# Yeast Pyruvate Decarboxylase Tetramers Can Dissociate into Dimers along Two Interfaces. Hybrids of Low-Activity D28A (or D28N) and E477Q Variants, with Substitution of Adjacent Active Center Acidic Groups from Different Subunits, Display Restored Activity†

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ABSTRACT: The tetrameric enzyme yeast pyruvate decarboxylase (YPDC) has been known to dissociate into dimers at elevated pH values. However, the interface along which the dissociation occurs, as well as the fundamental kinetic properties of the resulting dimers, remains unknown. The active sites of YPDC are comprised of amino acid residues from two subunits, a property which we utilize to address the issue as to which dimer interface is cleaved under different conditions of dissociation. Hydroxide-induced dissociation of the active site D28A (or D28N) and E477Q variants, each at least 100 times less reactive than wild-type YPDC, followed by reassociation of D28A (or D28N) and E477Q variants led to a remarkable 35−50-fold increase in activity. This result is possible only if the hydroxide-induced dissociation results in a cleavage along the interface between two subunits so that residues D28 and E477 are now separated. Upon reassociation, one of the two active sites of the hybrid dimer will have both residues substituted, whereas the second one will be of the wild-type phenotype. In contrast to the hydroxide-induced dimers, the urea-induced dissociation recently proposed results in dissociation along dimer−dimer interfaces, without separating the active sites, and therefore, on reassociation, these dimers do not regain activity. The significance of the results is discussed in light of a recently proposed alternating sites mechanism for YPDC. A preparative ion-exchange method is reported for the separation and purification of hybrid enzymes.

Pyruvate decarboxylase catalyzes the nonoxidative decarboxylation of pyruvate to acetaldehyde (Aa) and requires thiamin diphosphate (ThDP)[1] and Mg(II) as cofactors (Scheme 1). Yeast pyruvate decarboxylase (YPDC, EC 4.1.1.1) is an enzyme consisting of four identical subunits (*1*, *2*). The ThDP is bound at the interface created by two subunits that form a tight dimer. Two of these dimers form a loose tetramer usually characterized as a "dimer of dimers".

To account for the growing body of experimental data not fully explained by the previous phenomenological model for this enzyme (*3*), we recently proposed a model in which YPDC tight dimers ("functional dimers") form the minimal catalytic unit (*4*). In this model, two active sites of the functional dimer act in an antiphase manner during the reaction, with each active site eventually completing the full catalytic cycle, but, at any instant, the predecarboxylation phase of the reaction is taking place in one monomer and the postdecarboxylation phase in the other.

In a paper on the structure of YPDC saturated with 300 mM pyruvamide [a substrate analogue that cannot be decarboxylated but has been long presumed to act as a substrate surrogate (*3*)], it was suggested that the spatial constraint forcing two functional dimers to acquire the same conformation is responsible for the cooperativity of YPDC (*5*). However, that model invokes concerted conformational changes (*6*), and therefore, it fails to explain the negative homotropic cooperativity observed in our laboratory with some variants (*7*) and with certain wild-type YPDC conformational states (*8*).

Tetramers of YPDC are known to reversibly dissociate into dimers under alkaline conditions, and those dimers were formerly thought to be inactive (*9*). A recent study reexamined this issue and claimed that YPDC dimers could indeed be active (*10*), and at the same time, a new method for urea-induced dissociation of YPDC tetramers to dimers was proposed.

We here report that, depending on the experimental conditions selected, two types of stable YPDC dimers can be generated. Raising the pH of the solution to above 8.5 (hydroxide-induced dissociation) induces dissociation of the tetramers across the functional dimer interface and results in inactive dimers. In contrast, dilution and urea addition

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[1] Abbreviations: ADH, yeast alcohol dehydrogenase; ThDP, thiamin diphosphate; YPDC, pyruvate decarboxylase from the yeast *Saccharomyces cerevisiae* overexpressed in *Escherichia coli*; WT, wild-type YPDC; D28A, D28N, and E477Q, variants of YPDC with the indicated substitutions; HT or $(His)_6$ tag, C-terminally histidine-tagged YPDC.

