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Three of Four Cysteines, Including That Responsible for Substrate Activation, Are Ionized at pH 6.0 in Yeast Pyruvate Decarboxylase: Evidence from Fourier Transform Infrared and Isoelectric Focusing Studies†

Irina Baburina, David J. Moore, Alexander Volkov, Ara Kahyaoglu, Frank Jordan,* and Richard Mendelsohn

Department of Chemistry, Rutgers, the State University of New Jersey, 73 Warren Street, Newark, New Jersey 07102

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ABSTRACT: Oligonucleotide-directed site-specific mutagenesis was carried out on pyruvate decarboxylase (EC 4.1.1.1) from *Saccharomyces cerevisiae* at three of the four cysteines (152, 221, and 222), the fourth (69) being buried according to X-ray crystallographic results [Arjunan et al. (1996) *J. Mol. Biol. 256*, 590−600]. All of the variants still retained significant activity, and all could be purified to homogeneity. FT-IR experiments were run on the C221S, C222S, C221S/C222S and C152A variants, as well as on the wild-type enzyme. There is a band present at 2557 cm^{-1} in the spectra of *all* variants and the wild-type enzyme, except in the spectrum of the C152A variant. This frequency is appropriate to a cysteine S−H stretching mode. It was therefore concluded that C152 is the only undissociated cysteine on the enzyme at pH 6.0, the pH optimum of this enzyme, whereas C221, C222, and C69 are all ionized. Isoelectric focusing experiments were carried out on all of these variants, as well as on the H92A variant (H92 is across the domain divide on the α domain, from C221 located on the β domain). The variation in isoelectric points deduced from the data was consistent with removal of negative charges concomitant with the C221S, C222S, and C221S/C222S substitutions and removal of a positive charge with the H92A substitution when compared to that of the wild-type enzyme. The results of these two types of experiments are in good accord and suggest that the site of substrate activation at C221 [Baburina et al. (1994) *Biochemistry 33*, 5630−5635] is comprised of a $Cys221S^{-}$ $^{+}$HHis92 ion pair, not unlike that found in papain and glyceraldehyde-3-phosphate dehydrogenase. This finding suggests that the regulatory site of this enzyme has been optimized for nucleophilic reactivity between the thiolate of C221 and the keto carbon of the 2-oxoacid.

Pyruvate decarboxylase from brewers' yeast (PDC,[1] EC 4.1.1.1) is one of several enzymes that participate in nonoxidative functions of thiamin diphosphate (ThDP, the vitamin B1 coenzyme). PDC converts pyruvate to acetaldehyde and carbon dioxide [for reviews, see Krampitz (1969), Sable and Gubler (1982), Kluger (1987), Schellenberger and Schowen (1988), and Bisswanger and Ullrich (1991)]. The enzyme also has a requirement for a divalent

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* To whom correspondence should be addressed. Telephone: 201-648-5470. Fax: 201-648-1264. E-mail: frjordan@andromeda.rutgers.edu.

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[1] Abbreviations: FWHH, full peak width at half-height; ThDP, thiamin diphosphate; PDC, pyruvate decarboxylase (EC 4.1.1.1); pdc1, wild-type pyruvate decarboxylase isolated from *Saccharomyces cerevisiae*; WT, wild-type PDC; C221S, C222S, C221S/C222S, C152A, and H92A are variants of this enzyme; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride.

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cation best fulfilled by Mg(II). The presence of divalent cations as part of the diphosphate binding locus has been confirmed on the three ThDP-dependent enzymes whose structures are known to date (Müller et al., 1993). According to chemical modification of PDC (Ullrich, 1982), traditional thiol reagents inactivate PDC, and cysteines are believed to be the only highly reactive side chains that alter enzyme activity [see also Zeng et al. (1991, 1993) and references therein]. The three-dimensional structure of PDC revealed the presence of four subunits of 563 amino acids each. According to the amino acid sequence deduced from the gene sequence (Hohmann & Cederberg, 1990), in the enzyme isolated from *Saccharomyces cerevisiae*, each subunit possesses four cysteines at positions 69, 152, 221, and 222, none of which participates in disulfide bridges. The X-ray structures of PDC from *Saccharomyces uvarum* (Dyda, 1992; Dyda et al., 1993) and *S. cerevisiae* (Arjunan et al., 1996) have revealed that the Cys nearest to ThDP (C221) is >20 Å away from the reaction center, the C2 atom of the thiazolium ring on ThDP. PDC from yeast is subject to substrate activation (Boiteux & Hess, 1970; Hübner et al., 1978), and it was proposed that a cysteine side chain may be responsible for this activation (Sieber et al., 1983; Schellenberger et al., 1988). The ThDP resides in a cleft between the α and γ domains in different subunits. The Cys side chains at positions 221 and 222, most favorably positioned for a potential regulatory function, reside on the β domain (Dyda et al., 1993; Arjunan et al., 1996).

