Conversion of *Escherichia coli* pyruvate oxidase to an 'α-ketobutyrate oxidase'

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Escherichia coli pyruvate oxidase (PoxB), a lipid-activated homotetrameric enzyme, is active on both pyruvate and 2-oxobutanoate ('α-ketobutyrate'), although pyruvate is the favoured substrate. By localized random mutagenesis of residues chosen on the basis of a modelled active site, we obtained several PoxB enzymes that had a markedly decreased activity with the natural substrate, pyruvate, but retained full activity with 2-oxobutanoate. In each of these mutant proteins Val-380 had been replaced with a smaller residue, namely alanine, glycine or serine. One of these, PoxB V380A/L253F, was shown to lack detectable pyruvate oxidase activity *in vivo*; this protein was purified, studied and found to have a 6-fold increase in K_m for pyruvate and a 10-fold lower V_{max} with this substrate. In contrast, the mutant had essentially normal kinetic constants with 2-oxobutanoate. The altered substrate specificity was reflected in a decreased rate of pyruvate binding to the latent conformer of the mutant protein owing to the V380A mutation. The L253F mutation alone had no effect on PoxB activity, although it increased the activity of proteins carrying substitutions at residue 380, as it did that of the wild-type protein. The properties of the V380A/L253F protein provide new insights into the mode of substrate binding and the unusual activation properties of this enzyme.

Key words: acetolactate synthases, active-site mutagenesis, substrate specificity.

INTRODUCTION

Escherichia coli pyruvate oxidase (PoxB) is a peripheral membrane flavoprotein that catalyses the decarboxylation of pyruvate to acetate and CO_2 [1]. (The enzyme is a dehydrogenase and is misnamed.) The decarboxylation reaction requires thiamine pyrophosphate (TPP) bound to PoxB through a Mg^{2+} ion. The enzyme is a homotetramer consisting of four copies of a 62 kDa subunit (encoded by the *poxB* gene) each containing a tightly (but non-covalently) bound FAD. PoxB displays unusual activation phenomena [1–5]. In the presence of the substrate, pyruvate, and cofactors, the enzyme is activated by phospholipids (the physiologically relevant activator) or by detergents, resulting in a 20-fold increase in V_{max} and a 10-fold decrease in the K_m for pyruvate. This behaviour *in vitro* is thought to reflect the function of PoxB *in vivo*. *In vivo*, PoxB is thought to act by shuttling between the cytosol and inner membrane depending on the intracellular pyruvate concentration [1–3]. When the intracellular pyruvate concentration is low, the enzyme is inactive and located in the cytosol, whereas high concentrations of pyruvate trigger a conformational change that exposes the C-terminal lipid-binding domain. This C-terminal domain then inserts into the lipid bilayer, which gives the PoxB flavin moiety access to the physiological electron acceptor, ubiquinone [4,5]. Insertion of the C-terminal domain into the membrane bilayer also greatly increases enzyme activity by impeding the inhibitory C-terminus from interaction with the active site [1–5].

We previously reported that the PoxB amino acid sequence has a high degree of similarity to a group of enzymes defined by the acetohydroxy acid synthase (AHAS) isoenzymes of bacteria and plants together with *E. coli* glyoxylate carboligase (Gcl) [6–8]. The AHAS isoenzymes (called acetolactate synthases in plants) catalyse the first step of the synthetic pathway of the branched amino acids valine, isoleucine and leucine. *E. coli* contains two AHAS isoenzymes, AHAS I and III, whereas *Salmonella typhimurium* contains AHAS I and II. AHAS I functions mainly to catalyse the condensation of two pyruvate molecules to form acetolactate, an intermediate in the synthesis of valine, whereas the other two AHAS isoenzymes preferentially catalyse the condensation of 2-oxobutanoic acid ('α-ketobutyrate'; AKB) and pyruvate to form α-acetohydroxybutyrate, the isoleucine synthetic intermediate [9–12]. Another member of this protein family, Gcl [8], catalyses the condensation of two glyoxylate molecules to form tartronate semialdehyde plus CO_2, a reaction mechanistically identical with acetolactate formation by AHAS. Although the AHAS and Gcl enzymes use FAD as a structural rather than a catalytic element [6], protein sequence alignments and biochemical studies of these enzymes and of chimaeric PoxB–AHAS proteins indicated that these proteins are evolutionarily and functionally related [6]. Indeed, we have proposed that the AHAS and Gcl proteins were derived from PoxB or a similar protein [6].

In addition to these proteins, a pyruvate oxidase from *Lactobacillus plantarum* (lacto-Pox) has been crystallized and its high-resolution crystal structure determined [13,14]. Although this enzyme produces different products (acetyl phosphate and H_2O_2) from PoxB and lacks lipid activation and lipid-binding properties, there is strong conservation of amino acid sequence between lacto-Pox and PoxB [2,3]. The overall structure, cofactor requirements and sequence alignments all indicate that lacto-Pox belongs to the same enzyme family as PoxB, Gcl and the AHAS isoenzymes. Indeed, on the basis of our work, Ibdah [15] and Ott [16] and their co-workers used the lacto-Pox structure to model and successfully modify the active sites of *S. typhimurium* and plant AHASs.

In an effort to improve the quality of biopolymers containing poly-(3-hydroxyvalerate), an enzyme was sought that could produce a high yield of a key intermediate, propionate, from

Abbreviations used: AHAS, acetohydroxy acid synthase; AKB, 'α-ketobutyrate' (2-oxobutanoic acid); Gcl, *E. coli* glyoxylate carboligase; lacto-Pox, *Lactobacillus plantarum* pyruvate oxidase; PoxB, *E. coli* pyruvate oxidase; TPP, thiamine pyrophosphate.