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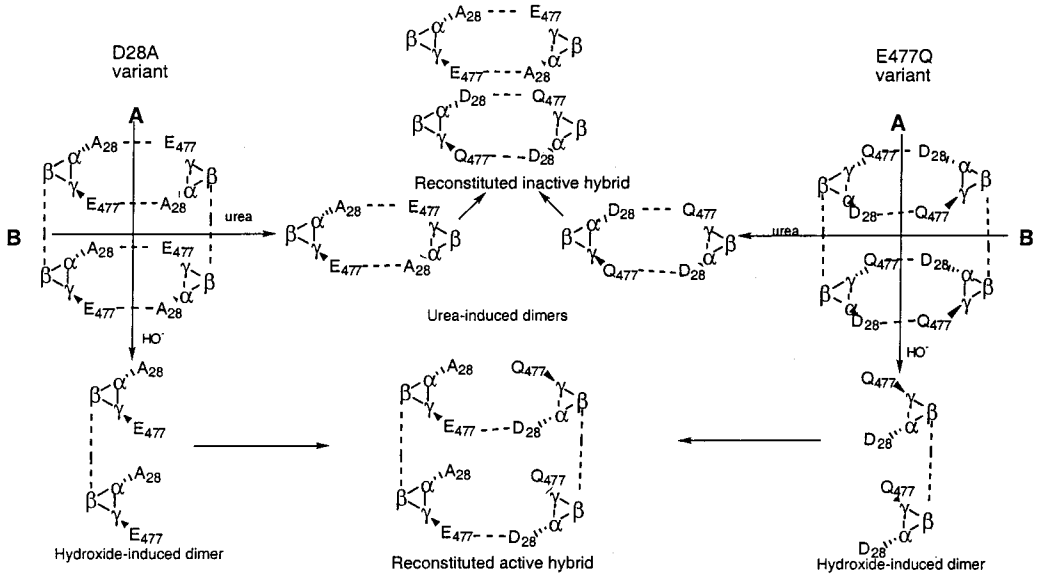
Scheme 1: Urea- and Hydroxide-Induced Dimer Formation and Reconstitution to Tetramers (α, β, and γ Denote Domains)




**Reconstitution of urea-induced dimers.**
Formation of tetramer from either two urea dimers of D28A or of E477Q individually, or of a hybrid tetramer created by mixing one D28A and one E477Q urea dimers, leads to inactive (low activity) species.

**Reconstitution of alkaline-induced dimers.**
Formation of tetramer from two hydroxide-induced dimers of D28A or of E477Q individually will lead to low activity species. However, formation of a hybrid tetramer created by mixing one D28A with one E477Q hydroxide-induced dimer would lead to active species; the activity could be reduced by symmetry considerations.

separates functional dimers of the tetramer resulting in the formation of active dimers. This demonstration is premised on our ability to reassociate two essentially inactive variants (created by substitution of the active site acid−base groups D28 and E477 located on different subunits but only 3.5 Å from each other) to fully active tetramers after hydroxide-induced dissociation. The work demonstrates that the active center variants with very low activity can complement each other to form active dimers with nearly the predicted activity and also argues that no monomers need to be formed in the dissociation process. The methods used provide entry to tetrameric hybrids of YPDC with any desired subunit composition.

## EXPERIMENTAL PROCEDURES

*Wild-type YPDC and the D28A, D28N, and E477Q variants* were obtained and purified as described elsewhere (*11*, *12*). Enzymes were stored at a concentration of 20−60 mg/mL in 50% glycerol at −20 °C in 50 mM MES buffer (pH 6.0), containing 5 mM $MgCl_2$ and 1 mM ThDP.

*The activity of YPDC* was assayed at 20 °C in 100 mM MES (pH 6.0), containing 20 mM pyruvate, 5 mM $MgCl_2$, and 1 mM ThDP, as well as 0.2−0.3 mM NADH and 10 units/mL alcohol dehydrogenase (*13*).