In earlier studies from these laboratories, it was first shown that C221 as the single cysteine in a pdc1−6 fusion enzyme (consisting of amino acids 1−45 derived from the *PDC1* and 46−563 derived from the *PDC6* gene) can still be activated by pyruvate (Zeng et al., 1993). Next, the C221S, C222S (one-site), and C221S/C222S (two-site) mutant yeasts were constructed, and then the genes were transferred into a high-expression *Escherichia coli* vector for overexpression. Both pre-steady state and steady state kinetic measurements demonstrated that substitution of C221, but not of C222, with serine led to the abolition of substrate activation, thereby identifying C221 as the site at which substrate or the substrate surrogate pyruvamide [that can also activate the enzyme but cannot be decarboxylated; see Hübner et al. (1978)] is bound. To date, it is not known whether the substrate is bound with a covalent or noncovalent linkage. A third cysteine, C152, while accessible, is not favorably positioned to interact either with ThDP or with any of the other protein domains. Since there was evidence that during the preparation of Hg derivatives for X-ray studies C152 was sometimes found labeled by the Hg, C152 was also substituted for an alanine for assignment purposes.

To gain further insight into the reactivity of C221, we have undertaken FT-IR and isoelectric focusing experiments on several variants of the PDC from *S. cerevisiae*. The sensitivity of protein S−H stretching modes in the IR spectrum to tertiary structure/hydrogen bonding state has been clearly established by Alben and his co-workers (Bare et al., 1975) in their studies of hemoglobin. Consequently, a detailed study was undertaken of the S−H stretching region (2500−2600 cm^{-1}) for PDC and its aforementioned variants to determine the state of cysteine ionization. Isoelectric focusing experiments were also conducted to provide independent confirmation of the conclusions drawn from the FT-IR studies. It is demonstrated that FT-IR is capable of detecting a single S−H bond and assigning cysteine ionization states in proteins as large as PDC (240 kDa, four identical subunits). The evidence suggests that of the four cysteines found in PDC (at positions 69, 152, 221, and 222) only C152 is un-ionized near pH 6. This information provides an important further step in delineating the substrate activation mechanism.

EXPERIMENTAL PROCEDURES

The construction of vectors and overexpression of C221S, C222S singly, and C221S/C222S doubly substituted variants was reported earlier (Baburina et al., 1994). Restriction enzymes were purchased from Promega and New England Biolabs. Sequencing was performed on an Applied Biosystems Model 373 DNA Sequencer using a dye-terminator sequencing kit.

Construction of the C152A and H92A Mutant PDC Genes. Mutagenesis reactions were performed according to the Chameleon Double-Stranded Site-Directed Mutagenesis Kit from Strategene (Papworth et al., 1995). A pET1120 vector containing the pyruvate decarboxylase gene (Baburina et al., 1994) was heat-denatured and annealed with two oligonucleotide primers. One primer (mutagenic primer), 5′AGGCTGAAATCGATAGA**GCT**ATCAGAACCAC3′, changed the cysteine at position 152 to alanine (TGT to GCT, bold) and created a *Cla*I restriction site for mutant screening, by changing nucleotide sequence ATTGAC to ATCGAT (underlined). Another primer (selection primer), 5′CTGTGACTGGTGCGTCAACCAAGTC3′, changed the *Sca*I restriction site on the Amp gene to a *Mlu*I restriction site. Annealed primers were extended with T7 DNA polymerase and treated with T4 DNA ligase. The resulting mixture was treated with *Sca*I restriction endonuclease and transformed into XLmutS competent cells. The transformed bacteria were grown overnight in liquid culture, and the plasmid DNA was isolated and digested with *Sca*I endonuclease again. The reaction mixture was transformed into Epicurian Coli XL1-Blue competent cells, and the cells were grown overnight on LB agar plates containing 100 u/mL ampicillin. Plasmid DNA from six ampicillin resistant clones were screened for the presence of the mutation by digestion with *Cla*I endonuclease. The correct mutation was identified in two out of six clones. The mutation was confirmed by sequence analysis using the primer 5′CCGCGAAATTAATACGACTCACTATAGGG3′. The mutated plasmids were transformed into the BL21(DE3) strain of *E. coli* for expression.

The H92A-mutated *PDC* gene was constructed following the same procedure. Mutagenic primer 5′CTTACGCTGAA**GCC**GTCGGTCTTTTGC3′ changed His at position 92 to Ala (codon 92 from CAC to GCC, bold) and eliminated the restriction site *Afl*III (underlined). Six ampicillin resistant clones were screened by digestion with *Afl*III endonuclease; one of six clones contained the mutation at position 92. Mutation was confirmed by sequence analysis using the same primer as for the C152A mutation.

Enzyme Purification and Assay. The enzyme was purified according to Farrenkopf's protocol (Farrenkopf & Jordan, 1992). Protein assay was performed by the Bradford (1976) method, and the enzyme was assayed by the alcohol dehydrogenase coupled assay (Holzer et al., 1956) by monitoring the depletion of NADH with time at 340 nm on the COBAS-BIO (Roche Diagnostics) automated assay

system. All kinetic assays were done at 25 °C. One unit of activity is defined as the amount of PDC required to convert 1 μmol of pyruvate to acetaldehyde per minute at 25 °C at pH 6.0.