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AKB. *E. coli* PoxB seemed a promising enzymic candidate for structural modification with this aim. Unlike other single-subunit enzymes that decarboxylate pyruvate, such as lacto-Pox [17], *E. coli* PoxB has appreciable activity with AKB [18]. We therefore used site-directed mutagenesis of PoxB to seek mutants with altered binding of AKB. Our strategy was based on the structure of Lacto-PoxB and comparisons with the bacterial AHAS isoenzymes plus Gcl. We report the isolation of mutants of *E. coli* PoxB that are defective in pyruvate utilization, but which retain normal activity with AKB. The properties of these mutants have allowed the refinement of our model of the PoxB activation process.

MATERIALS AND METHODS

Bacterial strains, media and chemicals

All bacterial strains were derivatives of *E. coli* K-12. Strain CG3 is Δ*aceEF pfl-1 pps-4 poxB1 recA*. Strain YYC404 is a derivative of RK4988 and its relevant markers are Δ*aceEF* Δ(*ara-leu-ilvIH*)*863* Δ*(ilvB-uhpA)2089 poxB::kan srl::Tn10* Δ*(srl-recA)*. Strain RK4988 is an Δ*(ilvB-uhpA)2089* derivative of MC4100. Strain YYC1290 is Δ*aceEF poxB::cm* and is derived from strain CY265 [19]. Genetic markers were introduced into strains by transduction with P1 phage. Maloy–Nunn medium [20] was used for selection of tetracycline-sensitive colonies when several cycles of Tn*10*-linked mutations were used.

The media used were the rich, minimal and TTC-pyruvate media described previously [19,23]. Strains with the Δ*aceEF* gene require acetate for growth and were supplemented with 10 mM sodium acetate. TTC-AKB medium was the same as the TTC-pyruvate medium except that 20 mM sodium AKB was used in place of sodium pyruvate. Different *poxB*-null mutant host strains were used for screening on the two TTC media because different competing pathways needed to be inactivated. Strain CG3 was used as the plasmid host when screening was done on TTC-pyruvate medium, whereas strain YYC404 was the host used for screening on TTC-AKB medium. The concentrations of antibiotics used to select plasmid maintenance were (in mg/l): tetracycline hydrochloride, 10; sodium ampicillin, 100; chloramphenicol, 25; kanamycin sulphate, 50. All chemicals were obtained from Sigma Chemical Co.

DNA manipulations

Plasmids were prepared by QIAprep Spin Miniprep kit (Qiagen). DNA sequencing and the synthesis of oligonucleotides were done at University of Illinois Keck Center. Site-directed mutagenesis was done by the *ung/dut* method of Kunkel et al. [21]. All mutations were confirmed by DNA sequence analysis of both strands.

Oligonucleotides used in site-directed mutagenesis

The altered nucleotide bases are underlined. The letter N represents an equimolar mixture of A, T, G and C nucleotides at that position. Primers used were as follows: primer 1 (A467K, M468Q, E469W, M470Q), 5′-GCCACCAGCTTTTTGCCATTGTTTCACAAAGCCCAGCAC-3′; primer 2 (M468Q), 5′-CTTTCATCTCTTGCGCCACAA-3′; primer 3 (E469W), 5′-AGCTTTCATCCACATCGCCAC-3′; primer 4 (L253X), 5′-CGAGAAGCCGATNNNCCCGGTCATTCC-3′; primer 5 (V380X), 5′-CGTTGGCGTACCNNNGTCACAGGTGAAAATAGC-3′; primer 6 (V466X), 5′-CATCTCCATCGCNNNAAAGCCCAGCAC-3′; primer 7 (*Bsp*HI), 5′-GTTTCATGATTCTCCATC-3′.

Plasmid constructions

All plasmids were derived from plasmid pYYC102 [22], which encodes wild-type PoxB. Plasmid pYYC213 (PoxB A467K M468Q, E469W, M470Q), plasmid pYYC214 (PoxB M468Q) and plasmid pYYC215 (PoxB E469W) were constructed by using oligonucleotide primers 1, 2 and 3 respectively. Plasmid pYYC219 was identical with pYYC102 except that a *Bsp*HI restriction site was introduced that overlapped the PoxB methionine initiation codon. Plasmid pYYC219 was constructed with primer 7 to facilitate the construction of plasmid pYYC220. In plasmid pYYC220 the *poxB* gene was inserted into the expression vector pSE380 (Invitrogen) by cutting plasmid pYYC219 with *Bsp*HI and *Sac*I and ligating the 1.7 kb DNA fragment encoding *poxB* into the *Nco*I and *Sac*I cloning sites of the vector.

Plasmids pYYC221, pYYC222, pYYC223 and pYYC224 were derived by mutagenesis of plasmid pYYC102 with primers 4, 5 and 6, which were added simultaneously to a site-directed mutagenesis reaction. Plasmid pYYC225 was constructed from plasmids pYYC221 and pYYC102. Plasmid pYYC221 was cut with *Hin*dIII and *Sal*I and a 0.4 kb DNA fragment carrying the V380A mutation was ligated to the 4.6 kb *Hin*dIII–*Sal*I fragment of pYYC102 to form plasmid pYYC225. Plasmid pYYC226 was similarly made by cutting plasmid pYYC221 with *Kpn*I and *Sal*I. The 0.4 kb *Kpn*I–*Sal*I fragment carrying the L253F mutation was ligated to the 4.6 kb *Kpn*I–*Sal*I fragment of plasmid pYYC102.

