Minimum Requirements for Protease Activation of Flavin Pyruvate Oxidase†

Byron L. Bertagnolli and Lowell P. Hager*
Roger Adams Laboratory, Department of Biochemistry, University of Illinois, Champaign—Urbana, Illinois 61801
Received January 22, 1991; Revised Manuscript Received June 10, 1991

ABSTRACT: Previous investigations have shown that the catalytic efficiency (k_{cat}/K_M) of pyruvate oxidase can be enhanced 450-fold by chymotrypic cleavage of a 23-residue peptide (α-peptide) from the carboxy terminus of the enzyme. The minimum requirement for proteolytic activation has been investigated by exposing pyruvate oxidase to a variety of carboxypeptidases, either singly or in combination. The extent of carboxypeptidase hydrolysis was followed by analyzing the release of amino acids and by mass spectral analysis of the truncated α-peptides which were derived from the carboxypeptidase-treated preparations. The results indicate that the removal of 7 carboxy-terminal residues does not activate the enzyme whereas the removal of 10 or 11 residues produces activated pyruvate oxidase. Activation of pyruvate oxidase by endoproteinase Glu-C confirms the carboxypeptidase results. Endoproteinase Glu-C specificity predicts hydrolytic cleavage of the peptide bond between Glu-561 and Val-562 with the removal of 11 residues from the carboxy terminus of the enzyme.

Pyruvate oxidase (POX, EC 1.2.2.2)[1] is a peripheral membrane-associated flavoprotein from *Escherichia coli*. Enzyme-bound FAD and TPP are required for the oxidative decarboxylation of pyruvate to carbon dioxide and acetate and the reduction of POX to E·FADH (Hager, 1957; Williams & Hager, 1966). The active enzyme species under steady-state conditions is a homotetramer. Each monomeric subunit is composed of 572 amino acids of known sequence (O'Brien et al., 1976; Russell et al., 1977a,b; Stevens & Gennis, 1980; Grabau & Cronan, 1984, 1986). The catalytic efficiency (k_{cat}/K_m) of POX, as measured by the standard ferricyanide spectrophotometric assay, is increased 450-fold either by binding lipid amphiphiles to a high-affinity site in the carboxyl domain (Hamilton et al., 1986) or by specific limited proteolytic removal of a 23-residue peptide (the α-peptide) from the carboxy terminus by α-chymotrypsin (Russell et al., 1977a,b; Recny & Hager, 1983; Recny et al., 1985; Zhang & Hager, 1987a,b).

In a continuing investigation on the activation of pyruvate oxidase by proteolysis, current research has focused on the minimum extent of cleavage in the α-peptide region necessary to achieve activation. The approach has been the incubation of POX with a combination of carboxypeptidases A, B, and Y. The products of the carboxypeptidase digestion were subjected to amino acid analyses, FAB mass spectrometric analysis of the truncated peptides cleaved from the treated enzyme by α-chymotrypsin, and quantitative determinations of the peptide yields. It was found that cleavage about halfway into the α-peptide region is sufficient to produce activated enzyme. Moreover, quantitative studies indicate that only 50% of the POX monomers are activated by treatment with the carboxypeptidase mixture. The other 50% of the monomers are partially degraded by carboxypeptidase hydrolysis, but, in this case, cleavage has stopped after approximately seven residues have been removed from the C-terminus of the enzyme.

Materials and Methods

Materials. All reagents were of analytical grade or higher. Ammonium formate, formic acid, potassium ferricyanide, glycerol, 2-propanol, zinc chloride, and sodium hydroxide were all from Fisher Scientific Co. Hydrochloric acid, mono- and dibasic potassium and sodium phosphates, enzyme-grade ammonium sulfate, sodium bicarbonate, and sodium chloride were all from Mallinckrodt, Inc. Acetonitrile was from EM Sciences Omni-Solv. Boric acid and trifluoroacetic acid were from Aldrich Chemical Co. Dialysis tubing of 50 000 molecular weight cutoff was from Spectrum Medical Industries, Inc. Magnesium chloride hexahydrate was from Baker Chemical Co. Thiamin pyrophosphate was from Fluka Chemicals-Biochemicals. Ultrafiltration membranes PLGC 10000 MWCO (25-mm diameter) and YM-100 (25-, 43-, and 76-mm diameters) were from Amicon Corp. Sephadex DEAE A-50 resin was from Pharmacia Fine Chemicals. A C_8 reverse-phase HPLC column (250 mm × 4.6 mm) was from Vydac. A Bio-Sil SEC-250 column [300 × 7.5 mm; 250-Å pore size, (10 ± 2)-mμ particle size], an HPLC column, gel filtration standards, and protein assay reagent were all from Bio-Rad. Sodium pyruvate, PMSF-treated carboxypeptidase A, DFP-treated carboxypeptidase B, carboxypeptidase Y, carboxypeptidase P, endoproteinase Glu-C, endoproteinase Asp-N, ubiquinone Q_0, phenylmethanesulfonyl fluoride, pepstatin A, Na_2EDTA, sodium lauryl sulfate molecular biology reagent quality, α-chymotrypsin, bovine albumin fraction V, and fluorescamine were all from Sigma Chemical Co.

Purification of Pyruvate Oxidase and Assay for Enzyme Activity. Preparation of the *E. coli* W-191-6 cell paste, sonication, heat step, ammonium sulfate fractionation, and Sephadex DEAE A-50 column chromatography were done essentially according to procedures outlined in Recny and Hager (1982). The enzyme was further purified by HPLC with a Bio-Sil SEC-250 sizing column (Bio-Rad) under nondenaturing conditions as given by Recny and Hager (1983). The eluted enzyme was concentrated by use of a YM-100 ultrafiltration membrane, made up to 25% (v/v) with glycerol, and stored in the dark at −15 °C. The specific activity of the purified enzyme, expressed as micromoles of ferricyanide re-

† This work was supported by Grant PHS GM 07768 from the National Institutes of Health.

