The role of residues Arg169 and Arg220 in intersubunit interactions of yeast D-amino acid oxidase

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D-Amino acid oxidase from the yeast *Trigonopsis variabilis* (EC 1.4.3.3, TvDAAO) exists as a dimer consisting of two identical subunits. The dimeric structure of the enzyme is stabilized by 12 (six pairs) hydrogen bonds, the residues Arg169 and Arg220 of each subunit being involved in eight hydrogen bonds. The Arg169Glu and Arg(169,220)Ala mutants of TvDAAO were prepared. Both mutant enzymes were expressed in *E. coli* cells as insoluble but catalytically active inclusion bodies. The introduction of amino acid substitutions at the intersubunit interface resulted in a change in the substrate specificity profile and a strong decrease in thermal stability.

Key words: D-amino acid oxidase, site-directed mutagenesis, quaternary structure, substrate specificity, thermal stability.

D-Amino acid oxidase (EC 1.4.3.3, DAAO) is the FAD-containing enzyme that catalyzes the oxidative deamination of D-amino acids to form α-keto acids, hydrogen peroxide, and an ammonium ion. In spite of the fact that the enzyme plays an essential role in the regulation of a number of very important processes in animal organisms (nervous activity, hormone secretion, *etc.*)[1,2] and is widely used in practice,[3—5] the structure—function relationship of DAAOs from different sources is little known. By 2008, the structures of only three D-amino acid oxidases were solved, *viz.*, those from porcine kidney (pkDAAO),[6,7] human kidney (hDAAO),[8,9] and the yeast *Rhodotorula gracilis* (RgDAAO).[10,11] The enzyme from the yeast *Trigonopsis variabilis* (TvDAAO) is the fourth best characterized DAAO. The latter enzyme was obtained[12] in a highly purified state in 1976 and is most widely used in practice.[3—5] One can assume that, in view of the practical importance of this enzyme, numerous attempts were made in the last 30 years to crystallize TvDAAO. However, no structural data on this enzyme are available in the Protein Data Bank (PDB) and there are no publications concerning this problem, which suggests that all the attempts were unsuccessful. We have also carried out a large-scale screening of the crystallization conditions for wild-type TvDAAO but failed to obtain crystals. Only after obtaining the Cys108Phe mutant of TvDAAO,[13] we succeeded in growing crystals of this enzyme and then solved its structure first at 2.8 Å resolution[14] and then at 1.8 Å resolution.[2]

Porcine kidney D-amino acid oxidase *in vivo* is a monomer, whereas the enzymes from other sources exist in nature as dimers. In the crystal, pkDAAO also exists as a dimer, in which two subunits are linked in a head-to-head fashion.[6,7] For human D-amino acid oxidase, two crystal modifications were grown. In one modification, like in the dimer of pkDAAO, the subunits are arranged in a head-to-head fashion,[9] whereas in another modification the subunits are arranged in a cross-like fashion.[8] The dimers of TvDAAO C108F and RgDAAO are arranged in a head-to-tail fashion. However, unlike the latter enzyme, the subunits of the TvDAAO C108F molecule do not lie in one plane and are shifted with respect to one another. The enzymes also differ in the mode of formation of the dimer interface. In the enzyme RgDAAO, the loops consisting of 14 amino acid residues located in the lower part of the FAD-binding domain play a key role in the formation of the dimer. These loops provide the necessary orientation and the efficient interaction between the positively charged residues Arg305 and Arg314 of this loop from one subunit and the negatively charged residues Asp269, Glu275, and Glu276 of the α-helices I3′ and I3″ from another sub-

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unit.[15] Mutants of the enzyme with deletions in this loop were prepared.[15–17] It appeared that five deletions in this loop are sufficient for RgDAAO to lose the ability to form dimers. The monomeric form of mutant RgDAAO has a much lower thermal stability compared to the wild-type enzyme, and the FAD binding constant of the mutant is 5 times lower.[15,17] These results indicate that the above-mentioned loop plays a key role in the existence of RgDAAO as a dimer *in vivo* and provides strong binding of the FAD molecule in the active site.

An analysis of the structure of the Cys108Phe TvDAAO mutant showed that the dimeric structure of the enzyme is stabilized by six pairs of hydrogen bonds between residues from different subunits, two residues, *viz.*, Arg169 and Arg220, forming two hydrogen bonds each (Fig. 1). The residue Arg169 forms two hydrogen bonds through the NH(1) and NH(2) atoms of the guanidinium group with the carbonyl oxygen atom of the residue Gly250 from the second subunit (the distances are 3.10 and 3.11 Å, respectively). The residue Arg220 forms hydrogen bonds through the NH(E) and NH(2) atoms of the guanidinium group with the carbonyl oxygen atom of the residue Glu32 (the distances are 2.86 and 3.06 Å, respectively) (see Fig. 1, *b*). Therefore, two pairs of the residues Arg169 and Arg220 of each subunit are responsible for the formation of eight of the twelve (67%) hydrogen bonds.

