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Co-purification of Microsomal Epoxide Hydrolase with the Warfarin-Sensitive Vitamin K_1 Oxide Reductase of the Vitamin K Cycle

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ABSTRACT. Vitamin K_1 oxide reductase activity has been partially purified from rat liver microsomes. A three-step procedure produced a preparation in which warfarin-sensitive vitamin K_1 oxide reductase activity was 118-fold enriched over the activity in intact rat liver microsomes. A major component of the multi-protein mixture was identified as a 50 kDa protein that strongly cross-reacts with antiserum prepared against homogeneous rat liver microsomal epoxide hydrolase. The reductase preparation also had a high level of epoxide hydrolase activity against two xenobiotic epoxide substrates. The K_m values for hydrolysis by the reductase preparation were similar to those for homogeneous microsomal epoxide hydrolase itself, and the specific hydrolase activities of the reductase preparation were 25–35% of the specific activities measured for the homogeneous hydrolase preparation. Antibodies prepared against homogeneous microsomal epoxide hydrolase inhibited up to 80% of reductase activity of the reductase preparation. Homogeneous microsomal epoxide hydrolase had no vitamin K_1 oxide reductase activity. This evidence suggests that microsomal epoxide hydrolase, or a protein that is very similar to it, is a major functional component of a multi-protein complex that is responsible for vitamin K_1 oxide reduction in rat liver microsomes. BIOCHEM PHARMACOL **55**;2:169–175, 1998.

KEY WORDS. epoxide hydrolase; vitamin K cycle; vitamin K_1 oxide; vitamin K_1 oxide reductase; microsomes

The role of microsomal epoxide hydrolase (EC 3.3.2.3; HYL1§ in the nomenclature system proposed by Beetham *et al.* [1]) in the metabolic detoxication of a wide variety of xenobiotic epoxides has been appreciated for over three decades [2]. Oxygenation of a variety of xenobiotic substrates by cytochrome P450 can result in the formation of electrophilic epoxides, whose covalent binding to cellular macromolecules may result in cytotoxicity, mutagenesis, or carcinogenesis. Because HYL1 converts many of these bioactivated epoxides to dihydrodiols, which are essentially nonreactive toward cellular macromolecules, this enzyme represents a major form of cellular protection against exogenously derived toxic or mutagenic metabolites [2–5].

Although the protective role of this enzyme against xenobiotic-derived reactive intermediates has received much scrutiny, relatively less is known about the participation of HYL1 in the metabolism of endogenous compounds. It has been suggested that HYL1 may play a role in the metabolism of steroid-derived epoxides, and two endogenously formed steroid epoxides, the 16,17 α- and β-oxides formed from estratrienol and androstadienone, have been shown to be good substrates for HYL1 [6]. However, these endogenous steroid epoxides have no known physiological function, and are present in cells in extremely small amounts, so that a major physiological role of this ubiquitous enzyme as a means of removing these metabolites is difficult to rationalize. A second physiological function for the HYL1 has been suggested to be that of a transport protein for bile acid derivatives. A taurocholate transporting protein on the sinusoidal hepatocyte plasma membrane was identified by antibodies that cross-reacted with HYL1. This protein has a molecular mass, N-terminal amino acid sequence, and proteolytic fragmentation pattern that were identical to those of HYL1 [7]. The transfection of HYL1 cDNA into hamster kidney fibroblasts resulted in no enhancement of bile acid uptake [8], but Madin-Darby canine kidney cells stably transfected with HYL1 cDNA do express an enhanced ability to take up bile acids [9].

Hydrolysis is an important metabolic conversion for several physiologically important epoxides produced by the oxygenation of endogenous precursors. EETs are produced by the cytochrome P450-catalyzed oxygenation of arachi-

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§ *Abbreviations:* ABO, allylbenzene 2′,3′-oxide; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; DIFP, diisopropylfluorophosphonate; EET, epoxyeicosatriene; HYL1, microsomal epoxide hydrolase; IgG, immunoglobulin G; STO, styrene 1′,2′-oxide; VK, vitamin K_1, phylloquinone; VKO, vitamin K_1 2,3-oxide; VKOR, vitamin K_1 2,3-oxide reductase.