SDS−PAGE. Polyacrylamide gel electrophoresis was run under denaturing conditions in the presence of sodium dodecyl sulfate (SDS−PAGE) according to the protocol of Laemmli (1970). The gel was stained with Coomassie Brilliant Blue R.

Isoelectric Focusing. Three milliliter samples of PDC (wild-type or variants) with initial concentrations of 1.0−2.5 mg/mL dissolved in 100 mM KP_i (pH 6.30) containing 10 mM ThDP, 2 mM PMSF, and 0.1% (w/v) sodium azide were centrifuged at 5000 rpm (3830*g*) for 30 min in the JA-14 rotor of a Beckman J2-21 centrifuge in Centricon 30 centrifugal concentrators purchased from Amicon. After the sample volume was reduced to 100−200 μL, the volume was increased to 3 mL with 10 mM KP_i (pH 6.30) for native or with 8 M urea for denaturing isoelectric focusing and then centrifuged again to reduce the volume to 100−200 μL. The procedure was repeated three or four times to ensure complete transfer of the sample to the desired medium. The resulting samples had a volume of 0.5−1.0 mL and a concentration of 4−6 mg/mL protein. A 0.5−2.0 μL aliquot was diluted to a 20 μL total volume with MilliQ water immediately prior to application to the gel.

Precast Immobiline Dry Plate purchased from Pharmacia/LKB was rehydrated for 2 h with MilliQ water for native isoelectric focusing or in 8 M urea for isoelectric focusing under denaturing conditions. Samples containing 0.5−1.5 mg of protein in an appropriate solvent were applied close to the cathodic end of the Immobiline Dry Plate on the LKB Multiphor electrophoresis system under the following conditions at 10 °C: pH 4−7 water, 2000 V, 6 mA, 11 W, 1h; pH 4−7, 8 M urea, 2000 V, 6 mA, 11 W, 1.5−2 h; pH 5−6, water, 1500 V, 6 mA, 11 W, 1 h.

FT−IR Sample Preparation and Data Collection. Three milliliter samples of PDC (wild-type or variants) with initial concentrations of 2.5−5.5 mg/mL dissolved in 100 mM KP_i (pH 6.30) containing 10 mM ThDP, 2 mM PMSF, and 0.1% (w/v) sodium azide were centrifuged at 5000 rpm (3830*g*) for 30 min in the JA-14 rotor of a Beckman J2-21 centrifuge in Centricon 30 centrifugal concentrators purchased from Amicon. The resulting samples had a volume of 0.1−0.25 mL and an enzyme concentration of 35−70 mg/mL. Samples were kept on ice to avoid precipitation.

A 30 μL sample of protein solution was placed between two CaF_2 windows separated with a 50 μm spacer. Spectra were acquired at room temperature on a Mattson RS1 FTIR spectrometer. Spectra were generated by coaddition of 1024 interferograms collected at 4 cm^{-1} resolution and Fourier transformed with triangular apodization; two orders of zero filling were employed, yielding spectra encoded every 1 cm^{-1}.

Processing of FT-IR Spectral Data. Temperature- and path length-matched IR spectra of pure buffer were subtracted from protein spectra between 2500 and 2600 cm^{-1}. In all cases (except the C152A variant), the resulting difference spectra contained a single peak at ∼2557 cm^{-1} due to a Cys S−H stretching mode. A two-point baseline was determined between minima in the difference spectra before further measurements were made. Integrated intensities, full peak widths at half-height (FWHH), and peak positions were measured from baseline-corrected difference spectra. In some experiments, several different PDC variants were dissolved in a sodium azide-containing buffer. The concentration of sodium azide was constant in all PDC samples, allowing the IR absorption band arising from the N_3 asymmetric stretch (∼2050 cm^{-1}) to be used as an internal path length standard for normalizing the S−H band. This protocol removes any variation in absorbance due to slight differences in path length between samples. However, with this approach, it is necessary to determine the PDC protein concentration of each sample from some standard chemical analysis (Bradford, 1976) to compare the integrated intensity of the S−H band in each spectrum. A more direct method of measuring protein concentration, and that utilized in comparative intensity measurements (Table 1), was to determine the integrated intensity of the spectral region from ∼1430 to 1480 cm^{-1}. The region contains protein vibrations which are assumed to be of a constant intensity among variants. Spectral integration was accomplished in this region above a two-point baseline connecting spectral minima at ∼1430 and 1480 cm^{-1}. Ratioing the integrated intensity of the S−H bands to this region thus normalizes the data for protein concentration. The precision of the S−H band intensities is such that any peak >5% of the band shown in Figure 1B would be detected.

