The Vitamin K-dependent Carboxylase Has Been Acquired by *Leptospira* Pathogens and Shows Altered Activity That Suggests a Role Other Than Protein Carboxylation*

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Leptospirosis is an emerging infectious disease whose pathology includes a hemorrhagic response, and sequencing of the *Leptospira interrogans* genome revealed an ortholog of the vitamin K-dependent (VKD) carboxylase as one of several hemostatic proteins present in the bacterium. Until now, the VKD carboxylase was known to be present only in the animal kingdom (i.e. metazoans that include mammals, fish, snails, and insects), and this restricted distribution and high sequence similarity between metazoan and *Leptospira* orthologs strongly suggests that *Leptospira* acquired the VKD carboxylase by horizontal gene transfer. In metazoans, the VKD carboxylase is bifunctional, acting as an epoxidase that oxygenates vitamin K to a strong base and a carboxylase that uses the base to carboxylate Glu residues in VKD proteins, rendering them active in hemostasis and other physiologies. In contrast, the *Leptospira* ortholog showed epoxidase but not detectable carboxylase activity and divergence in a region of identity in all known metazoan VKD carboxylases that is important to Glu interaction. Furthermore, although the mammalian carboxylase is regulated so that vitamin K epoxidation does not occur unless Glu substrate is present, the *Leptospira* VKD epoxidase showed unfettered epoxidation in the absence of Glu substrate. Finally, human VKD protein orthologs were not detected in the *L. interrogans* genome. The combined data, then, suggest that *Leptospira* exapted the metazoan VKD carboxylase for some use other than VKD protein carboxylation, such as using the strong vitamin K base to drive a new reaction or to promote oxidative damage or depleting vitamin K to indirectly inhibit host VKD protein carboxylation.

The vitamin K-dependent (VKD)* carboxylase converts Glu residues to γ-carboxylated Glu (or Gla) residues in VKD proteins. In mammals this post-translational modification is required for VKD protein activities in a wide range of physiologies that includes hemostasis, calcium homeostasis, and growth control (1). VKD protein carboxylation cannot occur in the absence of reduced vitamin K, as the carboxylase uses the energy of oxygenation of the reduced vitamin K to drive Glu carboxylation. Combined chemical modeling and biochemical studies indicate that the carboxylase deprotonates reduced vitamin K to generate a highly reactive vitamin K intermediate (*K*<sub>−</sub> in Fig. 1) with sufficient basicity (p*Kr ~25*) to deprotonate Glu (2). This Glu carbanion is then carboxylated to Gla by the addition of CO<sub>2</sub> while the vitamin K base is protonated to form the vitamin K epoxide product. The carboxylase, then, acts as both a vitamin K epoxidase and a Glu carboxylase and so is a bifunctional enzyme.

The carboxylase is characterized by complex interactions with VKD proteins. All mammalian VKD proteins contain a high affinity carboxylase-binding site, usually a propeptide that is cleaved subsequent to carboxylation, that targets them to the carboxylase. Propeptide tethering results in processive carboxylation so that multiple Glu residues in the Gla domains of VKD proteins are all converted to Gla residues (3, 4). Full carboxylation transforms the Gla domain into a calcium binding module that promotes binding of the VKD proteins to cell surfaces that contain exposed anionic phospholipids or to hydroxyapatite.

Carboxylation was first discovered in mammals; however, more recent studies show that carboxylase activity that depends upon vitamin K is also present in nonmammalian organisms, namely the marine snail *Conus* and *Drosophila* (5–8). *Conus* venom contains short VKD peptides that paralyze prey by antagonizing neurotransmission (5, 6, 9). The *Conus* VKD peptides and mammalian VKD proteins exhibit the same overall organization, i.e. they have a propeptide that is immediately upstream of the Gla domain. However, there is no obvious homology between *Conus* and mammals in either of these two domains. Nonetheless, interspecies cross-reactivity occurs: the *Conus* carboxylase can carboxylate a pentapeptide (FLEEL) derived from the mammalian Gla domain as well as a mammalian-derived propeptide-containing substrate (6). The *Drosophila* VKD carboxylase has also been shown to carboxylate FLEEL (7). *Drosophila* VKD proteins have not been identified, however, and so the functional consequences of VKD carboxylation in this organism are currently unknown. An important point regarding the *Conus* and *Drosophila* carboxylase orthologs is that in both cases they use vitamin K epoxidation to drive Glu carboxylation. Therefore, the fundamental role of vitamin K in these organisms is the same as that in mammals.