*pH-induced YPDC hybrids* were obtained by first raising the pH to 8.6 (creating the "hydroxide-induced dimers") and then lowering it back to 6.0 (creating the "pH-induced hybrids"). Typically, to the solution containing the two variant enzymes (total concentration 1 mg/mL) in 20 mM MES (pH 6.0) was added $^1/_{10}$ volume of the dissociation buffer (0.5 M Tris and 20 mM EDTA, pH 10.6) to a final concentration of 45 mM Tris and 1.8 mM EDTA. The resulting solution (pH = 8.6) was incubated at room temperature for 5 min; to the solution was then added $^1/_9$ volume of the reassociation buffer (0.5 M MES, 0.5 M acetate, pH 4.5, 100 mM $MgCl_2$, and 10 mM ThDP). This resulted in final concentrations of 50 mM MES, 50 mM acetate, 10 mM Mg(II), and 1 mM ThD, and a pH of 6.0. Control samples were carried through with the combination of premixed dissociation and reassociation buffers to reach the same final concentration of reagents.

*Separation of pH-induced YPDC hybrids from parental variant enzymes* was performed by ion-exchange chromatography. WT YPDC with a C-terminal $(His)_6$ tag and the D28A variant without the $(His)_6$ tag (10 mg of each) were dissolved in 7 mM MES and 1.5 mM EDTA (pH 6.0). The pH of the mixture was adjusted to 8.6 with slow addition of 1 M NaOH from a 1−20 μL pipet tip. After 30 min incubation on ice, $MgCl_2$ (to 4.6 mM) and ThDP (to 0.7 mM) were added, and the pH was adjusted to 5.5 with 1 M HCl in the same way as above. The enzyme solution was brought to room temperature and loaded onto a SP-Sepharose column (1 × 20 cm), equilibrated with 20 mM MES (pH 5.5), 2 mM $MgCl_2$, and 0.1 mM EDTA (buffer A). The column was washed with 25 mL of buffer A. Concentration of NaCl was raised from 0 to 100 mM with 10 mL of a linear gradient and then with 100 mL of a linear gradient of buffer A ranging from 100 to 400 mM NaCl. Fractions (1.5 mL) were collected into glass tubes with 1 mM ThDP (final concentration). Fractions were analyzed by SDS−PAGE according to Laemmli (*14*).

*Urea-induced YPDC dimers* were obtained by dissociating the tetrameric enzymes utilizing urea (*10*). The enzymes were dissolved at a concentration of 0.5−1 mg/mL in 20 mM MES (pH 6.0), containing 1 mM ThDP and 5 mM $MgCl_2$, and then urea was added to a final concentration of 0.5 M (resulting in the "urea-induced dimers"). After 15 min incubation, the solutions of the two variants (or one variant and wild-type YPDC) were mixed in the desired proportion (resulting in "urea-induced hybrids"). The urea was diluted

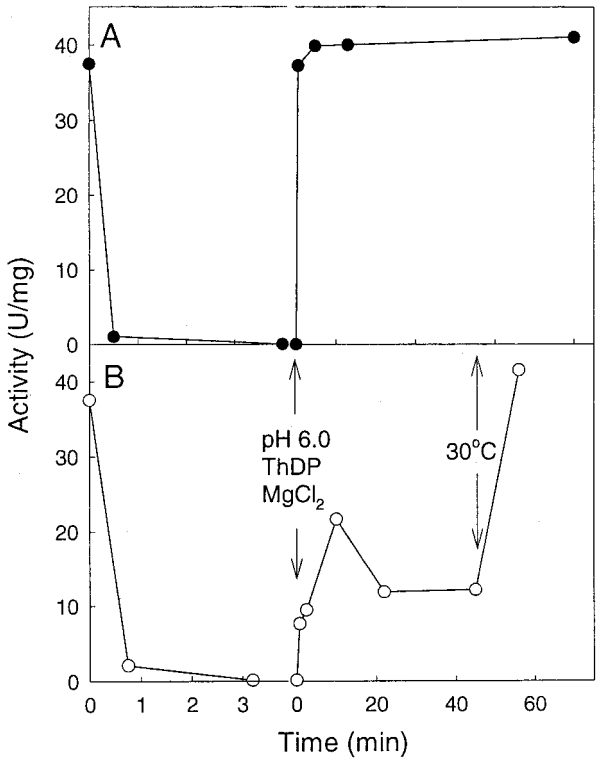



FIGURE 1: Effect of temperature on hydroxide-induced dissociation and reassociation of the WT YPDC. Enzyme was diluted to a concentration of 0.24 mg/mL in 20 mM MES (pH 6.0). The hydroxide-induced dissociation and reassociation of the tetramers was initiated by addition of appropriate buffers to reach pH 8.6 and then back to pH 6.0 as described under Experimental Procedures. The temperature was maintained at 25 °C (A) and 0 °C (B).

