Interdomain Information Transfer during Substrate Activation of Yeast Pyruvate Decarboxylase: The Interaction between Cysteine 221 and Histidine 92[†]

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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 two cysteines on the β domain (221 and 222) and at H92 on the α domain, across the domain divide from C221. While C221 has been shown to provide the trigger for substrate activation [Baburina, I., et al. (1994) *Biochemistry 33*, 5630−5635], the information must be transmitted from the substrate bound at this site [Arjunan, D., et al. (1996) *J. Mol. Biol. 256*, 590−600] to the active center thiamin diphosphate located at the interface of the α and γ domains. Substitution at H92 with G, A, or C leads to great reduction of the Hill coefficient (from 2.0 in the wild-type enzyme to 1.2−1.3), while substitution for Lys affords an active enzyme with a Hill coefficient of 1.5−1.6. Iodoacetate at 10 mM reduced the Hill coefficient from 2.0 to 1.1, while also causing significant inactivation of the enzyme, presumably by carboxymethylation of C221. 1,3-Dibromoacetone, a potential cross-linker when added to the H92C/C222S variant at 0.1 mM, abolished substrate activation while reducing the activity only by 30%. Therefore, 1,3-dibromoacetone may cross-link C92 and C221. It was concluded that H92 is on the information transfer pathway during the substrate activation process and the interaction between C221 on the β domain and H92 on the α domain is required for substrate activation. Extensive pH studies of the steady-state kinetic constants provide support for the interaction of C221 and H92 and the transmission of regulatory information to the active center via this pathway and pK_as for the two groups. This important interaction between the C221-bound pyruvate and His92 probably has both electrostatic and steric components.

Yeast pyruvate decarboxylase (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), Bisswanger and Ullrich (1991), and Bisswanger and Schellenberger (1996)]. The enzyme also has a requirement for a divalent cation, best fulfilled by Mg(II). The presence of divalent cations as part of the diphosphate binding locus has been confirmed for 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 were believed to be the only highly reactive side chains on the enzyme (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 gene sequence-derived amino acid sequence of pdc1 from *Saccharomyces cerevisiae* (scpdc1; Hohmann & Cederberg, 1990), each subunit possesses four cysteines at positions 69, 152, 221, and 222, none of which participate in disulfide bridges. The X-ray structures of PDC from *Saccharomyces uvarum* (supdc1; Dyda et al., 1993) and scpdc1 (Arjunan et al., 1996) have revealed that C221, the cysteine nearest to ThDP, 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 on 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).

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[1] Abbreviations: ThDP, thiamin diphosphate; PDC, pyruvate decarboxylase (EC 4.1.1.1); scpdc1, wild-type pyruvate decarboxylase isolated from *Saccharomyces cerevisiae*; WT, wild-type PDC; C221S, C222S, C221S/C222S, C152A, H92A, H92G, H92K, and H92C/C222S, variants of PDC; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; CPB, (*E*)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); *p*CMB, *p*-chloromercuribenzoic acid; n_H, Hill coefficient; DBA, 1,3-dibromoacetone.

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In earlier studies from these laboratories, it was first shown that a pdc1−6 fusion enzyme (consisting of amino acids 1−45 derived from the *PDC1* gene and 46−563 derived from the *PDC6* gene) with its single cysteine at position 221 can still be activated by pyruvate (Zeng et al., 1993). Next, the C221S, C222S (single-site), and C221S/C222S (two-site) mutant yeasts were constructed, and then the genes were transferred into a high-expression *Escherichia coli* vector for further mutagenesis studies. It was convincingly shown (Baburina et al., 1994) that substitution of C221, but not of C222, with serine (or for Ala, see below) 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, via either a covalent or a noncovalent linkage. A third cysteine, C152, while accessible, is not favorably positioned to interact with substrate. Later, FT-IR and isoelectric focusing experiments were carried out on several variants of scpdc1. An examination of the S−H stretching region (2500−2600 cm^{-1}) of PDC and its aforementioned variants, along with isoelectric focusing experiments, provided evidence that of the four cysteines found in PDC only C152 is undissociated near pH 6 (Baburina et al., 1996). According to isoelectric focusing experiments, the residue H92 is probably protonated at pH 6.0, the pH optimum of the enzyme. Molecular modeling suggested that pyruvate bound to C221 could reach over to and interact with H92.

In this paper, we report several experiments carried out with PDC variants at C221 and H92 to probe the nature of the interaction between these two residues and their respective domains. Replacement of H92 by Gly, Ala, or Cys reduces the Hill coefficient substantially, as does substitution at C221, suggesting that H92 participates in the transmission of information from the substrate bound at C221 to the active center ThDP. Two experiments were designed to mimic the interaction of pyruvate bound in a hemithioketal form at C221 with H92. Substitution of H92 with cysteine created the possibility of covalently linking C221 and C92 with 1,3-dibromoacetone (Dahl & McKinley-McKee, 1977), perhaps leading to the activated enzyme. Carboxymethylation of C221 with iodoacetate would introduce a two-carbon moiety with its carboxyl group potentially assuming the same locus as would the covalently bound pyruvate. The results point out the need for the 2-oxo group. In the following paper (Baburina et al., 1998), it is demonstrated that cysteine 221 is the most reactive cysteine in PDC and that any reaction at that site has significant consequences on the homotropic regulation of PDC.

EXPERIMENTAL PROCEDURES

Material. The Chameleon site-directed mutagenesis kit was purchased from Stratagene (the kit supplies DNA polymerase, DNA ligase, selection primer, and competent cells) (Papworth et al., 1995). *Afl*III restriction enzyme and the corresponding buffer were purchased form New England Biolabs. All other restriction enzymes and corresponding buffers, including Multicore buffer, were purchased form Promega. Agarose for DNA gels was purchased from Promega. BL21(DE3) strain *E. coli* cells were obtained from Novagene. Components of the LB medium (tryptone, yeast extract, and NaCl), various inorganic chemicals [Na_2HPO_4, K_2HPO_4, NaH_2PO_4, KH_2PO_4, KOH, HCl, $MgSO_4$, $(NH_4)_2SO_4$, etc.], β-mercaptoethanol, EDTA, and streptomycin sulfate were purchased from Fisher Scientific. ThDP, sodium pyruvate, NADH, ADH, PMSF, and Triton X-100 were purchased from Sigma. IPTG was purchased from U.S. Biochemicals or Promega. DEAE Sepharose Fast-Flow was purchased from Pharmacia. Components for the SDS−PAGE procedure were obtained from Bio-Rad, U.S. Biochemicals, or Promega. 1,3-Dibromacetone was purchased from ICN. The Wizard 373 DNA purification kit was supplied by Promega. ABI Prizm Terminator Ready Reaction Mix for DNA sequencing was supplied by Applied Biosystems−Perkin Elmer.