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donic acid, and possess a diverse variety of endocrine-like functions. They are potent mediators of membrane permeability, ion transport, vasodilation, and pituitary hormone release [10–12]. However, while EETs are good substrates for the soluble epoxide hydrolase, an enzyme functionally similar but structurally distinct from HYL1, HYL1 has no hydrolytic activity against any of the EETs [12]. Leukotriene A_4, an epoxide derived from arachidonic acid via the lipoxygenase pathway, is a precursor of leukotriene C_4, a potent mediator of the inflammatory response. Leukotriene A_4 is subject to hydrolysis by a highly substrate-specific epoxide hydrolase, but HYL1 plays no role in the hydrolysis of this endogenous epoxide [13]. An endogenously derived epoxide metabolite of cholesterol, cholesterol 5,6-oxide, is formed *in vivo*, and has been suggested as a possible mediator of vascular damage. This epoxide is also hydrolyzed enzymatically by a specific epoxide hydrolase, but HYL1 has been shown to have no catalytic activity toward this compound [14,15].

VKO is formed during the VK-dependent carboxylation of glutamic acid in the post-translational modification of several clotting factors [16–18]. The so-called vitamin K cycle starts with the reduction of vitamin K_1 to its hydroquinone form. In the presence of molecular oxygen and carbon dioxide, the hydroquinone is converted to VKO, with concomitant selective γ-carboxylation of glutamyl residues present in precursors of vitamin K-activated proteins, including prothrombin and several plasma clotting factors. The active reduced vitamin K_1 cofactor is regenerated by enzymatic reduction of VKO [16, 19]. This reduction very efficiently utilizes dithiothreitol as a reductant *in vitro* [16, 19, 20], but it is thought that reduced thioredoxin may be the physiological cofactor for this reaction [21]. VKOR activity is inhibitable by warfarin, and disruption of the vitamin K cycle by warfarin is the basis of its pharmacological anticoagulant activity [22, 23]. While VKO is readily reduced, both *in vivo* and *in vitro*, it is not known to be hydrolyzed to a dihydrodiol by any epoxide hydrolase, including HYL1 [24].

The enzyme complex that forms the vitamin K cycle has been partially dissected, but all individual components have not been identified definitively, especially those components responsible for VKO reduction. VKO reduction and concomitant γ-carboxylation are known to be catalyzed by integral membrane proteins. The carboxylase has been purified successfully by affinity chromatography [20], but all attempts to purify the reductase as a single, homogeneous protein have failed. The failure of these numerous attempts to isolate a single protein responsible for VKO reduction has prompted the conjecture that a complex comprising multiple membrane proteins is required for this activity. We have presented a protocol for the partial purification of VKOR activity from rat liver microsomes, which results in the isolation and enrichment of several individual proteins that collectively reduce VKO in the presence of dithiothreitol *in vitro*. This reduction is inhibited by warfarin. Separation of these proteins on an SDS–polyacrylamide gel shows a major component of the mixture with an apparent molecular mass of 50 kDa. We present data here showing that this 50 kDa protein cross-reacts with polyvalent antiserum raised against homogeneous HYL1, which also has an apparent molecular mass of 50 kDa. We have demonstrated the hydrolytic activity of the VKOR complex toward two xenobiotic epoxide substrates. The presence of xenobiotic epoxide hydrolase activity in the VKOR complex, combined with the detection of a protein with a 50 kDa molecular mass that cross-reacts with anti-HYL1 antiserum, provides strong evidence that HYL1, or a protein that is structurally and functionally similar to it, is associated with the VKOR complex.

MATERIALS AND METHODS

Materials

CHAPS, DIFP, Tris, benzamidine, ammonium sulfate, glycerol, protein A-agarose, purified rabbit IgG, and vitamin K_1 were obtained from the Sigma Chemical Co. Asolectin phospholipid preparation was obtained from the Fluka Chemical Corp. Sepharose 6B and Sephadex G25 were obtained from Pharmacia Biotech Inc. Hydroxyapatite and reagents used for SDS–PAGE were obtained from Bio-Rad Laboratories. Antiserum to purified homogeneous rat liver HYL1 was raised in adult male rabbits, as described. [25].