to a final concentration of less than 40 mM, and the activity was measured. This concentration of urea did not affect the activity of WT YPDC or of alcohol dehydrogenase, the coupling enzyme.

*Time-Dependent Dissociation and Reassociation of the YPDC*. Wild-type YPDC was diluted to a concentration of 0.24 mg/mL in 20 mM MES (pH 6.0). The hydroxide-induced dissociation and reassociation of the tetramers were initiated as described above. Activity was measured by addition of 990 $\mu$L of the assay buffer without Mg(II) and ThDP to the 10 $\mu$L aliquots. The progress curves measured under those conditions were linear, whereas addition of ThDP and $MgCl_2$ produced a pronounced activation phenomenon in the progress curves.

## RESULTS

*Time-Dependent Changes of Activity at Elevated pH*. Dissociation of the wild-type YPDC tetramers into hydroxide-induced dimers was initiated by increasing the pH to 8.6 and chelation of the Mg(II) ions with EDTA. Since the enzyme dissolved in 5 mM $MgCl_2$ was diluted at least 100-fold in the 20 mM MES buffer, to which 2 mM EDTA was added, the free Mg(II) concentration is expected to be less than 2 nM at pH 8.6 and 1.7 $\mu$M at pH 6.0. Upon alkaline dissociation, no activity of wild-type YPDC could be detected. Within the first 30 s, the activity decreased to 3−4% of the original value at 25 °C (Figure 1), giving an estimate of 0.11−0.12 $s^{-1}$ for the rate constant. When carried out on ice, the rate constant for dissociation was approximately one-half that value. The rate constant for the reassociation could be estimated as 0.08 $s^{-1}$ at 25 °C and only 0.0045 $s^{-1}$ at 0 °C. At the same time, the reassociation proceeded to completion at elevated temperature but only to 50% completion at low temperature. A longer incubation time at 0 °C caused the activity to decrease to a constant value at 25% of the original activity. Those changes were reversible, and once the temperature was raised, 100% activity was recovered. Increasing the temperature from 25 to 30 °C in the first case did not change the activity.

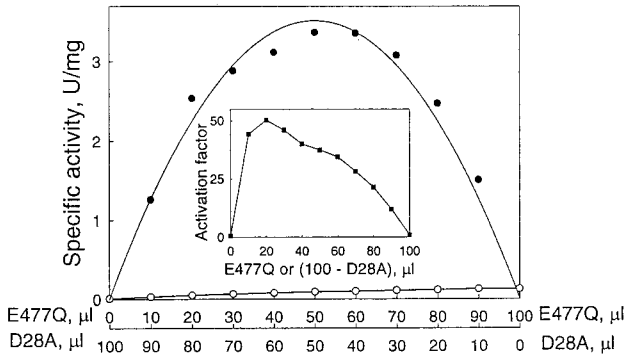



FIGURE 2: Job plot of hydroxide-induced hybridization of the E477Q and D28A YPDC variants. The solutions of two variants (1 mg/mL each) in 20 mM MES buffer (pH 6.0) were mixed in different molar ratios. To the mixture was added the dissociation buffer (closed circles) to a final concentration of 45 mM Tris and 1.8 mM EDTA and final pH of 8.6. The resulting solution was incubated at room temperature for 5 min, and to the solution was then added the reassociation buffer to give final concentrations of 50 mM MES, 50 mM acetate, 10 mM Mg(II), and 1 mM ThDP and a pH of 6.0. Control samples (open circles) were carried through with the combination of premixed dissociation and reassociation buffers to reach the same final concentration of reagents. Inset: ratio of activities in hybridized and control samples.

