Substrate Activation of Brewers' Yeast Pyruvate Decarboxylase Is Abolished by Mutation of Cysteine 221 to Serine†

Irina Baburina, Yuhong Gao, Zhixiang Hu, and Frank Jordan*

Department of Chemistry, Rutgers University, Newark, New Jersey 07102

Stefan Hohmann

Laboratorium voor Moleculaire Celbiologie, Instituut voor Plantkunde, Katholieke Universiteit te Leuven, Kardinaal Mercierlaan 92, B-3001 Leuven-Heverlee, Flanders, Belgium

William Furey

Biocrystallography Laboratory, Veterans Administration Medical Center, P.O. Box 12055, University Drive C, Pittsburgh, Pennsylvania 15240, and Department of Crystallography, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Received January 19, 1994®

ABSTRACT: Brewers' yeast pyruvate decarboxylase (EC 4.1.1.1), a thiamin diphosphate and Mg(II)-dependent enzyme, isolated from *Saccharomyces cerevisiae* possesses four cysteines/subunit at positions 69, 152, 221, and 222. Earlier studies conducted on a variant of the enzyme with a single Cys at position 221 (derived from a gene that was the product of spontaneous fusion) showed that this enzyme is still subject to substrate activation [Zeng, X., Farrenkopf, B., Hohmann, S., Jordan, F., Dyda, F., & Furey, W. (1993) *Biochemistry 32,* 2704–2709], indicating that if Cys was responsible for this activation, it had to be C221. To further test the hypothesis, the C221S and C222S single and the C221S–C222S double mutants were constructed. It is clearly shown that the mutation at C221, but not at C222, leads to abolished substrate activation according to a number of kinetic criteria, both steady state and pre steady state. On the basis of the three-dimensional structure of the enzyme [Dyda, F., Furey, W., Swaminathan, S., Sax, M., Farrenkopf, B., Jordan, F. (1993) *Biochemistry 32,* 6165–6170], it is obvious that while C221 is located on the β domain, whereas thiamin diphosphate is wedged at the interface of the α and γ domains, addition of pyruvate or pyruvamide as a hemiketal adduct to the sulfur of C221 can easily bridge the gap between the β and α domains. In fact, residues in one or both domains must be dislocated by this adduct formation. It is very likely that regulation as expressed in substrate activation is transmitted via this direct contact made between the two domains in the presence of the activator. One can, in addition, also speculate that covalent attachment of large molecules to C221 will result in larger distortions, which will lead to greatly diminished activity, as is seen with conjugated substrate analogs, such as $XC_6H_4CH{=}CHCOCOOH$, explored by the authors.

Pyruvate decarboxylase from brewers' yeast (PDC,[1] EC 4.1.1.1) is one of the simpler enzymes that involve nonoxidative functions of thiamin diphosphate (ThDP) and 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 cation, and Mg(II) can best fulfill the function. The presence of divalent cations as part of the diphosphate binding locus on all three ThDP-dependent enzymes whose structures have been determined to date has been confirmed (Müller et al., 1993). According to the three-dimensional structure of PDC, the protein consists of four subunits of 563 amino acids each, and according to the gene sequence, in the enzyme isolated from *Saccharomyces cerevisiae*, each subunit possesses four cysteines at positions 69, 152, 221, and 222 (Hohmann & Cederberg, 1990), none of which participate in disulfide bridges. We have been interested in the potential function of these Cys thiol groups for a number of years, since according to chemical modification of PDC (Ullrich, 1982), thiols are about the only side chains in the enzyme whose modification has reliably been shown to lead to altered enzyme activity [see also Zeng et al. (1991, 1993) and references therein]. While earlier several groups (including one of ours) had believed that there may be a thiol in the reactive center of PDC, the X-ray structure has once and for all dispelled that notion; the nearest Cys to ThDP is 20 Å away from the reaction center, the C2 atom of the thiazolium ring (Dyda, 1992; Dyda et al., 1993). Even more surprising is the finding that, while ThDP resides in a cleft between the α and γ domains is different subunits, the two Cys side chains at positions 221 and 222, most favorably positioned for a regulatory function, reside on the β domain. The finding that PDC from brewers' yeast is subject to substrate activation (Boiteux & Hess, 1970; Hübner et al., 1978), coupled to the hypothesis that a cysteine side chain may be responsible for this activation (Sieber et al., 1983; Schellenberger et al., 1988), makes a study of this problem of particular interest.

