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Characterization of the stabilizing effect of point mutations of pyruvate oxidase from *Lactobacillus plantarum*: Protection of the native state by modulating coenzyme binding and subunit interaction*

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Abstract

Point mutations in the gene of pyruvate oxidase from *Lactobacillus plantarum*, with proline residue 178 changed to serine, serine 188 to asparagine, and alanine 458 to valine, as well as a combination of the three single point mutations, lead to a significant functional stabilization of the protein. The enzyme is a tetrameric flavoprotein with tightly bound cofactors, FAD, TPP, and divalent metal ions. Thus, stabilization may be achieved either at the level of tertiary or quaternary interactions,or by enhanced cofactor binding. In order to discriminate between these alternatives, unfolding, dissociation, and cofactor binding of the mutant proteins were analyzed. The point mutations do not affect the secondary and tertiary structure, as determined by circular dichroism and protein fluorescence. Similarly, the amino acid substitutions neither modulate the enzymatic properties of the mutant proteins nor do they stabilize the structural stability of the apoenzymes. This holds true for both the local and the global structure with unfolding transitions around 2.5 M and 5 M urea, respectively. On the other hand, deactivation of the holoenzyme (by urea or temperature) is significantly decreased.

The most important stabilizing effect is caused by the Ala–Val exchange in the C-terminal domain of the molecule. Its contribution is close to the value observed for the triple mutant, which exhibits maximum stability, with a shift in the thermal transition of ca. 10 °C. The effects of the point mutations on FAD binding and subunit association are interconnected. Because FAD binding is linked to oligomerization, the stability of the mutant apoenzyme–FAD complexes is increased. Accordingly, mutants with maximum apparent FAD binding exhibit maximum stability. Analysis of the quaternary structure of the mutant enzymes in the absence and in the presence of coenzymes gives clear evidence that both improved ligand binding and subunit interactions contribute to the observed thermal stabilization.

Keywords: coenzyme binding; point mutations; pyruvate oxidase; quaternary structure; thermal stability

Proteins show only marginal free energies of stabilization. As has been shown by the comparison of homologous proteins from mesophilic and thermophilic organisms, enhanced stability requires only minute local changes in the spatial structure. Therefore, general strategies of stabilization cannot be established, especially because homologous proteins of different origin differ in numerous

* This paper is dedicated to Professor Robert L. Baldwin on the occasion of his 65th birthday.

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Abbreviations: CDTA, trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; FAD, flavin adenine dinucleotide; TPP, thiamine pyrophosphate; SE-HPLC, size-exclusion high-performance liquid chromatography.

amino acid positions (Jaenicke, 1991a). Because the correlation of structure and stability of proteins is still not at a stage where predictions can be made, the only way to come closer to an understanding of specific mechanisms of stabilization is to use point mutations differing in stability. In this connection, the monomeric phage T4 lysozyme has been investigated in great detail by crystallography, thermal analysis, and homology modeling (Matthews, 1987, 1991; Alber, 1989). In the case of oligomeric proteins, subunit interactions contribute to stability. This has clearly been shown for lactate dehydrogenase (Opitz et al., 1987; Jaenicke, 1991b). Apart from alterations at the protein level, extrinsic factors such as ions or cofactors may serve to enhance protein stability (Jaenicke, 1991b).

In this study, mutants of pyruvate oxidase from *Lactobacillus plantarum*, a tetrameric flavoprotein with tightly bound cofactors FAD, TPP, and a divalent metal ion, were used to answer the question of how both quaternary structure and ligand binding are affected by stabilizing point mutations. In the companion paper (Risse et al., 1992), the stability of the wild-type enzyme was characterized in detail. As has been demonstrated by the enhanced stability at high protein and FAD concentrations, quaternary contacts play a significant role in the stabilization of the enzyme. Because no structural data for the enzyme are presently available, there is no rational basis for site-directed mutagenesis rendering increased (thermal) stability. Therefore, random mutagenesis was used to select stable variants containing single point mutations under conditions where the wild-type enzyme is inactivated (Schumacher et al., 1991).

In the present study, three mutants of pyruvate oxidase containing single point mutations, as well as a fourth mutant that combines all three amino acid substitutions, were analyzed and compared with the wild-type enzyme. The objective of this study was to determine the influence of single or multiple amino acid substitutions on (1) the enzymatic properties of pyruvate oxidase, (2) the stability of the holoenzyme, (3) cofactor binding, and (4) the stability and quaternary structure of the apoenzyme. As a result, a mechanism for the stabilizing effect of single point mutations in the polypeptide chain of pyruvate oxidase is suggested that involves increased cofactor binding, as well as enhanced subunit interactions in the native tetrameric quaternary structure.

