Sulfhydryl Chemistry Detects Three Conformations of the Lipid Binding Region of *Escherichia coli* Pyruvate Oxidase[†]

Ying-Ying Chang*[,‡] and John E. Cronan, Jr.[‡,§]

Departments of Microbiology and Biochemistry, University of Illinois, Urbana, Illinois 61801

Received April 21, 1997[⊗]

ABSTRACT: Site-specific disulfide cross-linking experiments detected a conformational change within the C-terminal segment of *Escherichia coli* pyruvate oxidase (PoxB), a lipid-activated homotetrameric enzyme, upon substrate binding [Chang, Y.-Y., & Cronan, J. E., Jr. (1995) *J. Biol. Chem. 270*, 7896−7901]. The C-terminal lipid binding regions were cross-linked only in the presence of the substrate, pyruvate, and the thiamine pyrophosphate cofactor, indicating close proximity of a pair of C termini. We have now systematically substituted cysteine at 18 additional amino acid positions within the C-terminal region to obtain a panel of 21 proteins each having a single residue changed to cysteine. These proteins have been studied by disulfide cross-linking and by accessibility of the cysteine side chain to a variety of sulfhydryl agents. In the absence of pyruvate, the cysteine residues of the modified PoxB proteins failed to form disulfide bonds, generally failed to react with a large and rigid hydrophilic sulfhydryl reagent, 4-acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonic acid (IASD), and in some cases reacted weakly with a smaller more hydrophobic reagent, *N*-ethylmaleimide. Therefore, in this conformation, the C termini appear fixed in a rigid environment having limited exposure to solvent. In the presence of pyruvate, all of the C-terminal cysteine residues (except the two most distal from the C terminus) reacted with both sulfhydryl reagents and readily formed disulfide cross-linked species, indicating conversion to a structure having a high degree of conformational freedom. In the presence of lipid activators, Triton X-100 or dipalmitoylphosphatidylglycerol, a subset of the cysteine-substituted proteins no longer reacted with the membrane-impermeable IASD reagent, indicating penetration of these protein segments into the lipid micelles. For most of the proteins, similar extents of disulfide formation were seen upon addition of an oxidizing agent in the presence or absence of lipid activators. An exception was PoxB D560C which was much more readily cross-linked in the presence of lipid. Moreover, a subset of PoxB proteins that cross-linked to lower extents in the presence of lipids was found. The behavior of these proteins provides strong support for the model in which two C termini associate to form the functional lipid binding domain. These data are discussed in terms of three distinct PoxB conformers and the known crystal structure of a highly related protein.

Escherichia coli pyruvate oxidase (PoxB),[1] a soluble flavoprotein encoded by the *poxB* gene (Chang & Cronan, 1983, 1984; Grabau & Cronan, 1986a), consists of four identical 62 kDa subunits (Grabau & Cronan, 1986a; Gennis & Hager, 1976) having four active sites. PoxB catalyzes the decarboxylation of pyruvate to acetate plus CO_2 in a reaction requiring thiamine pyrophosphate (TPP) and results in the reduction of the tightly bound FAD moiety (Gennis & Hager, 1976). The enzyme stably interacts with phospholipid bilayers in the presence of the substrate, pyruvate, and TPP−Mg^{2+} and upon lipid binding undergoes a dramatic increase in enzyme activity. However, in the absence of pyruvate and TPP−Mg^{2+}, no association with lipids is seen; the lipid binding site is cryptic (Gennis & Hager 1976). Exposure of the PoxB lipid binding site involves a conformational change within the C-terminal region since this region becomes sensitive to specific cleavage by proteases. In the presence of both substrate and cofactors, pyruvate oxidase is specifically clipped by chymotrypsin between residues 549 and 550 to give fragments of 59 and 3 kDa (Recny et al., 1985). The cleaved enzyme has kinetic constants that mimic those of the lipid-activated form. Addition of pyruvate and TPP−Mg^{2+} results in reduction of the FAD moiety which in turn triggers the conformational change that exposes the lipid binding site (Russell et al., 1977a,b). Binding to phospholipid bilayers is essential for PoxB function in vivo, since ubiquinone, the natural electron acceptor of the enzyme, is dissolved within the membrane lipid bilayer (Koland et al., 1984). Moreover lipid binding increases the activity of the enzyme 10−20-fold and gives a 10-fold decrease in the Michealis constant for pyruvate (Gennis & Hager, 1976). Deletion of the C-terminal region and mutations within this region result in loss of lipid binding, indicating that the subunit C termini are the sites

[†] This work was supported by NIH Grant GM26156.

* Author to whom correspondence should be addressed: Department of Microbiology, University of Illinois, B103 Chemical and Life Sciences Laboratory, 601 S. Goodwin Ave., Urbana, IL 61801. Telephone: (217) 244-3466. Fax: (217) 244-6697. E-mail: yychang@pop.life.uiuc.edu.

[‡] Department of Microbiology.

[§] Department of Biochemistry.

[⊗] Abstract published in *Advance ACS Abstracts*, September 15, 1997.

[1] Abbreviations: PoxB, pyruvate oxidase; TPP, thiamine pyrophosphate; CuPHT, $CuSO_4$/1,10-phenanthroline; DTT_{ox}, oxidized dithiothreitol; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DDPC, didecanoylphosphatidylglycerol; NEM, *N*-ethylmaleimide; BMH, bis(maleimido)hexane; IASD, 4-acetamido-4′-[(iodoacetyl)amino]stilbene-2,2′-disulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

S0006-2960(97)00910-0 CCC: $14.00

of lipid binding (Grabau & Cronan, 1986b; Grabau et al., 1989). In previous work, we formed tetramers composed of mixtures of normal subunits and subunits lacking the C terminus and showed that a pair of C termini is required for lipid binding (Wang et al., 1991). We subsequently used site-specific disulfide cross-linking to study the C termini in greater detail (Chang & Cronan, 1995). Disulfide cross-linking of cysteine residues within the C-terminal lipid binding site was observed only when both pyruvate and TPP−Mg^{2+} were present. Moreover, the cross-linked species retained the ability to bind lipid, suggesting that a structure in which C termini are paired may be important in the lipid binding process (Chang & Cronan, 1995).

In the present paper, we have extended this approach and systematically substituted cysteine for 18 additional residues of the C terminus and used sulfhydryl chemistry to assay the conformations of the C termini. Several of these new constructs gave data which markedly strengthen the C-terminal pairing model of lipid binding and provide a view of the conformational changes within the C-terminal region of PoxB triggered by flavin reduction and the presence of lipid micelles.

MATERIALS AND METHODS

Strains, Media, and *Chemicals*. All bacterial strains were derivatives of *E. coli* K-12. The cysteine-substituted PoxB proteins were purified from strain CG3 (Δ*aceEF pfl-1 pps-1 poxB1 recA*) carrying the respective mutant plasmids. Other bacterial strains used for *ung*/*dut* site-directed mutagenesis and the unique site elimination method were the same as described previously (Chang & Cronan, 1995). $CuSO_4$ and 1,10-phenanthroline (CuPHT), *trans*-4,5-dihydroxy-1,2-dithiane (oxidized dithiothreitol, DTT_{ox}), *N*-ethylmaleimide (NEM), and other chemicals were obtained from Sigma Chemical Co. Bis(maleimido)hexane (BMH) was purchased from Pierce Chemical Co. 4-Acetamido-4′-[(iodoacetyl)-amino]stilbene-2,2′-disulfonic acid (IASD) was obtained from Molecular Probes Inc. Dipalmitoylphosphatidylcholine (DPPC) was from Sigma Chemical Co., whereas dipalmitoylphosphatidylglycerol (DPPG) and didecanoylphosphatidylglycerol (DDPG) were from Calbiochem. Oligonucleotide primers were synthesized by the University of Illinois Genetic Engineering Facility. The bacteriological media used in this study were described previously (Chang & Cronan, 1982).