Recently it became possible to further examine in greater detail the potential roles of cysteines in PDC. Our first

† F.J. is supported at Rutgers by NSF-DMB87-09758, NIH-GM50380, the Rutgers Busch Biomedical Fund, and Ciba-Geigy, Inc., Ardsley, NY. S.H. acknowledges receipt of a long-term EMBO fellowship and a fellowship from the Research Fund of the Katholieke Universiteit te Leuven. W.F. acknowledges support by NIH-GM-48195-01.

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* April 15, 1994.

[1] Abbreviations: ThDP, thiamin diphosphate; PDC, pyruvate decarboxylase (EC 4.1.1.1); pdc1, wild type pyruvate decarboxylase isolated from *Saccharomyces cerevisiae*; C221S, C222S, and C221S–C222S are mutants of this enzyme; pdc1–6, pyruvate decarboxylase isolated from the spontaneous fusion gene *Saccharomyces cerevisiae PDC1–PDC6*; CPB, (*E*)-4-(4-chlorophenyl)-2-oxo-3-butenoic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/94/0433-5630$04.50/0 © 1994 American Chemical Society

approach took advantage of the fact that in addition to the *PDC1* gene expressed in yeast, there appear to be a *PDC5* and a *PDC6* gene, and *PDC1* and *PDC6* undergo spontaneous gene fusion, the product of which has the first 45 N-terminal amino acids derived from *PDC1* and the 520 C-terminal ones derived from *PDC6*. One consequence of interest to us is that the enzyme expressed from this fusion gene has only a single Cys, that at position 221. The fusion enzyme was still subject to activation by the nondecarboxylatable substrate surrogate pyruvamide [known to activate the enzyme; see Hübner et al. (1978)]; therefore, if any Cys had any impact on catalysis and/or regulation, it would by necessity have to be C221 (Zeng et al., 1993). The obvious next question that one would like to resolve is What is the effect of single amino acid mutations involving Cys on PDC derived from the *PDC1* gene.

We have constructed yeast with the mutations C221S, C222S, and the double mutation C221S–C222S and here report kinetic studies on all three and on the wild type (WT) enzyme addressing this issue. Since C221 and C222 are adjacent to each other and at least partly exposed, mutation at both sites was deemed desirable, if for no other reason than to serve as a solid control experiment. Consistent with the behavior of the fusion PDC, Cys69 is totally buried and is not accessible from the protein surface. The residue Cys152, while accessible, is not favorably positioned to interact either with ThDP or with any of the other protein domains. For ease of producing significant amounts of protein, the genes were all transferred into a high expression vector in *Escherichia coli*, and all three mutant enzymes were purified to homogeneity, along with the wild type enzyme.

EXPERIMENTAL SECTION

Construction of in Vitro Mutated PDC1 Genes

The procedure followed essentially the instructions for the Altered Sites *in vitro* Mutagenesis System of Promega. In this system, a fragment of the gene of interest is subcloned into the plasmid pALTER1. This vector has a mutation in its ampicillin resistance gene, and transformants are selected on tetracycline plates. For the mutagenesis reaction two primers are used: one is designed to mutate the gene of interest; the other, supplied with the kit, repairs the ampicillin resistance. This procedure guarantees that a very high percentage of the transformants isolated subsequently on ampicillin plates contain the mutation.

A 3.4-kb *Sph*I/*Sal*I fragment of *PDC1* (Schaaff et al., 1989) was subcloned into the *Sph*I/*Sal*I digested vector pALTER1. The following primers were used to introduce the mutations from cysteine to serine at positions 221 and 222, respectively.

Ser^{221}: 5′ CTGGAACAAGAAGCATCAG 3′
changing codon 221 from TGT to TCT

Ser^{222}: 5′ GTGTCTGGAAGAACAAGCATC 3′
changing codon 222 from TGT to TCT

Ser^{221,222} (double mutant):
5′ CGTCGTGTCTGGAAGAAGAAGCATCAGCCAAG 3′
changing codons 221 and 222 from TGT to TCT

Ampicillin resistant clones were checked by sequence analysis on both strands using the following two primers: 5′ GACAGATGTATCAGAACC 3′ (nucleotide positions 448–466 of the coding region) and 5′ AAGAGAAAGAACCG-GTG 3′ (nucleotide positions 895–879). For the single mutants, the correct mutation was identified among the first two clones checked already; for the double mutant, eight clones had to be sequenced to identify the desired one.