Results and discussion

Stabilizing point mutations are localized in different sequence positions along the amino acid sequence of pyruvate oxidase

Figure 1 illustrates the amino acid sequence of pyruvate oxidase from *L. plantarum*, as translated from the cDNA sequence, highlighting the three amino acid exchanges

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  1  MVMKQTKQTN ILAGAAVIKV LEAWGVDHLY GIPGGSINSI MDALSAERDR
 51  IHYIQVRHEE VGAMAAAADA KLTGKIGVCF GSAGPGGTHL MNGLYDARED
101  HVPVLALIGQ FGTTGMNMDT FQEMNENPIY ADVADYNVTA VNAATLPHVI
151  DEAIRRAYAH QGVAVVQIPV DLPWQQIPAE DWYASANSYQ TPLLPEPDVQ
201  AVTRLTQTLL AAERPLIYYG IGARKAGKEL EQLSKTLKIP LMSTYPAKGI
251  VADRYPAYLG SANRVAQKPA NEALAQADVV LFVGNNYPFA EVSKAFKNTR
301  YFLQIDIDPA KLGKRHKTDI AVLADAQKTL AAILAQVSER ESTPWWQANL
351  ANVKNWRAYL ASLEDKQEGP LQAYQVLRAV NKIAEPDAIY SIDVGDINLN
401  ANRHLKLTPS NRHITSNLFA TMGVGIPGAI AAKLNYPERQ VFNLAGDGGA
451  SMTMQDLATQ VQYHLPVINV VFTNCQYGFI KDEQEDTNQN DFIGVEFNDI
501  DFSKIADGVH MQAFRVNKIE QLPDVFEQAK AIAQHEPVLI DAVITGDRPL
551  PAEKLRLDSA MSSAADIEAF KQRYEAQDLQ PLSTYLKQFG LDDQHQIGQ
601  GGF
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Fig. 1. Protein sequence of pyruvate oxidase from *Lactobacillus plantarum*. Substitution of Pro (178) → Ser (mutant 1), Ser (188) → Asn (mutant 2), and Ala (458) → Val (mutant 3) refer to the stabilized mutant pyruvate oxidases 201, 202, and 203, respectively. In mutant 2006 all three animo acid substitutions are combined. From sequence homology analysis (Hawkins et al., 1989) the prospective TPP binding site resides in the underlined amino acid sequence, 436–482.

obtained by random mutagenesis: Pro 178 → Ser (201), Ser 188 → Asn (202), and Ala 458 → Val (203), as well as the triple mutant (2006) combining the three point mutations. All four mutant proteins exhibit significantly enhanced stability, as monitored by deactivation kinetics under solvent conditions where the wild-type enzyme is fully inactive (cf. Schumacher et al., 1991). The enzyme from the triple mutant shows maximum stability compared to the wild-type enzyme and the three single-mutant proteins.

As taken from the sequence homology of pyruvate oxidase to conserved TPP binding sites of other TPP-dependent enzymes (Hawkins et al., 1989), the Ala → Val substitution at position 458 (mutant 203) may be assumed to be localized in the putative TPP binding site (Fig. 1). In the case of the amino acid substitutions at positions 178 (Pro → Ser; mutant 201) and 188 (Ser → Asn; mutant 202), sequence alignment does not yield any unambiguous correlation with potential coenzyme binding sites.

The structural and enzymatic properties of mutant pyruvate oxidases are unchanged

Increased stability of proteins by molecular adaptation or mutagenesis has been shown to be accompanied by reduced flexibility in the polypeptide backbone (Jaenicke, 1991a). In the case of enzymes, this may significantly interfere with the catalytic efficiency (cf. Wrba et al., 1990a,b). Therefore, the effects of the amino acid substitutions on the enzymatic activity of pyruvate oxidase were studied. The specific activities of the enzyme from wild type and from the four mutants show no significant dif-

Table 1. *Enzymatic properties of wild-type and mutant pyruvate oxidases*[a]

	Wild type	201	202	203	2006
Specific activity (U/mg)	5.1	5.1	5.4	5.5	5.4
Michaelis constant, K_m (mM), for					
Phosphate[a]	2.3	2.4	1.7	1.7	1.9
Pyruvate[b]	0.40	0.35	0.40	0.30	0.35

[a] In 0.05 M potassium phosphate (imidazole) buffer, pH 6, 20 °C. See Materials and methods.
[b] Data from H. Möllering, Boehringer Mannheim GmbH (pers. comm.).

ferences (Table 1). Similarly, the Michaelis constants (K_m) for both pyruvate and phosphate do not differ from those of the wild-type enzyme. Evidently, neither the single nor the cumulative amino acid substitutions exert a significant effect on the catalytic properties of pyruvate oxidase.

The single or multiple point mutations do not affect the secondary structure of the holoenzyme, as determined by far-UV circular dichroism (Fig. 2A). Similarly, there is no effect on the intrinsic protein fluorescence of the apoenzymes (Fig. 2B). Because the latter spectroscopic technique is extremely sensitive with respect to local structural differences in the vicinity of tyrosine and tryptophan residues, i.e., in tertiary interactions, the structural properties of the mutant enzymes must be identical with the parental wild-type protein.