Construction of Cysteine-Substituted PoxB Mutants. Plasmids pYYC170, pYYC173, and pYYC174 encoding D560C, V562C, and E564C oxidases were described previously (Chang & Cronan, 1995). The 18 remaining plasmids (pYYC186−pYYC203) were constructed with the *ung*/*dut* site-directed mutagenesis method (Kunkel et al., 1987). These plasmids were all derived from plasmid pYYC102 (Wang et al., 1991) which encodes the wild type oxidase. Plasmids pYYC186−pYYC195 which encode the PoxB proteins W570C, N569C, T568C, K567C, A566C, L565C, I563C, E561C, G559C, and R558C, respectively, were constructed by use of a series of 10 50-mer oligonucleotide primers of the same nucleotide sequence (5′-TA CCT TAG CCA GTT TGT TTT CGC CAG TTC GAT CAC TTC ATC ACC GCG TCC-3′) except that a cysteine anticodon, GCA, replaced one amino acid anticodon to be mutated (underlined) in each primer. The other mutagenic oligonucleotide primers used for mutagenesis were as follows: plasmid pYYC196 (A553C), 5′-TCCGCTGATGATGCAGCGCAGCATATA-3′; plasmid pYYC197 (L548C), 5′-GCGCAGCATATAGCAGCTGA-AACCTTT-3′; plasmid pYYC198 (A543C), 5′-GCTGAAAC-CTTTGCACTGTTCGAGTTT-3′; plasmid pYYC199 (I538C), 5′-CTGTTCGAGTTTGCACTGCGGTGGAAT-3′; plasmid pYYC200 (A533C), 5′-CTGCGGTGGAATGCATAACTCT-TCTTT-3′; plasmid pYYC201 (A528C), 5′-TAACTCT-TCTTTGCAGACCACCACATC-3′; plasmid pYYC202 (R572C), 5′-ATTCGAGCTCTAGCATAGCCAGTTTGT-3′; and plasmid pYYC203 (L571C), 5′-CGAGCTCTACCTG-CACCAGTTTGTTTTC-3′. The underlined bases are those giving the designed mutations.

The efficiency of the site-directed mutagenesis was generally 60−70%. The mutations in all plasmids were verified by DNA sequencing. Plasmid preparation and DNA sequencing were carried out with conventional methods (Sambrook et al., 1989).

Purification and Assay of Pyruvate Oxidase. We developed a rapid method for extracting the oxidase in a form suitable for cross-linking reactions. Cells from overnight cultures (0.2 mL) were harvested by centrifugation in microcentifuge tubes, and the pellets were suspended in 25 μL of 25 mM Tris-HCl buffer (pH 6.8) containing 10 mM EDTA and 4 mg/mL lysozyme. The suspensions were incubated at room temperature for 10 min and then frozen (−70 °C) and thawed. This cycle was repeated twice. To these suspensions was added 1 μL of 0.1 mg/mL DNAase I followed by incubation for 5 min at room temperature. The cell suspensions were then heated for 63 °C for 3 min and centrifuged at top speed in an Eppendorf microcentrifuge. The supernatant (6 μL) was carefully withdrawn. This volume of crude extracts was sufficient for detection of cross-linking by immunoblot analysis. This rapid cell lysis method was used to screen mutant candidates obtained from site-directed mutagenesis prior to DNA sequencing.

For more quantitative work, crude extracts were prepared by sonication of cells, and subsequently, the supernatants were heated at 63 °C for 3 min. This heat treatment removed most lipids and high-molecular mass proteins and gave a clean background on the gel in the molecular range of the oxidase dimers. The method for purification of the PoxB proteins was essentially as described previously (Chang & Cronan, 1995). Oxidase activity was assayed spectrophotometrically with $K_3Fe(CN)_6$ as the electron acceptor as described previously (Chang & Cronan, 1983).

Conditions for Sulfhydryl Chemistry. The conditions for cross-linking catalyzed by CuPHT were those used previously (Chang & Cronan, 1995). The concentration of CuPHT used was 0.2 mM $CuSO_4$ and 0.6 mM 1,10-phenanthroline, and the incubation time was 2 min at 37 °C. When cross-linking was catalyzed by DTT_{ox}, the crude extracts or purified proteins were mixed with 0.1 M sodium phosphate (pH 7.4), 0.1 M sodium pyruvate, 0.2 mM TPP, plus 10 mM $MgCl_2$ and incubated for 5 min at room temperature, and then DTT_{ox} was added to 0.1 M. The samples were then incubated for 1 h at 37 °C before analysis.

The IASD inhibition experiments (Figure 2) were peformed by addition of 1 mM IASD to the reaction mixture following the addition of substrate and cofactors as described above and incubation for 10 min at room temperature. After incubation, DTT_{ox} was added (to 0.1 M) followed by incubation for 1 h at 37 °C. Triton X-100 protection was

ensured by including 1% Triton X-100 in the reaction mixture before the addition of IASD.

The NEM inhibition experiments of latent forms of the cysteine-substituted PoxB proteins (Figure 1A) were performed by incubating purified PoxB (1.5 mg/mL) in the presence of TPP (0.2 mM) and $MgCl_2$ (10 mM) (with no pyruvate) and NEM (2 mM) for 10 min at room temperature. After incubation, 2-mercaptoethanol (3 mM) was added and the NEM and other solutes were removed either by dialysis or by a desalting column (2 mL KwikSep Excellulose column, Pierce Chemical Co.) equilibrated with 0.1 M sodium phosphate at pH 6.0. Dialysis was done by drop dialysis on a Millipore VSWP filter disk for 40 min against 0.1 M sodium phosphate at pH 6.0 and 0 °C. Aliquotes of the sample were then subjected to CuPHT-catalyzed oxidation in the presence of pyruvate and TPP−Mg^{2+} was described above. The IASD inhibition experiments (Figure 1B) were performed with the same protocol except that IASD (1 mM) was substituted for NEM. Following incubation, 2-mercaptoethanol was added to 14 mM and the samples were desalted on 5 mL D-Salt Polyacrylamide 6000 columns (Pierce Chemical Co.).

Electrospray mass spectrometry was used on the D560C and I563C oxidases. The conditions were similar to those of the IASD inhibition experiments of Figure 1B described above except in larger scale and in the presence of absence of pyruvate. Samples were either desalted on desalting columns equilibrated with 20 mM ammonium acetate or extensively dialyzed against a large volume of 20 mM ammonium acetate followed by lyophilization. Analysis was done on a VG Quatro mass spectrometer at the mass spectrometry facility of the University of Illinois at Urbana-Champaign.

When BMH was used as the cross-linking reagent, the incubation mixtures were the same as those described for DTT_{ox}-catalyzed cross-linking except that BMH (1 mM) replaced DTT_{ox} and the incubations were done at 37 °C for 5 min. Following incubation, an equal volume of a twice-concentrated reducing sample buffer containing 10% 2-mercaptoethanol (see below) was added and the samples were loaded on an SDS gel.

SDS−Polyacrylamide Gel Electrophoresis and Immunoblotting. The conditions for SDS−polyacrylamide gel electrophoresis were described previously (Chang & Cronan, 1995). After cross-linking, samples were withdrawn and an equal volume of NEM sample buffer (a sulfhydryl-quenching and nonreducing buffer) (Chang & Cronan, 1995) was added. For reduction of disulfides, the sample buffer contained 10% 2-mercapoethanol as described previously (Chang & Cronan, 1995). When the purified oxidase was used, the densities of the monomeric and dimeric protein bands were measured from dried gels stained with Coomassie blue. When the crude oxidase extracts were used, analysis was done by immunoblotting. The densities of monomeric and dimeric proteins on the membrane were measured by immersing the membrane in mineral oil which rendered the membrane semitransparent. Later, we found that the negatives of the photographs taken of the immunoblot membranes could be used to quantitate the protein band densities. The computer program used for integration of densities of protein bands was the GSXL program (Pharmacia, Inc.).