Until now, the VKD carboxylase was known to be present only in multicellular organisms. Although vitamin K is found in some bacteria and plants, it is used there in respiration or photosynthesis, respectively, and VKD proteins are not present in these organisms. It was therefore a surprise when a VKD carboxylase ortholog was observed in the bacterial
pathogen *Leptospira interrogans* (serovars Lai and Copenhageni) as a consequence of genome sequencing projects (10, 11). Pathogenic *Leptospira* species that include *L. interrogans* and *L. borgdorferi* cause leptospirosis, a global zoonotic disease characterized by the infection of a wide spectrum of animals and by the potential for severe morbidity and mortality in humans (12). The molecular mechanisms of leptospirosis are poorly understood due to the lack of a transformation method for studying the pathogenic strains (13). A notable phenotype of fulminating leptospirosis is the hemorrhagic response which, given the critical role of vitamin K in hemostasis, raised the question of whether the mammalian carboxylase in hemostasis, plays some role in the pathogenesis of this disease. We therefore expressed and analyzed the VKD carboxylase ortholog in *Leptospira* pathogenesis to determine whether the VKD carboxylase ortholog plays some role in the pathogenesis of this disease. We therefore expressed and analyzed the VKD carboxylase ortholog in *Leptospira* pathogenesis to determine whether the VKD carboxylase ortholog plays some role in the pathogenesis of this disease.

**EXPERIMENTAL PROCEDURES**

**Isolation and Expression of the L. borgdorferi VKD Carboxylase Ortholog ORF**—The VKD carboxylase ORF was isolated by PCR based on the genome sequence of the region from the *L. borgdorferi* serovar hardjo strain JB197. *L. borgdorferi* genomic DNA was used as template to amplify the ORF in two parts: a 5′-half using primers Lborg1S (CTGCAGCGATCGGTTCCGGTTATTATGCGA) and Lborg1AS (GGAGCATACTTCTGGAGATTTCAG) and two different 3′-halves that used primers Lborg2S (GGCACCTTGTGGTTTCCTCCTCCCCTCCCTCCGAGA) and either Lborg2AS (GGTACCCATTCTCTGCTTCCCCGAGAAAAC) or Lborg2NAS (TGCGGCCAGCTTCTGGTCTCCGGAAGAAAAC). The Lborg2S+Lborg2AS oligonucleotides amplify the 3′-ORF and stop codon, whereas the Lborg2S+Lborg2NAS oligonucleotides replace the stop codon with nine base pairs that encode 3 Alas and a NotI site. The three PCR products were separately cloned into pCR2.1-TOPO (Invitrogen) and sequenced. The correct 5′-PstI-BglIII product and 3′-BglIII-Acc65I product from the Lborg2S+Lborg2AS amplification were cloned into PstI-Acc65I-digested pBacPAK8 (Clontech). The resulting plasmid, Lepto.borg-pBacPAK8, was digested with Acc65I and BglIII, and the large fragment was ligated with the BglIII-NotI fragment from Lborg2S+Lborg2NAS and the annealed oligonucleotides NotFLAG (GGCGCTGACTACAAAAGCATGTACGACAAATGAG) and Acc65FLAG (GTACCCACTTGTGGTTCTGGAGATTTCAG) and Acc65I-NotI fragment (GTACCCACTTGTGGTTCTGGAGATTTCAG). The resulting plasmid, Lepto.borgFLAG-pBacPAK8, contains the *L. borgdorferi* VKD carboxylase ortholog ORF with 3 Alas followed by the flag epitope tag (DYKDDDDK), which was confirmed by resequencing the entire ORF. Both plasmids (Lepto.borg-pBacPAK8 and Lepto.borgFLAG-pBacPAK8) were used to generate baculovirus, as before, (14), and plaques were screened by a Western using anti-FLAG antibody or by activity assay. Preparative amounts of baculovirus were then generated by amplifying the virus in insect cells (15).