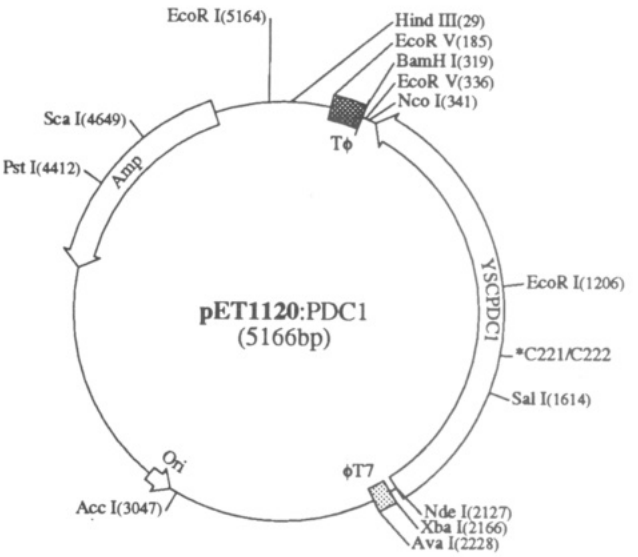

FIGURE 1: Restriction map of the expression plasmid pET 1120: PDC1. *ϕT7* and T*ϕ* represent the promoter and the transcription terminator of gene 10 of the bacterial phage T7. The gene coding for PDC (YSCPDC1) is cloned directly at the ATG starting codon of gene 10. The position of Cys221/Cys222 is also marked.

The mutated genes were originally cloned into the multicopy vector YEplac112 (Gietz & Sugino, 1988) on a 3.4-kb *Sph*I/*Bam*HI (*PDC1*) fragment. Then a 512-bp *Nde*I/*Sca*I fragment from pT7-*PDC1*-B1 (Candy et al., 1991) plus a 1273-bp *Sca*I/*Nco*I fragment from YEplac112 was ligated into the *Nde*I/*Nco*I double-digested pET1120 (Studier et al., 1990) to yield pET1120-*PDC1*, which gives an intact *PDC1* gene cloned under control of a T7 promoter (see plasmid map in Figure 1). Compared to the *PDC1* gene in pT7-PDC1-B1, a 1.4-kbp noncoding region is removed. The BL21(DE3) strain of *E. coli* was used for the expression, as recommended. Expression levels of PDC as high as 80% of total cellular protein could be achieved by inducing the cells grown in LB-amp-ThDP with 0.1 mM IPTG at its late log phase. The PDC was found active and in the soluble fraction of the cell lysate. Further details of this procedure, along with a purification protocol devised for the expressed protein, will be reported elsewhere.

Purification and Kinetic Studies

Enzyme Purification and Assay. The enzyme was purified according to Farrenkopf's protocol (Farrenkopf & Jordan, 1992), with an improved resolution over the procedure of Kuo et al. (1986). Protein assay was performed by the Bradford (1976) method, and the enzyme was assayed by the pH-stat method (Schellenberger et al., 1968) or the aldehyde dehydrogenase coupled assay (Holzer et al., 1956) by monitoring the depletion of NADH with time at 340 nm, either on the COBAS-BIO (Roche Diagnostics) automated assay system or on a HI-TECH Scientific PQ-SF53 stopped-flow spectrophotometer. All kinetic assays were done at 30 °C. One unit of activity is defined as the amount of PDC required to convert 1 μmol of pyruvate to acetaldehyde per minute at 30 °C at pH 6.0.

SDS–PAGE Electrophoresis. 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.

RESULTS AND DISCUSSION

The wild type (WT) PDC from *S. cerevisiae* and the mutant enzymes were all purified to homogeneity according to SDS–PAGE. The turnover numbers/subunit (s^{-1}) and specific activities (IU/mg) for the four enzymes were as follows: WT, 54.3 ± 0.77 and 55; C221S, 8.3 ± 1.7 and 4.8; C222S, 29.8 ± 0.36 and 30; C221S–C222S, 8.3 ± 1.06 and 5.5. The WT enzyme expressed and purified according to the protocol used was of sufficient quality to enable crystallization.

The steady-state v_0–S kinetic data are shown in Figure 2 for WT, C221S, and C222S. Data for the double mutant C221S–C222S are not shown since those were virtually superimposable within experimental error with those for C221S. It is instructive to look at three different representations of the data: top panels (v_0 vs S), Michaelis–Menten; middle panels, double reciprocal ($1/v_0$ vs $1/[S]$) or Lineweaver–Burk; and bottom panels ($v_0/[S]$ vs v_0), Eadie–Scatchard plots. In addition, the Hill equation was used to generate the following Hill coefficients: 2.05 ± 0.09 for WT, 0.82 ± 0.07 for C221S, 1.66 ± 0.06 for C222S, and 0.887 ± 0.067 for the C221S–C222S double mutant. The Hill coefficients deduced from nonlinear least squares fit of the progress curves (top, using the program Ultrafit from Biosoft) gave the same numbers within experimental error.