Plasmid pYYC231 was constructed by ligation of the 1.2 kb *Kpn*I–*Sac*I fragment of pYYC221 carrying the V380A and L253F mutations of PoxB to the 4.9 kb *Kpn*I–*Sac*I fragment of pYYC220. Plasmids pYYC232 and pYYC233 were constructed by ligating the 1.2 kb *Kpn*I–*Sac*I fragments of pYYC222 and pYYC223 respectively to the 4.9 kb *Kpn*I–*Sac*I fragment of plasmid pYYC220. Plasmid pYYC235 was made by ligation of the 1.2 kb *Kpn*I–*Sac*I DNA fragment of plasmid pYYC225 to the 4.9 kb *Kpn*I–*Sac*I fragment of plasmid pYYC220.

Plasmids pYYC236 and pYYC237 were made by ligation of the 0.4 kb *Kpn*I–*Sal*I DNA fragment carrying L253F of pYYC221 to the 5.7 kb *Kpn*I–*Sal*I fragments of plasmids pYYC232 and pYYC233 respectively.

Enzyme purification and assay of PoxB

PoxB activity was assayed spectrophotometrically with potassium ferricynanide as the electron acceptor [19]. The enzyme activities in Tables 1 and 2 were determined by assaying crude extracts of strain YYC404 carrying various plasmids. The preparation of the cell extracts was as described previously [19]; activities are expressed in μmol of pyruvate or AKB decarboxylated/min (1 μmol/min is defined as one unit) per mg of protein. PoxB expression was induced in the isopropyl β-D-thiogalactoside-inducible plasmid pYYC220 and its derivatives by the addition of 50 μM isopropyl β-D-thiogalactoside to exponential-phase cultures for 2.5 h at 37 °C.

The wild-type and V380/L253F PoxB proteins were purified as described previously [2,23,24]. Denaturing PAGE [23] was used to check the purity of the enzymes, which were at least 95 % pure. For kinetic determinations the enzyme was first activated by Triton X-100 in a mixture containing enzyme (0.1 mg/ml), 0.1 M sodium phosphate buffer, pH 6.0, 1 % (v/v) Triton X-100, 10 mM $MgCl_2$, 0.1 mM TPP and either 50 mM pyruvate or 50 mM AKB. These mixtures were incubated at room temperature for 10 min. Samples containing 10 μg of enzyme were then withdrawn and added to reaction mixtures as above (final volume 1 ml) except that the pyruvate or AKB concentrations were varied. The wild-type enzyme was fully activated by either

pyruvate or AKB under these conditions. Kinetic constants (and standard deviations) were derived by curve fitting to a linear equation by the least-squares algorithm implemented by the Cricket Graph III software (Computer Associates International).

FAD spectra

Scanning of the PoxB FAD spectra was done on a Hewlett Packard ChemStation UV/visible diode array spectrometer. Solutions of the purified PoxB proteins [1 mg in a final volume of 0.6 ml, containing 0.1 M sodium phosphate, pH 6.0, 20% (v/v) glycerol, 10 mM $MgCl_2$ and 1 mM TPP] were scanned. The sodium salt of pyruvate or AKB was added to the enzyme mixture to the final concentration given and a scan from 300 to 500 nm was begun immediately. Sequential scans were obtained every 20 s for 2 min and then every 1 min thereafter for a further 3 min.

Assay of AKB degradation

The methods for the labelling of AKB and its turnover followed those of LaRossa et al. [25], with some modifications. Cultures of strain YYC1290 carrying various plasmids were grown overnight in glucose-minimal medium E supplemented with acetate and ampicillin. These cultures were subcultured 1:20 in the same medium and grown to an A_{600} of 0.4–0.5. To 1 ml of cell culture, 1 μCi of L-[1-^{14}C]threonine (214.6 mCi/mmol from American Radiolabeled Chemicals), 0.1 μmol of non-radioactive L-threonine and 0.85 μmol of L-valine were added. The cells were labelled at 37 °C for 20 min and samples (0.2 ml) were withdrawn and used for determining the amount of labelled AKB. To the remaining culture was added 0.76 μmol of L-isoleucine; at 30 and 60 min after the addition, samples (0.2 ml) were withdrawn. All samples were mixed with 0.1% 2,4-dinitrophenylhydrazine in 2 M HCl. After 10 min at room temperature, 0.4 ml of ethyl acetate was added and the dinitrophenylhydrazones were extracted by vigorous vortex-mixing of the samples for 30 s. The phases were separated by centrifugation; samples (0.1 ml) were withdrawn from the upper phase and taken to dryness. The samples were spotted on cellulose TLC plates that were developed in butan-1-ol/ethanol/water (4:1:1, by vol.). Samples of non-radioactive pyruvate and AKB were converted to their dinitrophenylhydrazones as described above and served as standards. The plates were exposed to a PhosphoImager plate and the radioactivities of the AKB spots were measured.