**Enzyme Assays**—Micromoles (4 mg/ml) were solubilized with CHAPS (0.3% final concentration) in the presence of 0.05% factor X propeptide, followed by centrifugation at 10,000 *g* for 1 h, at 4 °C. The supernatants were either assayed directly or were first purified on anti-FLAG agarose (Sigma). Supernatant (0.5–1 ml) was adsorbed to resin (100 μl) by overnight incubation at 4 °C, and the resin was then washed with 25 mM Tris, 0.25% CHAPS, 0.25% phosphatidyl choline, and 150 mM NaCl, pH 7.4. Enzyme was eluted by incubating the resin with FLAG peptide (100 μg/ml; Sigma) for 1 h at 20 °C. As indicated under “Results,” in one experiment resin-bound protein was assayed, rather than eluted protein, because the *Leptospira* ortholog was not expressed at very high levels and assaying the resin-bound material allowed more concentrated samples to be analyzed.

Carboxylase activity was assayed in reactions containing final concentrations of 0.6 mM ammonium sulfate, 0.06% CHAPS, 0.06% sodium cholate, 0.06% phosphatidyl choline, 1.1 mM [14CO2]NaHCO3, 3 mM dithiothreitol, 9 μM propeptide, 130 μM vitamin K hydroquinone, 45 mM BES, pH 6.6, and 2.5 mM substrate (FLEEL (Sigma), EEL (Bachem), or TxIX (Anaspec)). Reactions were quenched with trichloroacetic acid and then processed for scintillation counting as previously described (14). Epoxidase activity was assayed using the same reaction mixture except that the NaHCO3 was unlabeled. The epoxidase reactions were quenched by the addition of 2.5 volumes of 3:2:2-propanol:hexane. Samples were extracted and analyzed by high pressure liquid chromatography as before (16) except for the addition here of a standard (2 nmol K25, GL Synthesis) added after reaction quenching, which controlled for variation in vitamin K recovery during extraction. Vitamin K epoxide formation was quanitated either by direct integration of the peak using the high pressure liquid chromatography system or by scanning the chromatogram and creating TIFF files that were then analyzed by ImageQuant (Amersham Biosciences). Both methods were validated using a vitamin K epoxide standard curve. To determine the specific activity of the *Leptospira* ortholog, the amount of protein was quantitated using a fluorescence-based assay. *Leptospira* ortholog samples were gel electrophoresed along with a standard curve of human carboxylase of...
known concentration as well as a control FLAG-BAP fusion protein (Sigma). The gel was then processed in a Western using anti-FLAG antibody (0.4 μg/ml), doubly purified anti-rabbit alkaline phosphatase conjugate (Bio-Rad), and AttoPhos substrate (Promega, used as instructed) followed by quantitation using a StormImager.

RESULTS

Leptospira Appears to Have Acquired the VKD Carboxylase from Metazoans by Horizontal Gene Transfer—Protein-protein BLAST searches using the human VKD carboxylase protein sequence as the query against the genome of the pathogen L. interrogans (11) identified a predicted protein (GenBank™ accession number NP_713762.1) with a BLAST score of 140 and a probability of a match by random chance (the E value) of 10\(^{-34}\). Orthologs from two additional Leptospira pathogens were subsequently identified: the sequenced genome of L. interrogans serovar Copenhageni was shown to encode a protein (REFSEQ: accession NC_005823.1) identical to the L. interrogans serovar Lai ORF except for an N238K substitution (10), and a genome sequencing project for the pathogen L. borgpetersenii (AY974602) revealed the presence of a protein with 82% sequence identity to the L. interrogans ORFs (supplemental Figs. S1 and S2). A reciprocal BLAST search using the L. interrogans sero-