The different v_0 vs [pyruvate] behavior of the two mutants with C221S (hyperbolic) compared to the WT or the C222S (both sigmoidal) is clear, but perhaps most dramatic are the differences according to the Eadie–Scatchard representation.

The plots observed for the wild type and C222S mutant enzymes are consistent with substrate activation or positive cooperativity (Segel, 1975; Hammes, 1982; Bell & Bell, 1988) whereas those for the C221S and C221S–C222S mutant enzymes show kinetic behavior devoid of substrate activation within experimental error. To the contrary, there is a hint of negative cooperativity resulting from the conversion of Cys221 to a Ser, more pronounced in the single mutant.

To gain further insight concerning the kinetic behavior of these enzymes, we performed a coupled enzyme assay for activation of the four PDC variants. We would load one syringe of the stopped flow with the PDC, the second with alcohol dehydrogenase, NADH, and pyruvate prior to mixing. The panels in Figure 3 demonstrate rather convincingly that the wild type PDC and the C222S mutant show substrate activation characterized by a lag in NADH depletion, while C221S and C221S–C222S show no such lag phase within the experimental error limits. Such results on the WT enzyme had been reported repeatedly by Schellenberger and his coworkers.

We conclude that C221 is the site of substrate activation in this strain of PDC.

Several other relevant issues need mentioning. PDC from some other sources (e.g., from *Zymomonas mobilis*) have not been reported to be subject to substrate activation. Also, protein chemical techniques have identified C221 as the site of alkylation of PDC that leads to substantial inactivation by "mechanism-based" inactivators of the CPB type (XC_6H_4-CH=CHCOCOOH, where X = *p*-Cl, Dikdan, unpublished results). Zeng et al. (1991) reported that inactivation by such compounds led to modification of one cysteine of the four per subunit. We now also suspect that our previous model, developed to convert PDC to pyruvate oxidase (an FAD-dependent enzyme that has in common with PDC the steps through decarboxylation) by covalently attaching 8-acetyl-10-methylisoalloxazine to PDC, resulted in covalent modification of C221 as well, since it totally abolished the activity for acetaldehyde formation, but resulted in the slow formation of acetate instead (Annan & Jordan, 1990). The 3D structure of PDC is consistent with this idea in that C221 and the C2 and S atoms of ThDP (Dyda, 1992) are clearly accessible to substrate and inhibitor.

It is worth recalling some of the accumulated evidence concerning possible consequences of substrate activation. Some of these effects were noted for pyruvate itself, some others for saturating pyruvamide. (1) In a comprehensive summary of the activation kinetics of the enzyme, Alvarez et al. (1991) summarized the rate constants for the formation of the activated PDC by substrate, deducing a slow unimolecular kinetic step for the interconversion of the unactivated to the activated forms of the enzyme. (2) It was shown that, from alternate conjugated substrates with the structure XC_6H_4-CH=CHCOCOOH, the enamine/2-α-carbanion intermediate that is the immediate product of decarboxylation is formed slowly in the absence of pyruvamide, but at rates consistent with the pyruvate turnover number in its presence; hence, for those substrates at least, decarboxylation is definitely accelerated by substrate activation (Menon-Rudolph et al., 1992). (3) Zeng demonstrated (Zeng et al., 1991; Zeng, 1992) that the enamine/2-α-carbanion formed from such conjugated alternate substrates can be protonated at both the α and γ positions (unlike that from pyruvate, which has only the α position available for protonation), leading to preference for protonation at the γ position in the absence of pyruvamide but for the α position in its presence. We interpreted this regiochemical paradox to mean that enamine protonation is misaligned in the absence of the activator/substrate surrogate and corrected in its presence. (4) In a very recent publication the formation of acetoin from acetaldehyde by PDC was reexamined and found to proceed at a modestly faster rate in the presence of pyruvamide than in its absence (Stivers and Washabaugh, 1993).

What is also evident from our results here presented is that PDC that is devoid of the ability to be activated by substrate is by no means a dead enzyme. This is consistent with the massive amount of chemical modification data directed to cysteines on this enzyme over a period of 50 years, which never resulted in total inactivation.