*pH-Induced Hybrid YPDC*. The E477Q and D28A variants of YPDC were mixed in different mole fractions but at a constant total concentration. Hydroxide-induced dimers of each variant were obtained by increasing the pH to 8.6; then a mixture of the two hydroxide-induced dimers was allowed to reassociate by lowering the pH to 6.0. The activity of the resulting samples was much higher (50-fold at the maximum) than of the control samples that did not undergo dissociation and reassociation but contained all of the same constituents. The final activity achieved depended on the molar ratio of the two variants (Figure 2) and displayed a maximum at a 1:1 molar ratio. When mixtures of the E477Q and D28N (the latter in place of D28A) variants of YPDC were utilized, the results were almost identical (data not shown).

*Urea-Induced Hybrid YPDC*. The E477Q and D28N variants were mixed in different molar ratios in the presence of 0.5 M urea and were then diluted at least 12-fold in the buffer without urea. Activity was assayed in the buffer without urea. No changes in activity were detected (Figure 3).

*Separation of pH-induced YPDC hybrids from parental proteins* was performed on a cation-exchange column. This approach is based on the different number of $(His)_6$ tags (HT) per tetramer in the original variants and the hybrid enzyme. In this experiment the mixture of WT YPDC and D28A variant enzymes was subjected to hydroxide-induced dissociation and reassociation. WT YPDC has a C-terminal $(His)_6$ tag in each monomer, whereas the D28A variant does

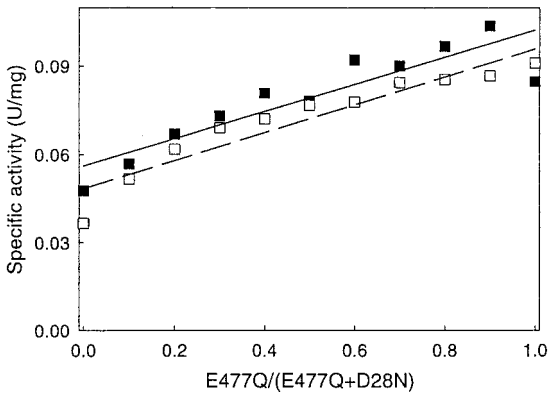



FIGURE 3: Job plot of urea-induced hybridization of E477Q and D28N YPDC variants. The enzymes were dissolved at a concentration of 1 mg/mL in 20 mM MES (pH 6.0), containing 1 mM ThDP and 5 mM $MgCl_2$, and then urea was added to a final concentration of 0.5 M. After 15 min incubation, the solutions of the two variants were mixed in the desired molar ratios. The urea was diluted to a final concentration of less than 40 mM, and the activity was measured. Samples are shown as open squares. Control samples contained no urea and are shown in closed squares.

not. The chromatogram presented in Figure 4A demonstrates a separation of the enzyme mixture into three discrete peaks with some enzyme passing through the column without significant binding (data not shown). The activity in the flow-through fraction was equal to 0.23 unit/mg, and that at the maximum of the first, second, and third peaks was 0.15, 17, and 27 units/mg, respectively. Electrophoretic analysis of the fractions (Figure 4B) revealed that both the flow-through fraction and the first peak contain only subunits with no His tag (of lower effective molecular weight on SDS−PAGE), with the bulk of the protein appearing in the flow-through fraction. The second major peak contains equal amounts of non-HT and HT subunits, as seen from an inspection of the SDS−PAGE (Figure 4B). The third peak contains only HT subunits.

## DISCUSSION

An examination of the X-ray structure of YPDC (*1*, *2*) indicates that the tetramer could potentially be dissociated to two different dimers, depending on the interface between subunits along which it is cleaved (Scheme 1). We will denote planes that dissect the tetramer into dimers as A (the plane that separates subunits interacting via the α and $\gamma$ domains) and B (the plane that separates subunits interacting via the $\beta$ domains). To differentiate the two types of dimers, we designate the asymmetric dimer (the tetramer is dissociated along plane B), with subunit interaction through the α and $\gamma$ domains, the "functional dimer". The second type of dimer (created through dissociation along plane A) is created by subunits in the same conformation and interacting through the $\beta$ domains as a "symmetric dimer" (Scheme 1).