FIGURE 2. ClustalW alignment of the L. borgpetersenii VKD carboxylase ortholog with four metazoan carboxylases. Multiple carboxylases have been identified in some of the metazoan classes (e.g. mammals), but only one evolutionarily divergent representative has been used in each case to avoid biasing one class of metazoan carboxylases over another. Residues that are identical in all five proteins are highlighted in red, those identical in four of five proteins are highlighted in black, and chemically similar residues present in at least four proteins are highlighted in gray.
carboxylase ortholog whose activity is analyzed in this work. All of the VKD carboxylase orthologs in the shaded groups show high sequence homology throughout the molecule. Carboxylase orthologs are not found in the four other kingdoms or in many Eubacteria, despite the presence of a large number of sequenced genomes from these groups in the GenBank™ data base. This restricted distribution and high sequence homology between the Leptospira and metazoan VKD carboxylase orthologs suggest that Leptospira acquired metazoan VKD carboxylase sequences by horizontal transfer.

var Lai ORF as the query against the non-redundant protein data base identified VKD carboxylases in fish (Opsanus tau), a marine snail (Conus textile), insect (Drosophila melanogaster), and in several mammals, with E values ranging from $10^{-22}$ to $10^{-26}$. Notably, the sequence similarities extended over most of the length of the proteins (Fig. 2). In addition, protein structural predictions reinforced the similarity between the metazoan and Leptospira VKD orthologs, as their sequences all predicted similar membrane topologies (supplemental Fig. S2) (5–8, 17). VKD substrates for the carboxylase were not detected in the L. interrogans genome as no significant matches were found (E values $>0.06$) to the human VKD proteins factor VII, factor IX, protein Z, protein C, protein S, matrix Gla protein, osteocalcin, PRGP1, PRGP2, TMG3, TMG4, and Gas6.

The existence of the VKD carboxylase ortholog in Leptospira was quite surprising because previously VKD carboxylases were only known to be present in the animal kingdom (i.e. metazoans). The BLAST search with the L. interrogans ORF as the query also revealed VKD carboxylase orthologs in Cytophaga hutchinsonii and in environmental sequences (i.e. sequences isolated from shotgun sequencing of random bacterial samples from the Sargasso sea) (18). This occurrence in bacteria is low, as scores of bacterial genomes have now been sequenced and VKD carboxylase orthologs are not detected in these genomes. In addition, similar VKD carboxylase orthologs are not observed in fungi, plants, protozoa, or archea bacteria despite the presence of a large number of completely sequenced genomes from these kingdoms (Fig. 3). This restricted distribution and the high degree of homology between these bacterial and metazoan orthologs strongly suggest horizontal transfer of genetic information between species (19), with VKD carboxylase mRNA from a Leptospira-infected animal being incorporated into the bacterial genome. This interpretation is supported by the presence of several other sequences in the L. interrogans serovar Lai and Copenhagheni genomes that are similar to mammalian genes, i.e. platelet-activating factor acetylhydrolase (NP_712325 and YP_001728), two von Willebrand factor A domain proteins: batA (NP_714598 and YP_003432) and batB (NP_714599 and YP_003433) and paraoxonase 3 (NP_710580 and YP_000337) (10, 11). The evolution of these Leptospira proteins and their relationships to metazoan counterparts provides a unique opportunity to examine their structure and function. Therefore, the L. borgpetersenii VKD carboxylase ortholog was expressed and analyzed as a first step in the characterization of these proteins.

The Leptospira VKD Carboxylase Ortholog Is a Membrane-bound Protein—The Leptospira VKD carboxylase ortholog was produced in SF21 cells because these cells do not express endogenous carboxylase activity but can synthesize active enzyme when cDNAs encoding carboxylase activity (mammals, insects, and molluscs (6, 7, 20)) or have been shown to possess VKD hemostatic factors, which requires that the known VKD carboxylase ortholog must have carboxylase activity (fish (32)). Leptospira is also highlighted to indicate the presence of the VKD carboxylase ortholog whose activity is analyzed in this work. All of the VKD carboxylase orthologs in the shaded groups show high sequence homology throughout the molecule.