Substrate Synthesis

Radiolabeled STO and ABO were prepared by reduction of precursor halohydrins with tritiated borohydride [5]. VKO was synthesized by peroxidation of VK, as described by Tischler *et al.* [26].

Enzyme Activity

Epoxide hydrolase activity of microsomal proteins toward two xenobiotic epoxide substrates, STO and ABO, was measured as previously described [5]. Radiolabeled diol products were separated by solvent extraction, and quantitated by liquid scintillation counting. Kinetic constants were determined by varying the substrate concentrations from 55 to 875 μM for STO, and 200 μM to 10 mM for ABO. Calculation of these constants was performed using a commercial statistics software package [27]. VKOR activity was determined as described [20], by measuring the dithiothreitol-dependent conversion of VKO to VK. The two compounds were separated by reverse-phase HPLC and quantitated by their absorbance at 254 nm. Incubations contained partially purified enzyme preparations, prepared as outlined below, or microsomes that were washed and resuspended in VKOR assay buffer (250 mM Sucrose, 10 mM Tris, 1 mM EDTA, 2 mM DIFP, pH 7.6). Warfarin inhibition of VKOR activity was determined by measuring the activity as described above, in the presence of warfarin dissolved in 10 μL dimethyl sulfoxide. Control incubations

TABLE 1. Partial purification of VKOR activity from rat liver microsomes

Fraction	Volume (mL)	Protein (mg)	Specific activity (U/mg)*	Total activity (U)*
Microsomes	18	176.4	0.14	24.7
$(NH_4)_2$ SO_4 Precipitate	6	46.8	0.52	24.3
Sepharose 6B eluate	10	6.0	3.9	23.4
Hydroxyapatite eluate	4.5	1.1	16.7	18.4

* One unit (U) catalyzes the reduction of 1 nmol VKO/min.

for these experiments also contained 10 μL dimethyl sulfoxide.

Partial Purification of VKOR Activity

Rat liver microsomes were suspended with a Dounce homogenizer in 1.5 vol. of buffer A (50 mM Tris, 1 M NaCl, 20% ethylene glycol, 5 mM benzamidine, 5 mM DIFP, 1% CHAPS, pH 7.85). All steps were carried out on ice, or at 4–7°. The suspension was centrifuged for 30 min at 100,000 × *g*, and $(NH_4)_2SO_4$ was added to the supernatant fraction to 43% saturation. The suspension was centrifuged at 10,000 × *g*, for 10 min, and the precipitate was dissolved in buffer B [0.1 M sodium phosphate, 20% glycerol, 10% $(NH_4)_2SO_4$, 0.4 mg/mL soybean phospholipid (Asolectin), 0.5% CHAPS, pH 7.85] at one-third the initial suspension volume. After centrifugation at 100,000 × *g*, the supernatant fraction was loaded onto a 70 × 3.5 cm Sepharose 6B column, equilibrated with buffer B. The void volume fraction, which contains the enzyme complex, was collected, made 0.75% in CHAPS, and desalted over Sephadex G25 equilibrated with buffer C (25 mM phosphate, 25 mM KCl, 20% glycerol, 0.2 mg/mL phospholipid, 0.75% CHAPS, pH 7.85). The void fraction was sonicated, filtered, and loaded onto a 6 × 1.5 cm hydroxyapatite column equilibrated in buffer C. VKOR activity was eluted with a 150 mL linear gradient of buffer C and buffer D (250 mM phosphate, 250 mM KCl, 20% glycerol, 0.2 mg/mL phospholipid, 0.75% CHAPS, pH 7.85). Active fractions were pooled and exchanged into buffer C by chromatography over Sephadex G25 as described above. This procedure results in a 118-fold purification of VKOR activity, as outlined in Table 1.

Purification of HYL1

HYL1 was purified from rat liver to apparent homogeneity, as determined by SDS–PAGE [28]. This preparation had a specific activity toward STO of 402 nmol styrene glycol formed/min/mg protein.