RESULTS

Isolation of mutants deficient in pyruvate utilization

Alignments of the sequences of *E. coli* AHAS isoenzymes I, II and III plus PoxB indicated that the AHAS residues corresponding to residues Ala-467 to Met-470 of PoxB were conserved within the three isoenzymes but not in PoxB. In their modelling of *S. typhimurium* AHAS II, Ihdah et al. [15] placed Trp-464 (which corresponds to PoxB residue Glu-469) at the active site of the enzyme. These workers showed that the replacement of Trp-464 with another residue (Tyr, Phe, Leu or Ala) caused a loss of the preferred utilization of AKB as substrate. On the basis of this work we altered residues of the putative PoxB active site in the hope of improving AKB utilization. In our first attempt, four residues were simultaneously changed (A467K, M468Q, E469W and M470Q) to those present in *E. coli* AHAS III (which, like *S. typhimurium* AHAS II, prefers AKB over pyruvate) and found that the resulting mutant PoxB protein was completely inactive with either pyruvate or AKB as substrate.

Table 1 Enzymic activities of PoxB mutant proteins

Mutations	PoxB activity (units/mg)		Activity ratio (Pyr/AKB)
	Pyruvate	AKB	
None (wild type)	11	1.6	6.9
A467K/M468Q/E469W/M470Q	< 0.005	< 0.005	–
M468Q	9.4	1.4	6.7
E469W	< 0.005	< 0.005	–
L253F/V380A	0.83	1.8	0.46
V380S	0.55	0.73	0.75
V380G	0.04	0.99	0.04
L253F/V466S	< 0.005	< 0.005	–

We then proceeded to construct two singly mutant PoxBs, M468Q and E469W. The M468Q enzyme had normal activity, whereas the E469W enzyme was completely inactive.

Given these disappointing results we threaded the *E. coli* PoxB amino acid sequence on the lacto-Pox crystal structure [13,14] using the SWISS-MODEL server (http://www.expasy.ch) and our prior sequence alignment [2]. This model, together with alignments of PoxB, lacto-Pox, Gcl and the bacterial AHAS isoenzymes, was used to identify residues that might account for the substrate specificities of these enzymes. The AHAS enzymes, like PoxB and lacto-Pox, utilize pyruvate [9,10,15,16] and all have a valine residue in the positions corresponding to Val-380 of PoxB, whereas the analogous Gcl residue was isoleucine. This pattern, together with the models of AHAS II [15] and PoxB which placed the side chain of this valine residue very close to the thiazolium ring of TPP, suggested that residue 380 might have a role in pyruvate binding; the notion was that the larger Gcl side chain allowed interaction with the smaller substrate, glyoxylate. In addition to Val-380 we also targeted PoxB residues Leu-253 and Val-466. Leu-253 of PoxB corresponds to Met-250 of AHAS II and Arg-264 of lacto-Pox. In lacto-Pox, residue Arg-264 was suggested to be involved in electron transfer to FAD [14], whereas PoxB Val-466 corresponds to AHAS II Val-461, which has been suggested to have a role in substrate specificity [15].

We designed three oligonucleotide primers with each primer corresponding to one of the target residues. Because we lacked information concerning the residues that could functionally substitute for the targeted residues, we designed each oligonucleotide primer to contain a equimolar mixture of all four nucleotides at the targeted codon. This gave the possibility of all 20 residues (plus termination codons) at each position. We then performed site-directed mutageneses with each oligonucleotide alone or with all three primers combined in a single mutagenesis reaction. The mutagenized plasmids were transformed into *poxB*-null mutant strains; the resulting colonies were screened for their phenotypes on both the TTC-pyruvate and TTC-AKB media. Four PoxB mutant proteins were obtained. The mutations in these proteins were V380A/L253F, V380S, V380G and V466S/L253F. Each mutant gave white colonies on TTC-pyruvate medium, indicating that the PoxB enzymes could not utilize pyruvate normally. However, when subsequently tested on TTC-AKB medium, the mutant colonies contained very small central red dots, indicating PoxB activity with AKB. The red dots of the mutant colonies were the same size as, or slightly larger than, those of the colonies that carried the wild-type PoxB, suggesting normal utilization of AKB.

The enzymic activities of the mutant and wild-type enzymes were assayed *in vitro* in crude extracts with either pyruvate or AKB as substrate (Table 1). These results showed that the three

Table 2 Effect of the L253F mutation on PoxB activity with pyruvate or AKB

Results are averages of at least two determinations obtained from extracts of different cultures. The plasmids used were derivatives of pTZ19 except for L253F/V380S, which was a pSE380 derivative.

	Activity (units/mg)		
Mutations	Pyruvate	AKB	Activity ratio (Pyr/AKB)
L253F/V380A	0.66	1.5	0.44
V380A	0.17	0.48	0.36
V380S	0.51	0.72	0.71
L253F/V380S	1.5	1.8	0.83
V380G	0.025	0.46	0.06
L253F/V380G	0.14	0.78	0.18
L253F	20.0	3.9	5.1
None (wild type)	7.2	1.1	6.5

V380 mutants, namely V380A/L253F, V380S and V380G, were deficient in pyruvate decarboxylation, whereas the rates of AKB decarboxylation were much higher. Thus the ratios of activity with pyruvate to activity with AKB for the mutant proteins were less than 1, whereas wild-type PoxB had a ratio of approx. 7. Moreover, we observed that PoxB V380A/L253F had higher enzymic activity than the two single mutants V380S and V380G (Table 1). These results indicated that the replacement of Val-380 with alanine, serine or glycine resulted in decreased activity with pyruvate. We also found, when pyruvate (10–50 mM) was added to an ongoing assay with AKB as substrate, that the rate of decarboxylation was unchanged or only slightly increased for V380A/L253F PoxB, whereas with the wild-type enzyme a marked increase in the rate was observed on the addition of pyruvate (results not shown). This suggested that PoxB V380A/L253F had little or no affinity for pyruvate.