Immunoblotting was done by transferring proteins from a SDS−PAGE gel to an Immobilon-P membrane (0.45 μm, Millipore Corp.). The membrance was treated first with anti-PoxB antibody and then with goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Zymed Laboratories, Inc.). The protein bands were detected using 1-step 4-CN (Pierce Chemical Co.).

RESULTS

Experimental Background. Several points concerning our PoxB studies utilizing cross-linking between introduced cysteine residues should be noted. First, the extent of cross-linking was found to be insensitive to the PoxB concentration over a 16-fold range and was not inhibited by addition of a large molar excess of wild type PoxB (which contains no C-terminal cysteine residues). The extent of cross-linking was also unaffected by addition of a large excess of an unrelated protein containing an exposed sulfhydryl group, the reduced form of the acyl carrier protein (which has a covalently bound 4′-phosphopantotheine moiety). These data indicate that cross-linking is intramolecular; monomers within the same tetramer are cross-linked rather than monomers of different tetramers. Second, the disulfide bond is formed between the cysteine residues introduced into the C terminus rather than between the introduced cysteine and a native cysteine residue. As previously reported (Chang & Cronan, 1995), no cross-linking of wild type PoxB (which contains 10 native cysteine residues) was observed even under very strongly oxidizing conditions. Only a single native cysteine residue is accessible to the small sulfhydryl reagent *N*-ethylmaleimide (Koland & Gennis, 1982), and this residue is protected upon binding of TPP−Mg^{2+} (the cysteine lies next to a TPP-binding residue). We failed to detect cross-linking of a C-terminal cysteine to this (or any other) native cysteine residue. Our experimental approach utilized the quantitative and specific cleavage of either the activated or unactivated forms of PoxB by the chymotrypsin treatment (Recny et al., 1985). In the presence of substrate pyruvate and cofactor TPP−Mg^{2+}, this protease cleaves PoxB between Tyr449 and Met550 to give a 59 kDa species plus the 3 kDa C-terminal peptide (the α-peptide) (Recny & Hager, 1983; Recny et al., 1985). In the absence of TPP−Mg^{2+}, chymotrypsin cleaves PoxB within the unstructured TPP domain to give a large molecular species of 51 kDa and a small 11 kDa C-terminal peptide (the β-peptide) (Recny & Hager, 1983). If disulfides had formed between an engineered C-terminal cysteine and a native cysteine residue (all of which lie within the 59 kDa chymotrypsin fragment), then the 62 kDa species would be found only under nonreducing conditions upon analysis by SDS−PAGE. In the absence of reductant, the 3 kDa C-terminal peptide would remain bound to the 59 kDa fragment by the disulfide bond to give a species with an electrophoretic mobility resembling that of intact PoxB. However, in the presence of the reductant, the disulfide would be cleaved to give the 59 kDa species. In all cases examined, quantitative yields of the 59 kDa species were obtained in the presence or absence of reductant. Moreover, chymotrypsin treatment of the unactivated form of PoxB gave only the expected 51 kDa species for all of the oxidized cysteine-substituted PoxB proteins tested either in the presence or in the absence of reductant. No larger species were detected (data no shown). Therefore, consistent with our earlier results (Chang & Cronan, 1995), all cross-linked species can be attributed to reaction between the introduced cysteine residues. Finally, the cross-linking that

we observed is less than quantitative, generally in the range of 20−60%. Inefficient disulfide cross-linking between engineered cysteine residues has often been reported and is more the rule than the exception [e.g., Whitley et al. (1993) and Gozzo et al. (1996)]. Koshland and co-workers (Stoddard et al., 1992) explain this inefficiency with the low stability of the engineered disulfide bonds due to their less than ideal geometries and bond lengths together with the possibility of oxidative cleavage of the disulfide resulting from formation of the unstable sulfenic acid. Indeed, even non-native disulfide bonds between introduced cysteine residues chosen on the basis of crystal structure information can be difficult to form and unstable once formed (Villafranca et al., 1987). It seems that some of the inefficient disulfide cross-linking we observe could be due to instability since somewhat higher levels of cross-linking were observed when bis(maleimido)hexane, a reagent that forms stable bonds, was used to couple the cysteine residues. Moreover, it is possible that a portion of the PoxB molecules in our purified enzyme preparations may have lost the native structure and thus be unable to form cross-links. Indeed, the lipid binding ability of PoxB preparations is known to decline upon prolonged storage (Russell et al., 1977b), and we have noted that storage gave a similar decline in cross-linking efficiency.

It should also be noted that blockage of disulfide formation, our standard assay for cysteine modification by monofunctional sulfhydryl reagents, is not a direct measure of the extent of modification because a single modification is sufficient to block disulfide formation.

Properties of the Cysteine-Substituted Oxidases. Our panel of 21 proteins each with a single introduced cysteine (Materials and Methods) was assayed for enzymatic activity (Chang & Cronan, 1982) both in the absence of a lipid activator and following activation either by Triton X-100 addition or by α-chymotrypsin cleavage. All of these enzymes were indistinguishable from the wild type protein except the E561C, W570C, and R572C oxidases. These three enzymes showed lower activities when Triton X-100 was the activator. Addition of Triton X-100 to the wild type oxidase gives an activity higher (about 1.6-fold) than that given upon proteolytic activation with chymotrypsin. The E561C, W570C, and R572C oxidases had ratios of Triton X-100 activity to chymotrypsin activity of about 0.7. The possibility of secondary mutations was excluded by substitution of the sequenced fragment of the mutant gene for the equivalent fragment within the PoxB wild type gene (the DNA segment downstream of the *Bss*HII restriction site) which had no effect on the properties of these three oxidases which were due to the cysteine substitutions. The mutant proteins were screened by using immunoblot assays of crude extracts to find the more interesting proteins which were then produced in bulk and purified to allow more quantitative analyses.

Latent Conformation of PoxB. We designate the PoxB conformation observed in the absence of pyruvate and the presence of TPP−Mg^{2+} as the latent form. This conformer has a characteristic flavin spectrum (see Discussion) and lacks lipid binding ability. As noted with the two cysteine-substituted PoxB proteins studied previously (Chang & Cronan, 1995), the C termini of this conformer fail to form disulfide cross-links. This failure could be attributed to the low reactivity of the sulfhydryl groups or to the distance between the sulfhydryl groups (or both). To investigate the reactivity of the introduced cysteine residues, we tested the ability of the sulfhydryl reagent *N*-ethylmaleimide (NEM) to block disulfide formation upon subsequent addition of pyruvate and TPP−Mg^{2+}. We found that NEM blocked the formation of disulfides in most of the proteins (data not shown) except A553C PoxB (Figure 1A). However, we found that another reagent, IASD, was much less efficient in blocking disulfide formation of most of the PoxB proteins, indicating that access of this reagent to the sulfhydryl groups was more restricted (Figure 1B). We attribute the relative ineffectiveness of IASD to its larger effective size due to the greater molecular mass and the rigidity imparted by the stilbene moiety. An exception was PoxB D560C where both NEM and IASD readily blocked cross-linking. We used electrospray mass spectrometry to provide a second means of assaying IASD modification of two of these proteins (D560C and I563C) and found that although D560C PoxB was completely modified by IASD (giving an increase in molecular mass of 451 Da) only about 10% of the I563C PoxB monomers were converted to the higher-molecular mass IASD-modified species under the same conditions (data not shown).