Construction of the C221S, C222S, and C221S/C222S PDC Variants. C221S, C222S, and C221S/C222S mutant yeasts were constructed by S. Hohmann at the Kathholieke Universiteit te Leuven (Belgium). The Altered Sites system from Promega was used for mutagenesis. For the detailed procedure, see Baburina et al. (1994).

The *PDC1* gene and its mutants from *S. cerevisiae* were transferred into the pET1120 plasmid (Baburina et al., 1994) according to Studier et al. (1990). For more efficient site-directed mutagenesis, the *PDC1* gene was also transferred into the pSIT expression vector (Andreansky & Hunter, 1994a,b).

The pSIT-*PDC1* vector was used to obtain the H92K and the C152A variants of PDC, since the mutagenesis was unsuccessful for these variants in the pET vector.

Construction of H92A, H92G, H92K, H92C/C222S, C221A/C222A, and C222A Variants of PDC. The procedure followed the instructions for the Chameleon Double-Stranded Site-Directed Mutagenesis Kit from Stratagene (Papworth et al., 1995; Baburina et al., 1996) and is described in the Supporting Information.

PDC overexpression and purification are described in the Supporting Information.

Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was used to check the purity of PDC samples with a 12% linear SDS−PAGE gel according to Laemmli (1970).

Assay for PDC. PDC activity was monitored at 340 nm with the coupled enzyme assay using alcohol dehydrogenase and NADH (Holzer et al., 1956). All components of the reaction mixture were dissolved in 0.1 M 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer (pH 6.0) containing 0.1% (w/v) bovine serum albumin (BSA) and freshly prepared β-NADH, pyruvate, alcohol dehydrogenase, and PDC to yield final concentrations of 0.5 mM NADH, 6 units/mL ADH, and 2−5 units/mL PDC. The final concentration of pyruvate varied from 0.02 to 100 mM. The reaction was initiated mostly by the addition of enzyme. Routine assays were performed on a Varian DMS 300 UV Visible Spectrophotometer at 25 or 30 °C. One unit of activity is defined as the amount PDC required to convert 1 μmol of pyruvate to acetaldehyde per minute at 30 °C and pH 6.0.

Determination of Accessible SH Groups on PDC. Excess ThDP was removed from all samples using Centricon 30 (Amicon) centrifugal concentrators. The protein was diluted into 0.1 M MES buffer (pH 6.0), containing 1 mM EDTA and 2 mM Mg^{2+}. It is important that the sample contains no excess ThDP, since even at pH 6.0 a small percentage of the thiazolium ring of ThDP is open and hence can be titrated

and will produce a background absorbance with DTNB (Brown, 1990; Washabaugh et al., 1992). An excess of DTNB was added to a sample containing 0.5 μM PDC, and the reaction was allowed to proceed for 2 h at room temperature. A similar solution without PDC was used as a reference. The absorbance at 412 nm (extinction coefficient = 14 400 cm^{-1} M^{-1}; Ellman, 1959) was used to estimate the concentration of SH groups.

Steady-state kinetic assays for v_o-S plots were carried out on a COBAS-BIO automatic centrifugal analyzer (Roche Diagnostics, Somerville, NJ) using the NADH/ADH coupled assay. The system combines 200 μL of a MES/ADH/NADH mix and 10 μL of pyruvate ranging from 0.02 to 40 mM in 25 vials. The reaction is initiated with the addition of 10 μL of PDC (0.2−0.5 unit). The total volume of the reaction mixture, including the 20 μL flushing volume after addition of substrate and protein, was 260 μL. The instrument can assay 25 samples simultaneously.

The pH dependence of the kinetic parameters was also determined on the COBAS-BIO instrument. All components, except for PDC, were dissolved in a particular buffer. PDC was kept at pH 6.0 to avoid pH-dependent denaturation. The concentration of NADH remained the same at all times; the concentration of ADH was increased 10-fold to prevent ADH from being rate-limiting.

The following 0.1 M buffer solutions were used (pH ranges in parentheses): sodium citrate (4.5−5.5), MES (5.5−6.5), and 3-(*N*-morpholino)propanesulfonic acid (MOPS, 6.5−7.5).

The v_o-S data were fitted to the Hill equation, $v_o = (V_{max}S^n)/(K_m' + S^n)$, and the parameters were determined from a linear least-squares fit using the UltraFit program from Biosoft and DeltaGraphPro4 from DeltaPoint. The K_m and k_{cat} for the WT were determined earlier in the presence of 40 mM pyruvamide (Farrenkopf & Jordan, 1992). Data from those experiments coincide within experimental error with the data obtained from a nonlinear least-squares fit. To unify the results, the parameters for all PDC variants, including WT, were deduced from the computer fit. The Hill coefficient (n_H) was also determined using computer fitting, and it coincided within experimental error with the Hill coefficient from the logarithmic form of the Hill equation.

The Eadie−Scatchard plot (v_o/[S] vs v_o) was used to detect differences in Hill coefficients among PDC variants (Segel, 1975; Hammes, 1982).

The kinetic data were also treated according to the equation below, specific to PDC, and assuming two pyruvate sites, as developed by Schowen and co-workers (Alvarez et al., 1991):

$$v_o = V_{max}S^2/(A + BS + S^2)$$

from which, instead of the conventional V_{max}, K, and V_{max}/K (for the current case, V_{max}, $S_{0.5}$, and $V_{max}/S_{0.5}$), the constants V_{max}, V_{max}/B, and V_{max}/A (and if accurate enzyme concentrations are known, k_{cat}, k_{cat}/B, and k_{cat}/A) corresponding to a collection of rate and equilibrium constants zero-, first-, and second-order in S, respectively, are obtained. These three constants are plotted against pH so that mechanistic interpretations can be deduced for the function of the variants. Because of the kinetic complexity of this regulated enzyme, both methods were useful.

Table 2: Titration of Accessible SH Groups on PDC[a]

PDC variant	[SH]/[PDC monomer]
WT	3.150
C221S	1.980
C222S PDC	2.015
C221S/C222S PDC	1.335
C152A	1.996

[a] DTNB was diluted in methanol. An excess of DTNB (2 mM) was added to the sample containing 0.5 μM PDC, and the reaction was allowed to proceed for 2 h at room temperature. The absorbance at 412 nm was measured, and the concentration of SH groups was calculated using the extinction coefficient of 14 400 cm^{-1} M^{-1} (Ellman, 1959).

pK_{app}s were determined from plots of log(kinetic constant) vs pH (when the quality of the data permitted) assuming a Dixon−Webb scheme for protonic equilibria. The curves drawn through the experimental data in the figures and tables were constructed with the deduced pK_{app}s listed in Table 9.

Reactions with 1,3-Dibromoacetone. 1,3-Dibromoacetone (DBA) was used as a potential cross-linking reagent (Dahl & McKinley-McKee, 1977). A 100 mM stock solution of DBA was freshly prepared in DMSO and added to the enzyme to final concentrations ranging from 2 μM to 20 mM in 0.1 M MES buffer (pH 6.0) with 0.01% (w/v) BSA in a total volume of 2 mL. The reaction mixture was incubated at room temperature for 80 min and the enzyme was assayed for activity with different concentrations of pyruvate to generate the v_o-S plot as described above.