Amino acid substitutions do not stabilize tertiary structure

In the companion paper it was shown that the tertiary structures of apo- and holo-pyruvate oxidase are indistinguishable (Risse et al., 1992). Therefore, the apoenzyme may be used to determine the effect of the amino acid exchanges on the stability of the tertiary interactions. Figure 3 illustrates the urea-induced denaturation transitions of wild-type and mutant pyruvate oxidase, as monitored by intrinsic protein fluorescence. Surprisingly, the thermal stabilities of all four mutant apoenzymes are closely similar. This means that the enhanced stability of the tetrameric holoenzyme is not caused by the stabilization of the tertiary structure. Instead, the various apoenzymes exhibit equal, or even decreased, stability compared to the wild-type apoenzyme. The first (local) unfolding transition, indicative for a structured intermediate, is characterized by a decrease in protein fluorescence (Risse et al., 1992). The wild-type protein and mutant proteins 201 and 202 do not exhibit significant differences in the formation of this intermediate (Fig. 3A). However, the enzyme from mutant 203 is found to be destabilized, with aggregation accompanying the unfolding transition (data not shown). In the case of the triple mutant (2006), the amino acid exchanges in the N-terminal domain of the molecule partially compensate for this destabilizing effect in the first transition, leading to only marginal destabilization compared to the wild-type enzyme. On the other hand, the (global) unfolding transition reflected by the change in the wavelength of maximum protein fluorescence does not show any detectable differences (Fig. 3B). The con-

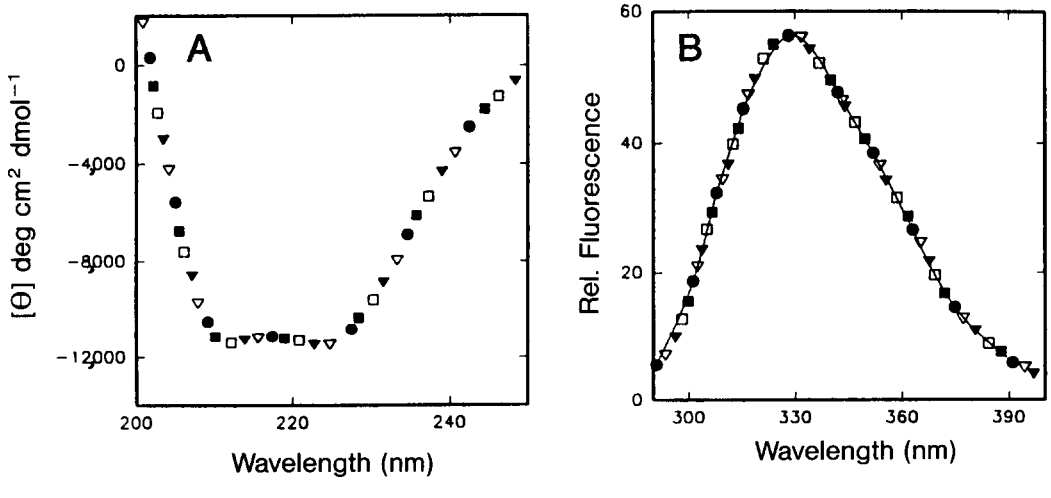

Fig. 2. Characterization of wild-type and mutant pyruvate oxidase. **A:** Far-UV circular dichroism of wild-type and mutant pyruvate oxidase holoenzymes in 0.05 M potassium phosphate, pH 6, containing 0.1 mM Mn^{2+}/TPP. Enzyme concentration 0.2 mg/mL; pathlength 0.1 cm. **B:** Intrinsic protein fluorescence (λ_{ex} = 280 nm) of wild-type and mutant pyruvate oxidase apoenzymes in 0.2 M potassium phosphate, pH 6, containing 20% (v/v) glycerol. Enzyme concentration 0.02 mg/mL. Wild type (○); mutant proteins 201 (▽), 202 (□), 203 (▼), and 2006 (■).

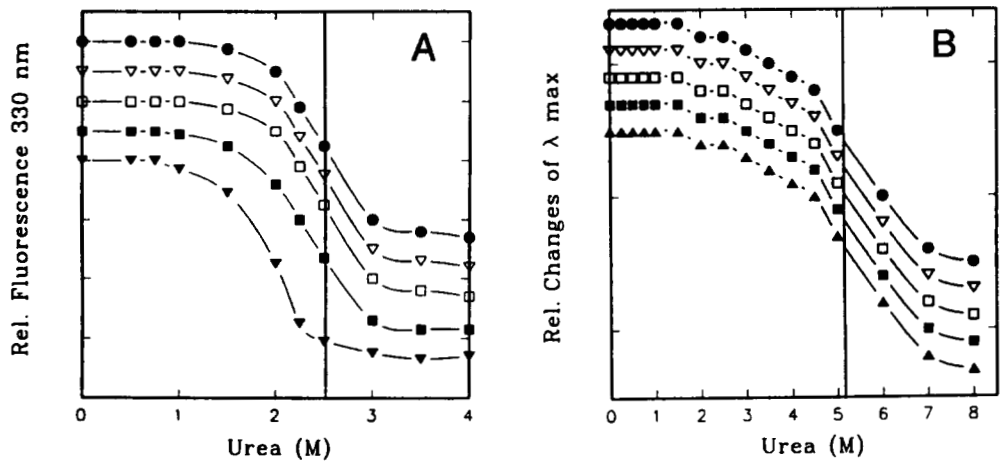

Fig. 3. Urea denaturation of wild-type and mutant apo-pyruvate oxidases. Urea denaturation was monitored by intrinsic protein fluorescence. Measurements were performed after 24 h incubation. In order to avoid confusion, the denaturation transitions were projected on top of each other in equal distances: wild type (●); mutant proteins 201 (▽), 202 (□), 203 (▼), and 2006 (■). **A:** Changes in fluorescence emission intensity at 330 nm (λ_{ex} = 280 nm). **B:** Shifts in fluorescence maximum (λ_{max}) at λ_{ex} = 280 nm.

clusion that may be drawn from these findings is that the point mutations under consideration do not contribute significantly to the overall stability of the tertiary structure of the enzyme. Thus, the question arises whether cofactor binding and/or enhanced subunit interactions are responsible for the stabilizing effect of the amino acid substitutions.

