutathione transferase. *Eur J Biochem* **189**: 493–497, 1990.
12. Liem HH, Grasso JA, Vincent SH and Muller-Eberhard U, Protein-mediated efflux of heme from isolated rat liver mitochondria. *Biochem Biophys Res Commun* **167**: 528–534, 1990.
13. Vincent SH and Muller-Eberhard U, A protein of the Z class of liver cytosolic proteins in the rat preferentially binds heme. *J Biol Chem* **260**: 14521–14528, 1985.
14. Rose MY, Thompson RA, Light WR and Olson JS, Heme transfer between phospholipid membranes and uptake by apohemoglobin. *J Biol Chem* **260**: 6632–6640, 1985.
15. Cannon JB, Kuo F-S, Pasternack RF, Wong NM and Muller-Eberhard U, Kinetics of the interaction of hemin liposomes with heme binding proteins. *Biochemistry* **23**: 3715–3721, 1984.
16. Tipping E and Ketterer B, The influence of soluble binding proteins on lipophile transport and metabolism in hepatocytes. *Biochem J* **195**: 441–452, 1981.
17. Correia MA and Meyer UA, Apocytochrome P-450: Reconstitution of functional cytochrome with hemin *in vitro*. *Proc Natl Acad Sci USA* **72**: 400–404, 1975.

Biochemical Pharmacology, Vol. 42, No. 1, pp. 190–192, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Inhibitory action of brovincamine on catecholamine secretion from cultured bovine adrenal medullary cells: possible relation to its blocking action on Ca^{2+} channels

(*Received* 22 *January* 1991; *accepted* 4 *March* 1991)

Brovincamine has been developed as a new derivative of vincamine, which has been reported to reduce vascular resistance, leading to an increase in the cerebrovascular blood flow [1–3]. Brovincamine is therefore expected to be clinically effective in the treatment of cerebral and cardiac ischemic disorders. Although the pharmacological actions of brovincamine as well as vincamine have been studied, details of the mechanisms underlying these actions remain to be elucidated. Brovincamine has recently been shown to cause a dose-dependent relaxation of the K^+-induced contraction of pulmonary arterial segments, and furthermore found to decrease the duration of slow action potentials in the guinea-pig papillary muscle [4]. Brovincamine therefore seems to produce its vasodilating action through blockade of slow Ca^{2+} channels in cerebral and cardiac blood vessels, but it is still questionable whether the Ca^{2+} antagonistic action of this drug is observable in other types of cell and tissue.

It is well established that Ca^{2+} plays a critical role in the secretory mechanisms of various neurotransmitters and hormones and that catecholamine secretion is initiated by an elevation of the intracellular free Ca^{2+} concentration [5], resulting from the increase in extracellular free Ca^{2+} influx into the cells [6–8]. Catecholamine secretion is therefore a suitable index to reflect an alteration in Ca^{2+} transport across the plasma membrane. Thus, the primary culture of adrenal chromaffin cells is considered to be a useful system to test the effects of various compounds on the Ca^{2+} transport mechanism. To examine whether brovincamine has a blocking action on the Ca^{2+} channels the effects of this drug on both catecholamine secretion and Ca^{2+} influx were investigated in cultured bovine adrenal chromaffin cells.

Materials and Methods

Chromaffin cells were enzymatically prepared from fresh bovine adrenal medulla, plated on 24-well cluster plates at a density of 5×10^5 cells/well, and maintained for 3 or 4 days as previously reported [9].

Cells were washed with 1 mL of balanced salt solution [135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM glucose and 20 mM 4-(2-hydroxyethyl)-1-