The L253F mutation enhances the PoxB activity of Val-380 mutants

The mutant protein having the highest activity with AKB as substrate contained two mutations, V380A and L253F. To determine the relative contributions of the two amino acid substitutions we separated the two mutations by subcloning each mutation into the wild-type gene. A strain carrying the plasmid with only the L253F mutation had a wild-type PoxB pyruvate-to-AKB activity ratio, whereas the strain carrying only the V380A substitution had the same ratio as the double mutant but lower enzymic activities with both substrates. These results indicated that L253F substitution by itself had no mutant phenotype but this mutation increased the activity of the V380A enzyme (Table 2). This effect was not specific to the V380A alteration because introduction of the L253F mutation into the V380G and V380S mutants had the same effect. The L253F mutation increased the activity of all of the Val-380 substitution mutants (V380A, V380S and V380G) by 2–5-fold with either AKB or pyruvate as substrate and seemed to have a similar, but more modest, effect on the wild-type enzyme.

The *poxB* promoter requires a special RNA polymerase sigma factor (sigma S), the level of which increases as cultures enter stationary phase and thus the expression of PoxB is dependent on growth phase [26]. Hence the PoxB activities we observed (Table 2) might have varied somewhat owing to our harvesting cells at different stages of stationary phase (and hence at different sigma S levels). To check this possibility we subcloned each of the *poxB* Val-380 mutant genes and the wild-type *poxB* gene into expression vector pSE380 to place *poxB* expression under control of the vector *tac* promoter and thus give defined induction conditions (see the Materials and methods section). We obtained very similar results with the two expression systems (results not shown).

Table 3 Kinetic values of the wild-type and V380/L253 pyruvate oxidases

Results were obtained with the purified enzymes as described in the Materials and methods section, and are shown as means ± S.D. The V_{max} (units/mg) values are shown per mg of PoxB protein.

	Pyruvate		AKB	
PoxB	K_m (mM)	V_{max} (units/mg)	K_m (mM)	V_{max} (units/mg)
Wild type	20 ± 0.7	447 ± 21	8 ± 1.0	26 ± 1.4
V380/L253	117 ± 4.0	47 ± 3.6	8 ± 0.4	29 ± 0.7

Purification and kinetic constants of the PoxB V380A enzyme

We purified the V380A/L253F mutant PoxB by the scheme used for the purification of wild-type PoxB. Because the purification scheme included a heat-treatment step, the stability of the mutant enzyme seemed normal. Note that PoxB must proceed through an activation step before full activity can be observed. This activation step has two stages. In the first stage, the conversion from the latent form to the low-activity form, the enzyme is incubated with substrate (pyruvate or AKB) and cofactors (TPP and Mg^{2+}). Under these conditions the FAD cofactor becomes reduced, resulting in a conformational change that exposes the C-terminal lipid-binding domain. This domain is required for the second stage, the binding of micellar activators to convert the low-activity form to the form with full activity. The natural activator *in vivo* is the phospholipids of the cell membrane [27], but *in vitro* either phospholipids or a variety of detergents will suffice [28]. In the present study we used the most potent activator *in vitro*, Triton X-100. After the activation process was complete, the electron acceptor (ferricyanide) was added and the catalytic reaction began. The K_m and V_{max} values of both wild-type and the V380A/L253F mutant PoxB proteins were obtained with either pyruvate or AKB (Table 3). When the enzyme was assayed with AKB (a concentrated enzyme solution containing 50 mM substrate was used in the activation step, followed by dilution into the assay mixture), the K_m and V_{max} values for AKB for the wild-type and mutant PoxB enzymes were very similar, approx. 8 mM and 28 units/mg of protein respectively (Table 3), indicating that the mutant enzyme decarboxylated AKB normally. In contrast, the mutant enzyme had a K_m for pyruvate of 117 mM and a V_{max} of 47 units/mg of protein. This is a 5-fold increase in K_m and a 10-fold decrease in V_{max} compared with the wild-type enzyme (Table 3). These properties of the V380A/L253F PoxB suggested a specific defect in pyruvate binding. This also occurred in the absence of a micellar activator: the V380A/L253F enzyme had no detectable activity with 50 mM pyruvate, whereas the wild-type enzyme had approx. 5% of the activity observed in the presence of Triton X-100 (results not shown).

There are no directly measured values for pyruvate binding to PoxB in the literature owing to the very low affinity of the enzyme for its substrate (only a very minor fraction of the substrate would be enzyme-bound). We therefore followed the kinetics of the spectral change of the PoxB FAD cofactor on the addition of pyruvate as an indirect indicator of binding, although this measured the rate of binding rather than a

