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In Vivo Function of *Escherichia coli* Pyruvate Oxidase Specifically Requires a Functional Lipid Binding Site†

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ABSTRACT: The pyruvate oxidase of *Escherichia coli* is a peripheral membrane flavoprotein that is dramatically activated by lipids. The enzyme strongly binds to phospholipid vesicles in vitro. In vivo, in addition to enzyme activation, binding is thought to be important to provide access of the enzyme to ubiquinone dissolved in the lipid bilayer. It was unclear if both or either of these attributes is needed for enzyme function in vivo. To differentiate between activation and lipid binding, we have constructed, using recombinant DNA techniques, a mutant gene that produces a truncated protein. The truncated protein lacks the last 24 amino acids of the C-terminus of the oxidase (due to introduction of a translation termination codon) and thus is closely analogous to the activated species produced in vitro by limited chymotrypsin cleavage [Recny, M. A., Grabau, C., Cronan, J. E., Jr., & Hager, L. P. (1985) *J. Biol. Chem. 260*, 14287-14291]. The truncated protein (like the protease-derived species) is fully active in vitro in the absence of lipid, and its activity is not further increased by addition of lipid activators. Moreover, the truncated enzyme fails to bind Triton X-114, a detergent that binds to and activates the wild-type oxidase. Strains producing the truncated protein were devoid of oxidase activity in vivo. This result indicates that binding to membrane lipids is specifically required for function of the oxidase in vivo; activation alone does not suffice.

We have chosen the lipid-activated enzyme *Escherichia coli* pyruvate oxidase as a model to study the activation of enzymes by lipid and the physiological importance of such interactions. Pyruvate oxidase is one of the better characterized lipid-activated enzymes. The oxidase, a peripheral membrane flavoprotein coupled to the electron-transport chain, catalyzes the conversion of pyruvate to acetate and CO_2 (Hager, 1957; Williams & Hager, 1966; Koland et al., 1984). The enzyme is composed of four identical subunits (M_r 62 000), each of which contains a tightly bound FAD[1] molecule and a loosely bound TPP[1] molecule (Koland et al., 1984; O'Brien et al., 1976; Grabau and Cronan, unpublished experiments). In vitro, the enzyme utilizes artificial electron acceptors such as ferricyanide. Pyruvate oxidase displays a dramatic activation by both monomeric and aggregated amphiphiles (Cunningham & Hager, 1971; Blake et al., 1978). The addition of any of a wide variety of lipids or detergents results in a 20-25-fold increase in the enzyme specific activity (Russell et al., 1977a,b; Recny & Hager, 1983) accompanied by a tight association of the activator with the protein (Russell et al., 1977b).

Pyruvate oxidase can also be activated by limited proteolysis of the enzyme when incubated with pyruvate and TPP. Proteolytic activation (the degree of activation is similar to that given by phospholipids) involves the clipping of a small M_r 2600 peptide from the C-terminus of the protein. Proteolytic treatment in the absence of pyruvate and TPP results in enzyme inactivation due to cleavage at a different site (producing a M_r ca. 51 000 subunit and a M_r ca. 11 000 peptide) (Russell et al., 1977a; Recny & Hager, 1983). Both activation phenomena are dependent on the presence of pyruvate and TPP. In the presence of substrate and cofactor, pyruvate oxidase undergoes a conformational change that exposes both the lipid binding site and the proteolytic cleavage site. The two activation phenomena are mutually exclusive.

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[1] Abbreviations: FAD, flavin adenine dinucleotide; TPP, thiamin pyrophosphate; kbp, kilobase pairs; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl.

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Activation by lipid protects against proteolytic cleavage, and moreover, the proteolytically activated oxidase can no longer bind to nor be further activated by lipid (Russell et al., 1977b). These characteristics are readily exploitable in the study of the activation of this enzyme by lipid and also strongly suggest that the C-terminus of the protein is involved in lipid binding.

Several mutants deficient in pyruvate oxidase activity in vivo have been described by Chang and Cronan (1983, 1984, 1986). Two of these mutants produce oxidases defective in both lipid binding and lipid activation. To test whether activation per se is sufficient for enzyme function in vivo, we have constructed, by recombinant DNA techniques, a mutant *poxB* gene that encodes a truncated protein analogous to the protease-activated oxidase made in vitro. This mutation results in the synthesis of an activated enzyme unable to bind lipid and thus allowed us to test the physiological roles of lipid binding vs. activation in vivo.