[1] Abbreviations: POX, pyruvate oxidase; E, enzyme; FADH, flavin adenine dinucleotide, reduced form; TPP, thiamin pyrophosphate; PMSF, phenylmethanesulfonyl fluoride; PTC, phenylthiocarbamyl; HPLC, high-performance liquid chromatography; FAB, fast atom bombardment; EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl fluorophosphate; N-Cbz, *N*-carbobenzoxy.

0006-2960/91/0430-8131$02.50/0

duced per minute per milligram of protein, was calculated to be 171. Protein concentrations were estimated by the Bradford (1976) method using commercially available protein dye reagent (Bio-Rad) and bovine serum albumin fraction V as standard.

A convenient and sensitive in vitro spectrophotometric assay for enzyme activity employs ferricyanide as electron acceptor monitoring the decrease in absorbance at 450 nm (Hager, 1957; Williams & Hager, 1966). Enhanced enzyme activity is expressed as the rate differential measured in the absence of (i.e., unactivated control) and in the presence of 35 μM SDS lipid activator (i.e. fully activated POX). The order of addition of cofactor, substrate, lipid activator, and ferricyanide as well as the conditions of the assay were essentially those as outlined by Blake et al. (1978).

Activation of POX by Carboxypeptidases at pH 6 and 22 °C. Aliquots of stock enzyme were dialyzed (50000 molecular weight cutoff tubing) over a 3-h period against two changes of 5 L of 0.5% ammonium formate adjusted to pH 6.0 with dilute HCl. This dialysis step was necessary to remove glycerol and to replace phosphate with a volatile buffer. The presence of the volatile buffer greatly aids in the lyophilization steps prior to amino acid and FAB mass spectroscopic analyses.

Reaction mixtures contained in 1-mL total volume the following reagents at their final concentration: 2 mM $MgCl_2$, 200 μM TPP, 30 mM sodium pyruvate, and dialyzed POX (5 nmol of tetramers; 20 nmol of monomers). The addition of TPP and pyruvate is necessary in order to give the reduced enzyme (E·FADH), the enzyme species which can be activated by proteolysis. Carboxypeptidases either singly or in combination were then added to the reduced enzyme preparations. Zinc chloride was added to carboxypeptidases A and B in a 1:1 molar ratio for experiments involving these enzymes. Carboxypeptidase Y was treated with 50 μM pepstatin A to inhibit contaminating proteinase A activity according to procedures outlined by Lee and Riordan (1978). Aliquots were removed from the incubation mixtures at various time periods and assayed by using the standard ferricyanide method. The degree of activation of POX by carboxypeptidase treatment is expressed as the percent activation relative to the 100% activation of the enzyme by 35 μM SDS.

The analysis of amino acids released by carboxypeptidase treatment was carried out by rapidly filtering the reaction mixtures through a prechilled Amicon concentrator fitted with a PLGC 10000 molecular weight cutoff membrane. The filtrate containing the released amino acids was rapidly frozen in liquid N_2, lyophilized, and then submitted for amino acid analysis. Amino acid analyses were carried out with a Waters PICO-TAG system. Isolation and analysis of the truncated α-peptides from the carboxypeptidase-treated samples were carried out by first adding 5 mM NaEDTA to the reaction mixture to stop the action of carboxypeptidases A and B. α-Chymotrypsin was then added for 30 min to cleave off the truncated peptides. The reaction was stopped by the addition of 50 μM PMSF, and the entire reaction mixture was subjected to HPLC fractionation to separate the truncated peptides from the other reaction mixture components. Peptide peak fractions collected by HPLC were frozen in liquid N_2 and lyophilized, and aliquots were submitted for FAB mass spectroscopic analyses. Other peptide aliquots were analyzed by fluorometric means to quantitate the yield of the various peptides.

Activation of POX by Specific Endoproteolytic Cleavage. The HPLC-purified endoproteinase Glu-C should cleave in the α-peptide region of POX on the carboxyl side of glutamic acids-561 and -564. Similarly, endoproteinase Asp-N would be expected to cleave on the amino side of aspartic acid-560. Activation experiments with these two endoproteases were carried out in identical fashion with those developed for the carboxypeptidase experiments with two exceptions. The weight ratio of POX to endoprotease was 100:1 with both enzymes, and in the experiments with endoproteinase Asp-N, the reaction mixtures were adjusted to pH 8.2, the pH optimum for this enzyme.

HPLC Separation of Peptides. The conditions for the HPLC separation of the truncated α-peptides and the other reaction components were essentially those given by Recny et al. (1985) except that a Vydac C_8 reverse-phase column was used. Synthetic α-peptide was used as an HPLC marker and as a standard for calculating the percent recovery of truncated peptides.

Synthesis and Analysis of α-Peptide. The 23-residue α-peptide derived from the C-terminus of POX (Met-550 through Arg-572) was synthesized by employing standard *t*-Boc solid-phase synthesis techniques (Sarin et al., 1981) using an Applied Biosystems 430A peptide synthesizer. The amino acid composition was determined by methods already given. The peptide was purified by HPLC and sequenced by automated Edman degradation on an Applied Biosystems 477A pulsed-liquid-phase sequencer and a 470A gas-phase sequencer both equipped with on-line PTH amino acid analyzers (Edman & Begg, 1967; Hewick et al., 1981).