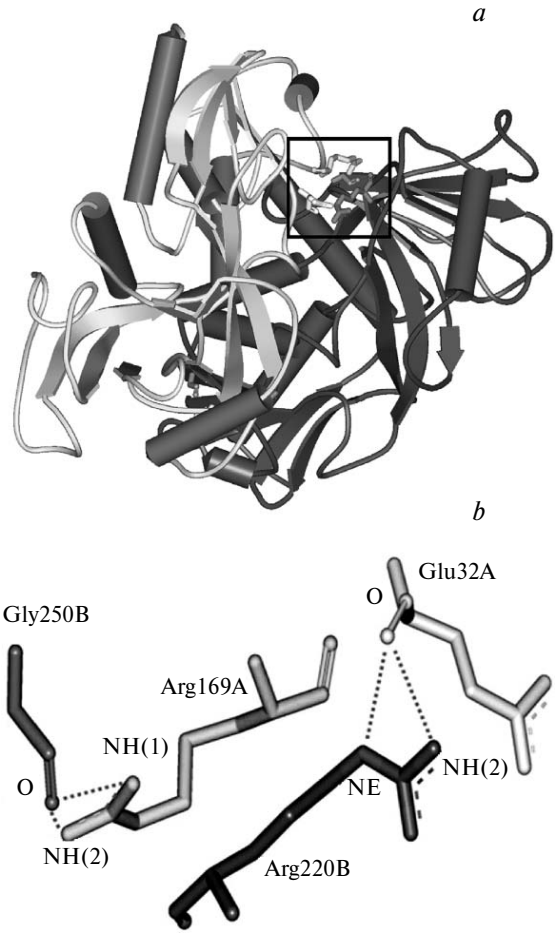

Fig. 1. Hydrogen bonds in the structure of TvDAAO formed by the residues Arg169 and Arg220: *a*, the positions of these residues in the protein globule (enclosed in the black square); *b*, an enlarged fragment.

The aim of the present study was to reveal the role of the residues Arg169 and Arg220 in the structure and stability of TvDAAO, primarily, to investigate the influence of these residues on the oligomeric structure of the enzyme. For this purpose, we prepared two mutants of the enzyme, Arg220Glu TvDAAO and Arg(169,220)Ala TvDAAO. In the former mutant, the substitution of the negatively charged residue Glu for the positively charged residue Arg should lead to the repulsion from the residue Glu32 of the adjacent subunit. The substitution of the residues Ala for two residues Arg enables the elimination of hydrogen bonds.

Experimental

All "Molecular Biology Grade" reagents were used in the gene engineering. Microbiological experiments were performed with the use of the following reagents: Bacto tryptone, the yeast extract and agar (Difco, USA), glycerol (99.9%, ultra pure), potassium hydrogenphosphate and sodium chloride (ultra pure), lysozyme (Fluka/BioChemika, Switzerland), potassium chloride and magnesium chloride (ultra pure, Merck, Switzerland), isopropyl β-D-thiogalactoside (IPTG), kanamycin and chloramphenicol (Sigma, USA), sodium hydrogenphosphate (analytical grade), and glucose (ReaKhim, Russia). Restriction endonucleases, bacteriophage T4 DNA ligase, and Taq DNA polymerase (Fermentas, Lithuania) were used for the DNA fragment cloning and site-directed mutagenesis. Oligonucleotides for the polymerase chain reaction and sequencing were synthesized by Sintol (Russia). The experiments were performed with MilliQ water (Millipore, USA).

Introduction of mutations into the *tvdaao* gene. Single mutations were introduced by the polymerase chain reaction (PCR) according to a procedure described previously.[14] The pDAAO2B plasmid was used as the matrix, in which the *daao* gene was under control of the strong promoter of the bacteriophage T7 RNA polymerase.[18] The mutations were introduced with the use of the forward (DAOFor1) and reverse (DAORev5) primers for the beginning and the end of the gene, respectively (Fig. 2, *a*), as well as of the forward (Pr1) and reverse (Pr2) primers carrying the required substitution in the *tvdaao* gene (Fig. 2, *b*).

The reaction mixture for the PCR reaction contained 2.5 μL of a 10× SE buffer for Taq DNA polymerase (60 *mM* Tris-HCl, pH 8.5, at 25 °C), 1.5 *mM* $MgCl_2$, 25 *mM* KCl, 10 *mM* 2-mercaptoethanol, 0.1% Triton X-100), 2.5 μL of a mixture of dNTP (dATP, dGTP, dTTP, dCTP; the concentration of each component was 2.5 μmol L^{-1}), 1 μL of the DNA matrix (≈10 ng μL^{-1}), 2 μL of each primer (5 nmol mL^{-1}), 1 μL of Taq DNA polymerase (5 U μL^{-1}), and deionized water (added to the total volume of the mixture of 25 μL).