An interesting question that will require further research is, How is the information relayed to the ThDP catalytic locus (located between the α and γ domains from different subunits) on binding to substrate to C221 (located on the β domain) in this allosteric kinetic system? Assuming that the α-keto acid or its amide forms a hemiketal with C221, we constructed a model based on the crystallographic coordinates of the native holoenzyme. First, it is clear that the covalently bound pyruvate can bridge the gap between the β and the α domain. Figure 4 shows the details of the interaction surface among the three domains. Several features should be pointed out. First, it is relevant to note that while the C221–SH points to the α domain, the C222–SH points away from the catalytic domains. Second, binding of additional atoms to the S atom of C221 will create unfavorably short contacts to H92 (α domain); thus, one or both of these residues must move. If the main chain of H92 is indeed affected, then the residue E91 would also be displaced somewhat. The side chain of E91 forms numerous van der Waals contact interactions with the γ domain, as well as a hydrogen bond to the main chain N atom of W412. Thus one can see how the binding of substrate

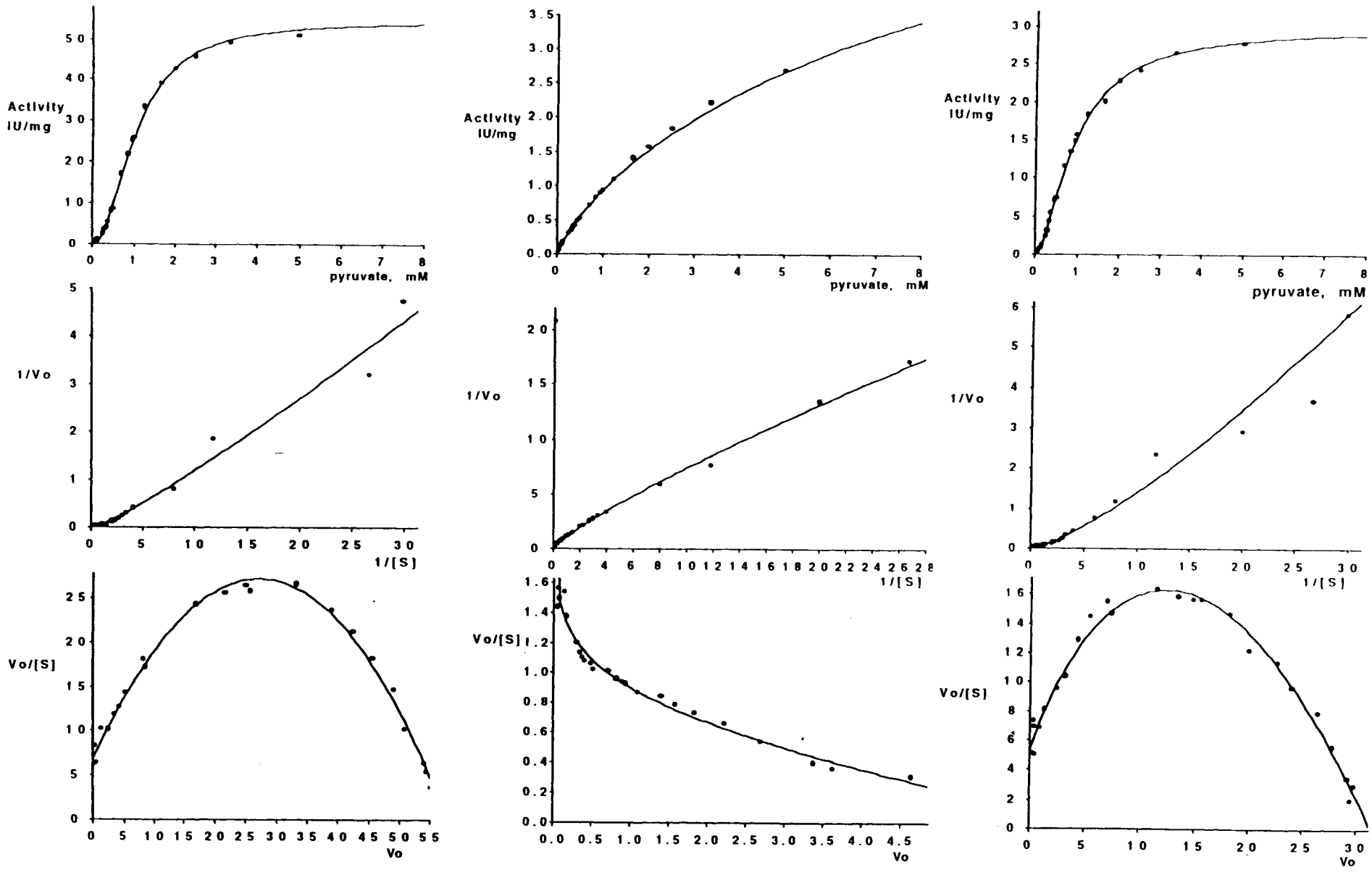

FIGURE 2: Steady-state v_0 vs pyruvate concentration data for the PDC variants. Left-hand panels, wild type (WT); center panels, C221S; right-hand panels, C222S. Top panels, v_0 vs [pyruvate]; middle panels, $1/v_0$ vs 1/[pyruvate]; bottom panels, v_0/[pyruvate] vs v_0. All kinetic data were obtained at 30 °C at pH 6.0, the NADH depletion being measured at 340 nm. All components including PDC were originally diluted into 0.1 M MES buffer, pH 6.0, containing 0.1% bovine serum albumin and 0.5 mM phenylmethanesulfonyl fluoride. Components were combined to yield a final concentration of 5.3 IU/mL of alcohol dehydrogenase and 0.5 mM β-NADH. Pyruvate concentrations varied from 0.025 to 15 mM. In the automated assay, 10 μL of the PDC was combined with 200 μL of the alcohol dehydrogenase/NADH mixture and 10 μL of the substrate to yield a total cuvette volume of 260 μL (including a 20-μL flush after the sample and the substrate addition). The final concentrations of PDC were 0.09 IU/mL for the WT, 0.025 IU/mL for C221S, 0.106 IU/mL for C222S, and 0.06 IU/mL for C221S–C222S.