These data indicate that the PoxB cysteine-substituted C termini are poorly accessible to a large reagent such as IASD, indicating that these residues are fixed to the protein surface. However, even the D560C oxidase which was readily accessible to IASD failed to form disulfide cross-links, indicating that the failure to form cross-links in the absence of pyruvate was due to the distance between the C termini or to the instability of the disulfides formed rather than to a lack of reactivity of the sulfhydryls (the thiolate anion is the active species in both the addition and oxidation reactions). To test these possibilities, we used the bifunctional cross-linker BMH to test if an extended (16 Å) cross-linker could span the distance between C termini. Cross-linking by BMH is readily distinguished from disulfide cross-linking because the Michael addition adducts formed by BMH are stable with respect to reductants. We found that BMH failed to cross-link the PoxB D560C C termini in the absence of pyruvate (BMH cross-linking was readily observed in the presence of pyruvate), and no reductant-insensitive cross-linking was observed over a range of BMH concentrations (the molar ratios of BMH to PoxB monomers ranged from 0.1 to 5). Like that of NEM, addition of BMH in the absence of pyruvate blocked subsequent disulfide formation upon addition of pyruvate. This inhibition indicated that at least one end of the reagent had reacted with a C-terminal cysteine. The partial nature of BMH inhibition of disulfide cross-linking seen at low BMH concentrations indicates that sulfhydryl groups remained available for reaction but failed to react with the remaining maleimide groups. We expect that, when one end of a BMH molecule has reacted and is thus tethered to the protein surface, the local effective concentration of maleimide near the tethering point should be very high. Therefore, at low BMH concentrations, we expect that an accessible sulfhydryl group on the protein surface should react much more readily (forming a reductant-insensitive cross-link) with the free end of a tethered molecule than with an unreacted BMH molecule recruited from solution. Our finding that no reductant-insensitive cross-linking is seen over a range of BMH concentrations indicates that the unreacted maleimide moieties of tethered

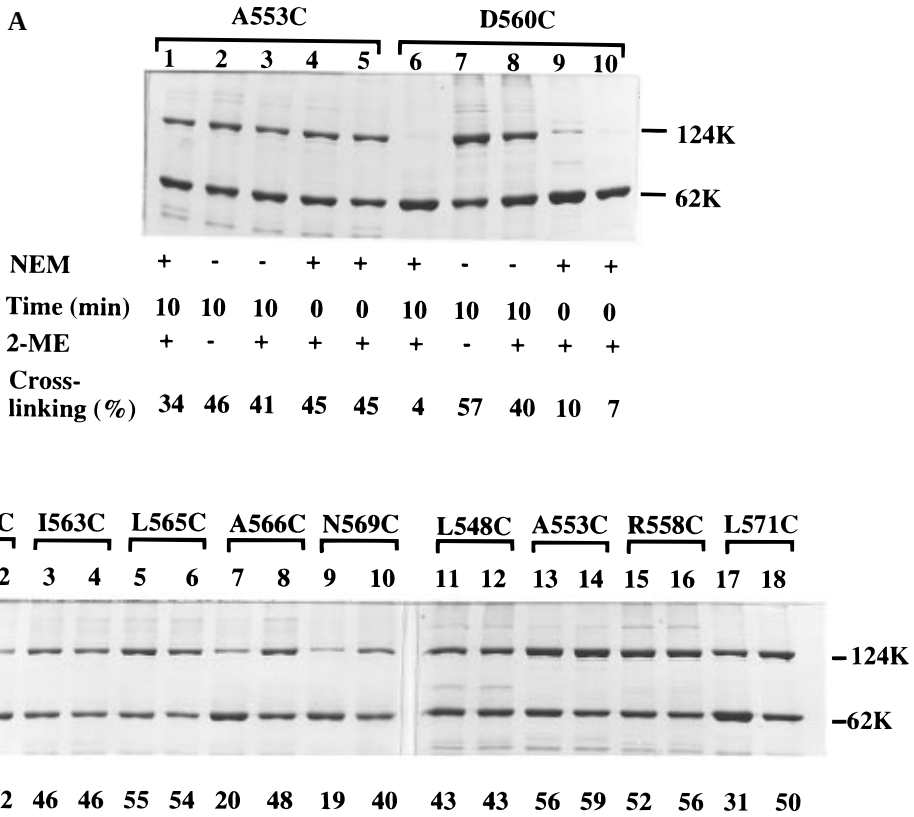

FIGURE 1: (A) Gel electrophoresis of cross-linking of the latent forms of the A553C PoxB and D560C oxidases after treatment with NEM. The purified PoxB proteins were incubated in the presence of TPP−Mg^{2+} (in the absence of pyruvate) plus NEM as described in Materials and Methods. Following addition of 2-mercaptoethanol (2-ME) to stop the modification reaction, the proteins were desalted (dialysis or a desalting column) and the desalted samples were then subjected to CuPHT-catalyzed oxidation in the presence of substrate and cofactors as described in Materials and Methods. SDS−PAGE was carried out, and the percentages of cross-linking were measured. In lanes 1−4 and 6−9, the samples were dialyzed, whereas in lanes 5 and 10, a desalting column was used. 62K denotes the 62 kDa PoxB monomer; 124K denotes the 124 kDa PoxB dimer. Reaction of D560C PoxB with NEM was sufficiently rapid that most of the cysteine residues were modified even in samples removed immediately after NEM addition (lanes 9 and 10). (B) Gel electrophoresis of cross-linking of the latent forms of nine cysteine-substituted PoxBs after treatment with IASD. The conditions were the same as in panel A except that IASD (1 mM) replaced NEM and all samples were desalted. In the odd-numbered lanes, the incubation time was 10 min, whereas in the even-numbered lanes, samples were taken immediately upon addition of IASD.

BMH molecules cannot reach a second C terminus. Moreover, since the Michael adducts formed by BMH are stable, this precludes the possibility that disulfides form, but are unstable.

Low-Activity Conformation of PoxB. Upon addition of pyruvate (and TPP−Mg^{2+}, if not already present) to PoxB, the flavin becomes reduced which triggers a conformation change. This low-activity conformer of the protein has a protease cleavage pattern distinct from that of the latent conformer and an exposed functional lipid binding site. The oxidase activity of this form is only a few percent of that of the lipid-activated form, and a 10-fold higher pyruvate concentration is required to reach maximal activity (Gennis & Hager, 1976). Our prior studies on two cysteine-substituted PoxBs showed that addition of pyruvate gave high levels of disulfide cross-linking of both proteins (Chang & Cronan, 1996). We now report that most of the cysteine-substituted PoxBs showed this same behavior, the only exceptions being those proteins having cysteine substitutions most distal from the C terminus, A528C and A533C (Table 1). PoxB A528C failed to cross-link, whereas the crosslinking of A533C was very inefficient. The facile cross-linking of the C-terminal cysteine residues downstream of this region indicates that this segment of the protein becomes highly mobile upon pyruvate addition. Moreover, these sulfhydryl groups were readily modified by IASD (Figure 2) and NEM (data not shown), indicating that the C termini do not form a tight complex prior to disulfide formation.