Mutations influence deactivation and release of the coenzymes

Data describing the long-term stability of wild-type and mutant apo- and holo-pyruvate oxidase are summarized in Table 2. Under all denaturation conditions applied, i.e., temperature, urea, and alkaline pH, the mutant enzymes show enhanced stability. The relative order in the case of thermal deactivation is wild type < 202 < 203 < 201 < 2006; the one following from deactivation by urea and alkaline pH is wild type < 202 < 201 < 203 < 2006. The results clearly show that the combination of the single point mutations results in increased stability of the enzyme compared to wild-type pyruvate oxidase or the three single substitution enzymes. However, there is no strict additivity of the stabilizing effects of the single point mutations.

A detailed comparison of the urea-induced deactivation of wild-type and mutant pyruvate oxidases, as well as the dissociation into subunits and free cofactors, is presented in Figure 4. As pointed out in the companion paper (Risse et al., 1992), both FAD release and dissociation of the holoenzyme into its subunits coincide. However, deactivation precedes FAD release and the concomitant dissociation of the tetramers (Risse et al., 1992). The slight difference in the deactivation and dissociation profiles was explained by the recombination of FAD with apoenzyme in the course of the urea-induced dissociation equilibrium, leading to inactive, tetrameric binary FAD complexes (Risse et al., 1992). As a consequence, the deactivation transition represents the release of TPP, whereas the transition monitored by FAD release accounts for the dissociation of the residual binary FAD complexes. Compared to the wild-type holoenzyme, mutants 201 and 202 show increased stability toward urea denaturation. Furthermore, the differences between the transitions of deactivation and FAD release are significantly more pronounced.

In the case of the enzyme from mutants 203 and 2006, both deactivation and FAD release coincide. The deactivation transitions of these holoenzymes parallel the first denaturation transitions of their respective apoenzymes (cf. Fig. 3A). This means that both quaternary structure and FAD binding of these mutant proteins are strongly stabilized so that inactivation necessitates partial unfolding. The relative order of stability for the individual mutant enzymes in the case of the urea-induced FAD release (Fig. 4) resembles that for the thermal deactivation transitions (Table 2).

Comparing the enzymes from mutants 201 and 202 with those from mutants 203 and 2006 reveals that the Ala → Val substitution in the prospective TPP binding site seems to exert a major stabilizing effect on the pyruvate oxidase holoenzyme complex, which may be attributed to increased TPP binding.

Stabilization by point mutations can only be observed in the presence of coenzymes

In contrast to the result obtained for the wild-type enzyme, mutant pyruvate oxidase holoenzymes show closely

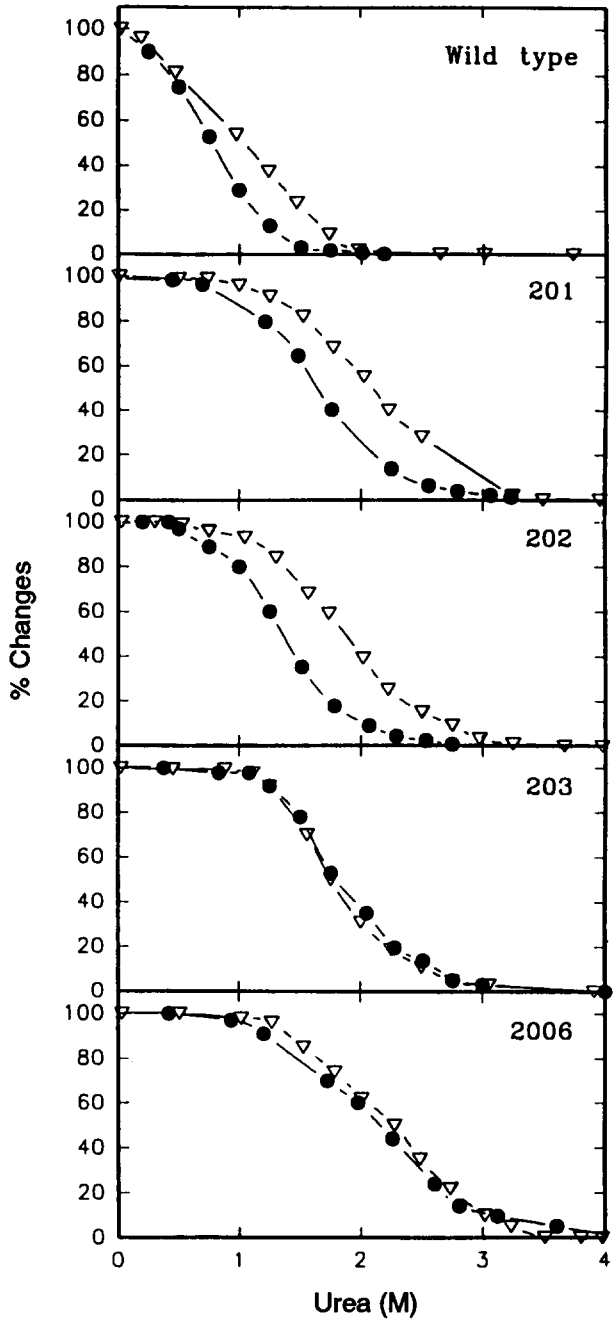

Fig. 4. Urea-induced deactivation and FAD release of wild-type and mutant holo-pyruvate oxidases. Urea denaturation of holoenzyme as monitored by activity (●) and FAD release (λ_{ex} = 450 nm/λ_{em} = 527 nm) (▽).