Immunoblotting

Proteins were separated by SDS–PAGE on 10% polyacrylamide gels, and transferred onto nitrocellulose membranes, as described [18]. Membrane-bound proteins were probed with IgG that was purified by protein A Sepharose chromatography of rabbit antiserum to pure rat HYL1. Detection of immune complex formation by peroxidase-linked second antibodies was carried out as described [18].

Antibody Inhibition of VKOR Activity

Ten micrograms of the purified VKOR complex, or 100 μg of suspended microsomes, was diluted in 500 μL assay buffer (see above). Control and immune IgG were reconstituted from the lyophilized state to 62.5 mg/mL in water. A total volume of 25 μL of reconstituted IgG solution, containing the amounts of immune IgG indicated in Fig. 3, was added, and the mixtures were incubated for 3 h on ice. Suspensions were then assayed for VKOR activity as described above.

Antibody Inhibition of ABO Hydrolase Activity

Enzyme complex was diluted in 500 μL VKOR assay buffer (see above) to a final protein concentration of 20 μg/mL. A total volume of 30 μL of reconstituted IgG solution, containing the amounts of immune IgG indicated in Fig. 4 was added, and the mixtures were incubated for 3 h on ice. Suspensions were then assayed for ABO hydrolase activity, as described above.

RESULTS

Table 1 shows the partial purification of VKOR activity from rat liver microsomes. The specific activity of the enzyme was enhanced 118-fold by this procedure. The VKOR activity of this preparation was inhibited 85% in the presence of 10 μM warfarin, and 92% in the presence of 25 μM warfarin, indicating that it indeed represents the VKOR activity of the vitamin K cycle. SDS–PAGE analysis of the eluted proteins shows a complex mixture, comprising a major protein component with apparent molecular mass of 50 kDa, and several other major components with lower molecular masses (Fig. 1). Obviously, VKOR activity is not purified as a single homogeneous protein, and attempts to further separate and isolate single proteins from the complex, whether by ion-exchange, hydrophobic, or dye affinity chromatography, have resulted in a complete loss of reductase activity upon recombination. When eluted fractions from the final hydroxyapatite column were probed with antibody to purified rat HYL1, positive immunostaining reactions were seen in those fractions with enzyme activity, and the degree of immuno-

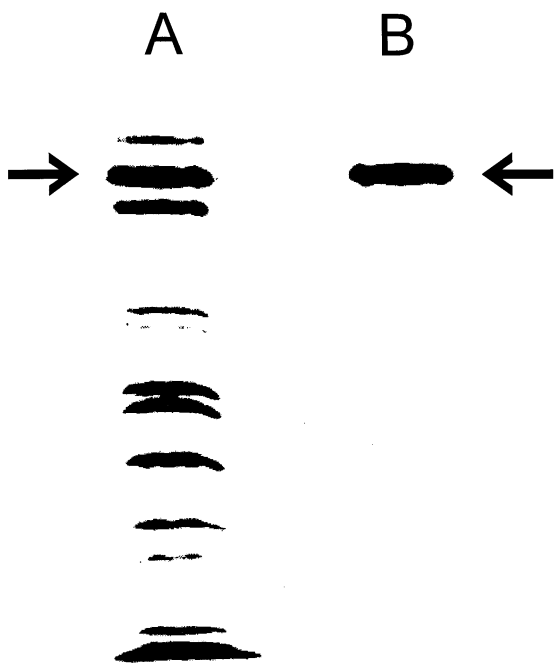

FIG. 1. SDS–PAGE of the partially purified VKOR complex. Lane A is a scanned image of a Coomassie blue-stained electrophoretogram of the combined high specific VKOR activity fractions obtained from the final hydroxyapatite column chromatography purification step. Lane B is a juxtaposed scanned image of a peroxidase-detected immunoblot obtained by reacting anti-HYL1 IgG with the same mixture of gel-separated proteins shown in lane A. The arrows indicate migration distance corresponding to proteins with a relative molecular mass of 50 kDa.

staining was proportional to the VKOR activity (Fig. 2). Epoxide hydrolase activity toward STO was also measured in the VKOR-containing fractions, and the elution profile of epoxide hydrolase activity corresponded exactly with the elution profile of VKOR activity from the hydroxyapatite column. HYL1 alone, separately purified to homogeneity from rat liver microsomes, had no detectable VKOR activity.