Table 1: Cys S−H Stretching Band Parameters of Wild-Type and Variant PDCs as Measured in FT-IR Difference Spectra

enzyme variant	peak position (cm^{-1})	FWHH[a]	intensity[b]
wild-type (WT)	2557.2	10.1	0.0047
WT + ketomalonate	2557.3	9.5	0.0048
C221	2557.5	9.9	0.0054
C222	2557.4	11.6	0.0064
C221/C222	2557.5	9.8	0.0043
C152A	−	−	0.0000 ± 0.0003

[a] Full width at half-height. [b] Reported as the integrated intensity of the S−H band divided by the integrated intensity of the protein bands between 1430 and 1480 cm^{-1}.

RESULTS

Two new variants of PDC, in addition to those reported earlier at C221 and C222 (Baburina et al., 1994), with substitution at positions C152 and H92 were prepared and overexpressed. The presence of the mutated genes was confirmed by both restriction analysis and DNA sequencing (Baburina, 1996). All of the variant enzymes were purified to homogeneity, giving a single band on SDS−PAGE. The motivation for the substitution at C152 is to provide a data base sufficient to rule in or out all four cysteines in the sequence. C69 appears to be buried (Dyda et al., 1993; Arjunan et al., 1996), and as will be shown below, the ionization states of all four cysteines could be determined at pH 6.0, the optimum pH of the enzyme, without making an explicit substitution at position 69. The residue H92 was substituted because of the possibility of a direct interaction of this side chain (located on the α domain) with C221, a residue facing it on the β domain (Arjunan et al., 1996; Baburina et al., 1994; Baburina, 1996). The significant activity of all PDC variants is listed in Table 2.

Original, difference and second-derivative spectra for the S−H stretching region of WT PDC are shown in Figure 1. A peak at 2557 cm^{-1} is evident in the spectrum. Single

Table 2: Kinetic Parameters of PDC Variants Used in This Study[a]

enzyme variant	specific activity (u/mg)	K_m[b] (mM)	n[c]
wild-type (C221)	50 ± 5	1.1 ± 0.2	2.1 ± 0.04
C221S	10 ± 3	7.6 ± 0.5	0.89 ± 0.02
C222S	45 ± 3	4.3 ± 0.6	1.8 ± 0.03
C221S/C222S	9 ± 4	8.8 ± 0.4	0.98 ± 0.02
C152A	40 ± 2	3.23 ± 0.5	1.98 ± 0.01
H92A	25 ± 3	10.1 ± 0.5	1.32 ± 0.05

[a] At 25 °C. [b] For Hill coefficient ≠1.0, it is an apparent value. [c] Hill coefficient.

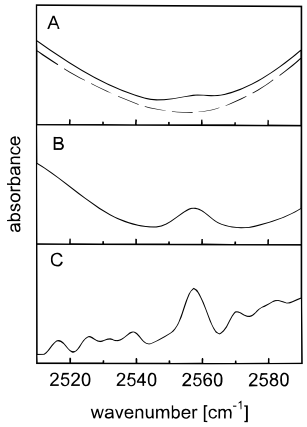


FIGURE 1: FT-IR spectrum of wild-type PDC in the S−H stretching region. Figure 1A displays original spectra of wild-type PDC in buffer (—) and buffer alone (- - -). Figure 1B shows the difference spectrum generated by subtracting the buffer spectrum from the PDC spectrum; the original spectra were obtained under identical conditions (see Experimental Procedures). The subtraction factor was determined such that the minima of the peaks were approximately level; thus, a horizontal two-point baseline could be drawn between the minima. Since path length, temperature, and IR windows were the same, the subtraction factor was routinely very close to unity. Figure 1C is the inverted second-derivative spectrum of the original unsubtracted wild-type PDC spectrum. The difference and derivative spectra are in excellent agreement; both reveal the presence of a single peak at ∼2557 cm^{-1}.

peaks at about the same position are observed in the FT-IR difference spectra for WT with ketomalonate, WT/KM [a "lock-on" activator (Alvarez & Schowen, 1991) that has been clearly shown to reside at C221 (Baburina, 1996)], C221, C222, and C221/C222 PDC. A somewhat narrower range of S−H frequencies for these molecules is displayed in Figure 2A,B on the left-hand side. Included for comparison in each case (Figure 2A,B, right-hand side) is the spectral contour between 1430 and 1480 cm^{-1}, which provides a direct measure of protein concentration. The difference spectrum of C152A (Figure 2A) however had no measurable band in the S−H stretching region greater than 5% of the 2557 cm^{-1} intensity in any other variant or the WT spectrum. This variant was examined at a protein concentration at least twice that of any other, as judged by the integrated intensity of the 1430−1480 cm^{-1} contour (Figure 2A). The peak position, full width at half-height (FWHH), and integrated intensity of the 2557 cm^{-1} peak did not vary, within experimental error, among all samples in which it was observed. Band parameters are collected in Table 1.