similar midpoints of thermal deactivation (Table 2) and aggregation profiles (Table 3). Accordingly, thermal denaturation (which is superimposed by aggregation) as well as TPP and FAD release must be correlated. As has been mentioned, this does not hold for the urea-induced unfolding transitions.

The apoenzymes of mutant pyruvate oxidases are found to be destabilized with respect to heat denaturation

Table 2. *Stability of wild-type and mutant pyruvate oxidases as determined by activity measurements*[a]

	Wild type	201	202	203	2006
Midpoint of deactivation by					
Temperature (°C)	42.0	50.0	48.5	49.0	51.5
pH	6.70	7.30	7.15	7.55	7.85
Urea (M)	0.75	1.65	1.35	1.85	2.15

[a] In 0.2 M potassium phosphate buffer, pH 6.0, 20% glycerol (temperature, urea) or 0.1 M potassium phosphate buffer (pH). See Materials and methods.

compared to the wild-type apoenzyme (Table 3). Regarding their midpoints of thermal denaturation, the apoenzymes belong to two groups: the enzymes from wild type and from mutants 201 and 202, with an aggregation transition at ca. 48 °C, and those from the less stable mutants 203 and 2006 with a transition midpoint at 46 °C.

Binding of TPP does not cause further stabilization of wild-type, 201, and 202 apoenzymes (Table 3). The thermal aggregation transitions of the binary apoenzyme–TPP complexes of mutants 203 and 2006 reveal enhanced stability compared to the respective coenzyme-free apoenzymes. In the case of the enzyme from mutant 203, bound TPP increases the stability, yielding a midpoint of denaturation (aggregation) characteristic for the wild-type apoenzyme. Similarly, the binary TPP complex of mutant enzyme 2006 exhibits a denaturation midpoint equal to that of the apoenzymes from mutants 201 or 202. The results show that TPP binding to apoenzyme alone does not contribute significantly to the stability of the mutant proteins.

As expected, FAD binding stabilizes all four mutant enzymes. The stabilizing effects observed in the case of mutants 203 and 2006 exceed those observed for mutants 201 and 202 (Table 3). Obviously, the Ala → Val substitution in the prospective TPP binding site affects FAD binding even in the absence of TPP. However, in this context, one

Table 3. *Thermostability of wild-type and mutant pyruvate oxidases as determined by thermal aggregation*[a]

	T_m (°C)				
State of the enzyme	Wild type	201	202	203	2006
Holoenzyme[b]	48.5	50.0	49.0	49.0	51.0
Apoenzyme	48.5	47.5	47.5	46.0	46.0
Binary TPP complex[b]	48.5	47.5	47.5	48.5	47.5
Binary FAD complex[c]	50.0	53.5	52.0	56.0	57.0

[a] In 0.2 M potassium phosphate buffer, pH 6.0, 20% glycerol. See Materials and methods; limit of error ±0.5%.
[b] Monitored in the presence of 1 mM Mn^{2+}/TPP.
[c] Monitored in the presence of 1 mM Mn^{2+}/0.1 mM FAD.

has to keep in mind that commonly thermal denaturation is an irreversible process. Therefore, kinetic effects, e.g., dissociation of cofactors, may also influence the midpoints of thermal aggregation. In order to discriminate between these alternatives, binding of FAD to the apoenzyme was determined independently.

Mutant enzymes show enhanced binding constants for FAD

FAD binding studies in the presence of 0.1 mM Mn^{2+} revealed that the apparent FAD binding constants of the pyruvate oxidase–FAD complex for all four mutants are increased relative to the wild-type apoenzyme (Fig. 5A). Under the given conditions, maximum apparent FAD binding constants are observed for the mutant enzymes 203 and 2006. In this context, one has to consider that FAD binding in the absence of TPP is linked to subunit association (Risse et al., 1992). However, at the given apoenzyme concentration (1 μM), the monomer predominates (see below).

In the absence of divalent cations (e.g., in the presence of 10 mM CDTA), wild-type pyruvate oxidase has been shown to exhibit decreased FAD binding with cooperative saturation characteristics depending on protein concentration (Risse et al., 1992). In the case of mutants 203 and 2006, in the absence of divalent cations, FAD binding to pyruvate oxidase is blocked. On the other hand, mutant enzymes 201 and 202 show increased FAD binding, exceeding the affinity of the wild-type apoenzyme (Fig. 5B). Removal of Mn^{2+} in the case of mutants 203 and 2006 has no influence on the structural stability of the apoenzymes. Therefore, removal of the metal ion must prevent FAD binding by imposing steric constraints.