Increasing the pH from 6.0 to 8.6 resulted in the total loss of WT YPDC activity within 1 min. Complete loss of activity suggests that all of the tetramers are dissociated under these conditions. Restoring the pH to the optimal value of 6.0, with concomitant addition of ThDP and Mg(II), led to a *complete recovery of the activity*. As was reported earlier, the YPDC tetramer undergoes dissociation into dimers at elevated pH (*9*).

The effect of temperature on the hydroxide-induced dissociation suggests that hydrophobic interactions are involved in the rate-limiting step of reassociation. The abundance of nonpolar amino acids in the contact region between monomers in the functional dimer (as opposed to the polar residues in the interdimer interface) (*10*) suggests that hydroxide-induced dissociation is destroying the functional dimers. The extent of reactivation also depends on the temperature, suggesting that hydrophobic interactions were involved not only in the rate-limiting step but also in the step determining the pathway. The initial increase in activity at low temperature changed to a decrease and then to a constant value of 10−11 units/mg, possibly trapping the enzyme in some conformation that is more stable at lower than at higher temperatures. Previously, two pathways were observed for reassociation of YPDC dimers to tetramers (although no state of oligomerization was determined under alkaline conditions) (*15*). It is conceivable that at both low and high temperatures the reassociation proceeds via only one of the pathways.

Hybridization of the hydroxide-induced dimers of the two variants with extremely low activity resulted in an increase of activity up to 3.5 units/mg. Potentially, two types of tetramers with distinct features might result from the reassociation of dimers created under different conditions (Scheme 1). But, only the dimers that resulted from dissociation of the active sites could give rise to the observed dramatic activity increase on reassociation. This feature is premised on the observation that amino acid residues from two different subunits comprise the YPDC active site. In this case, the monomer of each variant would contribute one substituted residue and one wild-type residue to the hybrid dimer. For example, the E477Q variant would have altered E477 but unaltered D28 residues, resulting in the creation of one active site of wild-type character per dimer (Scheme 1). This methodology was originally developed for aspartate transcarbamoylase, another enzyme whose active site is composed of amino acids from two subunits (*16*, *17*). For that enzyme, a hybrid catalytic trimer with a single intact active site displayed 33% of the activity intrinsic to the WT catalytic trimer.

The substitutions introduced at D28 and E477 are not expected to change the rate of dissociation or reassociation; therefore, the equilibrium constants of dissociation should be the same for both homo- and heterotetramers. Under these conditions, the molar ratio of the resulting homo- and heterotetramers will be present according to a binomial distribution: $([E477Q] + [D28])^2 = [E477Q]^2 + 2[E477Q][D28A] + [D28A]^2$; i.e., the relative concentration of the species $E477Q_2$:E477Q-D28A:$D28A_2$ is 1:2:1, when variants are present at equal concentrations. The maximum concentration of hybrid tetramers will be reached for a 1:1 molar ratio of the individual variants and will be equal to 50% of the total enzyme concentration. Therefore, the predicted activity of a 1:1 molar mixture of the E477Q and D28A variants would be 7 units/mg of the hybrid enzyme, twice the measured value of 3.5 units/mg. In turn, in the hybrid tetramers there are only two active sites of the wild-type phenotype, and the remaining two are of the doubly substituted phenotype. Therefore, the activity of the wild-type active sites present in the hybrid is 14 units/mg.