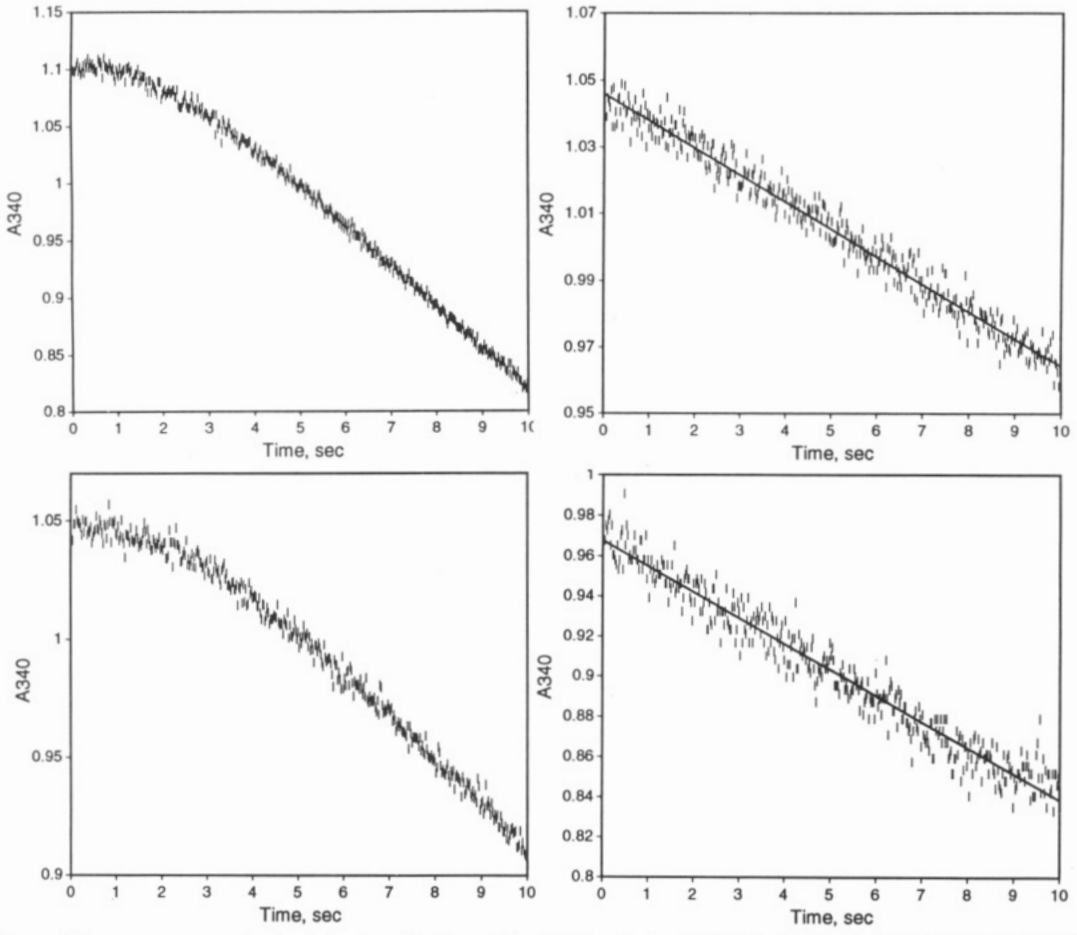

FIGURE 3: Stopped-flow measurements of substrate activation of the PDC variants at 30 °C. Clockwise from upper left: wild type, C221S, C221S–C222S, C222S. Conditions: All components, including PDC, were diluted into 0.1 M MES buffer, pH 6.0, containing 0.1% bovine serum albumin and 0.5 mM phenylmethanesulfonyl fluoride. One syringe contained PDC at a concentration of 0.075 IU/mL. The second syringe contained 10.6 IU/mL of alcohol dehydrogenase (specific activity 380 IU/mg), 1 mM β-NADH, and 60 mM pyruvate. On mixing, all of these concentrations were diluted by a factor of 2.

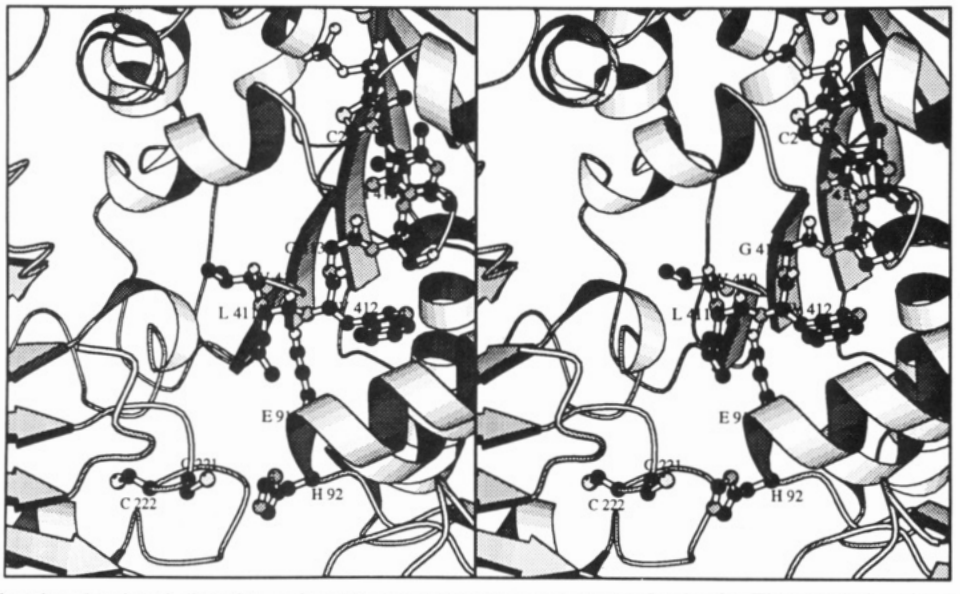

FIGURE 4: Stereo drawing showing the location and environment of C221 in a single PDC subunit. The protein backbone is shown as a ribbon representation, while all atoms are shown for the cofactor and the short chain segment (residues 411–415) leading to it from the activation site. Only side chain atoms are shown for C221, C222, E91, and H92. The residues E91 and H92 are in the α domain, C221 and C222 are in the β domain, and the stretch 411–415 is in the γ domain. The figure was created with the program MOLSCRIPT written by Kraulis (1991).

to C221 of the β domain has the potential to affect both catalytic α and γ domains as well. These contacts, especially to W412, may be important as they involve part of the short chain segment leading to the catalytic site. In particular, the main chain carbonyl O atom of G413 forms a conserved hydrogen bond to N4′ of the 4′-aminopyrimidnie ring of ThDP, while the side chain of I415 is wedged tightly between the cofactor rings, supporting the V conformation. The N atom

of I415 also forms a conserved hydrogen bond with N3′ of the cofactor.

The nature and degree of perturbation in these residues induced by binding substrate at the regulatory site C221, and how it results in enhanced activity, will be most interesting to examine by additional biochemical and crystallographic studies. Thus PDC presents an exciting case for studying the mechanism of homotropic activation in multidomain enzymes.

REFERENCES

Alvarez, F. J., Ermer, J., Hübner, G., Schellenberger, A., & Schowen, R. L. (1991) *J. Am. Chem. Soc. 113*, 8402–8409.

Annan, N., & Jordan, F. (1990) *J. Am. Chem. Soc. 112*, 3222–3223.