![FIGURE 3. Restricted distribution of VKD carboxylase orthologs in the kingdoms of life indicates horizontal transfer from metazoans to Leptospira. A rooted evolutionary tree based on homologies to the human L3 ribosomal protein was constructed as described in the supplemental data. The species highlighted with the shaded boxes have either been shown to contain VKD carboxylase orthologs with demonstrated in vitro carboxylase activity (mammals, insects, and molluscs (6, 7, 20)) or have been shown to possess VKD hemostatic factors, which requires that the known VKD carboxylase ortholog must have carboxylase activity (fish (32)). The tree based on homologies to the human L3 ribosomal protein was constructed as described in the supplemental data. The species highlighted with the shaded boxes have either been shown to contain VKD carboxylase orthologs with demonstrated in vitro carboxylase activity (mammals, insects, and molluscs (6, 7, 20)) or have been shown to possess VKD hemostatic factors, which requires that the known VKD carboxylase ortholog must have carboxylase activity (fish (32)). Leptospira is also highlighted to indicate the presence of the VKD carboxylase ortholog whose activity is analyzed in this work. All of the VKD carboxylase orthologs in the shaded groups show high sequence homology throughout the molecule. Carboxylase orthologs are not found in the four other kingdoms or in many Eubacteria, despite the presence of a large number of sequenced genomes from these groups in the GenBank™ data base. This restricted distribution and high sequence homology between the Leptospira and metazoan VKD carboxylase orthologs suggest that Leptospira acquired metazoan VKD carboxylase sequences by horizontal transfer.

![FIGURE 4. Expression of the Leptospira ortholog in SF21 cells. A, whole cell extract (WCE) prepared from baculovirus (Leptospira ortholog)-infected SF21 cells was centrifuged to separate cytoplasm (CYT) from microsomal (MICR) material. All three fractions were then analyzed in a Western using anti-FLAG antibody (0.4 µg/ml) against the FLAG-tagged Leptospira ortholog. A mock-infected sample (MOCK) was analyzed in parallel. B, microsomes from SF21 cells that were mock-infected or infected with baculoviruses containing human carboxylase (Hum) or the Leptospira ortholog (Lep) were detergent solubilized followed by centrifugation to remove unsolubilized material. Supernatants, either alone or mixed (Lep/Hum), were treated with endoglycosidase H (New England Biolabs) and then analyzed in a Western using anti-FLAG antibody, which showed that only the human carboxylase is glycosylated. The analysis also showed that detergent solubilization and centrifugation removed the lower molecular mass material observed in unsolubilized microsomes (i.e. MICR sample in panel A).]
Leptospira Vitamin K-dependent Epoxidase

Boxylases from several different species (mammals, *Conus*, and *Drosophila*) (6, 7, 20) are exogenously introduced. Baculoviruses containing the *Leptospira* ortholog with a C-terminal FLAG epitope was generated and used to infect SF21 cells, and the expression of this protein was monitored by Western analysis. Virtually all of the *Leptospira* ortholog was recovered in the microsomal fraction (Fig. 4A), which is the same result obtained with mammalian (bovine and human) VKD boxylases and is consistent with the hydrophobic sequences predicted by the *Leptospira* gene (supplemental Fig. S2). The gene also predicted a potential N-glycosylation site that would not result in glycosylation in the *Leptospira* bacterium but could conceivably be glycosylated during insect cell expression. We therefore tested for endoglycosidase H sensitivity. Treatment of human boxylase with endoglycosidase H resulted in a decrease in the size of the observed band (Fig. 4B), as expected. The *Leptospira* ortholog, however, was unaffected by endoglycosidase H treatment, indicating that N-glycosylation did not occur and therefore would not affect the function of the *Leptospira* ortholog expressed by insect cells.

**The Leptospira Ortholog Has Epoxidase Activity**—In all known VKD boxylases, the epoxidation of vitamin K hydroquinone provides the energy required for the carboxylation reaction, and so epoxidation is a prerequisite of carboxylation. We therefore tested whether the *Leptospira* ortholog could convert vitamin K hydroquinone to vitamin K epoxide. The results showed that the *Leptospira* ortholog, like the human VKD boxylase ortholog, had epoxidase activity that was dose-dependent (Fig. 5). Activity was not observed in mock-infected cells or in cells infected with an irrelevant virus (containing factor IX), showing that activity was specific to the *Leptospira* or human orthologs. The specific activity of the *Leptospira* epoxidase was compared with that of the human enzyme by performing epoxidase assays in parallel with protein determination by a quantitative Western. This analysis showed that the *Leptospira* ortholog specific activity was lower (18%, TABLE ONE) but still comparable with that of the human boxylase.