Epoxide hydrolase activity of the pooled VKOR complex fractions was investigated further using two different xenobiotic epoxide hydrolase substrates. Kinetic constants for the hydrolysis of STO and ABO by the partially purified VKOR were measured, and compared with those observed for a homogeneous HYL1 preparation. For ABO, a K_m of 805 μM was calculated for hydrolysis by the VKOR complex; in comparison, the K_m of a homogeneous HYL1 preparation for ABO was calculated as 868 μM. For hydrolysis of STO by the VKOR complex, a K_m of 65 μM was calculated. The K_m of the homogeneous HYL1 for STO hydrolysis was 177 μM. Comparison of the specific hydrolase activities, calculated as V_{max}, obtained for the VKOR complex and the homogeneous HYL1, showed specific activities for STO of 108 nmol/min/mg for the partially purified VKOR complex, and 402 nmol/min/mg for the homogeneous HYL1. Specific activities toward ABO of 718 nmol/min/mg for the VKOR complex and 2240 nmol/min/mg for the homogeneous HYL1 were calculated.

While the above data clearly indicate a copurification of HYL1 activity with the VKOR complex, they do not indicate whether or not HYL1 may play a functional role in the reduction of VKO. Such a role is suggested by data shown in Fig. 3, which shows that anti-HYL1 antibodies inhibited VKOR activity either in intact microsomal membranes or in the partially purified VKOR complex. The curve in panel A shows a concentration-dependent inhibition of VKOR activity upon prior addition of antibody to an incubation mixture containing intact microsomes. All incubations contained a total of 25 μL of reconstituted IgG solution, comprising an appropriate mixture of anti-HYL1 IgG and IgG prepared from non-immune serum. Panel B shows that anti-HYL1 also inhibited VKOR activity in the partially purified enzyme complex. Twenty-five microliters of anti-HYL1 IgG inhibited VKOR activity by about 80%, whereas 25 μL of control IgG had no effect on the enzyme activity. The anti-HYL1 antiserum had no effect on hydrolysis of STO by the homogeneous HYL1 preparation (data not shown). The inability of anti-HYL1 antiserum to inhibit hydrolase activity by homogeneous HYL1 is consistent with similar observations in several laboratories [29–31,*]. Of note is the fact that the antibody did inhibit epoxide hydrolase activity by the VKOR complex. Figure 4 shows a concentration-dependent inhibition of ABO hydrolase activity when increasing amounts of anti-HYL1 IgG were added to incubations containing the VKOR complex. An inhibition of 70% of the hydrolase activity was achieved at the highest antibody concentration (30 μL anti-HYL1 IgG).

DISCUSSION

The data presented clearly indicate a copurification of HYL1 activity with the VKOR complex. That HYL1, or a very similar protein, is a major component of the isolated mixture of proteins is evident from the relatively high abundance of the 50 kDa band on the SDS-polyacrylamide gel electrophoretogram, as well as from the high immunoreactivity of the 50 kDa protein with an anti-HYL1 antibody, as shown by the immunoblots in Figs. 1 and 2. The high relative abundance of HYL1 in this mixture of protein is also made evident by the relatively high specific hydrolase activity observed with the isolated protein mixture; on a per milligram of protein basis, the isolated mixture has a specific activity toward ABO that is approximately 32% that of purified homogeneous HYL1, and a specific activity toward STO that is approximately 27% that of purified homogeneous HYL1.

It appears that the hydrolase activity that copurifies with the VKOR complex is attributable to either HYL1, or to an enzyme that is structurally very similar to HYL1. Only one gene exists in the human or murine genome that codes for HYL1 [32, 33], and no post-translational modification of HYL1 is known to occur [2, 34]. Therefore, the existence of

*Guenthner TM, unpublished results.