Bell, J. E., & Bell, E. T. (1988) *Proteins and Enzymes*, pp 370–377, Prentice Hall, Inc., Englewood Cliffs, NJ.

Bisswanger, H., & Ullrich, J., Eds. (1991) *Biochemistry and Physiology of Thiamin Diphosphate Enzymes*, pp 1–453, VCH Publishers, New York.

Boiteux, A., & Hess, B. (1970) *FEBS Lett. 9*, 293–296.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Candy, J. M., Duggleby, R. G., & Mattick, J. S. (1991) *J. Gen. Microbiol. 137*, 2811–2815.

Dyda, F. (1992) Ph.D. Dissertation, University of Pittsburgh.

Dyda, F., Furey, W., Swaminathan, S., Sax, M., Farrenkopf, B., & Jordan, F. (1993) *Biochemistry 32*, 6165–6170.

Farrenkopf, B., & Jordan, F. (1992) *J. Protein Expression Purif. 3*, 101–107.

Gietz, R. D., & Sugino, A. (1988) *Gene 74*, 527–534.

Hammes, G. G. (1982) *Enzyme Catalysis and Regulation*, pp 152–186, Academic Press, New York.

Hohmann, S. (1991a) *Curr. Genet. 20*, 373–378.

Hohmann, S. (1991b) in *Biochemistry* and *Physiology of Thiamin Diphosphate Enzymes* (Bisswanger, H., & Ullrich, J., Eds.) pp 106–114, VCH Publishers, New York.

Hohmann, S., & Cederberg, H. (1990) *Eur. J. Biochem. 188*, 615–621.

Holzer, H., Schultz, G., Villar-Palasi, C., & Juntgen-Sell, J. (1956) *Biochem. Z. 327*, 331–344.

Hübner, G., Weidhase, R., & Schellenberger, A. (1978) *Eur. J. Biochem. 92*, 175–181.

Kluger, R. (1987) *Chem. Rev. 87*, 863–876.

Krampitz, L. O. (1969) *Annu. Rev. Biochem. 38*, 213–240.

Kraulis, P. (1991) *J. Appl. Crystallogr. 24*, 946.

Kuo, D. J., Dikdan, G., & Jordan, F. (1986) *J. Biol. Chem. 261*, 3316–3319.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Menon-Rudolph, S., Nishikawa, S., Zeng, X., & Jordan, F. (1992) *J. Am. Chem. Soc. 114*, 10110–10112.

Müller, Y. A., Lindquist, Y., Furey, W., Schulz, G. E., Jordan, F., & Schneider, G. (1993) *Structure 1*, 95–103.

Sable, H. Z., & Gubler, C. J., Eds. (1982) *Ann. N.Y. Acad. Sci. 378*, 7–122.

Schaaff, I., Green, J. B. A., Gozalbo, D., & Hohmann, S. (1989) *Curr. Genet. 15*, 75–81.

Schellenberger, A., Hübner, G., & Lehmann, G. (1968) *Angew. Chem., Int. Ed. Engl. 11*, 886–887.

Schellenberger, A., & Schowen, R. L., Eds. (1988) *Thiamine Pyrophosphate Biochemistry*, CRC Press, Boca Raton, FL, Vols. 1–2.

Schellenberger, A., Hübner, G., & Sieber, M. (1988) in *Thiamin Pyrophosphate Biochemistry* (Schellenberger, A., & Schowen, R. L., Eds.) pp 113–121, CRC Press, Boca Raton, FL, Vol. 1.

Segel, I. H. (1975) *Enzyme Kinetics*, Wiley Interscience, London, pp 346–397.

Sherman, F., Fink, G. R., & Hicks, J. B. (1986) *Methods in yeast genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sieber, M., Kõnig, S., Hübner, G., & Schellenberger, A. (1983) *Biomed. Biochim. Acta 42*, 343–349.

Stivers, J. T., & Washabaugh, M. W. (1993) *Biochemistry 32*, 13472–13482.

Studier, W., Rosenberg, A. R., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60.

Ullrich, J. (1982) *Ann. N.Y. Acad. Sci. 378*, 287–305.

Zeng, X. (1992) Doctoral Dissertation, Rutgers University Graduate Faculty, Newark, NJ.

Zeng, X., Chung, A., Haran, M., & Jordan, F. (1991) *J. Am. Chem. Soc. 113*, 5842–5849.

Zeng, X., Farrenkopf, B., Hohmann, S., Dyda, F., Furey, W., & Jordan, F. (1993) *Biochemistry 32*, 2704–2709.