FAB Mass Spectrometry of Isolated Peptides. The instrument used was a ZAB-SE Vacuum General (Manchester, England) high-resolution double-focusing mass spectrometer operated with xenon gas at an accelerating potential of 8 kV and probe temperature of 30 °C. Lyophilized peptide samples were dissolved in a minimum volume of 0.1% TFA and loaded onto the probe tip coated with thioglycerol matrix and then inserted into the source chamber.

Quantitation of Peptides by Fluorometric Analyses. Standard techniques were used for analyzing aliquots of the peptide fractions separated by HPLC. Aliquots were incubated with 0.2 M sodium borate buffer, pH 8.2, and freshly prepared fluorescamine solution (Weigele et al., 1972; Undenfriend et al., 1972; Yokosawa et al., 1983). After rapid mixing, the resultant fluorescence intensity was measured by using an excitation wavelength at 390 nm and emission at 475 nm, 10-s integration time, on a Perkin-Elmer MPF-66 fluorescence spectrometer. All data were corrected for the appropriate blanks. Calculations for the loss of truncated peptides during HPLC separations were made by determining the percent recovery of the synthetic α-peptide which had been coinjected in the HPLC runs.

Visible Absorption Spectra of Unactivated and Proteolytically Activated POX. Spectra were recorded for POX activated to the 50% level by carboxypeptidase A, B, and Y treatment and for POX fully activated by endoproteinase Glu-C. The carboxypeptidase activation reaction mixtures were stopped after a 3-h incubation period by the addition of EDTA and PMSF to final concentrations of 5 mM and 50 μM, respectively. The endoproteinase Glu-C activation reaction mixture was stopped after a 30-min incubation period by the addition of PMSF to a final concentration of 50 μM. Cofactors, substrates, proteases, and inhibitors were removed from the experimental and control reaction mixtures by dialysis in 50000 molecular weight cutoff tubing against 100 mM sodium phosphate buffer, pH 5.8, containing 20% glycerol (v/v) for 3 h. Ubiquinone Q_0 at a final concentration of 500 μM was then added to each dialysate in order to convert the reduced enzyme to its oxidized form. The reactions were then

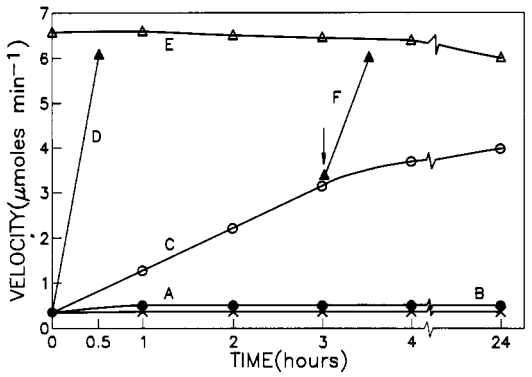

FIGURE 1: Activation of POX by exoprotease treatment. The activity curves shown are as follows: (A) Control (×), POX not incubated with protease or POX incubated with either carboxypeptidase A or B r carboxypeptidase P or Y (●); (B) control (×) or POX incubated with carboxypeptidase Y for 24 h (●); (C) POX incubated with a combination of carboxypeptidases A, B, and Y (○); (D) POX incubated with α-chymotrypsin (▲); (E) POX incubated for 2 min with lipid activator prior to addition of ferricyanide during the standard spectrophotometric enzyme activity assay (△), i.e., this curve represents fully activated POX; (F) addition of α-chymotrypsin after 3-h incubation of POX with carboxypeptidases A, B, and Y. The weight ratio of POX to α-chymotrypsin was 80:1, and in experiments involving POX/carboxypeptidases, the ratio was 160:1 (for each exopeptidase).

dialyzed for another 3-h period, and the ubiquinone Q_0 treatment was repeated. The final dialysis step was carried out overnight. Visible absorption spectra were recorded for the unactivated control and activated POX preparations in a Cary-Varian 219 spectrophotometer.

RESULTS

Activation of Pyruvate Oxidase by Carboxypeptidases. When POX is incubated separately with either carboxypeptidase A, B, P, or Y, no clearly measurable degree of activation of the enzyme is achieved over a 4-h period (Figure 1, curve A). All experiments were carried out at pH 6 and 22 °C. These conditions are most favorable for maintaining the stability of POX over extended periods of time. Carboxypeptidases A, B, and Y are known to have functional ranges which include pH 6. However, carboxypeptidase P would prefer a more acidic pH for its catalysis (Barouch, 1990). Therefore, in a separate experiment, POX was incubated with carboxypeptidase P at pH 4.2, but again no activation was obtained. Pyruvate oxidase also was incubated over an extended period of 24 h (Figure 1, curve B) with carboxypeptidase Y, which does function well at pH 6, but this treatment also failed to give any significant activation. Samples from the carboxypeptidase Y incubation mixture were prepared for amino acid analysis to determine if any residues had been cleaved from the carboxy terminus of POX by carboxypeptidase Y treatment. As shown in Table I, these analyses indicated that carboxypeptidase Y had cleaved seven residues from Arg-572 through Ala-566.

Activation of POX occurred when the enzyme was incubated with a combination of carboxypeptidases A, B, and Y (Figure 1, curve C). A linear increase in activity was observed over a 3-h period. At the 3-h time point, the level of activation was approximately 50% of that obtained when POX is assayed in the presence of a saturating concentration of lipid activator (Figure 1, curve E).