Kinetic Measurements in the Presence of Pyruvamide. Pyruvamide was added to final concentrations ranging from 20 to 80 mM in a MES (0.1 M, pH 6.0)/ADH/NADH mixture. Pyruvamide was synthesized from pyruvonitrile. Into 30 mL of dry ether in a three-neck flask was bubbled N_2 for 10 min at 0 °C. Seven milliliters of pyruvonitrile was added to the cold ether solution, and then HCl gas was bubbled into it for 20 min. Next, 6 mL of water was added and HCl addition continued for another 30 min at 0 °C. A white precipitate formed, which was filtered, washed with cold ether, and recrystallized from 2-propanol.

RESULTS AND DISCUSSION

Optimization of Expression

Optimization of scpdc1 in *E. coli* and purification of wild-type and variant enzymes is described in the Supporting Information (Table S1 and Figures S1 and S2). The C221S, C222S, C221S/C222S, C222A, C221A/C222A, H92A, H92G, H92K, and H92C/C222S variants were purified to homogeneity. The number of SH groups titrated with DTNB (Table 2) is consistent with one of four cysteines being inaccessible (C69) in the native state of PDC (Arjunan et al., 1996) and confirms the success of both the mutagenesis and expression protocols. The reaction of PDC with DTNB led to partial inactivation [see Baburina et al. (1998)].

Kinetic Studies with the Variant Enzymes

Steady-State Kinetic Data for the C221 and C222 Variants of PDC. The v_o vs [pyruvate] plots for these variants and WT are presented in Figure 3, along with the Eadie−Scatchard plots in the insets. The calculated steady-state kinetic parameters are summarized in Table 3. The results

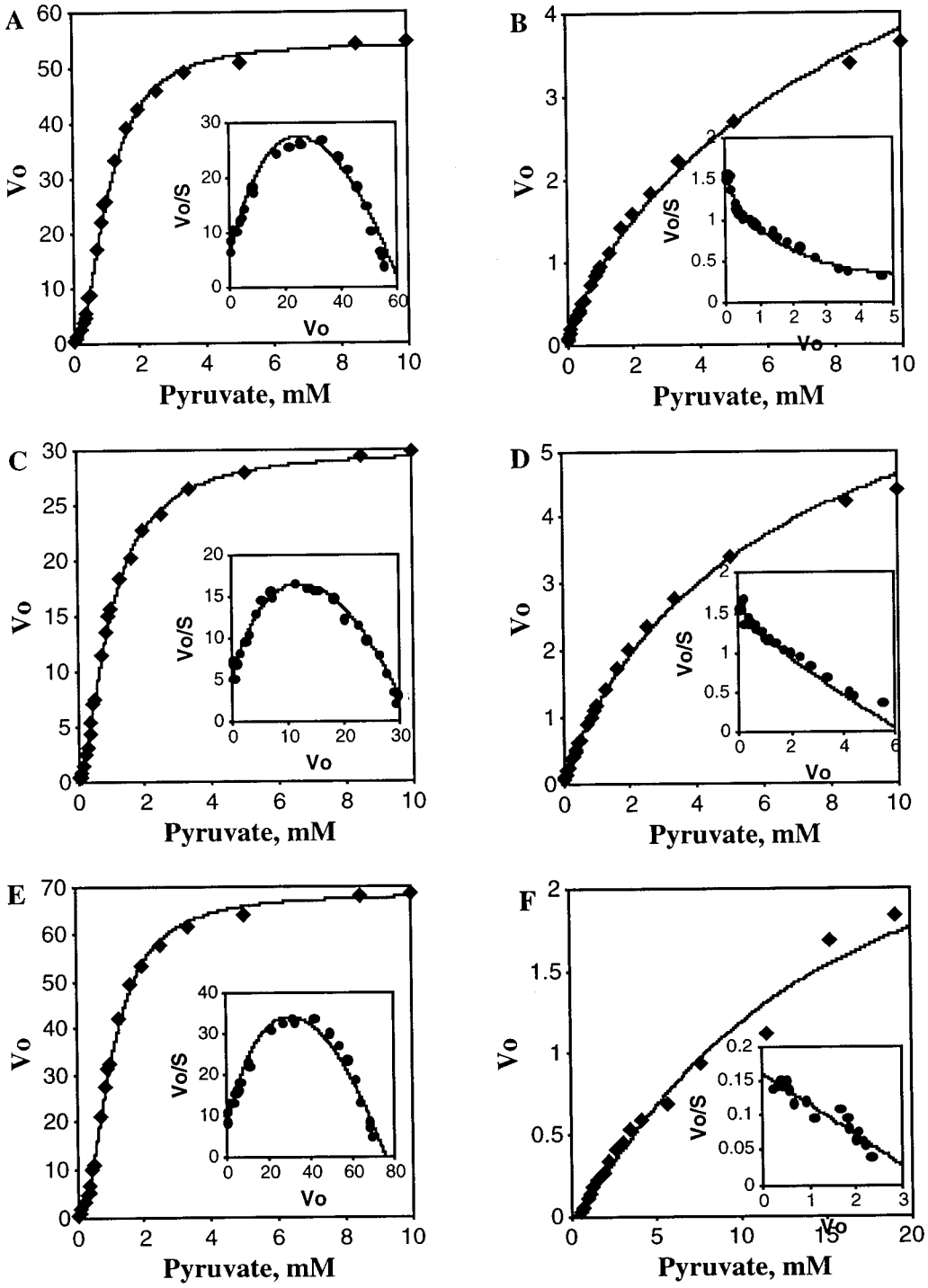


FIGURE 3: v_o vs [S] data for WT (A), C221S PDC (B), C222S PDC (C), C221S/C222S PDC (D), C222A PDC (E), and C221A/C222A PDC (F). Insets: Eadie−Scatchard coordinates.

with the alanine substitutions confirm the data with the serine-substituted enzymes (Baburina et al., 1994). The Hill coefficients of nearly 2.0 for WT and for both the C222S and C222A variants are consistent with substrate activation [positive cooperativity; see Segel (1975) and Hammes (1982)]. The Hill coefficients of less than 1.0 for the C221S and C221S/C222S variants are consistent with slightly negative cooperativity, showing that substitution in position 221, but not in position 222, results in the loss of positive cooperativity. The C221A/C222A variant exhibits neither substrate activation nor negative cooperativity. The C222A and C221A/C222A variants provide further proof of regulatory function of the C221 residue on PDC. The K_ms of the C221S and C221A variants are 4−6-fold greater than the K_m' of the WT. Similar dramatic changes on cooperativity as a result of single-site substitutions were recently reported for the aspartate receptor (Kolodziej et al., 1996).