The data presented above clearly show that differences in the stabilities of various pyruvate oxidase mutants are correlated with cofactor binding. However, up to this point it remains unresolved whether improved coenzyme binding shifts the association equilibrium, or whether stabilization of the tetrameric quaternary structure increases the affinities for FAD. Furthermore, a combination of both mechanisms may be involved. Experiments focusing on the state of association of the mutant apoenzymes may help clarify the stabilization mechanism.

Amino acid substitutions affect quaternary structure

Wild-type apoenzyme has been shown to undergo concentration-dependent association/dissociation according to

$$4M \rightleftharpoons 2D \rightleftharpoons T$$

(Risse et al., 1992). The corresponding SE-HPLC analysis of the various mutants of apo-pyruvate oxidase is summarized in Figure 6. The determination of the quaternary structure reveals an increased association tendency for the two mutant proteins (201 and 202), as compared to the parental wild-type protein (Fig. 6A). The elution profiles show significantly higher amounts of dimers and tetramers if the concentrations of the apoenzymes are raised beyond 0.2 mg/mL. The maximum amount of tetramers is observed for the apoenzyme of mutant 201. In the case of mutants 203 and 2006, the elution profiles resemble those for the wild-type enzyme (data not shown). In per-

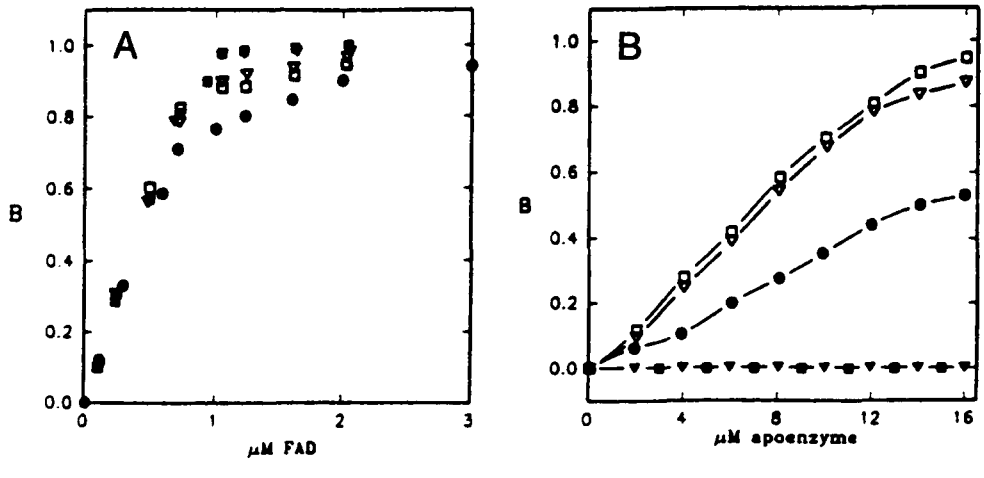

Fig. 5. Binding of FAD to the apoenzymes of wild-type and mutant pyruvate oxidase. **A:** FAD binding to 1 μM apoenzyme in the presence of 0.1 mM Mn^{2+}. Separate samples were prepared for each FAD concentration. After 24 h incubation, FAD binding was determined by fluorescence (λ_{ex} = 450 nm; λ_{em} = 527 nm). The ordinate (B) stands for the ratio of bound FAD per total apoenzyme concentration. **B:** FAD binding to apoenzyme in the presence of 10 mM CDTA, as determined by fluorescence quenching of 10 μM FAD (λ_{ex} = 450 nm/λ_{em} = 527 nm). The ordinate (B) stands for the ratio of bound FAD per total FAD concentration. Wild type (●); mutant proteins 201 (□), 202 (▽), 203 (▼), and 2006 (■).