It should be noted that the addition of α-chymotrypsin at the 3-h time point leads to nearly full activation of POX (Figure 1, curve F). This result proves that POX remains a fully activatable tetrameric enzyme after a 3-h incubation with carboxypeptidases A, B, and Y. A variety of different incubation conditions were examined in attempts to significantly raise the level of activation beyond 50%, but none were successful. In particular, raising the concentrations of the three carboxypeptidases proved ineffective. Aliquots of the reaction mixtures containing 50% activated POX were removed for amino acid analyses, FAB mass spectrometric analyses, and quantitation of the truncated α-peptides cleaved from POX by α-chymotrypsin. α-Chymotrypsin rapidly activates POX to a level comparable to that achieved by lipid activator (Figure 1, curve D) by cleavage between Tyr-549 and Met-550 to give a 23-residue α-peptide stretching from Met-550 through the carboxy-terminal Arg-572. Incubation of the carboxypeptidase-activated POX preparations with chymotrypsin gives the same cleavage (Tyr-549) and releases truncated α-peptides produced by carboxypeptidase hydrolysis.

Table I: Carboxypeptidase Y Treatment of Pyruvate Oxidase[a]

amino acid released	position from C-terminus	position from N-terminus	nmol of residue/nmol of Trp
(R) Arg	1	572	0.82
(L) Leu	2, 8	571, 565	0.89
(W) Trp	3	570	1.00
(N) Asn	4	569	0.84
(T) Thr	5	568	0.88
(K) Lys	6	567	0.91
(A) Ala	7	566	1.04
(E) Glu	9	564	0

[a] Pyruvate oxidase was incubated with carboxypeptidase Y for 24 h under the conditions given under Materials and Methods. The amino acids released by this treatment were quantitated by PTC amino acid analyses (see Materials and Methods). The values shown are the mean values calculated from five determinations.

Interestingly, activation of POX to the same 50% level also can be achieved by varying the sequence and timing of the additions of the exopeptidases. For example, although the incubation of POX with carboxypeptidase Y alone for 24 h does not yield significant activation (Figure 1, curve B), the addition of carboxypeptidases A and B at the 24-h time point promotes a rapid rate of activation and yields the same 50% activated POX. Conversely, incubation of POX with carboxypeptidase A and B for 24 h yielded an approximate 30% activated enzyme. The addition of carboxypeptidase Y at the 24-h time point also raised the level of activation to 50%. In both of these sets of experiments, continued incubation for a second 24-h period produced additional activation but at a much slower rate. The maximum activation observed in the 48-h time period was approximately 70%.

Amino Acids Released by Carboxypeptidase Treatment. The amino acid analytical data on the residues from 50% activated POX following incubation with carboxypeptidases A, B, and Y are given in Table II. The data clearly show that 11 residues from Arg-572 through Val-562 were cleaved, i.e., about halfway through the 23-residue α-peptide sequence. A rather dramatic decrease in the mole ratio yield of amino acids occurs at Glu-561 and continues for all other upstream residues. In contrast, amino acid analyses on unactivated POX preparations which had been incubated with only carboxypeptidase Y for 24 h showed that only the first seven residues from Arg-572 through Ala-566 were cleaved from the carboxy terminii of POX (Table I). No significant activation of POX occurred in this experiment (Figure 1, curve B).

Mass Spectral Analysis of Truncated α-Peptides. In order to further define the extent of carboxypeptidase hydrolysis, a second approach was used. After activation of POX with carboxypeptidases A, B, and Y, the truncated carboxy-terminal chains were cleaved from the enzyme with α-chymotrypsin.

Table II: Carboxypeptidase A, B, and Y Treatment of Pyruvate Oxidase[a]

amino acid released	position from C-terminus	position from N-terminus	nmol of residue/nmol of Trp
(R) Arg	1, 15, 21	572, 558, 552	0.91
(L) Leu	2, 8, 22	571, 565, 551	1.68
(W) Trp	3	570	1.00
(N) Asn	4	569	0.89
(T) Thr	5	568	1.03
(K) Lys	6	567	0.93
(A) Ala	7, 20	566, 553	1.07
(E) Glu	9, 12	564, 561	0.69
(I) Ile	10, 18, 19	563, 555, 554	0.53
(V) Val	11	562	0.48
(D) Asp	13	560	0.06
(G) Gly	14, 16	559, 557	0.05
(S) Ser	17	556	0.04
(M) Met	23	550	0

[a] Pyruvate oxidase was incubated with carboxypeptidases A, B, and Y for 3 h under the conditions given under Materials and Methods. Amino acid analyses were carried out as described in Table I. The values shown are the mean values calculated from five determinations.

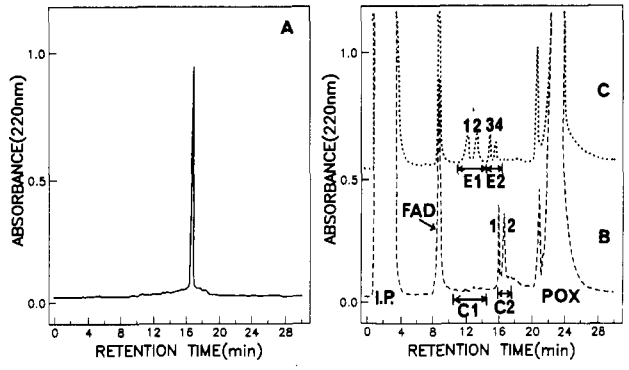

FIGURE 2: HPLC chromatography of the synthetic 23-residue α-peptide and peptide mixtures derived from α-chymotrypsin or carboxypeptidase-activated POX. The injection volumes were 500 μL. The chromatograms are as follows: (A) 300 nmol of synthetic α-peptide used as a marker and standard for calculations on percent recovery of peptides under the HPLC conditions employed; (B) POX incubated with α-chymotrypsin for 30 min; I.P. = injection peak, FAD = FAD removed from POX during HPLC, POX = main body of the enzyme minus cleaved peptides; peptide peak fractions are labeled C1 (small peaks) and C2 (two large peaks 1, 2); (C) POX incubated with a combination of carboxypeptidases A, B, and Y for 3 h followed by cleavage of the truncated peptides from the enzyme by α-chymotrypsin; peptide peak fractions are indicated by E1 (peaks 1, 2) and E2 (peaks 3, 4).