High-Activity Conformation of PoxB Where Disulfide Cross-Linking of Several PoxB Proteins Is Markedly Altered upon Addition of Lipid Activators. We tested all 21 cysteine-substituted PoxB proteins in immunoblot-crude extract experiments using DTT_{ox} as the oxidant rather than CuPHT (the extent and rate of disulfide formation are more readily controlled with DTT_{ox}). These studies were done both in the presence and in the absence of a lipid activator. Triton X-100 was generally used as a model lipid, since PoxB binds micelles of this detergent very tightly and this detergent is an excellent activator of enzyme activity (Blake et al., 1978). The extents of disulfide formation for most of the cysteine-substituted PoxB proteins were very similar upon addition of oxidant to the protein either in free solution or when bound to Triton X-100 micelles (Table 2). However, the G559C, L565C, A566C, K567C, and T568C PoxB proteins showed decreased cross-linking when oxidized while bound to Triton X-100 micelles. The largest decreases were seen with two consecutive residues, 566 and 567 (Table 2). In order to preclude the possibility that a decrease in disulfide formation was due to the inability of the oxidant to penetrate the Triton X-100 micelles, we mixed the Triton X-100 and DTT_{ox} in a chloroform/ethanol solution followed by evaporation of the solvent, dispersion of the mixture into buffer, and immediate

Table 1: Cross-Linking of Various Cys-Substituted PoxB Proteins[a]

PoxB	% cross-linking	
	CuPHT	DTT_{ox}
experiment 1		
R558C	46	43 (47)
C559C	46	51
D560C	48 (60)	43 (56)
E561C	32	40
V562C	56	53
I563C	53 (65)	57 (60)
E564C	41	44
L565C	48 (69)	61 (65)
A566C	46	55 (58)
K567C	46	57
T568C	48	54
N569C	46	52 (50)
W570C	46	50
L571C	47 (77)	54 (62)
R572C	45	42
experiment 2		
A553C	57	27 (20)
L548C	43	45 (45)
A543C	37	34
I538C	36	24
A533C	19	20
A528C	0	0

[a] The results of cross-linking catalyzed by CuPHT in experiment I were obtained means of immunoblot assays of crude extracts prepared by rapid cell lysis as described in Materials and Methods. The remainder of cross-linking results were from crude extracts prepared by sonication and subsequent heat treatment. The values in parentheses were obtained with purified enzyme preparations. Other experimental conditions are described in Materials and Methods. The oxidant used is given.

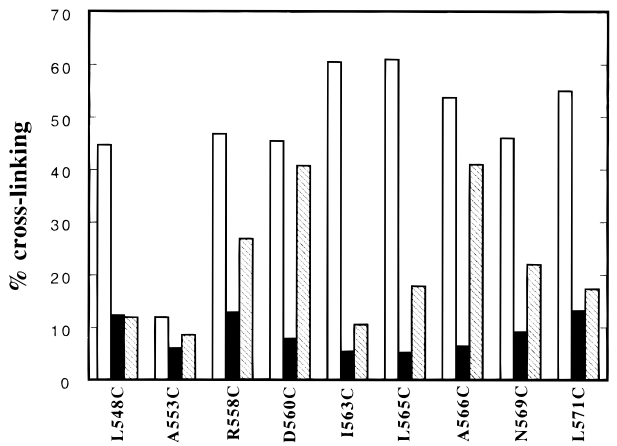

FIGURE 2: IASD inhibition of disulfide cross-linking of various Cys-substituted PoxB proteins and protection by Triton X-100. Each purified Cys-substituted PoxB protein (0.5 mg/mL) was incubated with pyruvate and TPP−Mg^{2+} as described in Materials and Methods excepto that IASD (1 mM) was added (filled columns) or omitted (open columns). The incubations of the stippled columns were done by adding 1% Triton X-100 (final concentration) prior to the addition of 1 mM IASD. DTT_{ox} was the oxidant, and the mixtures were incubated for 1 h at 37 °C. SDS−PAGE was carried out, and the percentates of cross-linking (averages of two experiments) were measured. Since the percentage of cross-linking of some of the Cys-substituted PoxB proteins was decreased in the presence of Triton X-100, all of the values in the stippled columns were normalized to the values as though Triton X-100 had not been added (see the text).

use. The results were very similar to those obtained upon adding DTT_{ox} to the preformed PoxB−micelle complexes, and therefore, these data cannot be explained by oxidant inaccessibility.

Table 2: Effect of Triton X-100 on Disulfide Cross-Linking[a]

amino acid residue	% cross-linked PoxB		
	no TX-100	with TX-100	ratio (±)
experiment 1			
R558C	42.2 ± 2.2	39.5 ± 1.0	0.94
G559C	40.9 ± 1.7	27.0 ± 2.9	0.66
D560C	42.3 ± 0.26	39.2 ± 2.2	0.93
E561C	35.8 ± 1.4	32.1 ± 2.6	0.90
V562C	42.6 ± 1.9	38.8 ± 1.5	0.91
I563C	45.0 ± 1.7	42.2 ± 2.1	0.94
E564C	26.4 ± 3.3	28.4 ± 2.5	1.08
L565C	35.3 ± 2.1	23.4 ± 1.2	0.66
A566C	44.3 ± 4.0	17.8 ± 0.9	0.40
K567C	47.5 ± 0.84	22.0 ± 2.2	0.46
T568C	42.9 ± 1.7	27.6 ± 1.3	0.64
N569C	48.6 ± 2.2	39.1 ± 1.8	0.80
W570C	42.0 ± 4.7	37.3 ± 5.2	0.89
L571C	50.1	36.4	0.73
R572C	37.0	34.4	0.93
experiment 2			
A553C	22.3	20.8	0.93
L548C	45.9	48.0	1.05
A543C	34.1	25.9	0.76
I538C	24.0	20.5	0.85
A533C	12.1	–	–
A528C	<1.0	–	–

[a] The cross-linking results were obtained from immunoblot experiments of crude extracts prepared from sonicated cells as described in Materials and Methods. The first column gives the results of cross-linking by DTT_{ox} done for 30 min in the absence of Triton X-100 (no TX-100), whereas the second column gives the results of DTT_{ox} cross-linking for 30 min in the presence of 0.8% Triton X-100 (with TX-100). The last column gives the ratio of the value obtained in the presence of Triton X-100 to that obtained in the absence of the detergent. Experiment 1 is the mean of three trials, whereas experiment 2 is the average of two trials. Other experimental conditions are described in Materials and Methods.

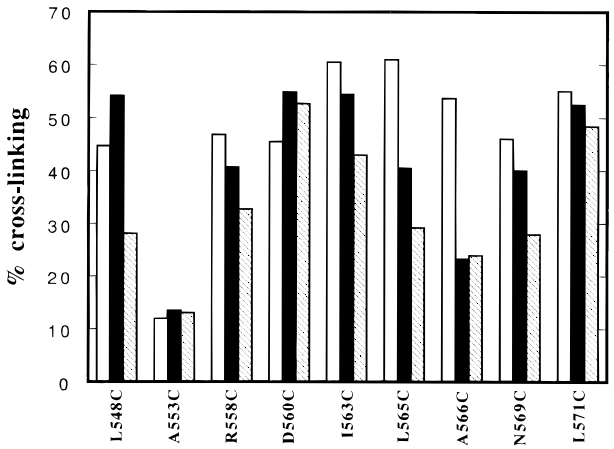

FIGURE 3: Effect of Triton X-100 and DPPG on cross-linking of various purified Cys-substituted PoxB proteins. Each purified Cys-substituted PoxB protein (0.5 mg/mL) was incubated with pyruvate and TPP−Mg^{2+} as described in Materials and Methods plus either with no addition to lipid activator (open columns) or after addition of Triton X-100 to 1% (filled columns) or after addition of DPPG to 1 mM (stippled columns). DTT_{ox} was then added as the oxidant, and the mixture was incubated for 1 h at 37 °C. Other experimental conditions were as in Figure 2.