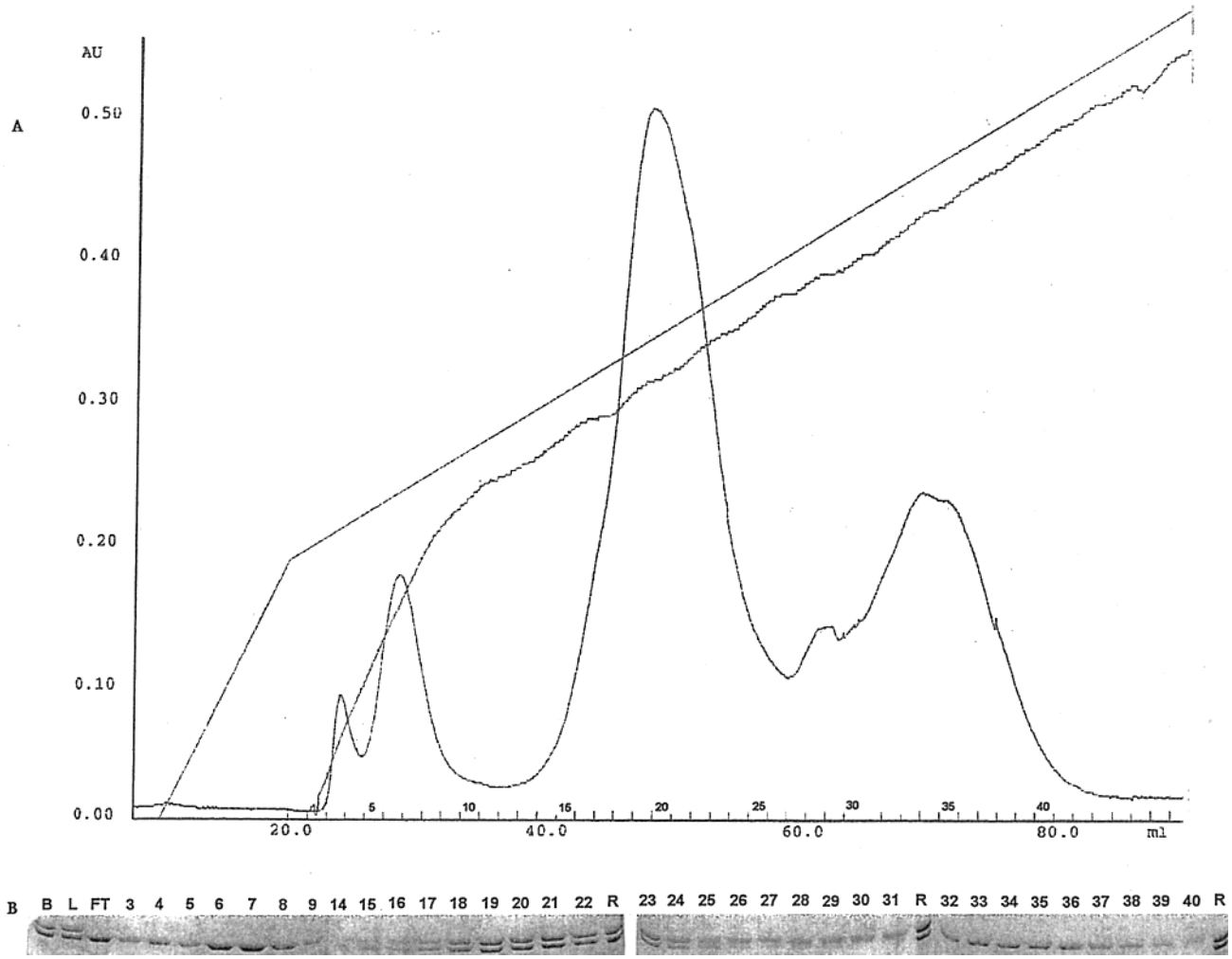



FIGURE 4: Chromatographic separation of pH-induced YPDC hybrids on a cation-exchange SP-Sepharose FF resin. A mixture of WT YPDC with the C-terminal $(His)_6$ tag in each monomer and the D28A variant [without the C-terminal $(His)_6$ tag] was subjected to hydroxide-induced dissociation and reassociation. The enzyme mixture was separated into three peaks (A). Electrophoretic analysis of the fractions (B) reveals that the flow-through and the first peak (fractions 3−9) only contain subunits of the D28A variant with lower effective molecular weight due to the absence of the $(His)_6$ tag in these constructs. The second major peak (fractions 16−25) contains equal amounts of the D28A variant and WT YPDC subunits. The third peak (fractions 30−39) only contains subunits of WT YPDC. Samples for SDS−PAGE are as follows: B, sample prior to dissociation; L, sample after reassociation; FT, flow-through; R, reference sample with equal concentrations of HT and non-HT enzymes.