The PCR reaction was carried out in a 0.5 mL thin-wall plastic tube (Eppendorf, Germany). To prevent the evaporation

a

DAOFor1 5′-ATA TAC CAT GGC TAA AAT CGT TGT TAT TGG TGC-3′
DAORev5 5′-GTT TGG ACG AGT AAG AGC TCT TTC GAC-3′,

b

Substitution Arg220Glu

Pr1 5′-GTC GTC CTT GTT **GA**A AAC TCT CTT CCT TTT ATG GCC TC-3′
Pr2 5′-CAT AAA AGG AAG AGA GTT T**TC** AAC AAG GAC GAC TTG TCC TCG-3′

Substitution Arg220Ala

Pr1 5′-GTC GTC CTT GTT **GC**A AAC TCT CTT CCT TTT ATG GCC TC-3′
Pr2 5′-CAT AAA AGG AAG AGA GTT T**GC** AAC AAG GAC GAC TTG TCC TCG-3′

Substitution Arg169Ala

Pr1 5′-ACG GTG GTT AAA **GC**T CGA GTG AAC CAT ATC AAG GAT GC-3′
Pr2 5′-TT CAC TCG A**GC** TTT AAC CAC CGT GGC GCC G-3′

Fig. 2. Forward (DAOFor1) and reverse (DAORev5) primers for the beginning and the end of the *daao* gene, respectively (*a*) and the forward (Pr1) and reverse (Pr2) primers carrying the required substitution in the *tvdaao* gene (*b*). The nucleotide substitutions providing the mutation are in the boldface type.

of the reaction mixture and its condensation on the cap, 30 μL of mineral oil was added to the tube. The tube was heated at 95 °C for 5 min, and then the PCR reaction was carried out using the following program: in the first step, 95 °C, 30 s; in the second step, 56—60 °C, 60 s; in the third step, 72 °C, 2 min; a total of 25—35 cycles. Then the reaction mixture was kept at 72 °C for 10 min. The temperature in the second step was 3—5 °C lower than the melting point of the duplexes (T_m) formed by the primers. The melting points T_m were determined by the empirical formula:

$$T_m = 2(n_A + n_T) + 4(n_G + n_C),$$

where n_X is the number of nucleotides X (X = A, T, C, or G) in the primer.

To obtain fragments containing the required mutation, we carried out two PCR reactions using pairs of the primers DAOFor1/Pr2 (fragment 1) and Pr1/DAORev5 (fragment 2). The PCR products, fragment 1 and fragment 2, were purified using 1% agarose gel. In the third step, PCR was conducted with the primers DAOFor1 and DAORev5 and the previously prepared fragments 1 and 2 used as the DNA matrix. The product of the third PCR reaction was purified analogously to fragments 1 and 2 and treated with Bsp119 I and EcoICR I restriction endonucleases. Then the DNA fragments were purified and ligated with the plasmid pDAAO2, which was treated with the same restriction endonucleases. After the ligation, *E. coli* TG1 cells were transformed with the reaction mixture, seeded into Petri dishes with an agar medium containing kanamycin (30 μg mL^{-1}), and kept for 16 h. For each mutant, six colonies were taken from the dishes, and plasmids were isolated from the colonies. The introduction of the requires mutations was monitored by sequencing the plasmid DNA.

Expression of TvDAAO and its mutants in *E. coli* cells. The expression of TvDAAO and its mutants was carried out in *E. coli* BL21 (DE3) pLysS Codon Plus cells. The producer strain of *E. coli* BL21 (DE3) pLysS Codon Plus cells was transformed with the corresponding plasmid and seeded into Petri dishes with an agar medium containing the required antibiotics. To prepare the inoculate, a single colony was taken from the Petri dish and cultured for 16 h on a 2YT medium (16 g L^{-1} Bacto tryptone, 10 g L^{-1} yeast extract, 1.5 g L^{-1} sodium dihydrogenphosphate, 1 g L^{-1} sodium chloride, 1 g L^{-1} potassium hydrogenphosphate, pH 7.5) in the presence of 30 μg mL^{-1} kanamycin and 25 μg mL^{-1} chloramphenicol. After overnight incubation, the cells were transferred into a fresh medium (the dilution was 1 : 1000) and cultured at 30 °C until the absorbance $A_{600} \approx 0.6$—0.8. The inoculate was placed into flasks for cultivation in an amount of 10% of the total volume of the medium (the modified LB medium containing 10 g L^{-1} yeast extract, 5 g L^{-1} Bacto tryptone, 10 g L^{-1} glucose, 1.5 g L^{-1} sodium dihydrophosphate, and 1 g L^{-1} potassium hydrogenphosphate, pH 7.5) containing 30 μg mL^{-1} kanamycin. The cultivation was carried out in 1 L conical flasks equipped with paddle impellers (the volume of the medium was at most 10—15% of the flask volume). The temperature of cultivation varied in the range of 18—27 °C; the shaker speed was 120—160 rpm. After the achievement of the absorbance $A_{600} \approx 0.7$—0.8, the expression of the enzyme was induced by adding IPTG to the medium. After the induction, the cells were cultured for 24 h and then precipitated on a Beckmann J-21 centrifuge (8000 rpm, a J-20 rotor, 30 min, 4 °C). The precipitate was resuspended in a 0.02 *M* Tris-HCl buffer (pH 7.5, 8.0, or pH 8.5) in a ratio of 1 : 4 (w/w) and subjected to sonication. The suspension was kept at −20 °C.