Experimental Procedures

Strain CY265 is a derivative of *E. coli* K-12 that carries a deletion of the *aceEF* (pyruvate dehydrogenase) genes (Chang & Cronan, 1983). Strain CG3 is a *recA poxB1* mutant derived from strain CY265, which also carries mutations in the *pps* and *pfl* loci (Grabau & Cronan, 1984). These strains were grown in rich broth (Grabau & Cronan, 1984) supplemented with 10 mM sodium acetate unless otherwise specified. The triphenyltetrazolium chloride containing medium was that of Bochner and Savageau (1977) containing 0.5% sodium pyruvate and 0.01% sodium acetate. The plasmid pCG7 (Grabau & Cronan, 1984) carries the wild-type *poxB* gene on a 3.2-kbp fragment inserted into plasmid pBR322. Strain JM103 and the M13 phage vector mp11 have been described by Messing (1983).

Recombinant DNA Techniques. Plasmid preparations, agarose gel electrophoresis, transformations, and handling of DNA were as previously described (Grabau & Cronan, 1984). DNA sequencing was by the chain termination method of Sanger et al. (1977, 1980). End labeling of DNA with [γ-^{32}P]ATP was by the method of Maxam and Gilbert (1980).

In Vitro Mutagenesis and Hybridization. The oligonucleotide 5′-GCCTGTAAATGCTGCG-3′ was synthesized with an Applied Biosystems 380 DNA synthesizer by the University of Illinois Genetic Engineering Facility and was purified from smaller contaminants by 20% polyacrylamide electrophoresis under denaturing conditions (Maniatis et al., 1982). The conditions for the annealing of the oligonucleotide to the single-stranded template (an mp11 derivative carrying the *poxB* gene) and for the elongation reactions were those of Gillam et al. (1979). The DNA was then used to transfect strain JM103, and the transformed cells were plated on RB agar.

The plaques (zones of infected cells) were transferred to nitrocellulose filters, and plaque hybridizations were carried out as described by Maniatis (1982) using the ^{32}P-labeled oligonucleotide as probe. Three different hybridization temperatures, 50, 55, and 60 °C, were used, corresponding to the calculated T_m (Meinkoth & Wahl, 1984) of the mutant, the T_m plus 5 °C, and the T_m plus 10 °C, respectively. Phage in those plaques that gave a positive signal at all three temperatures were grown in microtiter plates, transferred to nitrocellulose filters, and again hybridized to the probe.

Enzyme Purification. Strain CG3 carrying the plasmid pCG20 as the source of the mutant oxidase was grown in a broth medium supplemented with glycerol and 10 mM sodium acetate (Chang & Cronan, 1984). The enzyme was purified by the method of O'Brien et al. (1976) except that the affinity column was replaced by a second DEAE-cellulose column. Purified wild-type pyruvate oxidase was kindly provided by P. Porter and R. Gennis.

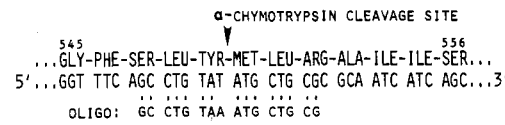

FIGURE 1: DNA sequence and deduced amino acid sequence of a portion of the *poxB* gene near the carboxyl terminus of pyruvate oxidase. The bottom line gives the sequence of the mutagenic oligonucleotide used to cause the T to A alteration at position 1647 (Recny et al., 1985).

Enzyme Analysis. The methods for extract preparation and the spectrophotometric enzyme assay were described by Chang and Cronan (1984). One unit of activity is 1 nmol of pyruvate decarboxylated/min. The assay for in vivo decarboxylation of pyruvate was also previously described (Chang & Cronan, 1984). Treatment of the purified protein with the detergent Triton X-114 was by the method of Bordier (1981) except that the sucrose cushion was omitted. The samples were analyzed by 10% SDS–polyacrylamide gels (Laemmli, 1970).

Results and Discussion

Oligonucleotide-directed mutagenesis of the *poxB* gene produced a single base change (T to A) at nucleotide 1647 (Figure 1). The change, verified by DNA sequence analysis of two isolates (not shown), generates a TAA translation termination codon in place of Tyr-549 (Recny et al., 1985). This mutation (allele *poxB6*) was expected to cause early termination of the protein, resulting in a truncated protein missing the last 24 amino acids of the protein. This base change was chosen because it would produce a protein closely analogous to the α-chymotrypsin-activated form of pyruvate oxidase, which lacks the last 23 amino acids. Despite the single amino acid difference we expected the truncated protein to have properties identical with those of the α-chymotrypsin-clipped form, since an essentially identical activation occurs upon clipping by other proteases (e.g., trypsin) expected to leave different termini (Russell et al., 1977a; Recny & Hager, 1983). Also, by alteration of this particular position within the gene, only a single base change was required to generate the same termination codon (TAA) used in the wild-type gene (Recny et al., 1985). The mutant *poxB6* gene was cloned on a 3.2-kbp *PstI* fragment into the *PstI* site of plasmid pBR322. A strain carrying the resulting plasmid, pCG20, was the source of the mutant enzyme and was also used in physiological experiments.