The results obtained with purified samples of several proteins (Figure 3) were consistent with the results of the immunoblot−crude extract experiments. The presence of Triton X-100 gave decreased cross-linking of the purified L565C and A566C proteins compared to cross-linking reactions done in the absence of detergent. In addition, the presence of DPPG vesicles also inhibited cross-linking of

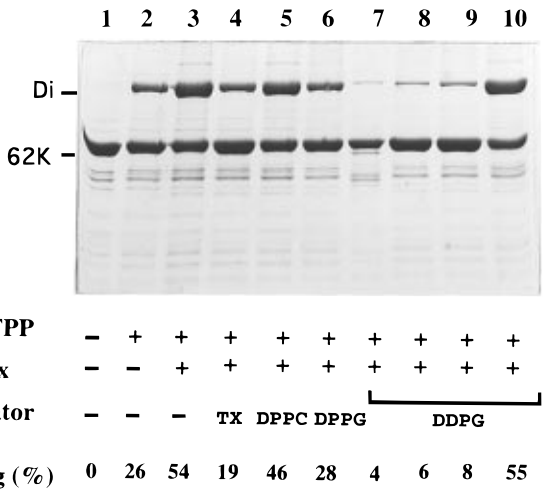

FIGURE 4: Gel electrophoresis of the cross-linking of purified A566C PoxB in the presence or absence of lipids. Purified A566C PoxB was incubated in the presence or absence of pyruvate (Pyr) and TPP−$MgCl_2$ (TPP) and subjected to DTT_{ox} oxidation as described in Materials and Methods. Triton X-100 (TX) or another lipid activator was added to the incubations (lanes 4−10) as shown. The final concentration of Triton X-100 was 2% (lane 4), whereas the final concentration of DPPC and DPPG was 1 mM (lanes 5 and 6). The concentrations of DDPG in lanes 7−10 were 1.25 mM, 0.125 mM, 62.5 μM, and 12.5 μM, respectively. Samples were analyzed by SDS−PAGE. The percentages of cross-linking were measured and are given. Di denotes the 124 kDa PoxB dimer.

this same subset of proteins upon subsequent addition of oxidant. As seen with Triton X-100, the L565C and A566C proteins showed the greatest effects (Figure 3). Since cross-linking of the A566C protein showed the greatest inhibition by lipid activators, we studied this protein in more detail (Figure 4). In the presence of pyruvate and TPP−Mg^{2+}, 54% cross-linking was seen, whereas upon addition of Triton X-100, only 19% of the protein was cross-linked (Figure 4). Moreover, addition of DPPG also resulted in decreased cross-linking of purified A566C PoxB (Figure 4). To test if lipid inhibition of cross-linking was reversible, we incubated the purified A566C oxidase with didecanoylphosphatidylglycerol (DDPG), a phospholipid having a high critical micelle concentration. At high concentrations, DDPG forms micelles, whereas in solutions with lower concentrations, the lipid is present as the monomer. At concentrations (1.25 mM to 62.5 μM) where DDPG forms a micelle, cross-linking of the A566C oxidase was blocked (lanes 7−9 of Figure 4). However, if the lipid concentration was decreased below the critical micelle concentration before addition of the oxidant, cross-linking was seen (lane 10 of Figure 4). Therefore, the decreased cross-linking of the A566C oxidase is reversible and can be attributed to interaction of PoxB with lipid bilayers rather than with discrete lipid molecules. It should be noted that DPPG is in the gel phase at the incubation temperature used in these experiments whereas DDPG would be in the liquid crystalline phase. Therefore, in agreement with earlier data (Blake et al., 1978; Chang & Cronan, 1984), the interactions of PoxB with lipid bilayers are insensitive to the physical state of the phospholipid acyl chains.

PoxB D560C, one of the first cysteine-substituted proteins we constructed (Chang & Cronan, 1995), was shown to cross-link efficiently either when bound to Triton X-100 micelles or in aqueous solution. Moreover, the cross-linked species formed in the absence of lipid activator retained the

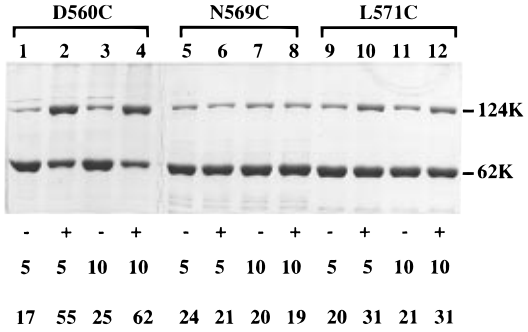

FIGURE 5: Gel electrophoresis of the cross-linking of Cys-substituted PoxB proteins formed by Triton X-100 in the absence of added oxidant. The purified D560C, N569C, and L571C PoxB oxidases were incubated in the presence of pyruvate and TPP−Mg^{2+} with or without Triton X-100 (TX-100) (1%) for either 5 or 10 min and then subjected to SDS−PAGE. Other experimental conditions were as in Figure 1.

ability to bind detergent micelles. Those reactions were carried out in the presence of externally added oxidizing agents and proceeded very rapidly. As a control in experiments described below, we examined a slower reaction, disulfide cross-linking in the absence of added oxidants (although atmospheric oxygen was present), and found that addition of Triton X-100 micelles or DPPG liposomes markedly stimulated spontaneous formation of disulfide-linked D560C PoxB (Figure 5). Indeed, it seemed that only minimally oxidizing conditions were necessary for formation of this disulfide, since degassing the solutions in an anaerobic hood gave only a small decrease in Triton-triggered cross-linking. Therefore, the paired C termini structure not only is compatible with lipid binding but also seems driven by lipid binding. We attribute the alterations in the efficiencies of disulfide cross-link formation of this subset of the cysteine-substituted proteins to alterations in the structure of the C termini resulting from the change in environment from the aqueous phase to a hydrocarbon-like phase. Depending on the residue involved, these alterations could either aid disulfide formation by giving a favorable geometry or inhibit cross-linking by giving an unfavorable geometry.

Protection of Cysteine Residues by Lipid Activators. We used the polar membrane-impermeable reagent, IASD, and the nine purified PoxB proteins to study interactions of the C termini with lipids. Reaction with IASD was assayed by inhibition of disulfide cross-linking by blocking the sulfhydryl groups with IASD, and we expected lipid vesicles to protect those C-terminal residues that reside within the nonpolar interior of the vesicles. Triton X-100 failed to block cross-linking of the two proteins having substitutions most distal from the C terminus, L548C and A553C, and thus, this protein segment does not seem to be in direct contact with the detergent micelle. Triton X-100 protected the sulfhydryl groups of the other cysteine-substituted PoxB proteins from IASD and therefore allowed various extents of disulfide formation (Figure 2). Triton X-100 almost completely protected the D560C protein from IASD (see below), and the A566C protein was similarly protected. Three other proteins, R588C, L565C, and N569C, showed intermediate levels of protection by Triton X-100, whereas I563C and L571C were not protected.

Additional controls were required in the IASD modification of those PoxB proteins having altered cross-linking behavior in the presencve of Triton X-100. IASD failed to

block disulfide formation when added to preformed PoxB D560C−Triton X-100 complexes but efficiently blocked disulfide formation when the reagent was dissolved together with Triton X-100 followed by micelle formation and addition of PoxB. Thus, the failure of IASD to block cross-linking when added to the PoxB D560C−Triton X-100 complex was due to a lack of accessibility of the sulfhydryl groups rather than to a failure of the reagent to compete with disulfide formation. In the case of those proteins that showed inhibition of cross-linking by Triton X-100, the control cross-linking reactions were carried out in the presence of Triton X-100 rather than in aqueous solution.