The truncated peptides were then separated by HPLC and their masses determined by FAB mass spectrometry. Since the sequence of the α-peptide is known, masses were easily correlated with particular sequences. The yield of these truncated peptides was quantitated by standard fluorometric analyses.

When native POX is incubated with α-chymotrypsin for 30 min, two major peptides, labeled peaks 1 and 2 in chromatogram B of Figure 2, are separated by HPLC. Amino acid and sequence analyses performed on peptide 1 revealed it to be the 21-residue α-peptide extending from Met-550 through Trp-570 while peak 2 peptide is the 23-residue sequence from Met-550 through the C-terminal Arg-572 (Figure 7). Furthermore, when a pooled fraction containing the peptides in peaks 1 and 2 was submitted to FAB mass spectrometry, the average molecular ions of 2372 and 2645, shown in spectrum B of Figure 3, match the masses calculated for the 21- and 23-residue α-peptide sequences, respectively. The minor peptide peaks collected in zone C1 of chromatogram B of

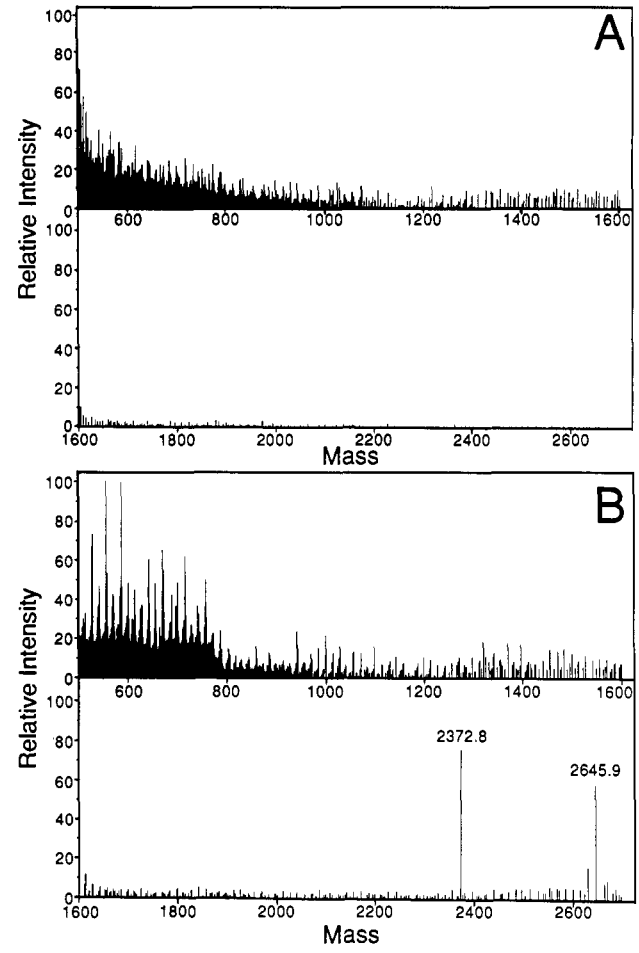

FIGURE 3: FAB mass spectroscopic analyses of HPLC-purified peptide fractions from the incubation of native POX with α-chymotrypsin. The spectra are (A) pooled minor peak fractions eluting with retention times between 10 and 14 min in zone C1 (Figure 2, chromatogram B) and (B) pooled major peak fractions 1 and 2 eluting with retention times between 16 and 16.8 min in zone C2 (Figure 2, chromatogram B).

Figure 2 failed to give any mass peaks above the background noise level in spectrum A of Figure 3.

The incubation of carboxypeptidase-activated POX with α-chymotrypsin to cleave off the truncated peptides produced four major peptides. Peptide peaks (1–4) were separated by HPLC (Figure 2, chromatogram C). FAB mass spectrometry of peptide peaks 1 and 2 gave two prominent mass peaks above background (Figure 4, spectrum A). The mass 1317 of peptide 1 corresponds to the sequence from Met-550 through Glu-561 while the mass 1416 for peptide 2 is in agreement with a mass calculated for the sequence Met-550 through Val-562 (Figure 7). These peptides represent about half of the chain length of the 23-residue α-peptide sequence and agree with the amino acid analyses which indicate that the removal of 10 or 11 amino acid residues produces activated enzyme. Peptide peaks 3 and 4 gave masses of 1772 and 1972 (Figure 4, spectrum B), corresponding to the sequences Met-550 through Leu-565 and Met-550 through Lys-567, respectively (Figure 7). These peptides must arise from POX monomers which had only been shortened by carboxypeptidase treatment by five or seven residues. These latter monomers must account for the presence of the 50% unactivated POX species since the carboxypeptidase Y experiment shows that the removal of seven residues does not produce activated enzyme.