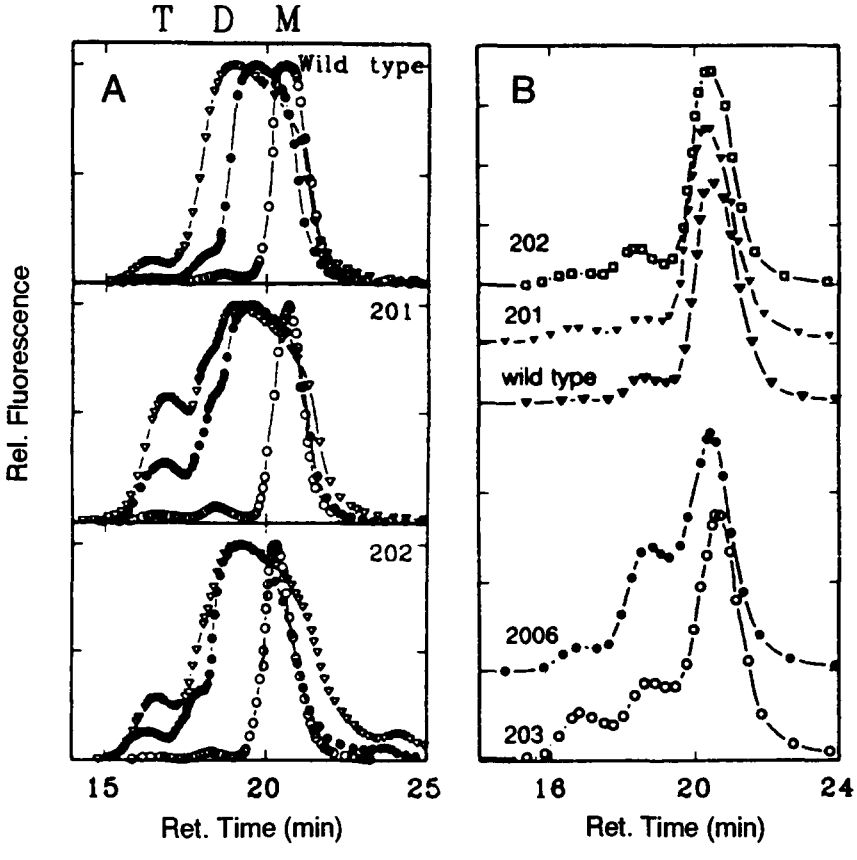

Fig. 6. State of association of apo-pyruvate oxidases. **A:** SE-HPLC of apoenzyme at varying protein concentration. Wild type: 0.02, 0.75, and 1.5 mg/mL; 201: 0.01, 0.75, and 1.5 mg/mL; 202: 0.01, 0.75, and 1.5 mg/mL. In each frame the same symbols are used for the apoenzyme at lowest (○), middle (●), and highest (▽) protein concentrations. Flow rate 0.05 mL/min at 20 °C. Peak broadening with increasing protein concentrations is due to dissociation/association equilibrium of the dimers during chromatography. T, D, and M refer to the tetrameric, dimeric, and monomeric apoenzymes, respectively. **B:** SE-HPLC of the binary TPP complex without Mn^{2+}/TPP present in the elution buffer. Enzyme concentration: 0.066 mg/mL; 0.1 M potassium phosphate, pH 6, containing 5% glycerol. The elution profiles were projected on top of each other: 203 (○), 2006 (●), wild type (▽), 201 (▼), and 202 (□).

forming this kind of (nonequilibrium) analysis, one has to consider that the apparent change in the state of association may be caused either by an increase in the subunit association constants or by altered dissociation/association kinetics. Furthermore, the solutions are diluted during the analysis by a factor of >10, which may also affect the quaternary structure.

Figure 6B illustrates the SE-HPLC analysis of the binary TPP complexes of the wild-type and the mutant enzymes. The binary TPP complex exhibits an association equilibrium between dimers and tetramers. At the given protein concentration, the tetramer predominates in the case of the wild-type protein (Risse et al., 1992). Without Mn^{2+}/TPP present in the elution buffer, the binary enzyme-TPP complexes of wild-type enzyme and mutants 201 and 202 dissociate during chromatography. This results in a distribution with monomers as the predominant species. In the case of mutants 203 and 2006, the amount of dimers is increased (Fig. 6B). These results indicate that the stabilizing effect of the Ala 458 → Val substitution is caused by a decrease in the dissociation rate of the binary TPP complex.

Conclusions

As pointed out in the companion paper, the stability of wild-type pyruvate oxidase from *L. plantarum* depends on both protein and FAD concentration (Risse et al., 1992). As a consequence, stabilization of pyruvate oxidase may not only be achieved by stabilizing tertiary interactions but also by enhancing subunit association or coenzyme binding. In the present case, only preliminary structural data for the enzyme are available (Y. Muller & G.E. Schulz, pers. comm.). Therefore, the method of plasmid random mutagenesis was used to obtain pyruvate oxidase derivatives characterized by a significant increase in functional stability (Schumacher et al., 1988, 1991).

The characterization of a number of mutant pyruvate oxidases shows that the enhanced stability of three amino acid substitutions, Pro (178) → Ser, Ser (188) → Asn, and Ala (458) → Val, and their combination in a triple mutant, resides in the stabilization of the active tetrameric quaternary structure of the enzyme. All four mutant proteins are unchanged in their enzymatic properties, with increased stabilities reflected by shifts in the thermal or chemical deactivation transitions of the holoenzymes.

The stabilization mechanism for the mutant enzymes can be outlined as follows None of the given amino acid substitutions enhances the stability of the tertiary structure of the dissociated apoenzyme. In the case of the enzyme from mutant 203, comparison with the sequence data for other TPP-dependent enzymes (Hawkins et al., 1989) suggests that the Ala → Val substitution at position 458 results in altered TPP binding. As taken from the SE-

HPLC analysis for this mutant enzyme, the dissociation rate of the binary apoenzyme–TPP complex is reduced. In the presence of Mn^{2+}, FAD binding is increased, whereas in the absence of the metal ion, the order of FAD binding affinities is reversed. Because the mutation seems to affect neither the equilibrium nor the kinetics of subunit dissociation of the apoenzyme, the altered stability cannot be explained by a direct effect of the amino acid substitution on the subunit interactions. Rather, the native quaternary structure must be stabilized indirectly by improved cofactor binding. In contrast to mutant 203, the apoenzymes of mutants 201 and 202 exhibit enhanced subunit association. Both mutations have no effect on TPP binding. The apparent increases in FAD binding may be attributed to improved subunit interactions. However, a direct effect of the amino acid substitutions on FAD binding cannot be ruled out at this point. In contrast to other examples in the literature, the stabilizing effects of the single point mutations do not show strict additivity: the enzyme from triple mutant 2006 closely resembles mutant 203 regarding its subunit interactions and the state of association of the apoenzyme. Obviously, the stabilizing effect of the Ala 458 → Val substitution dominates over the increments gained by the Pro 178 → Ser and Ser 188 → Asn point mutations.