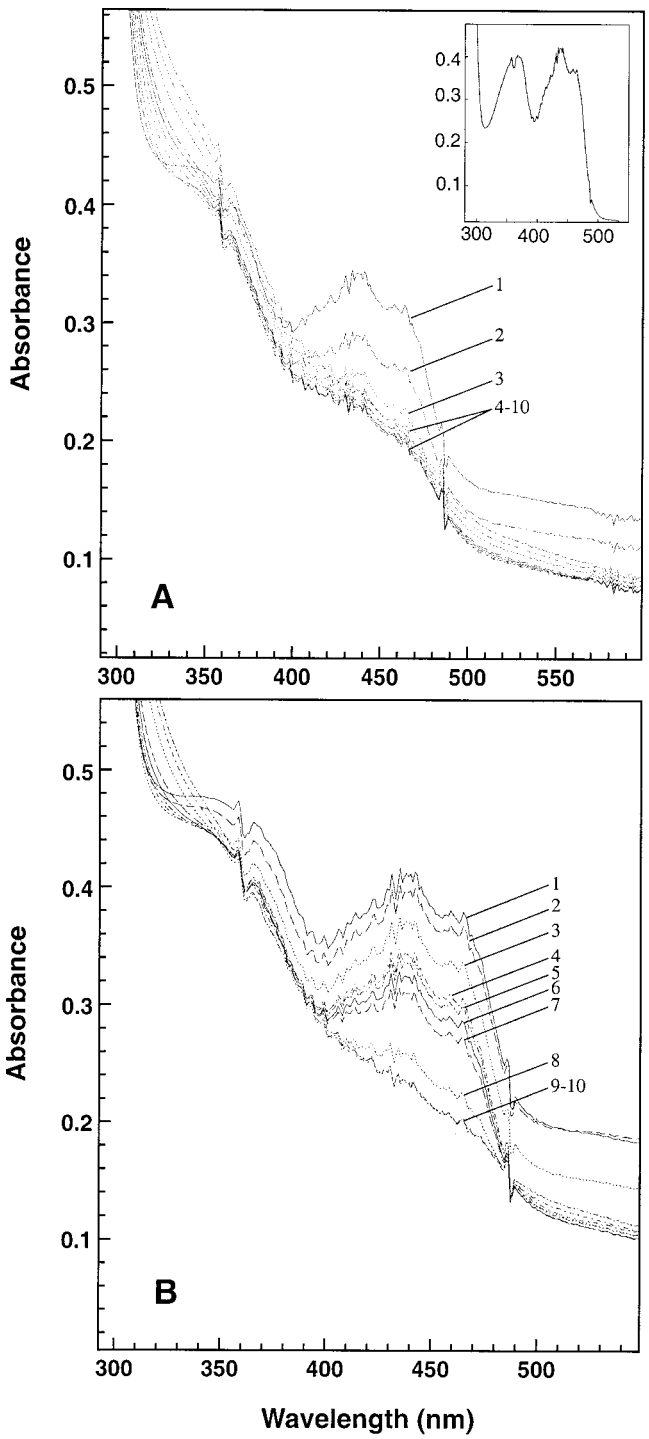

Figure 1 Kinetics of substrate binding assayed by flavin reduction

Spectral analysis of the purified wild-type PoxB (**A**) and the purified mutant V380A/L253F PoxB (**B**) from 300 to 500 nm. The first scan was done immediately on the addition of 5 mM pyruvate to the PoxB enzyme samples; successive scans were then taken at intervals: trace 1, 0 min; traces 2–7, increments of 20 s; traces 8–10, increments of 1 min until a total of 5 min elapsed time. The inset in (**A**) shows the oxidized spectrum of the wild-type PoxB (the spectrum of the mutant protein was identical with that of the wild-type enzyme). Note that the wavelength scales of (**A**) and (**B**) differ slightly.

thermodynamic binding constant. In the presence of pyruvate and TPP, PoxB forms a lactyl-TPP intermediate, which is decarboxylated to give the 2-(1-hydroxyethyl)-TPP intermediate [29]. The oxidation of this intermediate (accompanied by the release of acetate) and the concomitant reduction of the flavin has been shown to be the rate-limiting step in the formation of the high-activity conformer [29]. Because the mechanisms of FAD reduction by pyruvate and AKB should be identical, the rates of flavin reduction should reflect the relative rates of binding of these substrates to the enzyme [29].

The PoxB spectrum shows a characteristic oxidized FAD spectrum with peaks at 370 and 445 nm and a shoulder at 467 [29–32] (Figure 1). When pyruvate is added to the wild-type enzyme, reduction of the FAD moiety results in a loss of these absorption peaks [30,31]. The FAD spectra of the wild-type and mutant enzymes were examined after the addition of 5 mM pyruvate. The half-time of reduction of the wild-type enzyme was 20 s, whereas the V380A/L253F enzyme required about 120 s to reach this extent of reduction (Figure 1). In contrast, the rate of flavin reduction on the addition of 3.3 mM AKB to the V380A/L253F enzyme was only moderately slower than that of the of the wild-type enzyme (half-time of 80 s for the V380A/L253F enzyme, compared with 40 s for the wild-type enzyme; results not shown). It therefore seems likely that the differences between the wild-type and mutant enzymes can be attributed to a specific defect in the rate of binding of pyruvate to the V380A/L253F latent conformer. However, effects of the mutation on the thermodynamics of pyruvate binding are not precluded.

The V380A/L253F enzyme had low activity after incubation for 10 min in 50 mM pyruvate (Table 2), although the flavin spectra (Figure 1) showed that in the presence of 5 mM pyruvate the mutant enzyme was completely reduced within 5 min. This apparent inconsistency can be readily explained by the slow reduction of the PoxB V380A/L253F latent conformer. Although the mutant enzyme was fully reduced at the beginning of the enzyme assay, after the first cycle of catalysis the enzyme returned to the latent conformer. This form must again be reduced to bind the micellar activator again and reacquire full activity. The abnormally low rate of reduction of PoxB V380A/L253F by pyruvate means that at any given time a large fraction of the enzyme in the assay is present as the inactive latent conformer, thus accounting for the decreased V_{max} of V380A/L253F PoxB. The decreased rate of binding suggests a weakening of binding affinity that would be reflected in the increased K_m (and vice versa).