The human and *Leptospira* orthologs were both tagged at the C terminus with a FLAG epitope, which we showed did not have any effect upon their specific activities. Thus, we previously showed that FLAG-tagged and untagged human boxylases have the same specific activity (15) and in the current study performed similar analysis with an anti-C-terminal *Leptospira* ortholog antibody, which showed that the FLAG epitope did not change *Leptospira* activity (data not shown). Another condition that was tested for effect on *Leptospira* activity was pH value, because epoxidation is initiated by a catalytic base that requires deprotonation for reactivity (Fig. 1) and therefore is dependent upon pH values. Comparison of the activities of the *Leptospira* and human orthologs at several pH values showed that the response of activity to pH was similar for both enzymes (data not shown).

**Leptospira Vitamin K Epoxidation Does Not Result in Detectable Carboxylation**—Human boxylase and the *Leptospira* ortholog were assayed for carboxylase activity by measuring 14CO2 incorporation into peptide substrates, and epoxidase assays were carried out in parallel. Activity was measured using either solubilized microsomes or samples concentrated and purified on anti-FLAG antibody resin. When human boxylase was assayed using mammalian-derived substrates (EEL, FLEEL), the amount of epoxidation observed was almost identical to that of carboxylation (i.e. giving an epoxidation:carboxylation ratio of 1.1:2, TABLE TWO). These data for human boxylase were obtained with enzyme assayed in the presence of propeptide. However,

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**TABLE ONE**

The *Leptospira* VKD epoxidase specific activity is similar to that of the human ortholog

<table>
<thead>
<tr>
<th>Ortholog</th>
<th>Specific activity</th>
<th>pmol KO epoxide/pm mol enzyme/h</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td>1171</td>
<td>100</td>
</tr>
<tr>
<td><em>Leptospira</em></td>
<td></td>
<td>215</td>
<td>18</td>
</tr>
</tbody>
</table>

**TABLE TWO**

The *Leptospira* VKD enzyme does not show detectable carboxylase activity

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Ortholog</th>
<th>Substrate</th>
<th>Epoxidase activity</th>
<th>Carboxylase activity</th>
<th>Activity ratio (epoxidation:carboxylation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Human</td>
<td>EEL</td>
<td>4487</td>
<td>4250</td>
<td>1.1</td>
</tr>
<tr>
<td>2</td>
<td><em>Leptospira</em></td>
<td>EEL</td>
<td>218</td>
<td>4</td>
<td>&gt;55</td>
</tr>
<tr>
<td>3</td>
<td>Human</td>
<td>FLEEL</td>
<td>12870</td>
<td>10456</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td><em>Leptospira</em></td>
<td>FLEEL</td>
<td>380</td>
<td>4</td>
<td>&gt;95</td>
</tr>
<tr>
<td>5</td>
<td>Mock (−K)</td>
<td>FLEEL</td>
<td>ND</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Human</td>
<td>TxIX</td>
<td>1266</td>
<td>381</td>
<td>3.3</td>
</tr>
<tr>
<td>7</td>
<td><em>Leptospira</em></td>
<td>TxIX</td>
<td>600</td>
<td>4</td>
<td>&gt;150</td>
</tr>
</tbody>
</table>
this enzyme is regulated differently than the mammalian ortholog. The epoxidase activity of bovine VKD carboxylase has been shown to be regulated by VKD substrate such that very little epoxidation occurs when Glu substrate is absent (21). This feedback mechanism, which is not well understood, prevents the generation of an undesirable vitamin K base (K’ in Fig. 1) that could react with other molecules if Glu is not available for carboxylation. The lack of detectable carboxylase activity in the Leptospira ortholog (TABLE TWO) suggested the possibility that epoxidation and carboxylation are not coupled in this enzyme. We therefore tested this possibility by determining the amount of epoxidase activity in the presence or absence of VKD substrate. Human epoxidase activity was barely detectable when assayed in the absence of Glu substrate, and the presence of Glu substrate increased epoxidase activity 60-fold (Fig. 6). In contrast, the Leptospira enzyme showed considerable epoxidase activity in the absence of substrate and was unaffected by the presence of Glu substrate. Thus, the Leptospira enzyme shows a significant difference from the human enzyme in that a high level of epoxidation occurs in the absence of VKD substrate.