To show the mutant gene indeed encoded a truncated protein, SDS–acrylamide gel electrophoresis was performed on the purified mutant and wild-type pyruvate oxidases. The mutant subunit was distinctly smaller than the wild-type oxidase subunit (M_r 62 000) (Figure 2) and comigrated in other gel electrophoresis experiments (data not shown) with the α-chymotrypsin-activated form of the wild-type oxidase (M_r 59 400). Also, the protein encoded by the mutant gene in a maxicell system (Grabau & Cronan, 1984) (where only plasmid-encoded proteins become radioactively labeled) also migrated as though about M_r 2600 smaller than the protein encoded by the wild-type gene (data not shown).

Characterization of the Truncated Oxidase. Crude extracts of the *poxB1* strain CG3, harboring either plasmid pCG20 (*poxB6* allele) or plasmid pCG7 (wild-type allele), were prepared and assayed spectrophotometrically with ferricyanide. The level of pyruvate oxidase activity from cells harboring the *poxB6* plasmid was 4–5-fold greater than that of a strain carrying one chromosomal copy of the wild-type gene, whereas

Table I: Pyruvate Oxidase Activity of Crude Extracts of Various Strains

strain	chromosome	plasmid	crude extracts (units/mg of protein), no addition	lipid-depleted extracts[a] (units/mg of protein)		
				no addition	+α-chymotrypsin	+Triton X-100
CY256	*pox*$^+$		215	57	297	651
CG3	*poxB1*		<1	<1	<1	<1
CG3 (pCG7)	*poxB1*	*poxB*$^+$	1362	215	1756	4444
CG3 (pCG20)	*poxB1*	*poxB6*	922	2532	2294	2315

[a] The extracts were depleted of endogenous lipid by heating to 60 °C followed by centrifugation. The concentrations of activators were 25 μg/mL for α-chymotrypsin and 1% by volume for Triton X-100.

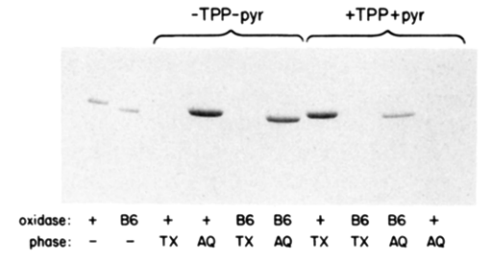

FIGURE 2: SDS–10% polyacrylamide gel electrophoresis of purified wild-type and mutant pyruvate oxidases. The first two lanes are wild-type (+) and mutant (B6) enzymes, respectively, untreated. The remaining lanes correspond to the phase into which the protein partitions (Experimental Procedures). Symbols: TX, Triton X-114 phase, and AQ, aqueous phase.

a 6-fold overproduction of activity was observed in the isogenic strain carrying the wild-type *poxB* plasmid (Table I). Removal of most of the endogenous lipids by heating the crude extracts to 60 °C resulted in >90% loss of the wild-type activity, which [as previously observed (Chang & Cronan, 1984)] could be restored by the addition of either the lipid activator Triton X-100 or the proteolytic activator α-chymotrypsin. In contrast, heat treatment of the *poxB6* mutant extracts did not affect the activity nor did the addition of Triton X-100 or α-chymotrypsin [the apparent increase in specific activity upon heating is due to the decrease (ca. 2.5-fold) in total extract protein]. These results indicated that the *poxB6* enzyme was produced in normal amounts but was in the activated conformation and was refractory to both α-chymotrypsin cleavage and lipid activation. It should be noted that the *poxB6* enzyme reduced the water-soluble ubiquinone UQ-1 and thus retained a functional ubiquinone reductase (Koland et al., 1984) activity (data not shown).

To study these interactions under more defined conditions, we purified the *poxB6* oxidase to >90% homogenity. Unfortunately, the *poxB6* oxidase was more labile during purification than either the wild-type oxidase or other mutant oxidases (O'Brien, 1976; Chang & Cronan, 1984, 1986); the final specific activities obtained were only about one-fourth that of the purified wild-type oxidase activated by chymotrypsin treatment. The lability of the truncated enzyme was not unexpected since the proteolytically activated wild-type oxidase was known to be unstable (Russell et al., 1977a; Recny & Hager, 1983), perhaps due to irreversible dissociation of flavin from the tetramer (Recny & Hager, 1983). Given the caveat of the low specific activity, the purified *poxB6* oxidase functioned exactly as expected from the experiments with crude extracts and the previous work on the protease-activated wild-type oxidase. Neither Triton X-100 nor α-chymotrypsin activated the *poxB6* oxidase (Table II), and the *poxB6* oxidase was completely unable to bind the Triton X-100 analogue Triton X-114.