Study of the PoxB V380A/L253F mutant *in vivo*

Use of the TTC-pyruvate medium indicated that the V380A/L253F PoxB was inactive in pyruvate oxidation *in vivo*. This was confirmed by a second approach. Under aerobic growth conditions, *E. coli* strains lacking pyruvate dehydrogenase are acetate auxotrophs. The acetate requirement can be bypassed by the introduction of a multicopy plasmid carrying a functional *poxB* gene [33]. We therefore transformed a strain carrying null mutations in *poxB* and *aceEF* (which encode two of the three pyruvate dehydrogenase subunits) with plasmids carrying either the wild-type *poxB* gene, the V380A/L253F *poxB* gene, a *poxB* gene encoding a totally inactive PoxB protein, or the vector plasmid. Only the strain carrying the wild-type plasmid grew in the absence of acetate (results not shown); the V380A/L253F protein therefore had insufficient activity to support growth.

Because the V380A/L253F PoxB was defective in conversion of the latent conformer to the low-activity conformer, we also tested the effects of pyruvate addition on growth. If the addition of pyruvate to the medium raises the intracellular concentration

of this metabolite, then V380A/L253F PoxB would be slowly converted to the low-activity conformer and, if the enzyme did not return to the latent conformer *in vivo*, the enzyme defect should be bypassed (the high copy number of the encoding plasmid should more than compensate for the lower activity of the mutant PoxB). The addition of low extracellular concentrations of pyruvate to an *E. coli* strain blocked in the major pathways of pyruvate metabolism is known to result in an intracellular pyruvate concentration of 8 mM that persists for at least 20 min before decay [34]. Although this pyruvate concentration and time interval are sufficient to activate the V380A/L253F latent conformer *in vitro* (Figure 1 and Table 1), pyruvate supplementation failed to allow growth of the strain carrying the plasmid encoding PoxB V380A/L253F in the absence of acetate (results not shown).

We also studied the AKB decarboxylation activity of V380A/L253F PoxB *in vivo* in the hope of finding a better screening or selection method. AKB accumulation is toxic to *S. typhimurium* and its close relative *E. coli* [25,35]; an increased ability to degrade AKB should therefore relieve this toxicity. To test whether the V380A/L253F PoxB mutant had an increased rate of AKB degradation *in vivo* we examined strain YYC1290 (*aceEF poxB*::*cm*) carrying either plasmid pYYC102 encoding wild *poxB* or plasmid pYYC221 encoding the V380A/L253F gene using the procedure of LaRossa et al. [25]. The strains were labelled with [1-^{14}C]threonine for 20 min in the presence of valine. Threonine deaminase converts the radioactive threonine to AKB; the consumption of AKB by AHAS I and III is inhibited by the addition of valine. Threonine deaminase is subsequently inhibited by the addition of isoleucine and the rate of the disappearance of the accumulated labelled AKB was followed by TLC of the dinitrophenylhydrazone (see the Materials and methods section). The half-life of AKB degradation was the same in both strains and in a strain carrying only the vector plasmid (results not shown). These results indicate that the major route of AKB metabolism does not involve PoxB and that if V380A/253F PoxB has an increased activity towards AKB *in vivo*, it is masked by other pathways that metabolize AKB.

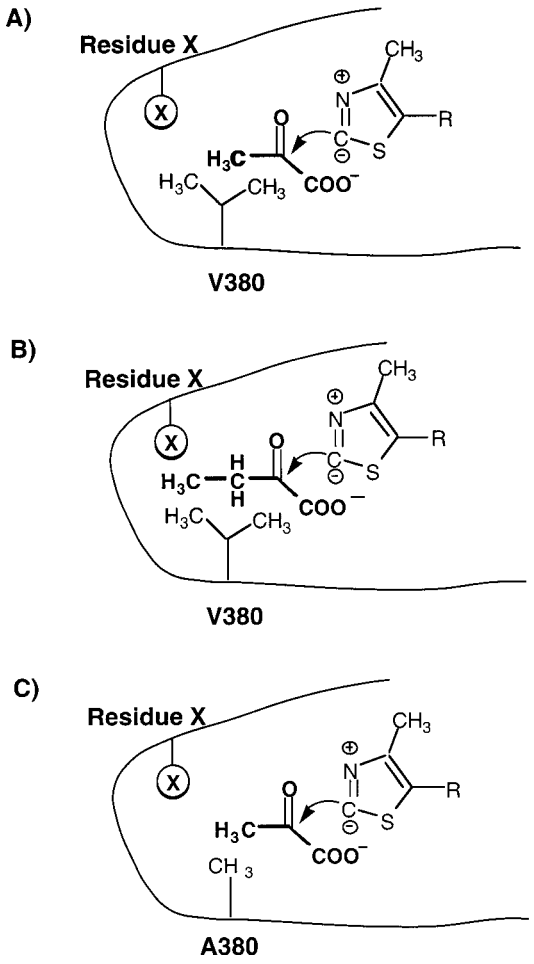

Figure 2 A model for the altered substrate specificity of PoxB A380

In these sketches of the active site the substrates are shown in bold. The thiazolium ring of TPP (deprotonated), the side chain of residue Val-380 (or Ala-380), and the side chain of an unknown residue (residue X) are also shown. The arrow depicts the nucleophilic attack of the reactive thiazolium C-2 atom on the substrate carbonyl carbon to form the first intermediate of the catalytic cycle (which, for pyruvate, is lactylthiamine pyrophosphate). (**A**, **B**) The wild-type active site with pyruvate (**A**) or AKB (**B**) bound. (**C**) The V380A mutant with pyruvate bound.