The isoelectric focusing experiments were performed under several conditions to optimize the resolution. The working hypothesis was that, for an un-ionized S−H group, substitution by Ser-OH will lead to no changes in the isoelectric point, whereas if the cysteine is ionized at the same pH, the

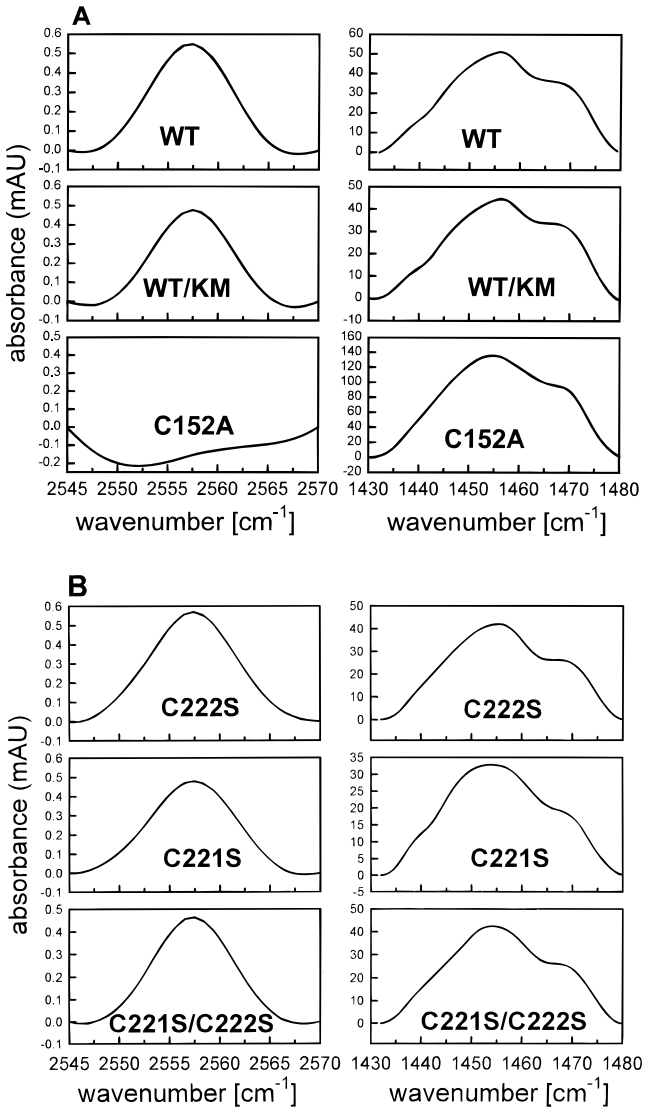


FIGURE 2: FT-IR spectra of wild-type and variant PDCs. The S−H stretching region of the protein spectra is displayed in the difference spectra on the left side. In all cases, these spectra were generated by subtraction of a pure buffer spectrum collected under conditions identical to those of the protein, i.e., same scan duration, temperature, path length, and IR windows. In all samples except the C152A variant, a single peak at ca. 2557 cm^{-1} was observed in the difference spectra. It is evident from these spectra that the band at 2557 cm^{-1} is an S−H stretching mode originating from C152, while the other cysteines must be ionized at this pH. The spectra displayed on the right-hand side are from a different region of the same spectrum in the same sample and were used to normalize S−H intensities for protein concentration; these bands arise from a variety of amino acid modes. The spectra have been baseline corrected between minima that occur at ∼1430 and 1480 cm^{-1}. Panels A and B show the behavior of the different variants examined. WT is the wild-type PDC, whereas WT/KM is in the presence of 1 mM ketomalonate.

isoelectric point may undergo a measurable change. Panel B of Figure 3 shows the results of a typical experiment. The experimentally determined isoelectric points are 5.61, 5.70, 5.66, 5.84, 5.64, and 5.51 for the WT PDC and the C221S, C222S, C221S/C222S, C152A, and H92A variants, respectively. Most shifts are ca. 0.09−0.10 unit per integral charge. The C221S, C222S, and C221S/C222S substitutions shift the isoelectric point in a direction that is consistent with removal of a negative charge at these positions, compared to the WT PDC. By contrast, the H92A variant exhibits a shift in the isoelectric point in a direction consistent with a decrease in