Effect of Pyruvamide on WT and Its Cysteine Variants. Since pyruvamide is believed to convert PDC into an activated state, its effects were tested on the variants (Table 3). While pyruvamide changes the kinetic behavior of the WT and of the C222S variant PDCs, the kinetic parameters of the C221S and C221S/C222S variants are unaltered by pyruvamide. Repeated efforts to locate pyruvamide at C221 crystallographically have failed to date, although some changes in the quaternary structure of the enzyme were noted

Table 3: Steady-State Kinetic Parameters of C221 and C222 Variants[a]

PDC variant	specific activity (u/mg)	K_m[b] (mM)	n_H
WT	50 ± 5 (52)	1.1 ± 0.2 (0.93)	2 ± 0.2 (1.12)
C221S	15 ± 5 (7)	7.6 ± 0.5 (4.4)	0.89 ± 0.02 (0.98)
C221S/C222S	14 ± 5 (7)	8.8 ± 0.4 (4.8)	0.98 ± 0.02 (0.84)
C221A/C222A[c]	16	5.7	1.00
C222S	45 ± 6 (40)	1.2 ± 0.2 (0.82)	1.9 ± 0.2 (1.16)
C222A[c]	41	1.2	1.85

[a] In parentheses are values in the presence of 80 mM pyruvamide. Presents averages of data obtained from purification of PDC and its variants over a period of 4 years. [b] For Hill coefficients different from unity, these represent apparent values. [c] C221A/C222A and C222A variants were purified only once.

on addition of pyruvamide. We also note that pyruvamide can be potentially hydrolyzed to pyruvate during the time required for crystal growth.

The substitutions at position 221 (or 222) do not influence significantly the active site of the enzyme; certainly, the activity of the variants indicates no significant impairment of tertiary structure. It is concluded that C221 is the site at which the substrate activation cascade is triggered. It of course is anticipated that the homotropic activation mechanism involves much more than just this single amino acid. After all, the information must be sensed at the catalytic center, in this case at a distance >20 Å from C221.

Molecular modeling was used to identify a probable signal transmission pathway from C221 on the β domain to the active site located between the α and γ domains, using the crystallographic coordinates and the assumption that pyruvate forms a hemithioketal with C221 (Figure 4A). Were pyruvate covalently bound to C221 on the β domain, it would form a very short contact with H92 located 4.5 Å from C221 on the α domain. H92 would be unfavorably positioned and would be forced to dislocate. The movement of H92 itself could initiate a chain of events involving movement of residues E91, W412, G413, S414, and I415; the loop extending from 411 to 415 is intimately involved in ThDP binding (Baburina et al., 1994; Figure 4B).

Steady-State Kinetic Data for the H92 Variants of PDC. According to the model, H92 should be the first residue affected by addition of pyruvate to C221. H92 was substituted with A, G, and K to test (1) whether H92 participates in the substrate activation pathway and (2) what kinds of interactions occur between pyruvate bound to C221 and H92. The H92C/C222S variant was also prepared in an attempt to convert PDC to a permanently activated form of the enzyme.

Michaelis−Menten plots for the H92 variants are presented in Figure 5, and Table 4 lists the kinetic constants. The H92A and H92G substitutions reduce the Hill coefficient from 2 to 1.2−1.3. With the H92K substitution, the positive charge is conserved and a Hill coefficient of 1.5−1.6 results, intermediate between that for the WT (2.0) and the H92G variant (1.2), suggesting that the positive charge at H92 is important.

During the work with the H92 variants, it was noticed that the H92A, H92G, and H92C/C222S variants are very unstable under the usual storage conditions (see Experimental Procedures); these proteins lost some of their activity and cooperativity within 2 weeks. However, the kinetic parameters of the H92K variant remained stable for several months (Table 4), and this variant retains its positive cooperativity. The results provide a clear indication of the importance of a positive charge at position 92 in the signal transmission between domains, in helping to stabilize the protein, in addition to maintaining positive cooperativity. Lysine would likely be protonated in the entire pH range of interest, and the k_{cat} vs pH profile for the H92K variant does show differences from that of the WT (see below).

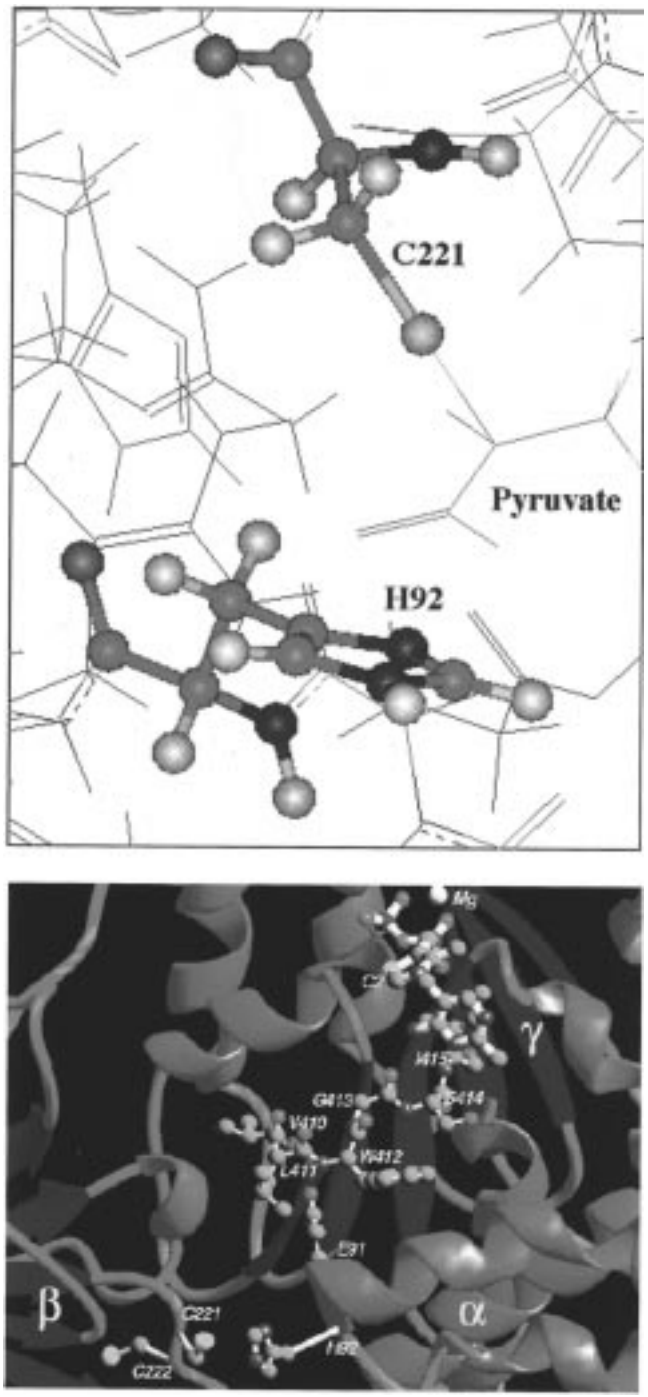


FIGURE 4: (A) Possible binding of pyruvate to cysteine 221 and its relation to H92. (B) Possible amino acid residues involved in the activation pathway of PDC.