Search for improved PoxB AKB utilization mutants starting from PoxB V380A/L253F

Starting with the V380A/L253F PoxB mutant, we attempted to obtain an enzyme with an increased rate of AKB utilization by using our model to identify residues close to the C-2 atom of TPP. Two residues, Val-380 and Met-408, were located within 5 Å of TPP C-2 and an additional seven residues (Gly-381, Thr-382, Gly-406, Leu-463, Phe-465, Val-466 and Glu-469) were within 7–10 Å. We chose residues Met-408, Thr-382, Phe-465, Val-466, Glu-469 and Thr-384 as targets for mutagenesis. Using the methods described above we synthesized DNA primers to mutagenize each codon and screened several hundred colonies for each primer. No mutant with a significantly increased AKB decarboxylation activity was found.

DISCUSSION

Our goal was to isolate a PoxB mutant with an increased activity towards AKB. Although our mutageneses did not yield a mutant with these properties, it did give mutants with decreased activity towards pyruvate while retaining normal activity with AKB. It should be noted that in a physiological context these mutants might be at least as useful in propionate production as the intended mutant. This is because environments *in vivo* are likely to contain much higher levels of pyruvate than of AKB. Therefore an enzyme that readily used pyruvate would be occupied with acetate production rather than being engaged in propionate production. Indeed, pyruvate would behave as a competitive inhibitor of propionate production. Thus an enzyme that discriminates against pyruvate should be a superior producer of propionate *in vivo*.

The best mutant that we obtained contained two mutations, V380A and L253F, but only the V380A mutation is involved in the utilization and binding of pyruvate. The L253F mutation increases the specific activity of the V380A protein by an unknown mechanism, but the effect is not specific to this mutant because V380S and V380G are similarly affected. Val-380 and its neighbouring residue, Gly-381, are conserved in lacto-Pox and all AHAS isoenzymes and were therefore not targeted in the studies of Ibdah et al. [15]. However, Gcl, which binds glyoxylate, a substrate smaller than pyruvate, has an isoleucine residue at

position 380. This fact seems enlightening when coupled with our finding that of all 20 possible amino acid substitutions at position 380, the only mutant proteins isolated had the small residues Gly, Ala or Ser at position 380. Because residue 380 in lacto-Pox is very close to the reactive C-2 atom of the TPP thiazolium ring, the nucleophile that attacks the carbonyl of the substrate, it seems reasonable that C-3 of pyruvate normally interacts with the Val-380 side chain. When the side chain is missing or reduced in size (as in the Gly, Ala and Ser substitution mutants) the resulting loss of hydrophobic surface area greatly weakens pyruvate binding (Figure 2). It follows from this model that the longer isoleucine side chain is able to reach C-2 of glyoxylate to account for binding of this substrate to Gcl. However, C-4 of AKB must interact with one or more other active-site residues (denoted X in Figure 2) sufficiently strongly for the loss of the interaction with C-3 to have little effect on AKB utilization. The fact that the K_m of wild-type PoxB for AKB is lower than that for pyruvate is consistent with this suggestion. We cannot suggest candidate residues for the proposed interaction with the AKB methyl group owing to the small size of the substrate, the free rotation around the bonds and the fact that AKB is an extremely poor substrate for lacto-Pox [17], the enzyme on which our PoxB model is based. Note that the rate of binding of AKB as measured by flavin reduction is somewhat slower in V380A/L253F PoxB than wild-type PoxB, showing that Val-380 does have a minor role in the binding of this substrate. This slower reduction is not reflected in the maximal velocity with AKB, as would be expected from the transient kinetic experiments of Bertagnolli and Hager [29], who showed that on the binding of PoxB to a micellar activator, the rate-limiting step in the overall oxidase reaction shifts from 2-(1-hydroxyethyl)-TPP oxidation to one of the partial steps of the decarboxylation reaction. Indeed, when the micellar activator was omitted from the assay the V_{max} of the V380A/L253F PoxB with AKB was about half that of the wild-type enzyme (results not shown), a value consistent with the FAD reduction results (Figure 1) and those of Bertagnolli and Hager [29].

PoxB exists in at least three conformers, as deduced from thiol chemistry, enzyme kinetics and spectral studies [2,3,29,30]. In the latent form the flavin is in the oxidized state and the small C-terminal domain acts as though tightly clamped to the protein surface. In the presence of TPP and Mg^{2+} the addition of pyruvate results in reduction of the flavin, which frees the C-terminal domain as detected by the accessibility of this region to modifying reactions and proteases. The C-terminal domain has an inhibitory role in the low-activity conformer because its removal by proteolytic clipping or cross-linking results in conversion to the high-activity form. Partition of the C-terminal domain into membrane lipids *in vivo* (or into micelles *in vitro*) also gives the high-activity conformer by preventing the C-terminus from interacting with the active site. From the present results we must modify our model of PoxB action. We previously proposed that, in addition to blocking access to the flavin moiety in the latent and low-activity conformers, the C-terminal domain might block pyruvate binding [3]. The properties of the V380A/L253F mutant protein argues that this is not so; the pyruvate-binding site is fully accessible but binds the substrate poorly. The accessible nature of the site is shown by the finding that the rate of AKB binding to PoxB V380A/L253F is relatively normal. This binding plus the greater size of AKB argues that the characteristic high K_m for pyruvate of the low-activity conformer cannot be attributed to steric occlusion.

This work was supported by a Joint Development Contract from the Monsanto Company.

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Received 18 July 2000/4 September 2000; accepted 3 October 2000