Quantitation of Truncated Peptides by Fluorometric Analyses. Quantitation of the truncated peptides gave ad-

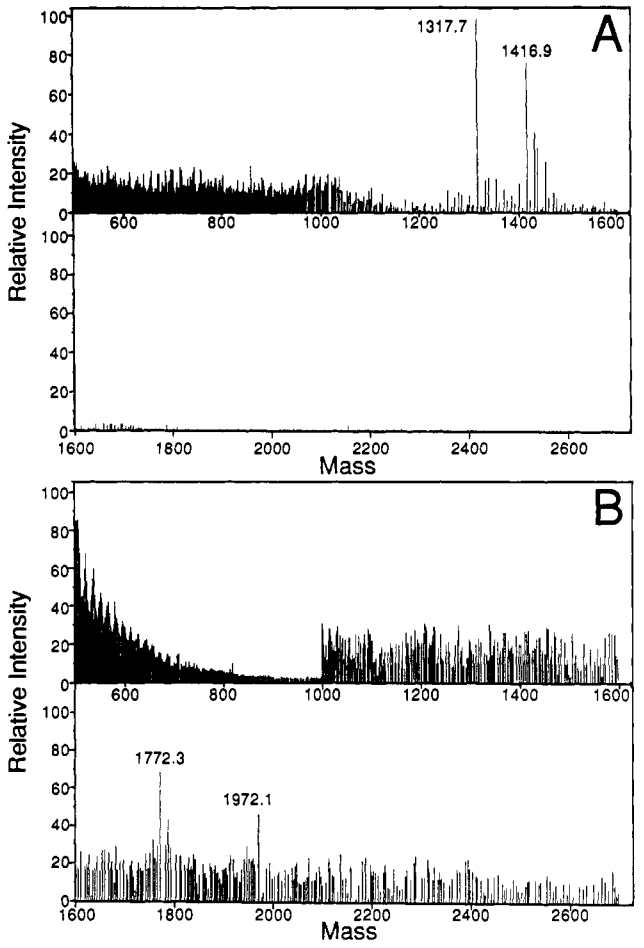

FIGURE 4: FAB mass spectroscopic analyses of HPLC-purified peptides from POX samples exposed to the combined action of carboxypeptidases A, B, and Y. The spectra are (A) pooled peak fractions 1 and 2 (11.5–13.5-min retention time) eluting in zone E1 (Figure 2, chromatogram C) and (B) pooled peak fractions 3 and 4 (14–15.5-min retention time) eluting in zone E2 (Figure 2, chromatogram C).

ditional information on the extent of cleavage of the carboxy-terminal chains of POX by the proteases. The yield of the two major α-peptides (21- and 23-mers) produced by α-chymotrypsin activation of POX matches closely to the theoretical value. Chymotryptic hydrolysis of native POX produces an activated enzyme species which is 93% activated compared to lipid-activated POX (compare curves D and E, Figure 1). The α-chymotryptic activation of 20 nmol of POX produced 18.21 nmol (91% yield) of the 21- plus 23-mer α-peptides (peaks 1 and 2, Figure 2B). Similar quantitation of the truncated α-peptides isolated from 20 nmol of carboxypeptidase-activated POX yielded 9.63 nmol of peptide 1 plus 2 (peaks 1 and 2, Figure 2C) and 7.57 nmol of peptides 3 and 4 (peaks 3 and 4, Figure 2C). Peptides 1 and 2 represent the truncated peptides which are produced from the carboxypeptidase-activated monomers while peptides 3 and 4 represent peptides derived from unactivated monomers which have been only partially cleaved by removal of five to seven C-terminal amino acid residues. Thus, for the 50% activated enzyme, the theoretical yield for peptides 1 and 2 would be 10 nmol, and the theoretical value for peptides 3 and 4 also would be 10 nmol. The experimental value of 9.63 nmol for the activated monomers is very close to unity. The yield from the carboxypeptidase-treated but unactivated monomers is less but still consistent with the data from amino acid analyses.

Activation of Pyruvate Oxidase by Endoproteinase Glu-C. In contrast, to the slow activation of POX catalyzed by the

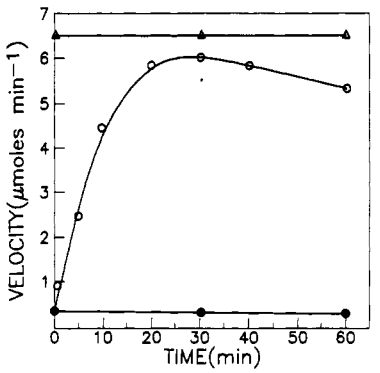

FIGURE 5: Activation of POX by endoproteinase Glu-C. The curves plot the enzyme activity as a function of time for POX alone (●), POX plus endoproteinase Glu-C (○), and POX plus 35 μM SDS (△). The assay conditions are described in Figure 1. The weight ratio of POX to endoproteinase Glu-C was 100:1.

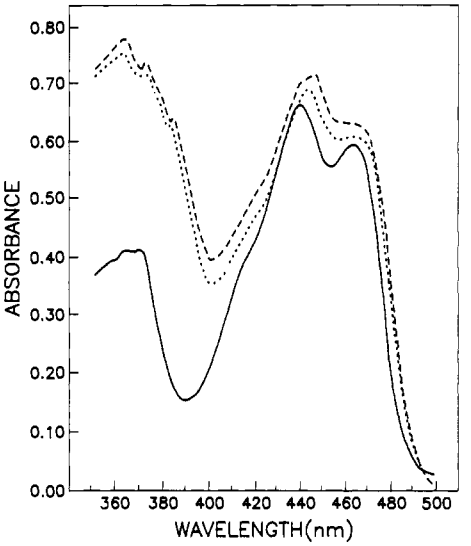

FIGURE 6: Visible absorption spectra of native and activated POX. The spectra are native untreated POX (—), carboxypeptidase-activated POX (···), and endoproteinase Glu-C activated POX (- - -). The concentration of POX is approximately 11 μM.

carboxypeptidases, endoproteinase Glu-C rapidly promotes essentially full activation of POX in 30 min (Figure 5). On the basis of its known specificity, endoproteinase Glu-C should catalyze the activation of POX by hydrolyzing the peptide bond between Glu-561 and Val-562. Endoproteinase Asp-N also should cleave POX in the α-peptide region by hydrolyzing the peptide bond between Gly-559 and Asp-560. However, incubation of POX with endoproteinase Asp-N either at pH 6 or at 8.2 pH gave very limited activation. Control experiments indicated that endoproteinase Asp-N catalyzes the inactivation of POX, presumably by cleaving peptide bonds in sensitive areas of the enzyme.