DISCUSSION

From the present studies and those reported earlier, it is clear that PoxB can exist in three different conformations depending on the presence of pyruvate and lipid vesicles.[2] It should be noted that extensive physical studies have failed to detect changes in the overall shape or size of PoxB for the various conformers (Raj et al., 1977; Stevens & Gennis, 1980; O'Brien et al., 1982), and thus, the structural changes seem to involve only a small and restricted portion of the protein. Indeed, the conformational changes seem largely localized to the C-terminal segment.

Our data obtained using sulfhydryl chemistry to probe the structure of the PoxB conformers can be readily summarized. In the latent conformer, the last 30 or so residues of the C termini are immobile and largely protected from IASD (and in some cases from NEM). In the low-activity conformer, the C termini become highly mobile and thus freely accessible to IASD and readily able to form disulfide cross-links. In the high-activity form, two C termini pair (as shown by the lipid-facilitated cross-linking of PoxB D560C) and enter the lipid bilayer which acts as a barrier to IASD and can alter the rate of disulfide formation.

We shall utilize the known 2.1 Å crystal structure of a *Lactobacillus* pyruvate oxidase (refered to as lacto-Pox) (Muller & Schulz, 1993; Muller et al., 1994) to model these data. Although lacto-Pox catalyzes a somewhat different reaction than PoxB, we previously argued (on the basis of amino acid sequence alignments and other data) that lacto-Pox provides a valid model for PoxB (Chang & Cronan, 1995). The appearance of the crystallographic data in the Brookhaven Protein Data Bank has allowed further analyses that strengthen this argument. The lacto-Pox quaternary structure is accurately described as a loose dimer of two tightly bonded catalytic dimers (Muller et al., 1994). The subunits that comprise the catalytic dimers are very strongly bonded and make up the active sites at the subunit interfaces (the TPP moiety is bound by both subunits of the catalytic dimer). In contrast, the association between two catalytic dimers giving the native tetrameric species is relatively loose. The quaternary structure of PoxB appears to be essentially the same as that of lacto-Pox. Virtually all of the regions where the amino acid sequences of PoxB and lacto-Pox fail to align are surface loops in lacto-Pox, and many of the hydrogen bond donor and acceptor pairs that stabilize lacto-Pox intersubunit interactions are conserved in PoxB. Moreover, pairs of hydrophobic residues involved in intersubunit interactions in lacto-Pox are also found as pairs of hydrophobic residues in PoxB, although the specific amino acids can be transposed (or differ) between the two sequences. The lacto-Pox quaternary structure places various restraints on interpretation of our PoxB data. For example, the monomer C termini are on opposite faces of the lacto-Pox catalytic dimers, and thus, the disulfide cross-linking of PoxB C termini we observe seems very unlikely to occur between the subunits of a catalytic dimer. Therefore, we believe that the cross-links are formed between subunits of two different catalytic dimers across the center of tetramer symmetry (i.e., between the monomers of the lacto-Pox crystallographic asymmetric dimer). Also, the subunit interfaces of lacto-Pox are extremely tightly packed (Muller et al., 1994), suggesting that intrasubunit movements are unlikely for PoxB. This is consistent with the lack of cooperativity seen for the PoxB enzymatic reaction (Wang et al., 1991) (each monomer acts as a separate kinetic entity) and the lack of alterations in the global structure of PoxB (see above). The only observed cooperativity is in binding a tetramer to lipid vesicles where two functional C termini are required (Wang et al., 1991).

Each lacto-Pox monomer is composed of four domains, an N-terminal core domain involved in subunit interactions plus binding of the TPP pyrimidine ring (Muller et al., 1993), a centrally located FAD binding domain, a domain that binds the bulk of the TPP cofactor (called the TPP domain), and a small C-termini "extension" of unknown function (Muller & Schulz, 1993; Muller et al., 1994). Each of the first three domains is conserved in PoxB. The only markedly different segments of the two proteins are within the PoxB region that we have studied, the C terminus where there is no sequence conservation with the lacto-Pox sequence downstream of the TPP domain. The lack of C-terminal similarity is expected since lacto-Pox lacks the lipid binding properties of PoxB. Given the divergence in sequence and function between PoxB and lacto-Pox, we are restricted to using the lacto-Pox structure as a scaffold upon which to place the C terminus of PoxB and as a set of constraints upon the possible interactions of the PoxB C termini. At the point where the lacto-Pox and PoxB sequences diverge (the end of the well-conserved TPP domain), the lacto-Pox C-terminal extension begins as an independently folded minidomain that is not tightly bound to the body of the protein. Indeed, although there is extensive hydrogen bonding within the extension (and also within the neighboring region of lacto-Pox), no hydrogen bonds link the extension with the body of the protein. If we conceptually excise the lacto-Pox extension and tether the PoxB terminus at the excision point, the tethering point is adjacent to and only about 20 Å removed from the active site. The lacto-Pox active site lies at the bottom of a "funnel" on the protein surface with only those portions of the FAD and TPP coenzymes involved in the chemical reactions being exposed to solvent (Muller et al., 1994). This "access funnel" arrangement appears to be characteristic of TPP enzymes (Muller et al., 1993), and thus, the active site of PoxB seems almost certain to have a structure very similar to that of lacto-Pox. The validity of using the lacto-Pox to model PoxB has been demonstrated by the recent work of others (Ibdah et al., 1996; Ott et al., 1996) who successfully used the lacto-Pox structure to model the active site of acetolactate synthase, an amino acid

[2] The loosely bound cofactor TPP (bound together with Mg^{2+}) is also required for activity; however, both components are abundant in vivo and for purposes of simplicity, TPP binding will be omitted from this discussion.

biosynthesis enzyme. Our prior work (Chang & Cronan, 1988) showed that the acetolactate synthases are derived from a PoxB-like protein, and this information plus the lacto-Pox structure provided a rational basis for altering the substrate and inhibitor specificities of two acetolactate synthases (Ibdah et al., 1996; Ott et al., 1996).

Model of the PoxB Conformational Changes. We will first discuss our picture of the process of the conversion of the latent form to the low-activity form. The latent conformation is found in the absence of pyruvate and is characterized by (i) the cryptic nature of the lipid binding site, (ii) a characteristic flavin absorption spectrum, and (iii) the inaccessibility of introduced cysteine residues to sulfhydryl reagents. These data indicate that in the latent form of PoxB the C-terminal region is tightly clamped to the protein surface.

The low-activity conformation is formed upon addition of pyruvate to the latent form in the absence of lipid vesicles. This conformer is characterized by (i) a high K_m for pyruvate, (ii) chymotrypsin cleavage between residues 449 and 450, (iii) exposure of the lipid binding site, and (iv) free accessibility of introduced cysteine residues as shown by reaction with sulfhydryl reagents and by disulfide formation. The trigger of this conformational change is reduction of the flavin which normally happens upon pyruvate addition but can be mimicked in the absence of pyruvate by chemical reduction of the flavin moiety. Flavin reduction seems to trigger a structural change within the FAD domain which we believe is then transmitted to the TPP domain and frees the C terminus of the protein. Transmission of the flavin domain conformational change to the TPP domain is indicated by the requirement for TPP$-Mg^{2+}$ to free the C terminus even when the flavin is reduced by chemical means (Russell et al., 1977a). Freeing of the C terminus is evident from the exposure of the protease clip site within this segment, the appearance of lipid binding ability, and the greatly increased C-terminal mobility we detected by sulfhydryl chemistry. The limits of this mobility were defined by those PoxB cysteine positions that failed to form disulfide cross-links. These positions were located at the extreme C-terminal end of the conserved TPP domain, a region that is highly structured and immobile in lacto-Pox (Muller et al., 1994).