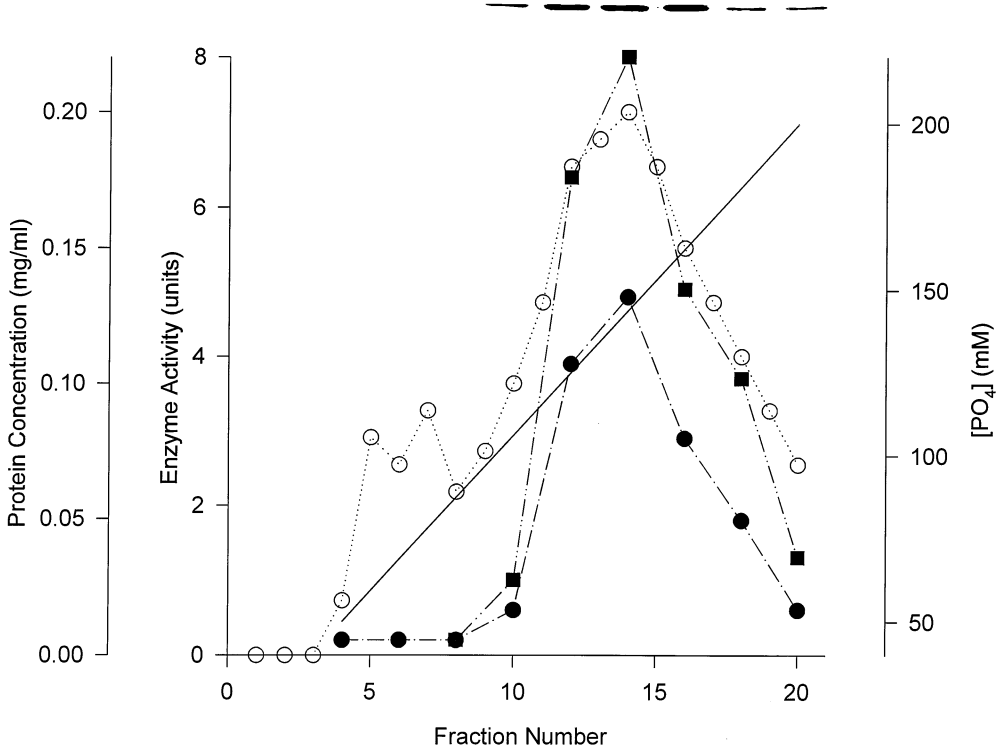

FIG. 2. Elution profile of proteins with high VKOR activity from a hydroxyapatite column. Following the final chromatography step described in the text, individual eluted fractions were assayed for VKOR activity, and subjected to SDS–Page and immunoblotting with anti-HYL1 antibodies, as described. The farthest left-hand axis indicates total protein concentration (○) and the second left-hand axis indicates enzyme activity. Both epoxide hydrolase (●) and VKOR (■) activities were plotted using this axis. One enzyme unit catalyzes the conversion of 1 nmol of substrate/min. The right-hand axis indicates phosphate concentration in the eluant, shown on the graph as a solid line. The bands shown above the graph indicate 50 kDa proteins detected by immunoblotting with anti-HYL1 antibody, and are placed directly above the corresponding eluted fractions indicated on the horizontal axis.

two different HYL1-like gene products with similar enzymatic activities, similar molecular weights, and sufficient structural similarity to provide antibody cross-reactivity appears to be less likely than the possibility that the protein we have identified is identical, or nearly so, to HYL1. Splice variants to HYL1 mRNA are known to exist in the rat [35], but the differences introduced are upstream from the coding region and appear to be related to tissue-dependent expression of identical enzyme proteins; no variations in the HYL1 coding sequence have been observed. Nevertheless, the possibility that the protein we have identified here is highly similar to, but not identical to, HYL1 cannot be ruled out by our data.