Importantly, the activity of the hybrid consisting of the D28N and E477Q variants was the same as that created by the D28A and E477Q variants, even though the specific activity of the D28N variant is at least twice as high as that of the D28A variant at 20 mM pyruvate (*12*). This finding supports the idea that restored activity originates from the active site of the WT phenotype and not from a doubly substituted one.

Interestingly, the activity of the WT phenotype active sites in the hybrid enzyme is approximately one-third of the activity of the WT homotetramer. Under the conditions employed, the dissociation−reassociation protocol results in full recovery of the activity of the WT enzyme. This suggests that the activity of the WT active site is significantly lower in the hybrid on account of the interactions of the active sites. According to the recently developed alternating sites mechanism for YPDC (*4*), two active sites in the functional dimer act in an antiphase manner during the reaction, with each active site eventually completing the full catalytic cycle; however, at any instant, the predecarboxylation phase of the reaction is taking place in one monomer and the postdecarboxylation phase in the other. Also, decarboxylation in one of the active sites is believed to promote product release in the second active site. In the hybrid tetramers, one active site of the functional dimer has substitutions at both D28 and E477. Both amino acids are important in the postdecarboxylation phase of the reaction (*12*), and D28 is also believed to participate in the decarboxylation step (*4*). This would predict that loss of catalysis in one active site should inhibit catalysis in the second site of the functional dimer, consistent with our observations. As a positive control for such experiments, one could utilize experiments with aspartate transcarbamoylase, which led to the conclusion that interaction between active sites is unimportant in the steady-state catalysis of that enzyme.

An alternative model proposed for YPDC predicts that a concerted mode of action of two subunits from neighboring functional dimers is responsible for the positive cooperativity of the enzyme (*5*). However, this model would predict 100% activity of the WT phenotype active sites in the tetramer created upon hybridization along plane A, not consistent with our results.

In addition to the active center complementation studies, we have also developed a method for the preparative separation of the hybrid enzyme created from two singly substituted enzyme variants, in which enzyme forms with non-HT phenotype pass through the column under the conditions described, due to loose binding. Hybrid enzymes interact with the column more tightly, and enzyme species with 4 HT per tetramer are the last ones to be eluted. The peaks corresponding to different enzyme species are well separated and therefore are not expected to lead to any significant cross-contamination. The separation described can be repeated for any enzyme form to further eliminate undesired oligomeric species.

Recently, a protocol to dissociate YPDC by urea was reported (*10*), apparently leading to cleavage of the tetramer along plane B into two functional dimers. It was also reported that $K_m$ for pyruvate is ca. 10-fold higher for urea-induced dimers than for the original tetrameric WT YPDC, whereas $V_{max}$ does not change upon tetramer dissociation. As expected from that report, treatment with urea resulted in active dimers that failed to give an increase (or decrease) in activity when we mixed the dimers of the two variants so created. This supports the notion that the active sites are untouched in the urea-induced dissociation. These data also predict that interactions between functional dimers are less important than those between subunits of the dimer. However, the reported difference in kinetic properties between those of the original tetramers and those of the urea-induced dimers suggests that interdimer interactions have an effect at least on the stability of the enzyme−substrate complex.

The methodology here reported is important for further studies of the interaction among subunits of the YPDC tetramer. The ability to dissociate and reassociate the tetramer along two planes, and to separate hybrid enzymes from parental ones on a preparative scale (Figure 4), enables the creation and study of hybrid tetramers with any combination of regulatory and catalytic sites. As an example, one may be able to create a tetrameric enzyme with only one subunit with a WT-phenotype active site and a C221A or C221S regulatory site. A second subunit would have a defective active site and a WT-phenotype regulatory site. The two remaining subunits would be deficient in both active and regulatory sites. Depending on the respective orientation of the subunits with intact active or regulatory sites, one could learn about the significance of intersubunit interactions in the very complex activation mechanism of YPDC (*8*).

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