Conversion of the low-activity form to the high-activity conformation results upon addition of appropriate lipid vesicles to the low-activity conformer, but can be mimicked by removal of the C terminus. Both conditions result in greatly increased enzyme activity and a similarly decreased K_m for pyruvate. In the case of the native protein, the low-activity conformer binds tightly to lipid vesicles and (if introduced cysteine residues and oxidant are present) disulfide bonds can be formed. The truncated protein lacks the ability to bind lipid. If pyruvate is removed from the truncated form and the flavin is reoxidized, the adsorption spectrum of the FAD moiety is found to differ from that of the latent conformer (Mather et al., 1982; Recny & Hager, 1983; Bertagnolli & Hager, 1991). This spectral change indicates that the flavin environment has changed from a hydrophobic pocket to one that is more open and hydrophilic in character (Mather et al., 1982; Recny & Hager, 1983; Bertagnolli & Hager, 1991).

The difference in the low- and high-activity conformers seems to be due to an inhibitory role played by the C terminus. This protein segment seems to directly hinder function of the PoxB active site since its removal converts the low-activity form to the high-activity form (Russell et al., 1977a,b). A similar activation is given by disulfide cross-linking of the C termini (Change & Cronan, 1995). We propose that lipid binding and disulfide cross-linking play the same role as truncation because all three processes provide a means of preventing association of the C terminus with the active site. Removal of the C terminus changes both the FAD environment (Mather et al., 1982; Recny & Hager, 1983; Bertagnolli & Hager, 1991) and changes the rate-limiting step of the catalytic cycle from that of flavin reduction to one of the steps prior to decarboxylation of pyruvate (Bertagnolli & Hager, 1991). These data are readily explained by direct or indirect interactions of the C terminus with the flavin ring system. Similar interactions of the C terminus with the pyruvate binding site could explain the higher concentrations of pyruvate needed to saturate the active site of the low-activity conformer. Thus, the C terminus might enter or block the funnel that gives access to the active site.

The constraints imposed by the detailed lacto-Pox structure and the data reported in this paper have defined our model of PoxB function. Our current model of the lipid activation process is one in which each PoxB subunit C terminus directly interacts with and alters the environment of the FAD moiety bound to that subunit. The C terminus is tethered sufficiently close to the active site that interaction seems to be a reasonable and straightforward explanation of our data and prior data. In the latent form of PoxB, the C terminus seems to be tightly bound to the protein surface as shown by the relative inaccessibility of the cysteine side chains. Upon binding pyruvate (and flavin reduction), there is a localized change within the FAD domain that frees the C terminus and allows interaction with (and inhibition of) the active site. In the presence of suitable lipid vesicles, the termini of the subunits of the crystallographic asymmetric dimer become paired (as demonstrated by the lipid-facilitated cross-linking of D560C PoxB) and the paired structure partitions into the lipid bilayer (as shown by lipid protection from IASD), thus releasing the active site from inhibition and activating the enzyme. If the lipid bilayer resides in a biological membrane, lipid binding also provides access for the active site funnel to ubiquinone, the mobile shuttle that carries electrons from PoxB to the electron transport chain. Therefore, in this model, the C terminus plays both a negative role in blocking the active site and a positive role in binding to lipid vesicles. Lipid binding cancels the inhibitory effect and provides access to the physiological electron acceptor, ubiquinone. The ambivalent role of the C terminus is illustrated by the in vivo behavior of PoxB mutant proteins lacking the lipid binding region (Grabau & Cronan, 1986; Grabau et al., 1989). Although these proteins have very high oxidase activities when assayed in vitro by using an artificial electron acceptor, the proteins are inactive in vivo because they lack access to ubiquinone. The physiological advantage of the ligand-triggered lipid binding properties of pyruvate oxidase is that the enzyme is membrane-bound only when intracellular pyruvate concentrations are high, and thus, PoxB competes for membrane binding with other such shuttle enzymes only when the enzyme is functioning.

REFERENCES

Bertagnolli, B. L., & Hager, L. P. (1991) *Biochemistry 30*, 8131−8137.

Blake, R., Hager, L. P., & Gennis, R. B. (1978) *J. Biol. Chem. 253*, 1963−1971.

Chang, Y. Y., & Cronan, J. E., Jr. (1982) *J. Bacteriol. 151*, 1279−1289.

Chang, Y. Y., & Cronan, J. E., Jr. (1983) *J. Bacteriol. 154*, 756−762.

Chang, Y. Y., & Cronan, J. E., Jr. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4348−4352.

Chang, Y. Y., & Cronan, J. E., Jr. (1988) *J. Biol. Chem. 263*, 3937−3945.

Chang, Y. Y., & Cronan, J. E., Jr. (1995) *J. Biol. Chem. 270*, 7896−7901.

Gennis, R. B., & Hager, L. P. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) Vol. 2, pp 493−504, Plenum, New York.

Grabau, C., & Cronan, J. E., Jr. (1986a) *Nucleic Acids Res. 14*, 5449−5460.

Grabau, C., & Cronan, J. E., Jr. (1986b) *Biochemistry 25*, 3748−3751.

Grabau, C., Chang, Y. Y., & Cronan, J. E., Jr. (1989) *J. Biol. Chem. 264*, 12510−12519.

Guzzo, A., Lee, M. H., Oda, K., & Walker, G. C. (1996) *J. Bacteriol. 178*, 7295−7303.

Ibdah, M., Bar-Ilan, A., Livnah, O., Schloss, J. V., Barak, Z., & Chipman, D. M. (1996) *Biochemistry 35*, 16282−16291.

Koland, J. G., & Gennis, R. B. (1982) *J. Biol. Chem. 257*, 6023−6027.

Koland, J. G., Miller, M. J., & Gennis, R. B. (1984) *Biochemistry 23*, 445−453.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.

Mather, M., Schopfer, L. M., Massey, V., & Gennis, B. (1982) *J. Biol. Chem. 257*, 12887−12892.

Muller, Y. A., & Schulz, G. E. (1993) *Science 259*, 965−967.

Muller, Y. A., Schumacher, G., Rudolph, R., & Schulz, G. E. (1994) *J. Mol. Biol. 237*, 315−335.

O'Brien, T. A., Shelton, E., Mather, M., & Gennis, R. B. (1982) *Biochim. Biophys. Acta 705*, 321−329.

Ott, K., Kwagh, J., Stockton, G. W., Sidorov, V., & Kakefuda, G. (1996) *J. Mol. Biol. 263*, 359−368.

Raj, T., Russell, P., Flygare, W. H., & Gennis, R. B. (1977) *Biochim. Biophys. Acta 481*, 42−49.

Recny, M. A., & Hager, L. P. (1983) *J. Biol. Chem. 258*, 5189−5195.

Recny, M. A., Grabau, C., Cronan, J. E., Jr. & Hager, L. P. (1985) *J. Biol. Chem. 260*, 14287−14291.

Russell, P., Hager, L. P., & Gennis, R. B. (1977a) *J. Biol. Chem. 252*, 7877−7882.

Russell, P., Schrock, H. L., & Gennis, R. B. (1977b) *J. Biol. Chem. 252*, 7883−7887.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.

Stevens, D. J., & Gennis, R. B. (1980) *J. Biol. Chem. 255*, 379−383.

Stoddard, B. L., Biu, J. D., & Koshland, D. E. (1992) *Biochemistry 31*, 11978−11983.

Villafranca, J. E., Howell, E. E., Oatley, S. J., Xuong, N. H., & Kraut, J. (1987) *Biochemistry 26*, 2182−2189.

Wang, A. Y., Chang, Y. Y., & Cronan, J. E., Jr. (1991) *J. Biol. Chem. 266*, 10959−10966.

Whitley, P., Nilsson, L., & von Heijne, G. (1993) *Biochemistry 32*, 8534−8539.

BI9709102