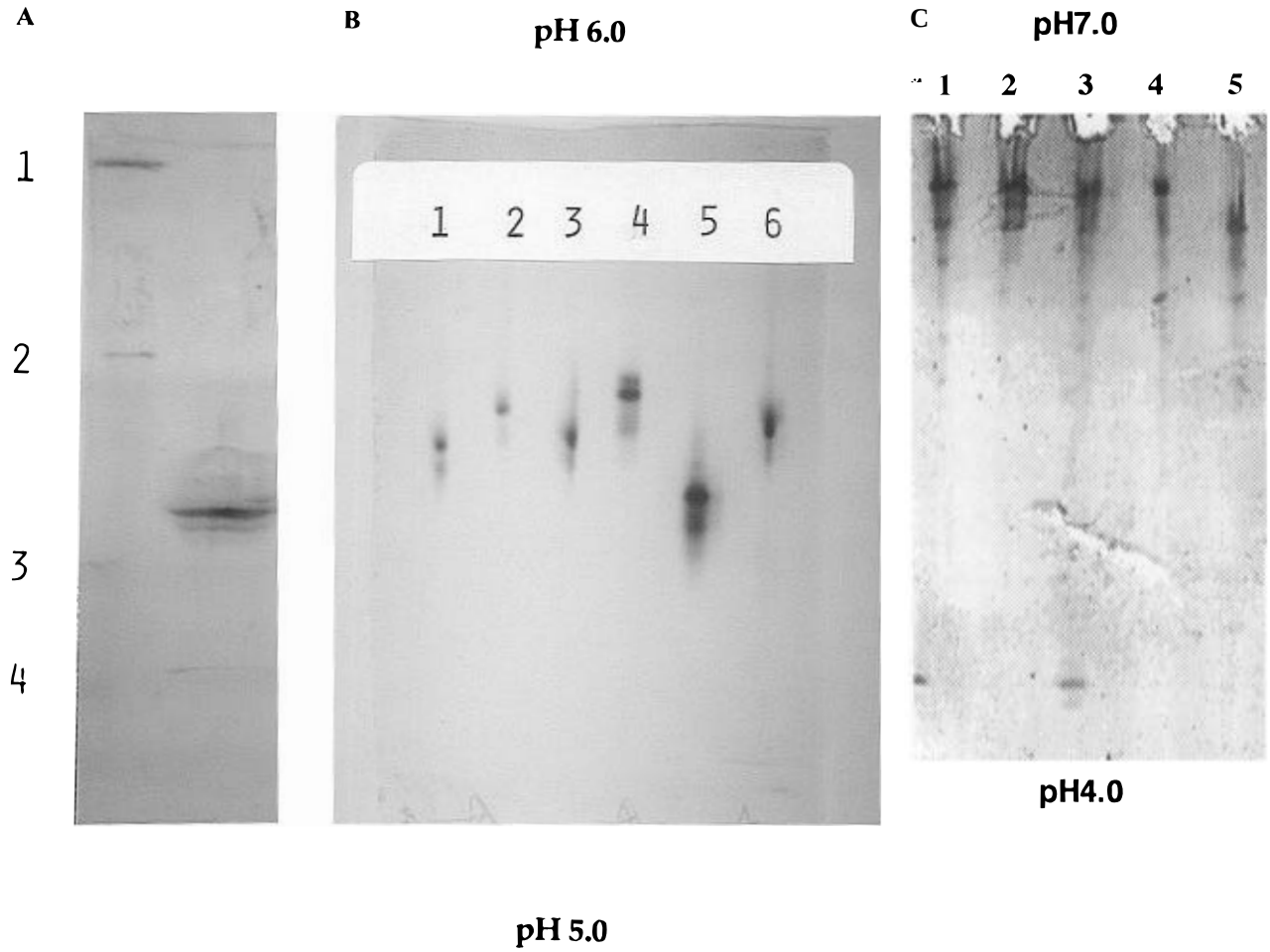


FIGURE 3: Isoelectric focusing of wild-type and variant PDCs. Panel A shows wild-type PDC on the right-hand side and standards at pH 6.85, 6.55, 5.20, and 4.55 from top to bottom on the left-hand side run under native conditions in an Immobiline pH 4.0 to 7.0 gradient. Panel B shows a native Immobiline pH 5.0 to 6.0 gradient for WT PDC (1) and the C221S (2), C222S (3), C221S/C222S (4), H92A (5), and C152A (6) variants. Panel C shows Immobiline pH 4.0 to 7.0 gradients in 8 M urea for WT PDC (1) and the C221S (2), C222S (3), C221S/C222S (4), and H92A (5) variants.

positive charge and strongly suggests that H92 is in its imidazolium ionization state in the WT at pH 6.0. Also supporting this interpretation are the isoelectric focusing results obtained in 8 M urea (Figure 3, panel C), according to which under denaturing conditions all cysteine variants have the same mobility but the H92A variant still reflects removal of a positive charge. This affirms that the perturbed p*K* values of the three cysteines are a result of secondary/tertiary structural interactions, whereas H92 is protonated at pH 6.0 under both native and denaturing conditions. In the urea gel, there is evidence of other bands. Since there are no additional bands present in the SDS−PAGE and native IEF gels, we conclude that these additional bands in urea do not represent breakdown products; rather, they may result from carbamoylation of lysines in PDC, a known side reaction resulting from long exposure to urea-derived cyanate (Righetti, 1983).