The pH dependence of steady-state kinetic parameters was determined for WT and the C221S, C222S, C221S/C222S, H92G, and H92K variants. To avoid redundancy (the C222S behaves as WT and the C221S/C222S as the C221S variant),

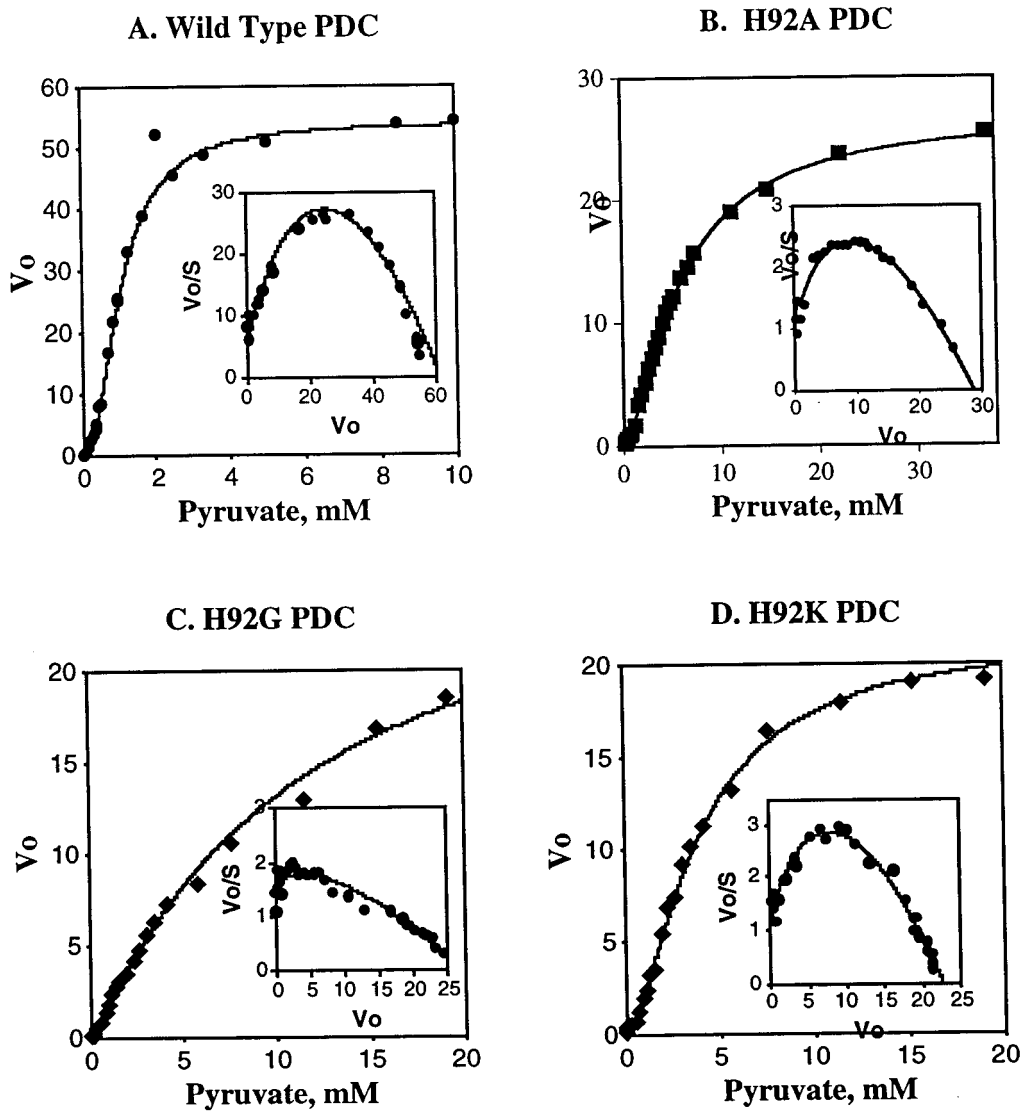


FIGURE 5: v_o-S plots for WT (A), H92A PDC (B), H92G PDC (C), and H92K PDC (D). (Insets) Same data in Eadie−Scatchard coordinates.

Table 4: Kinetic Parameters of H92 Variants of PDC[a] and after 3 Months of Storage (in Parentheses)

PDC variant	specific activity (u/mg)	K'_m (mM)	n_H
H92A	25 ± 3 (5)	4 ± 1 (4)	1.3 ± 0.1 (1.05)
H92G	23 ± 4 (4.5)	8.5 ± 1 (9)	1.2 ± 0.1 (1.09)
H92K	25 ± 2 (25)	5 ± 1 (5)	1.7 ± 0.1 (1.78)
H92C	23 ± 3 (5.2)	4.5 ± 1 (4.5)	1.3 ± 0.1 (0.99)

[a] Results of three purification procedures.

results for the C221S, H92G, and H92K variants are compared with those for WT (detailed kinetic results on *V*, *V*/*A*, *V*/*B*, *n*, and $S_{0.5}$ are presented in Tables S5−S8). A summary of the pH dependencies of steady-state kinetic parameters is presented in Figures S6−S9. There are significant changes in several parameters.

Perhaps most striking is the behavior of the Hill coefficients. While the Hill coefficients for WT and the C222S variant are pH-dependent and bell-shaped with the maximum at pH ~6.0, those for the C221S, H92G, and H92K variants are nearly pH-independent (Figure 10). A comparison of the Hill coefficients of the H92A and H92G variants with those of WT suggests that the length of the side chain at position 92 may be important, while a comparison with the

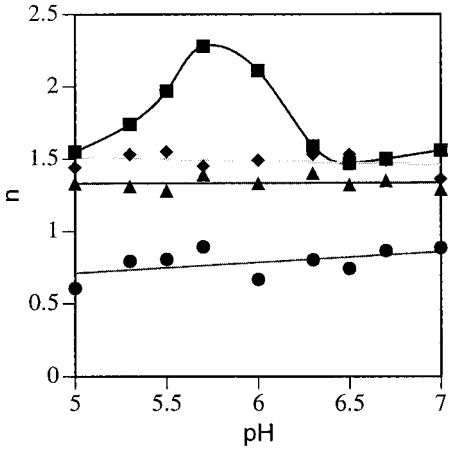


FIGURE 10: pH dependence of the Hill coefficient: WT (■), C221S PDC (●), H92G PDC (▲), and H92K PDC (◆).