Triton X-114 has the same structure as Triton X-100 but has a slightly longer population of poly(oxyethylene) chains than Triton X-100. This results in a useful phase separation. At 0–20 °C, aqueous solutions are homogeneous, but at 20 °C or above, a phase separation into an aqueous phase and a detergent phase occurs. Bordier (1981) has used this property as a means of separating hydrophobic and hydrophilic proteins. We used partition into the Triton X-114 detergent phase as a measure of detergent binding by the *poxB6* oxidase since the standard lipid binding assay (protection of the oxidase from α-chymotrypsin cleavage) was not possible. Partition of the purified proteins between the aqueous and Triton X-114 phases showed that the wild-type pyruvate oxidase partitioned into the aqueous phase upon phase separation (Figure 2). However, in the presence of pyruvate and TPP (conditions which cause the conformational change that exposes the lipid binding site) pyruvate oxidase quantitatively partitioned into the detergent phase. In contrast, the *poxB6* oxidase partitioned into the aqueous phase even in the presence of the substrate and cofactor, indicating that the enzyme lacked detergent-binding ability (reconstruction experiments indicated that >2.5% of the *poxB6* oxidase added would have been detected if present in the detergent). Similar results were found when crude extracts containing either the wild-type oxidase or the *poxB6* oxidase were examined by enzyme assays. Again, <2% of the *poxB6* oxidase activity partitioned into the detergent phase whereas 72–95% of the wild-type oxidase activity was found in the detergent phase.

Table II: Activities of the Purified Mutant and Wild-Type Oxidases[a]

activator	activity (units/mg of protein)	
	wild type	mutant
none	3670	12170
α-chymotrypsin	45870	13230
Triton X-100	142200	13580

[a] The concentrations of activators were as in Table I.

Table III: In Vivo Pyruvate Oxidase Activities of Various Strains[a]

strain	chromosome	plasmid	$^{14}CO_2$ released [nmol min^{-1} (10^9 cells)$^{-1}$]
CY265	*poxB*$^+$		20, 26
CG3	*poxB1*		4.6
CG3 (pCG7)	*poxB1*	*poxB*$^+$	160
CG3 (pCG20)	*poxB1*	*poxB6*	4.1

[a] The cells were grown in a minimal medium containing 0.4% succinate, 10 mM acetate, and 10 mM pyruvate and then assayed for release of $^{14}CO_2$ from [1-^{14}C]pyruvate as described under Experimental Procedures.

The poxB6 Oxidase Does Not Function in Vivo. We measured the ability of the *poxB6* enzyme to function in vivo by assaying $^{14}CO_2$ production from [1-^{14}C]pyruvate administered to intact cells (Table III). The plasmids were used to transform a bacterial strain blocked in all pyruvate utilization pathways. Introduction of the *poxB6* plasmid, pCG20, gave no detectable pyruvate oxidase activity in vivo (a rate of $^{14}CO_2$ release activity identical with that of the plasmid-free *poxB1* parental strain) whereas introduction of a plasmid

carrying the wild-type oxidase gave a much greater activity (Table III). A second indication of the lack of function of the *poxB6* enzyme in vivo was the inability of colonies growing on pyruvate indicator plates to reduce the tetrazolium indicator dye (all colonies remained white).

Conclusions

The truncated form of pyruvate oxidase fails to function in vivo. This finding indicates that activation is not sufficient for function in vivo. Since the properties of the truncated enzyme so closely resemble those of the lipid-activated enzyme (Russell et al., 1977a,b; Recny & Hager, 1983), it follows that lipid binding by the oxidase must play a role in addition to that of activation. It seems likely that lipid binding is essential for reoxidation of the flavin cofactor following pyruvate oxidation. The physiological electron acceptor for oxidation of the reduced flavin is ubiquinone 8, thought to be dissolved in the lipid bilayer of the bacterial inner membrane (Koland et al., 1984). A mutant oxidase lacking lipid binding ability therefore would have no access to the ubiquinone and thus becomes locked in the catalytically inactive reduced state.

The lack of in vivo activity of the truncated enzyme also argues strongly that the proteolytic activation observed in vitro has no physiological role. Indeed, Chang and Cronan (1984) found no enzyme the size of the protease-treated species present in extracts of wild-type cells. Our data indicate that the clipped protein would be stable in vivo and would copurify with the native protein. Thus, its absence from purified preparations of pyruvate oxidase constitutes further evidence that no protease-clipped protein is present in vivo.

Registry No. Triton X-114, 9036-19-5; Triton X-100, 9002-93-1; pyruvate oxidase, 9001-96-1.

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