Materials and methods

Chemicals

Ultrapure urea was purchased from Schwarz/Mann Biotech (Cleveland, Ohio), FAD from Boehringer Mannheim (Penzberg, Germany), and TPP from Sigma (St. Louis, Missouri). All other reagents were analytical grade substances from Boehringer Mannheim or from Merck (Darmstadt, Germany). Quartz-bidistilled water was used throughout.

Generation of pyruvate oxidase with enhanced stability

Pyruvate oxidase from *L. plantarum* was cloned and expressed in *Escherichia coli*. Under suitable fermentation conditions, the enzyme is obtained in native, fully active form from transformed *E. coli* cells (Schumacher et al., 1991). Plasmid pBP200 (European patent application EP-A0365836), coding for *L. plantarum* pyruvate oxidase, was subjected to in vivo mutagenesis as described by Schumacher et al. (1988). After isolation of plasmid DNA and transformation into *E. coli*, colonies were grown, replica-plated onto nitrocellulose filters, and assayed for pyruvate oxidase activity under conditions (pH 8.0, 0.2 M NaCl) where the wild-type enzyme is deactivated. Positive colonies were isolated, and the mutants were localized and characterized by DNA sequencing. The effect of single point mutations was verified by reintroduction of the mutation in the wild-type gene and subsequent analysis of stability. Using this approach, several point mutations with significantly increased stability were identified (Fig. 1) (Schumacher et al., 1991). Furthermore, starting from a given point mutation in the pyruvate oxidase gene, a second round of mutagenesis/screening was performed. Increased stability was measured and described in EP-A0365836. Both wild-type and mutant pyruvate oxidases were purified to homogeneity using conventional chromatographic procedures.

Preparation of the apoenzyme was performed according to Strittmatter (1961) as modified by Risse et al. (1992).

Determination of protein concentration and of enzyme activity

Protein concentration and enzymatic activity of pyruvate oxidase were determined as described by Risse et al. (1992).

Determination of the Michaelis constant (K_m) for phosphate

The activity of a 1.2-μg/mL solution of holo-pyruvate oxidase at varying phosphate concentrations (0–100 mM) was measured in imidazole buffer at 25 °C. The holoenzymes were dialyzed against (2 × 500 mL) 50 mM imidazole, pH 6, 20% (v/v) glycerol, plus 0.5 mM Mn^{2+}/TPP. Quantitative evaluation made use of Lineweaver–Burk plots.

Size-exclusion chromatography (SE-HPLC)

SE-HPLC was performed using a Pharmacia Ultrapac TSK 3000 column (7.5 mm × 300 mm) with a precolumn of 7.5 mm × 100 mm. All separations were carried out isocratically at 20 °C in 0.1 M potassium phosphate, pH 6.0, plus 5% (v/v) glycerol at a flow rate of 0.5 mL/min. The column was calibrated with α_2-macroglobulin (M_r = 725,000), β-galactosidase (M_r = 465,000), IgG (M_r = 150,000), Fab (M_r = 50,000), and myoglobin (M_r = 17,800). The apoenzymes as well as the binary TPP complexes of wild-type pyruvate oxidase and of all mutant proteins were analyzed at varying protein concentrations in the range of 0.1–1.5 mg/mL.

Urea-induced deactivation and release of cofactors

In the case of the apoenzymes, urea denaturation was monitored by intrinsic protein fluorescence at 330 nm (λ_{ex} = 280 nm). In the case of the holoenzymes, denaturation is accompanied by the release of FAD, which can be followed by fluorescence emission at 527 nm (λ_{ex} = 450 nm). Deactivation was determined at a final protein concentration of 30 μg/mL. In addition, dissociation was analyzed by SE-HPLC. Deactivation, dissociation, and

denaturation in 0.02 M potassium phosphate, 20% (v/v) glycerol, pH 6 (for the holoenzyme, plus 0.1 mM Mn^{2+}/TPP), were performed by dilution into buffers containing increasing concentrations of urea. To reach equilibrium, final values were measured after 24 h.

Deactivation at alkaline pH

Holo-pyruvate oxidase was incubated in 0.1 M potassium phosphate containing 0.1 mM Mn^{2+}/TPP at different pH values ranging from pH 6 to pH 9. The residual activity was determined after 24 h incubation.

Thermal deactivation and unfolding

Thermal denaturation was performed at an enzyme concentration of 30 μg/mL in 0.2 M potassium phosphate buffer, 20% (v/v) glycerol, pH 6. Apo-TPP and apo-FAD complexes were prepared by adding 1 mM Mn^{2+}/TPP and 1 mM Mn^{2+}/0.1 mM FAD, respectively. Thermal transitions were monitored by enzyme activity and light scattering at 450 nm. Measurements at 20–70 °C made use of a heating rate of 0.5 °C/min.

Cofactor binding

Cofactor binding was determined either by equilibrium dialysis (TPP) or by measuring fluorescence quenching (FAD) (Risse et al., 1992).

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