H92K variant shows that the positive charge at position 92 can compensate, in part, for the loss of the histidine. On the basis of the pH dependence of the Hill coefficient in WT and its pH independence in the C221S, H92G, and H92K variant PDCs, and the finding that C221 is dissociated at pH 6.0 (i.e. its pK_a is lower than 6; Baburina et al., 1996),

Scheme 1

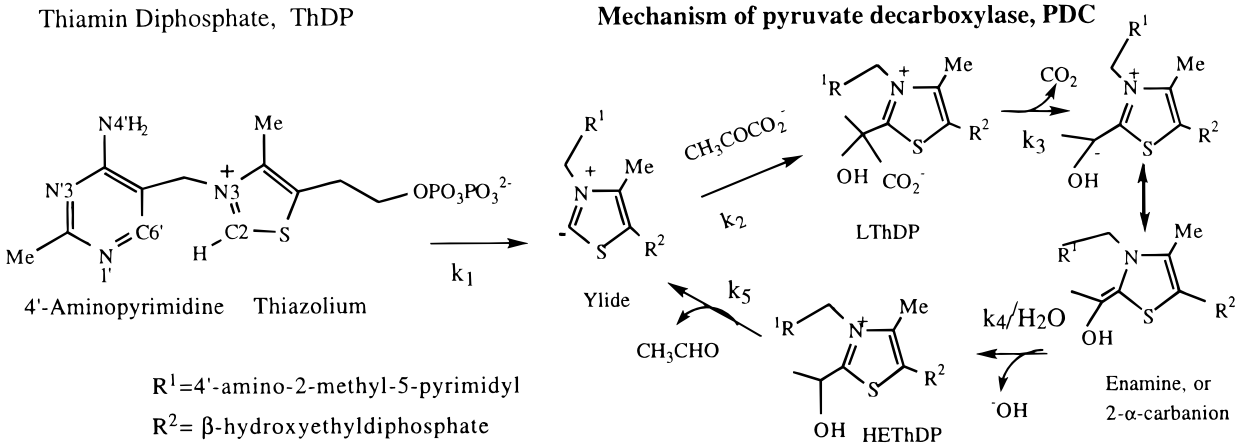


Table 9: pK_{app} of PDC Variants Estimated from pH-Dependent Kinetic Parameters[a]

	WT		C221S		H92G		H92K	
	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}	pK_{a1}	pK_{a2}
log(k_{cat})	5.5	6.6	5.6	6.5	5.5	6.8	<5.0	6.8
log($k_{cat}/S_{0.5}$)	5.1	6.6	ND[b]	ND[b]	5.2	6.3	ND[b]	6.2
log(V/A)	ND[b]	6.3	<5.0	6.5	5.5	6.3	ND[b]	ND
log(V/B)	ND[b]	6.0[b]	5.4	6.1	ND[b]	6.4	ND[b]	6.1

[a] Estimated error ± 0.3 unit, since the results were obtained from log(kinetic constant)−pH plots; pK_{a1} and pK_{a2} refer to the apparent values determined on the acid and alkaline side of the curves, respectively. The curves in Figures S6−S9 were constructed with the pK_{app}s estimated by a least-squares fit to the data tabulated in Tables S5−S8. [b] ND, no values could be estimated from the data.

we suggest that the pK_as apparent from the pH dependence of the Hill coefficient in the WT pertain to C221 (∼5.2) and H92 (∼6.4). As pointed out by Baburina et al. (1996), there are ample nearby positively charged residues, other than H92, to account for the pK_a suppression of C221.

The pH dependence of the steady-state kinetic parameters provides considerable insight into the substrate activation pathway (see Table 9 for the deduced pK_as, where statistically meaningful numbers can be extracted). In the WT, there is clear evidence of both V and $V/S_{0.5}$ being modulated by at least two ionizable groups according to the bell-shaped profiles. In the Dixon−Webb formalism, these would pertain to protonic equilibria involving the E·S complex and free E, respectively. The $V_{max}/S_{0.5}$−pH plots have virtually the same shape as the V_{max}/A−pH plots. According to the mechanism derived by Alvarez et al. (1991), V_{max}/A reflects the energetics of transition states starting with the addition of the first pyruvate to PDC (presumably at the regulatory site) and terminating with the first irreversible step, i.e. decarboxylation. The term V_{max}/B reflects the energetics of transition states starting with the E·S complex and terminating with decarboxylation. Finally, V_{max} (or k_{cat}) reports on transition-state energies for the decarboxylation step (formation of the enamine/2-α-carbanion), followed by product release (broadly defined), including protonation of the enamine and release of acetaldehyde from ThDP (Scheme 1).

When comparing the plots of WT with those of the C221S variant, one can conclude the following. (1) The k_{cat}−pH profiles have similar shapes, but the pK_as are somewhat closer to each other in the variant than in the WT. There is some perturbation in the pK_as of the active center residues. (2) A larger change is sensed in the V/A (or $k_{cat}/S_{0.5}$)−pH and V/B−pH plots, as expected from the above-mentioned dramatic changes in the Hill coefficients. It is evident from the results that an ionizable group with a pK_a in the acidic region is being perturbed by the substitution.

The shapes of the k_{cat}−pH and $k_{cat}/S_{0.5}$−pH (or V/A−pH and V/B−pH) plots for the H92G variant are very similar to those for the WT, signaling that there was no major perturbation in the rate-limiting steps. There is one notable difference. The pH optima of the $k_{cat}/S_{0.5}$−pH (or V/A−pH) plots and the single pK_{app} in the V/B−pH plot have experienced a shift to the acidic region by perhaps as much as 0.5 unit. This provides further evidence that the binding of pyruvate to the regulatory site can sense the changes in ionization state at position 92.

The results for the H92K variant were surprising. The k_{cat} does not decrease with increasing acidity to at least pH 5.0, in contrast to those of the other variants studied. This result provides a striking confirmation for the participation of H92 in the transmission of signal to the active center, since k_{cat} reflects energetics of transition states in decarboxylation and product release. A plausible explanation of the observation is that the group(s) responsible for the acid limb in the WT k_{cat}−pH profile has (have) experienced enhanced acidity so that even at pH 5 it is (they are) fully dissociated. In other experiments (Y. Gao, F. Guo, J. G. Wang, and F. Jordan, unpublished), there is evidence accumulating that several residues surrounding ThDP, and not near C221 or H92 (E51, E477, D28, H114, and H115), have an impact on this region of the k_{cat}−pH profile. As can be seen in Figure 4B, the pathway from H92 to E91 to W412 and the 411−415 loop leads to the active center and involves residues that are intimately involved in ThDP binding. Furthermore, these residues are in close proximity to the active center acid−base groups mentioned above.

The observation that substitutions at C221 and H92 have their most dramatic effects on cooperativity rather than on activity suggests that changes in the tertiary structure of the enzyme are not significant.