Comparison of the Leptospira VKD Enzyme with Metazoan VKD Carboxylases Shows Divergence in a Glu Binding Region—The sequence of the Leptospira VKD enzyme was aligned with metazoan VKD carboxylases that have been shown either to exhibit carboxylase activity (mammals, Drosophila, and Conus) or to contain VKD substrates (mammals, Conus, and bony fish), which implicates carboxylase activity. The metazoan VKD carboxylases all have a 12-amino acid region of complete identity (Fig. 2, residues 454–465) that has been indicated by mutagenesis studies to be important to Glu binding (24). Interestingly, the Leptospira VKD enzyme is divergent in this region, including divergence in 2 residues (Fig. 2, Leu-455 and Tyr-456) implicated as functionally important in the human VKD carboxylase. This loss of sequence identity and consequent possible loss of Glu binding may therefore explain why carboxylase activity was not detected with the Leptospira enzyme (TABLE TWO). Metazoan carboxylases also have a second site of VKD substrate interaction, i.e. the propeptide-binding site, but whether the Leptospira enzyme shares homology in this region is difficult to assess because this region is not well defined.

DISCUSSION

Leptospirosis results in multiorgan infection characterized by endothelial damage, inflammatory infiltrates, and hemorrhaging, and recent genome sequencing of the pathogen L. interrogans (11) revealed an ortholog of the VKD carboxylase, an enzyme previously observed only in multicellular organisms and known to be important to hemostasis. As described under “Results,” the Leptospira ortholog appears to have been acquired by horizontal transfer, and several lines of evidence strongly suggest that the ortholog is expressed and functions somehow in Leptospira physiology. The ortholog is a large ORF (503 amino acids) that is retained despite selective pressure, is part of an operon containing ORFs for three membrane proteins that includes one (drpA) known to be expressed in bacteria (25), is observed in at least two other Leptospira pathogens, and has an epoxidase specific activity similar to that of human VKD carboxylase (TABLE ONE), which indicates the retention of functional residues. In metazoans, the VKD carboxylase uses vitamin K epoxidation to carboxylate Glu residues in VKD proteins (Fig. 1), which renders them active. The function of the Leptospira ortholog, however, is unknown. We showed that Leptospira vitamin K epoxidation did not result in detectable Glu carboxylation (TABLE TWO) and was unregulated, i.e. occurring efficiently even in the absence of VKD substrate (Fig. 6). Alignment of the Leptospira enzyme with metazoan VKD carboxylases (mammals, Conus, Drosophila, and fish) indicated

FIGURE 6. Leptospira but not human VKD enzyme shows high levels of epoxidase activity in the absence of VKD substrate. Microsomes from SF21 cells infected with baculoviruses containing Leptospira (Lep) or human (Hum) VKD enzyme were solubilized, adsorbed to anti-FLAG antibody resin, and eluted with FLAG peptide as described under “Experimental Procedures” except for the absence of propeptide during isolation. The enzymes were then assayed for vitamin K epoxidation in cocktails that were identical except for the presence or absence of propeptide and FLEEL. Duplicate assays gave values within 12% of the mean.
Leptospira Vitamin K-dependent Epoxidase
deviation in a region of identity important for Glu-substrate interaction, and a BLAST search of the L. interrogans genome did not reveal the presence of any VKD substrates. The combined results, then, suggest that the Leptospira VKD enzyme has been adapted for some other role than VKD protein carboxylation.

The acquisition of the VKD carboxylase by a pathogen raises the question of whether this enzyme contributes to Leptospira pathology, and the properties of the acquired enzyme suggest several different mechanisms. One possibility is that the unfettered formation of the highly reactive intermediate (K− in Fig. 1) is being harnessed to cause oxidative damage to the vasculature to promote, for example, host invasion. Alternatively, the vitamin K base may have been adapted to drive a new, unknown reaction that generates a product contributing to Leptospira pathology. A third possibility is that the role of the Leptospira VKD epoxidase is in the consumption of vitamin K. Mammals depend upon a continual supply of dietary vitamin K, which is adsorbed from the gut and delivered through blood to tissues. Virtually all cells synthesize at least one VKD protein and therefore require vitamin K and carboxylation for function (1). Consequently, the location of Leptospira in blood (either circulating or present on the host cell surface and therefore interfacing with blood) could globally impact host VKD protein function by preventing vitamin K delivery to tissues.