The question remains whether this protein plays a required role in the VKO reduction reaction, or whether it is simply fortuitously copurified with the reductase activity. Again, the relatively high abundance of this protein in the mixture suggests that it may be important. Furthermore, the fact that anti-HYL1 antibody inhibits VKOR activity is strong evidence that perturbation of this protein disrupts the enzyme complex, and that HYL1 plays an important role in VKO reduction. We also observed that anti-HYL1 antibody inhibits the hydrolytic activity of the VKOR complex; ABO hydrolase activity was inhibited by 70%. This is a novel finding, since several laboratories, including our own, have observed that anti-HYL1 antibodies are normally non-inhibitory to epoxide hydrolase activity, whether measured with purified HYL1 or with intact microsomes [29–31,*]. This observation suggests that if HYL1 is present as an integral component of the VKOR complex, it may be present in a form that has a very different conformation to that of the isolated HYL1.

A possible contribution of HYL1 to the VKOR complex may be as a VKO binding protein. HYL1 binds a variety of epoxides, and not all of them are hydrolyzed efficiently [4]. It is possible that VKO binds to the same epoxide binding site to which hydrolyzable epoxides bind, but that this site is altered sufficiently by its association with other proteins in the complex such that VKO is not hydrolyzed. However, the observation that the K_m values of the VKOR complex for hydrolysis of both ABO and STO are similar to that of purified homogeneous HYL1 suggests that the catalytic site for hydrolysis is not greatly altered in the multi-protein complex. Alternatively, the data may indicate the presence of a binding site for VKO that is different from the site to which xenobiotic epoxides bind. In this regard, it is also noteworthy that TCPO, an inhibitor of epoxide hydrolase activity in intact microsomes, does not inhibit VKOR activity in intact microsomes [24], suggesting that VKO may be binding to a site different from that which interacts

*Guenthner TM, unpublished results.