Visible Spectroscopy of Activated Pyruvate Oxidase. The activated flavoprotein shows red-shifted FAD absorption bands in the visible region (Figure 6) similar to those observed by Recny and Hager (1983) for the α-chymotrypsin-activated POX. Carboxypeptidase-activated POX showed a band shift maximum from 437–438 to 441 nm, and for the endoproteinase Glu-C activated enzyme, the band peak shifted to 443 nm.

Discussion

The C-terminal amino acid sequence of pyruvate oxidase which encompasses the α-peptide domain of the enzyme is given in Figure 7. Previous work has shown that α-chymo-

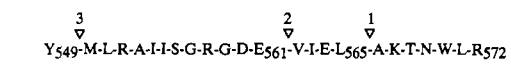

FIGURE 7: Exopeptidase trimming in the α-peptide domain of POX. The arrow at 1 marks the extent of cleavage by carboxypeptidase Y alone. Carboxypeptidase Y alone does not produce activated enzyme. The arrow at 2 marks the maximum cleavage achieved by the combined action of carboxypeptidases A, B, and Y. The arrow at 3 marks the site of α-chymotrypsin cleavage which produces the α-peptide.

tryptic cleavage at site 3 (Figure 7) releases the 23-residue α-peptide and completely activates the enzyme for catalysis (Recny et al., 1985). The carboxypeptidase experiments reported in this paper were designed to define the minimum requirement for protease activation of POX. Two major carboxypeptidase stop sites have been identified in this C-terminal region. Stop site 1 is seven amino acid residues upstream from the C-terminal Arg (R_{572}). POX monomers which have been trimmed back by carboxypeptidase action only to site 1 remain in the unactivated form. Stop site 2 is 11 amino acid residues upstream from the C-terminus. POX monomers which have been trimmed back to site 2 appear to be fully activated. We conclude from these results that the minimum requirement for protease activation of POX is the removal of 8–11 amino acid residues from the C-terminus of the enzyme.

Under the conditions employed in this study, no significant activation of POX could be achieved by the reaction of the enzyme with either carboxypeptidases A, B, P, or Y separately. Even incubation of POX over an extended 24-h period with carboxypeptidase Y, which is known to have a rather broad specificity toward carboxy-terminal amino acids (Kuhn et al., 1987), did not result in activation. The failure to achieve activation was not due to any great decline in POX activity since assay of the reaction mixtures both in the absence and in the presence of lipid activator showed that POX activity remained relatively undiminished even at the 24-h time point. Nor was the failure to achieve activation due to the loss of carboxypeptidase Y activity. Assay of aliquots of the reaction mixture by standard methodology measuring the decrease in absorbance at 224 nm upon hydrolysis of *N*-Cbz-L-phenylalanine-L-leucine (Hayashi, 1976) showed that carboxypeptidase Y also remained quite active at the 24-h time point. Amino acid analyses determined the extent of release of residues from POX by exposure to carboxypeptidase Y for 24 h and identified stop site 1. Quantitation showed that all monomers were cleaved to the same extent by carboxypeptidase Y.

In contrast, a significant degree of activation of POX could be achieved by incubation of the enzyme with a combination of carboxypeptidases A, B, and Y. Experiments have shown that the order of addition of the three carboxypeptidases, A, B, and Y, is not important in achieving 50% activation. The most rapid rate of activation was obtained when POX was incubated simultaneously with all three exopeptidases. However, the same degree of activation could be achieved by incubating POX with carboxypeptidase Y followed by A and B or, conversely, by incubating POX first with carboxypeptidases A and B followed by Y. This success was probably due, at least in part, to the fact that at pH 6 carboxypeptidase A would be expected to cleave mainly neutral residues while carboxypeptidases B and Y would act predominantly on acidic and basic amino acid residues, respectively (Barouch, 1990). However, the carboxypeptidase A, B, and Y treatment never yielded completely activated POX. The time course of the reaction showed a linear increase in POX activity up to approximately 50% of fully activated enzyme and then leveled off. Complete activation of POX can be obtained, however, by adding α-chymostrypsin or endoproteinase Glu-C to POX which has been activated to the 50% level by carboxypeptidase treatment. Previous experiments have shown that α-chymotrypsin is capable of cleaving the α-peptide from each of the four monomers which constitute the homotetrameric POX enzyme (Recny et al., 1985). Proteolytic activation of POX by a variety of endoproteases essentially produces 100% activated enzyme (Russell et al., 1977a). In this study, full activation of POX by endoproteinase Glu-C provides additional support for the conclusion reached in the carboxypeptidase experiments. That is, endoproteinase Glu-C would be expected to remove 11 residues from the carboxy terminus of POX (Val-562 to Arg-572). The removal of these same 11 residues by the combined action of carboxypeptidases A, B, and Y also produces an activated POX. Endoproteinase Asp-N should also activate POX by hydrolytic removal of the 12 C-terminal amino acid residues of POX. The incubation of POX with endoproteinase Asp-N does yield a partially activated enzyme, but the results are complicated by a significant inactivation of POX by endoproteinase Asp-N. Thus, it appears at this point that endoproteinase hydrolysis is capable of modifying all of the POX monomers, but in the carboxypeptidase case, two of the four monomers in the homotetramer are or become recalcitrant to cleavage. The chemical and mass spectral analyses of the carboxypeptidase digestion products support this conclusion. Direct amino acid analyses performed on the carboxypeptidase A, B, and Y reaction mixtures were difficult to interpret because of uneven trimming. In order to more clearly identify the modifications introduced by carboxypeptidase A, B, and Y digestion, a mass spectral analysis of truncated α-peptides was introduced. Chymotrypsin cleaves between Tyr-549 and Met-550 to give the 23-residue α-peptide. Treatment of the 50% carboxypeptidase-activated monomers with α-chymotrypsin yielded two sets of truncated peptides. Mass spectral analysis of 1 set of truncated peptide indicated that 10 or 11 amino acid residues had been removed from the C-terminus of the enzyme. Mass spectral analysis of the second truncated peptide set indicated that only five or seven residues had been removed. Thus, the analytical data support the conclusion that half of the C-terminal residues in POX are inherently resistant or become resistant to carboxypeptidase action during the course of the experiment. Since the C-terminal sequences of all POX monomers are identical, the half and half observation suggests that activation of two of the four subunits in the homotetramer produces a conformational change which makes the two remaining monomers resistant to carboxypeptidase action. Grabau et al. (1989) have pointed out that if the quaternary structure of POX is the common isologous square motif, two of the C-terminal helices may be sufficiently close to pair and give a two-stranded structure having hydrophilic faces in contact with each other and with hydrophobic faces exposed to a lipid bilayer. It is possible that such a two-stranded structure would behave differently in its reactivity to carboxypeptidase digestion. In this connection, it should also be mentioned that the removal of 10 or 11 C-terminal residues which produces the activated enzyme cuts away the putative membrane-binding amphipathic helical sequence between Gly-559 and Thr-568 (Recny et al., 1985). It has been postulated that the loss of this helical domain, either via lipid binding or via proteolysis, freezes the reduced enzyme in its activated state (Recny et al., 1985; Zhang & Hager, 1987a,b; Grabau et al., 1989). An alternative but less likely interpretation would be that 50% of the homotetramers can be fully activated by exopeptidase treatment