A separate point with regard to the function of the VKD ortholog is whether the enzyme has been adapted for the same use in all microorganisms. As described under “Results,” VKD orthologs have also been observed in Cytophaga, which is a fish pathogen, and in environmental sequences that are derived from an unknown mixture of organisms that reside in an extreme environment (18). It will be of interest to determine whether these orthologs also have enzymatic activity and how the activity compares with that of the Leptospira VKD enzyme. A few bacteria have also been shown to contain a portion of the carboxylase significantly smaller than that observed in Leptospira, Cytophaga, and the environmental sequences, and these sequences have been proposed to comprise a transmembrane domain (26). Determining whether these predicted proteins possess enzymatic activity will also be of interest.

At present, the source of the vitamin K used in Leptospira VKD epoxidation is unknown. Some bacteria synthesize vitamin K, i.e. menaquinones. However, when we searched the L. interrogans genome for menA, the gene that encodes an enzyme (1,4-dihydroxy-2-naphthoate octaprenyltransferase) unique to and absolutely required for menaquinone biosynthesis (27), no menA Leptospira ortholog was detected. Another unknown is how the vitamin K becomes reduced, which is required for generating the active cofactor form (Fig. 1). The L. interrogans genome sequence indicated the presence of an ortholog of the mammalian vitamin K epoxide reductase (28, 29), and future studies that address whether this ortholog functions in concert with the Leptospira VKD enzyme, as it does in mammals, will be of interest.

The fact that the Leptospira ortholog has VKD epoxidase activity is of interest with regard to identifying vitamin K-interacting regions. Functional metazoan VKD carboxylase residues have been difficult to define because there is no structural information or homology to other proteins to provide clues to function. One unknown is the catalytic base that deprotonates reduced vitamin K to generate the highly reactive vitamin K intermediate (Fig. 1). Our recent studies indicate that the catalytic base is an activated amine rather than a Cys residue as earlier suggested by inhibitor studies: a human carboxylase mutant with all 10 Cys residues substituted by Ala is active, and the unusual N-ethylmaleimide reactivity of the mutant as well as the wild type enzyme implicates an amine (15).

Alignment of the Leptospira enzyme with the metazoan VKD carboxylases indicates only a limited number of shared amines, which should facilitate the identification of this critical residue. Another unknown is the location of the vitamin K-binding site. Deletion of 82 C-terminal residues of bovine carboxylase (i.e. all of the residues downstream of Pro-756 in Fig. 2) resulted in a decrease in both catalytic efficiency toward vitamin K hydroquinone and epoxidase activity, which led to the proposal that the vitamin K epoxidase domain may reside near the C terminus (30). However, this region is not present in the Leptospira enzyme (Fig. 2), indicating that most if not all of the epoxidase domain lies elsewhere. A likely location for the vitamin K epoxidase domain is the hydrophobic region at the N terminus (supplemental Fig. 52) because vitamin K is hydrophobic and therefore likely interacts with membrane-associated parts of the epoxidase, analogous to other integral membrane enzymes with hydrophobic substrates (31). There are several N-terminal regions of identity or similarity between the metazoan VKD carboxylases and the Leptospira enzyme, and so the Leptospira enzyme may be valuable in guiding structure-function analyses to identify the epoxidase domain. A final area where the Leptospira enzyme may be useful is in understanding how epoxidation and carboxylation are coupled in metazoan VKD carboxylases. Human carboxylase showed a substantial increase in epoxidation in the presence of VKD substrate, whereas the Leptospira enzyme was unaffected (Fig. 6); a comparison of the orthologs may help in defining the mechanism that links the two reactions in the metazoan VKD carboxylases.

REFERENCES
Leptospira Vitamin K-dependent Epoxidase


The Vitamin K-dependent Carboxylase Has Been Acquired by *Leptospira* Pathogens and Shows Altered Activity That Suggests a Role Other than Protein Carboxylation

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