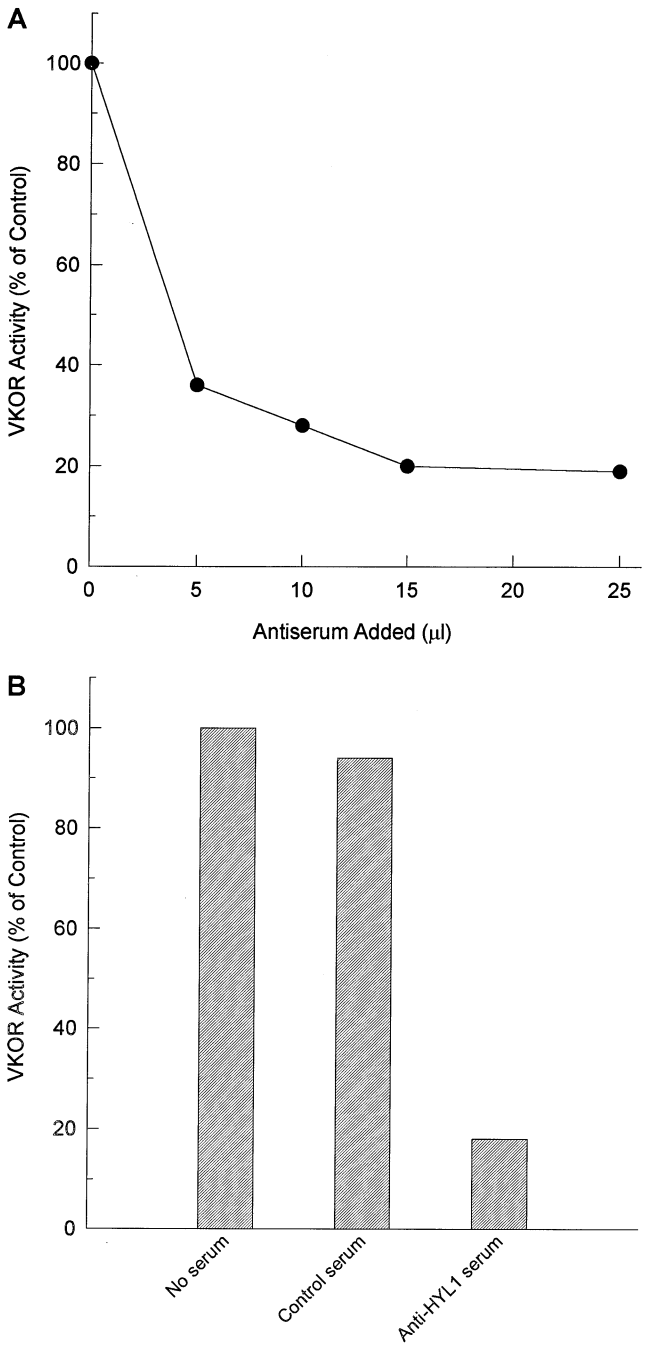

FIG. 3. Inhibition of VKOR activity by anti-HYL1 IgG. Panel A shows VKOR activity in intact microsomes following preincubation with varied proportions of anti-HYL1 IgG. Microsomes (100 μg protein) were preincubated for 3 hr at 4° with a total of 25 μL of reconstituted rabbit IgG solution (62.5 mg/mL), containing control IgG and indicated amounts of anti-HYL1 IgG. VKOR activity was then measured as described. The values are shown as percent of uninhibited activity (no antibody); values are means of duplicate determinations of enzyme activity. Panel B shows VKOR activity in the partially purified enzyme preparation (10 μg protein) following preincubation with either 25 μL of control IgG solution or 25 μL of immune IgG solution. Values are shown as a percent of uninhibited activity (no IgG); values are means of results of 3 separate antibody incubations. Uninhibited activities were 12 pmol/min/100 μg protein for microsomes, and 150 pmol/min/10 μg protein for purified enzyme preparation.

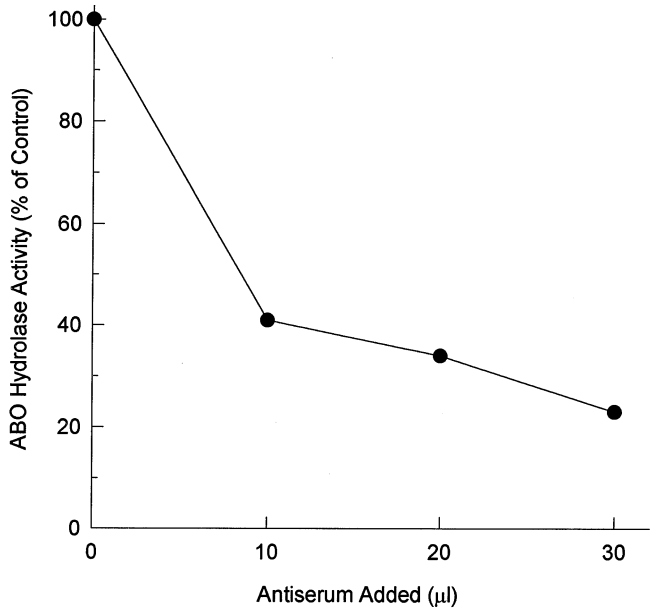

FIG. 4. Inhibition of ABO hydrolase activity in the purified VKOR complex by anti-HYL1 IgG. Enzyme preparations (10 μg protein) were preincubated for 3 hr at 4° with a total of 30μL reconstituted rabbit IgG solution (62.5 mg/mL), containing control IgG and indicated amounts of anti-HYL1 IgG. ABO hydrolase activity was then measured as described. The values are shown as percent of uninhibited activity (no antibody); values are means of duplicate determinations of enzyme activity. Uninhibited activity was 7 nmol/min/10 μg enzyme protein.

with TCPO. The site to which VKO binds could be a specific epoxide-binding site, or it could also conceivably be a non-specific hydrophobic binding site, since HYL1 has many hydrophobic domains.

In summary, we have shown the copurification of xenobiotic epoxide hydrolase activity with a multi-protein complex derived from liver microsomes that reduces VKO. This hydrolase activity corresponds to a protein that is structurally and catalytically indistinguishable from HYL1. If HYL1 is, in fact, an integral component of the VKOR complex, this activity would represent a clear example of a physiological role for HYL1, an enzyme that has been identified primarily with metabolism of exogenous compounds. Although the correlation of hydrolase activity with reductase activity is strong, definitive proof of the role of HYL1 in VKO reduction will require direct proof by reconstitution experiments. This has proven so far to be difficult, due to lack of recovery of reductase activity following further fractionation of the partially purified VKOR complex.

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