Using the DELPHI module from BIOSYM, an attempt was made to trace the origin of the electrostatic field that is responsible for the dramatic p*K* perturbations suggested by the results (the pK values of C69, C221, and C222 are deduced to be lower than 5.6, compared with the aqueous value near 8.5). Figure 4 shows the distribution of positive charges surrounding C221 and C222. In Table 3 are presented the maximum pK_a lowerings (i.e., a dielectric constant of 2) at each of the four cysteines by four of the nearest positive charges, assuming that His, Arg, and Lys are all positively charged. The predicted positive field is largest around C221. Clearly, given a low enough dielectric constant, there is ample positive field to induce the observed pK_a lowering. An inspection of the X-ray structure also indicates that the backbone carbonyl oxygen from E148 is 3.3 Å from the sulfur of C152, and it is poised to participate in a hydrogen bond with the C152 S−H group. This interaction may explain why this cysteine alone may have a more normal p*K*, not suppressed as those of the other three cysteines. As to why C69 is dissociated and C152 is not, one can surmise that, in addition to the aforementioned specific hydrogen bond to C152, C69 is buried deep inside the protein (hence, its local dielectric constant is bound to be low), while C152 is near the surface of the protein.

The Hill coefficients were measured for all variants in the entire pH range of activity, providing complementary information. For C221S, the Hill coefficient *n* is pH-independent at $\leq$1.0; for the H92A variant, it is pH-independent at 1.2−1.3. In contrast, for the WT PDC, *n* is 2.0−2.2 (this represents the average of many measurements made by at least six different investigators in Newark on many different preps from both yeast and *E. coli*) and experiences a monotonic decrease with increasing pH, from 5.6 to 7.0 and higher.

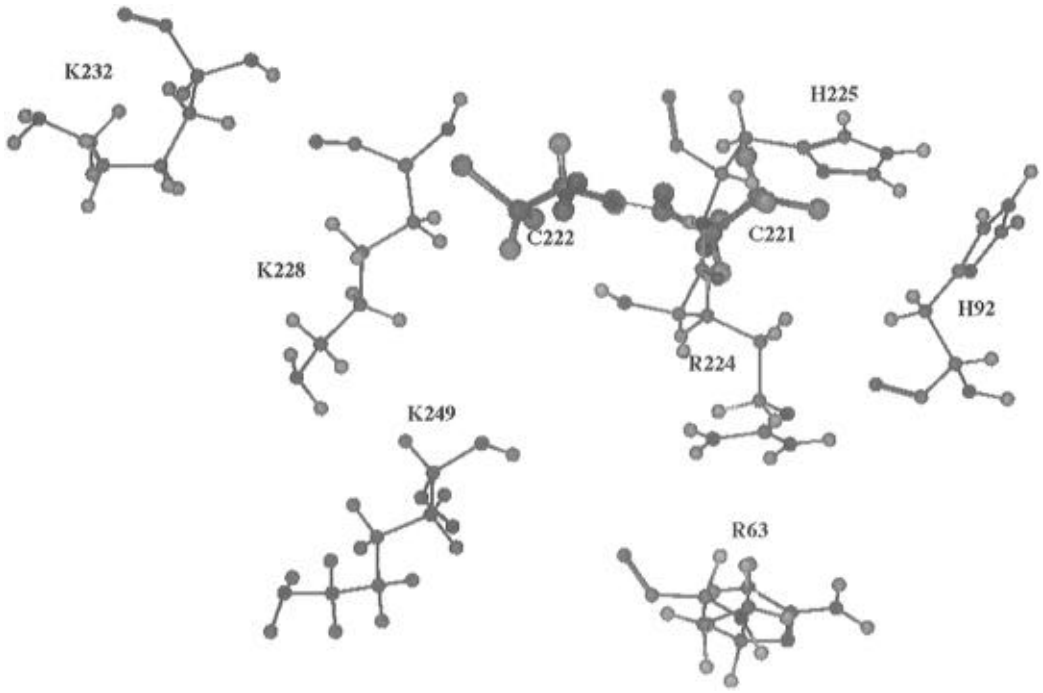


FIGURE 4: Positively charged amino acid side chains in the vicinity of C221 and C222 [from Arjunan et al. (1996)].

Table 3: Maximum pK_a Lowering Predicted for Cysteines in PDC under the Influence of Nearby Positively Charged Side Chains[a]

residue	pK_a shifts[b]			
C69	0.78 (R63)	1.77 (K65)	5.92 (H97)	0.57 (R459)
C152	0.47 (R13)	2.52 (H97)	0.86 (H126)	6.63 (R151)
C221	2.08 (R63)	3.35 (H92)	5.54 (R224)	7.71 (H225)
C222	1.67 (R224)	2.87 (K228)	4.75 (K232)	1.56 (K249)

[a] Assuming positively charged histidines, lysines, and arginines. Conditions: full Coulombic boundary condition, protein dielectric = 2, border space = 10 Å, I = 0.25 M, maximum grid points = 65, D = 80, and I_r = 2 Å. The water molecules are deleted in the 1pvd.pdb file of the Brookhaven Protein Data Bank entry. Delphi (Honig et al., 1993) and InsightII (BIOSYM Technologies, San Diego, CA) modules were used for the electrostatic calculations. [b] In parentheses is the residue whose contribution is calculated.