Interactions that trigger the chain of events leading to the conversion of PDC to its activated form after addition of pyruvate may be related to the chemistry of thiol addition to carbonyl groups (Jencks, 1969; Torchinski, 1977), perhaps

Table 10: Kinetic Properties of WT and H92C/C222S PDC after Interaction with 1,3-Dibromoacetone for 80 min at Room Temperature

[DBA] (mM)	% activity		$S_{0.5}$ (mM)		n_H	
	WT	H92C/C222S	WT	H92C/C222S	WT	H92C/C222S
0	100	100	0.52	2.43	1.97	1.47
0.01	85	100	0.52	2.82	1.80	1.29
0.1	60	68	0.65	2.00	1.57	1.00
1	20	56	1.11	1.45	1.06	1.01

involving addition of C221 to pyruvate resulting in a hemithioketal carrying a negative charge. It appears that the imidazole of H92 is positively charged at pH 6.0, the pH optimum of PDC (Baburina et al., 1996). This positive charge may stabilize the negatively charged hemithioketal. However, the possibility of simple mechanical interactions cannot be excluded due to the short distance (4.5 Å) between C221 and H92. Formation of a hemithioketal between pyruvate and C221 could possibly displace the H92 residue according to molecular modeling (Baburina et al., 1994).

Attempts to Mimic the Substrate-Activated State of PDC

The H92C/C222S variant was constructed in an attempt to cross-link the two domains (C221 on the β domain with C92 on the α domain) and to mimic the activation process. We hypothesized that the H92C/C222S variant on reaction with DBA at both cysteines would be converted into its activated form. Modeling showed that the 2-oxopropyl chain derived from DBA could bridge the gap between the 221 and 92 residues, as would bound pyruvate. To avoid potential complications arising from a side reaction at C222, the H92C substitution was performed on the C222S variant. We then compared the reactivity of WT and the H92C/C222S variant with DBA. DBA inhibits both WT and the H92C/C222S variant PDC at high ($\geq$1 mM) concentrations. At low concentrations of DBA, the H92C/C222S variant retains considerable activity, while the WT is still significantly inhibited.

The v_o-S plots and cooperativity for the two enzymes are different under the influence of DBA. WT loses both activity and cooperativity with increasing concentrations of the reagent (Table 10). The H92C/C222S variant, however, retains 100% activity at low concentrations of DBA, but its kinetic behavior changes from sigmoidal to hyperbolic and is consistent with an activated enzyme at low concentrations of pyruvate (Table 10).

There is further kinetic evidence for potential cross-linking of the H92C/C222S PDC variant by DBA from a comparison of the effects of this compound with that of 1-bromo-2-butanone (incapable of cross-linking, but electronically very similar to DBA). An at least 50-fold higher concentration (5 mM) of 1-bromo-2-butanone was required to produce the same extent of reduction in the Hill coefficient to near 1.0 (Table 11).

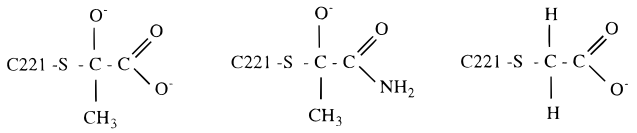
WT was inhibited by increasing concentrations of iodoacetate (data not shown). The value of n_H decreased and the K_m' increased with increasing iodoacetate concentration. Significant inhibition (to 20% activity remaining) is observed at 10 mM iodoacetate, with a resulting Hill coefficient near 1.0. Iodoacetate is very likely also reacting at C221, leading

Table 11: Kinetic Properties of WT and H92C/C222S PDC after Interaction with 1-Bromo-2-butanone[a]

[bromobutanone] (mM)	% activity		$S_{0.5}$ (mM)		n_H	
	WT	H92C/C222S	WT	H92C/C222S	WT	H92C/C222S
0	100	100	0.52	2.43	1.97	1.47
1	72	69	0.52	2.08	1.87	1.45
5	45	42	0.70	1.46	1.45	1.05
10	31	36	0.84	1.22	1.10	1.00

[a] Wild-type PDC was reacted for 40 min and the C222S/H92C variant for 15 min at room temperature at pH 6.0.

to carboxymethyl-Cys221, since the activity remaining after reaction with iodoacetate is similar to the reduced activity observed in the C221S and C221A variants. The following scheme compares possible covalent attachment of pyruvate or pyruvamide or carboxymethylation of C221. Carboxymethylation and pyruvate would introduce a negative charge nearer to H92, a charge that pyruvamide lacks. Pyruvamide is an activator at very high concentrations only, perhaps signaling this missing charge. The finding that carboxymethylation of C221 reduced the activity is probably telling us either that the presence of the 2-oxo group is crucial to activation or that iodoacetate is also reacting at a different site, near ThDP, or both.



Pyruvate **Pyruvamide** **Iodoacetate**

Reaction of WT with DBA is consistent with a reaction at a cysteine or histidine, and according to changes in the Hill coefficient, this residue is probably C221. Husain and Lowe (1968) found that DBA alkylates both partners of the cysteine-histidine pair on papain and concluded that cysteine and histidine are 5 Å from each other. However, the reaction of WT with DBA under similar conditions is consistent with modification of cysteine only (although there is no direct proof for this), even though the histidine residue is 4.5 Å away. The enzyme glyceraldehyde-3-phosphate dehydrogenase possesses a cysteine-histidine ion pair in its active site (Moras et al., 1975), as does papain, and its reaction mechanism is very similar to the events that are proposed for the regulatory site of PDC (Baburina et al., 1996). DBA reacts with glyceraldehyde-3-phosphate dehydrogenase by selectively modifying the cysteine residue in the active site, but does not react with a histidine residue (Moore & Fenselau, 1972a,b).

The following comparisons suggest that DBA cross-linked the H92C/C222S variant and that reaction with DBA produced an activated form of PDC.

(1) Both WT and the H92C/C222S variant PDC are inhibited by DBA in a time-dependent manner; the extent of inhibition at 1 mM DBA is greater for WT (80%) than for the variant (40−50%).

(2) DBA was much more powerful than 1-bromo-2-butanone at reducing the Hill coefficient to near 1.0 for the H92C/C222S variant ($\leq$0.1 mM for DBA, >5 mM for the

latter), and 1-bromo-2-butanone exhibits similar inhibition of both WT and H92C/C222S PDC.

(3) DBA at 0.1 mM reduced n_H to 1.0 in 80 min for the H92C/C222S variant; 1 mM DBA even at 180 min was insufficient to reduce n_H to 1.0 with WT.

(4) While $S_{0.5}$ decreases (improves) as n_H tends to unity for H92C/C222S, $S_{0.5}$ increases as n_H tends to unity for WT, whether treated with DBA or 1-bromo-2-butanone.