while the other 50% are completely resistant.

Activated POX preparations, activated either by carboxypeptidase or by endoproteinase Glu-C treatment, show significant changes in their visible absorption spectra. In both preparations, the second electronic absorption band (near 380 nm) associated with the FAD prosthetic group of POX is red-shifted. A similar absorption band shift has been detected with α-chymotrypsin-activated POX (Recny & Hager, 1983) and with lipid-activated POX (Mather & Gennis, 1985). The red-shifted absorption spectrum is consistent with a less rigid, more solvent-exposed environment for the flavin prosthetic group in the activated enzyme. The oxidized spectrum of α-chymotrypsin-activated POX is very similar to the absorption spectrum of free oxidized FAD in aqueous solution. Since previous circular dichroism studies failed to find major secondary structural alterations associated with the activation of POX (O'Brien et al., 1982), it appears that activation of POX primarily involves a conformational change which moves the FAD prosthetic group from a hydrophobic pocket to a hydrophilic environment.

Acknowledgments

We thank Mr. Saw Kyin and associates of the Genetic Engineering Facility/Protein Sequencing Lab, University of Illinoise, Champaign—Urbana, for the amino acid analyses, peptide synthesis, and peptide sequencing analyses; Dr. Richard Milberg and staff of the Mass Spectroscopy Lab, University of Illinois, Champaign—Urbana, for the FAB mass spectroscopic analyses; Ron Reinhart of the Drafting Facility of Roger Adams Laboratory and Jim Gray of the Graphic Services of Noyes Laboratory for preparation of the figures; and Terri George for preparation of the manuscript.

Registry No. POX, 9001-96-1.

References

Barouch, W. W. (1990) *Pept. Res. 3*, 73.

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

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Edman, P., & Begg, G. (1967) *Eur. J. Biochem. 1*, 80.

Grabau, C., & Cronan, J. E., Jr. (1984) *J. Bacteriol. 160*, 1088.

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

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

Hager, L. P. (1957) *J. Biol. Chem. 229*, 251.

Hamilton, S. E., Recny, M. A., & Hager, L. P. (1986) *Biochemistry 25*, 8178.

Hayashi, R. (1976) *Methods Enzymol. 45*, 568.

Hewick, R. M., Hunkapiller, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990.

Kuhn, R. W., Walsh, K. A., & Neurath, H. (1976) *Biochemistry 15*, 4881.

Lee, H.-M., & Riordan, J. F. (1978) *Biochem. Biophys. Res. Commun. 85*, 1135.

Mather, M. W., & Gennis, R. B. (1985) *J. Biol. Chem. 260*, 10395.

O'Brien, T. A., Schrock, H. L., Russell, P., Blake, R., & Gennis, R. B. (1976) *Biochim. Biophys. Acta 452*, 13.

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

Recny, M. A., & Hager, L. P. (1982) *J. Biol. Chem. 257*, 12878.

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

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

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

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

Sarin, V. K., Kent, S. B. H., Tam, J. P., & Merrifield, R. B. (1981) *Anal. Biochem. 117*, 147.

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

Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science 178*, 871.

Weigele, M., De Bernardo, S. L., Tengi, J. P., & Leimgruber, W. (1972) *J. Am. Chem. Soc. 94*, 5927.

Williams, F. R., & Hager, L. P. (1966) *Arch. Biochem. Biophys. 116*, 168.

Yokosawa, H., Ito, H., Murata, S., & Ishii, S.-I. (1983) *Anal. Biochem. 134*, 210.

Zhang, T.-F., & Hager, L. P. (1987a) *Arch. Biochem. Biophys. 255*, 201.

Zhang, T.-F., & Hager, L. P. (1987b) *Arch. Biochem. Biophys. 257*, 485.