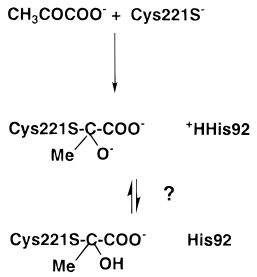
DISCUSSION

The relative integrated intensities of the S−H stretching peaks for WT PDC and for the C221S and C221S/C222S variants were the same within the experimental uncertainty estimated (from multiple runs of a single sample) at about 20%. The intensity for the C222S derivative is slightly higher than the others but nearly the same within the experimental uncertainties. We attribute the increased intensity to random error since reduction in the putative number of S−H groups should decrease the S−H peak intensity. The most startling result of these experiments is the essentially complete loss of S−H stretching intensity in the C152A variant. *This result, along with isoelectric focusing data, directly indicates that, of the four Cys residues present in PDC, the only un-ionized Cys (i.e., existing as an S−H) is that at position 152*. Li and Thomas (1991) have investigated the effects of solvent polarity and hydrogen-bonding characteristics on model compound S−H frequencies. On the basis of their studies, and those of Bare et al. (1975), the peak position of the C152 S−H stretching mode at ~2557 cm^{-1} indicates that the S−H forms a fairly strong hydrogen bond. Furthermore, the relatively narrow half-width of this band in all derivatives suggests that the C152 S−H is not participating in a fluctuating hydrogen-bonding environment since such behavior would produce an inhomogeneous broadening of the S−H band contour. Inspection of the X-ray structure (Arjunan et al., 1996) indicates that the sulfur atom of C152 is within 3.3 Å of the main chain carbonyl oxygen of Glu148, a distance that is certainly appropriate for a hydrogen bond. The perturbation of this delicate environment by the C152A substitution may be responsible for the perturbed isoelectric point that results in Figure 3B, i.e., that it is not identical to that of the wild-type PDC.

The results presented are of significance for two very different reasons. On one hand, they clearly demonstrate that directed mutagenesis in combination with FT-IR and isoelectric focusing can lead to a determination of the state of ionization of specific cysteine side chains in proteins. Additionally, it could be demonstrated that C221, the site of interaction of PDC with substrate in the substrate activation pathway, has a dramatically perturbed pK on PDC, compared to the value in model compounds. According to the FT-IR results, the pK_a is <6.0. Such a pK_a lowering would be of very great benefit to PDC, were the C221 sulfur to form a tetrahedral hemithioketal adduct with the substrate (the precise chemical nature of this interaction is so far unproven). It has been well-established and accepted that the thiolate ionization state of cysteine is orders of magnitude better as a nucleophile than the un-ionized thiol conjugate acid [for example, in its reaction with methyl methanethiosulfonate, 2-mercaptoethanol was found to be at least 5×10^9 times less reactive than its conjugate thiolate form; see Roberts et al. (1986)]. Such high reactivity would be indispensable for the mechanism of substrate activation suggested by Schowen and co-workers (Alvarez et al., 1995) that requires formation and dissociation of the hemithioketal for each turnover (k_{cat} in this lab for the purified enzyme/subunit is ca. 60 s^{-1} at 30 °C).

The ionization characteristics of H92, i.e., an imidazolium ion at pH 6.0, when considered in conjunction with the

thiolate at C221, suggest that the nucleophilicity at this site has been optimized as a thiolate−imidazolium ion pair, in a manner revealed by several groups for papain (Polgár, 1973; Lewis et al., 1981; Migliorini & Creighton, 1986). Accordingly, the C221-SH would have an unusually low p*K*, while the H92 imidazole would be more basic than in model compounds. For the papain active center, a p*K* of 3.3 for Cys25 and 8.5 for His159 was reported (Lewis et al., 1981). The pH dependence of the Hill coefficient for the wild-type PDC shows a monotonic decrease from 2.2 at pH 5.6 to 1.4 at pH 6.8; for the H92A variant, it is pH-independent at ca. 1.3, and for the C221S variant, it is near 1.0 in the entire pH range of activity. It is tempting to speculate that the diminished Hill coefficient with increasing pH is due to neutralization of the histidinium positive charge in WT PDC.



It is also noteworthy that there is a second ionized cysteine (C222) with no obvious function adjacent to the C221, the site of substrate activation. C222 may also aid in the "supernucleophilicity" of C221, as suggested for the role of adjacent cysteines 31 and 32 in reduced seminal ribonuclease (Parente et al., 1985). The presence of surrounding positive charges to enhance SH ionization/reactivity has also been noted on a histone (Bode et al., 1982).

It should be recalled that such p*K* perturbations on enzymes have been documented for 25 years. An early example was reported on the catalytic lysine of acetoacetate decarboxylase, whose p*K* was suppressed to 6, presumably to very much enhance its nucleophilicity near pH 6, the optimum pH of that enzyme (Kokesh & Westheimer, 1971).

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