To date, no structural evidence has been obtained for domain cross-linking. Both unmodified and DBA-modified proteins (WT and H92C/C222S PDC) showed the same electrophoretic mobility under native and denaturing conditions on PAGE, and elution profile on a gel-filtration column. Since the residues that would be cross-linked are in the same subunit, the change in protein conformation could be insignificant, and might not be detectable on the native gel (or gel filtration). If the C221 and C92 were cross-linked, they would form a loop of 128 residues, and a loop of this size may go undetected under denaturing conditions on SDS−PAGE as well.

GENERAL DISCUSSION AND CONCLUSIONS

Identification of C221 as the substrate activation trigger of PDC (Baburina et al., 1994) has prompted additional research on PDC. Lobell and Crout (1996) reported modeling studies suggesting that pyruvate can form two hydrogen bonds, to the NH and CO of S311, a residue near C221. These bonds could hold pyruvate in position for the formation of a hemithioketal. Substitution of pyruvate by pyruvamide allows the same interactions, but carboxymethylation of C221 would not. Results with iodoacetate, 1,3-dibromoacetone, and 1-bromo-2-butanone tend to support the importance of a recognition site for the keto group. (1) Iodoacetate has a much lower reactivity at the regulatory site than the two bromoketones. (2) Reaction of the H92C/C222S variant with the two bromoketones leads to *improved* $S_{0.5}$, whereas all such alkylation of the WT increases $S_{0.5}$. The bromoketones introduce the keto function that apparently converts the variant to its activated form (n tends to 1.0, and $S_{0.5}$ decreases with added bromoketone). Unfortunately, the C221 and H92C variants tend to be denatured with time; hence, there is no structural confirmation of this hypothesis to date.

Extensive kinetic isotope effect studies by Schowen and co-workers (Alvarez et al., 1995) were interpreted in terms of a series of events at the regulatory and active sites of PDC as a result of pyruvate addition. Addition of C221 to pyruvate triggers opening of the active site, and entry of substrate to the active site, followed by elimination of pyruvate resulting in the shutting down of the active site, allowing the decarboxylation of pyruvate to take place at the active site. Subsequent addition of pyruvate to the regulatory site results in the re-opening of the active site and the release of reaction products (Alvarez et al., 1995). Modeling studies by Lobell and Crout (1996) suggested that "docking" of pyruvate near C221 could facilitate this reaction, since the pyruvate could be bound in-place by hydrogen bonds between additions and eliminations. However, there is no apparent "lid" that can regulate access to the active site according to the crystal structure of PDC (Dyda et al., 1993; Arjunan et al., 1996), although some sequence fragments that could not be identified in the crystal structure might carry out this function. While structural evidence for such a lid is lacking, were such a lid indeed identified, one may speculate that in the C221S or C221A variants this lid may stay open at all times and this could explain the enhanced reactivities observed with some reagents for the C221S variant compared to WT [see Baburina et al. (1998)]. This could be the case even though the C221S or C221A substitutions eliminate substrate activation without fatally damaging the enzyme.

It is usually claimed that PDC from yeast is inactive in the absence of substrate (Alvarez et al., 1991). This is based on pre-steady-state kinetic experiments in which the reaction rate (monitored by the decrease in NADH absorbance in the coupled assay) exhibits a lag phase. This lag phase is no longer seen in the C221S variant (Baburina et al., 1994), nor is the lag phase totally flat at early times in the WT; rather, it exhibits a continuous curvature. It may also be more physiologically useful if PDC is capable of decarboxylating pyruvate at all times, and the excess of the substrate (as a result of highly active glycolysis) causes an additional increase in catalytic activity, since more pyruvate molecules can occupy both the active and regulatory sites of PDC.

PDC is among a large group of enzymes whose activity is being regulated by a ligand that binds in a locus distinct from the active site (the allosteric site). There are two models that describe allosteric behavior of proteins (Segel, 1975; Hammes, 1982; Bell & Bell, 1988). Generally, it is believed that regulated enzymes exist in "activated" and "nonactivated" forms, and the transformation from one to another for allosteric enzymes begins with the binding of a ligand (substrate, product, metabolic intermediate, phosphate residue, etc.) to the regulatory (allosteric) site which causes conformational changes leading to more efficient reactions at the catalytic site.

Some enzymes, such as aspartate carbamoyltransferase and phosphofructokinase (Hammes, 1982), respond to ligand binding by dissociation and association of subunits. In fact, most of the glycolytic enzymes, enzymes in nucleotide and amino acid metabolism, show evidence of dissociation in the presence of allosteric effectors (Traut, 1994). However, subunits of PDC from brewers' yeast do not dissociate in the presence of pyruvate (Printz & Gounaris, 1972). But the response of tetrameric PDC to regulation remains an open question, since it is not yet known whether all four active sites of the molecule are equivalent, and the number of regulatory sites needed to be occupied to achieve 100% activation is also not known. The aldehyde product $X-C_6H_4-CH=CH-CHO$, derived from conjugated substrate analogs, is bound with a stoichiometry of two per tetrameric holoenzyme, and binding is at C221 [see Baburina et al. (1998)]. This suggests the possibility of only two out of four regulatory sites being occupied for full PDC activation.

The enzyme tryptophan synthase is an $\alpha_2\beta_2$ tetramer and is subject to substrate activation. Binding of the substrate to the active site on the α subunit causes a displacement of two amino acid residues and initiates conformational changes that are transmitted to the active site on the β subunit (Strambini et al., 1992). The cAMP binding protein of *E. coli* is a dimer and is negatively regulated by cAMP. Addition of cAMP to one subunit of the protein causes conformational changes of the entire molecule (Heyduk et al., 1992). Detailed conformational changes in response to

ligand binding have been documented on phosphofructokinase (Auzat & Garel, 1992), glycogen phosphorylase (Barford & Johnson, 1989), and hemoglobin (Perutz, 1970), among others.

From our results on PDC, and from some of the examples described above, one can make some generalizations. Binding of a ligand at the regulatory site causes a displacement of a residue located nearby, by mechanical and/or charge−charge interactions. That distortion initiates a chain of rearrangements that leads to the activation of the enzyme, whether it causes only local changes of the active site or global changes of the entire molecule. On PDC, a solid case can now be made that the first substrate is bound at C221, although the evidence of covalent interaction with substrate will have to await further studies. In the following paper, it is shown by protein chemical means and with the variants that several known inhibitors and activators do interact at C221. The information from the substrate bound at C221 is transmitted to H92 and then is sensed at the active center as well, as shown with the H92 variants. Elucidation of the pathway from H92 to the active center and of the concomitant structural changes remains an important goal.

SUPPORTING INFORMATION AVAILABLE

Description of the construction of the H92A, H92G, H92K, H92C/C222S, C221A/C222A, and C222A variants of PDC and overexpression and purification of the variants; Table S1 and Figures S1 and S2 showing the results of the purification protocol; and Tables S5−S8 and Figures S6−S9 showing the results of steady-state kinetic studies at different values of pH for WT, C221S, H92G, and H92K PDCs, respectively (16 pages). Ordering information is given on any current masthead page.

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