Determination of activity and kinetic parameters of DAAO. The activity of D-amino acid oxidase was determined with the use of the DAAO—horseradish peroxidase two-enzyme system. D-Methionine and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were used as substrates for these two enzymes, respectively. The activity was determined at 30 °C from the accumulation of the oxidation product of ABTS (the absorbance at 414 nm, $\varepsilon_{414} = 36000$ L mol^{-1} cm^{-1}) using a Shimadzu UV-1601PC spectrophotometer (Germany). A measurement cell of the spectrophotometer (working volume was 1 mL, optical path was 1 cm) was filled with 870 μL of an air-saturated 50 *mM* potassium phosphate buffer (PPB), pH 8.0, 100 μL of a 100 *mM*

D-Met solution in 50 *mM* PPB, 20 μL of an ABTS solution in water (16 mg mL^{-1}), and 10 μL of a solution of peroxidase in 50 *mM* PPB (5 mg mL^{-1}). The cell was stored under temperature-controlled conditions for 10 min at 30 °C and then a sample of wild-type TvDAAO or the corresponding mutant was added (30—40 μL).

To determine the maximum reaction rate and the Michaelis constant, the concentration of the corresponding D-amino acid was varied from 0.5 to 5 K_M. The approximate value of K_M was determined in a separate experiment by measuring the reaction rate at concentrations of the corresponding D-amino acid of 0.1, 0.5, 1.0, 5.0, 10, and 50 mmol L^{-1}.

The kinetic data were analyzed using the OriginPro 7.0 SR4 program by linear and non-linear regression methods.

Study of stability at different temperatures. The thermal stability of the mutants and wild-type TvDAAO was studied in a 0.1 *M* potassium phosphate buffer, pH 8.0. For each experiment, 0.5 mL plastic tubes containing 100 μL of the enzyme solution were placed into a water thermostat preheated to the required temperature (the accuracy of the temperature setting was ±0.1 °C). At certain time intervals, the tubes were withdrawn and cooled in ice for 1—2 min, and then the activity was measured as described above. The sampling interval was chosen so that the activity of the enzyme in the course of the experiment decreased to 10—15% of the initial value. The rate constants for inactiviation were determined by plotting the residual activity *versus* time. The plots were processed by the non-linear regression method using the OriginPro 7.0 SR4 program (OriginLab).

Results and Discussion

Preparation of Arg220Glu and Arg(169,220)Ala TvDAAO mutants. Nucleotide substitutions providing the required mutations were introduced by the polymerase chain reaction. The Arg(169,220)Ala TvDAAO double mutant was prepared in two steps. First, the Arg169Ala TvDAAO mutant was prepared, and then the Arg220Ala substitution was made. For each of the three mutants, six plasmids were selected for sequencing. The results of sequencing showed that only required mutations were present in all plasmids in the *tvdaao* gene, whereas other nucleotide substitutions were absent.

Plasmids containing the Arg220Glu and Arg(169,220)Ala substitutions in the *tvdaao* gene were transformed into the BL21 (DE3) Codon Plus/pLysS strain. The recombinant strains thus prepared were cultured for 24 h according to the procedure described in the Experimental section.

The cultivation showed that the Arg220Glu and Arg(169,220)Ala mutants of TvDAAO were synthesized completely in the insoluble form as inclusion bodies (Fig. 3) with retention of the enzymatic activity.

It is known that a decrease in the concentration of the inductor and the temperature of cultivation allows the preparation of the target recombinant protein in the soluble form. In the present study, we carried out experiments on the optimization of cultivation by varying the concentration of the inductor IPTG from 0.005 to 0.1 mmol L^{-1}, and reduced the temperature of cultivation from 25 to 18 °C. A decrease in the concentration of IPTG from 0.1 to 0.05 mmol L^{-1} was shown to increase the yield of the enzyme by a factor of 1.5—2.5. However, both mutants were still expressed only in the insoluble form.

We also made efforts to solubilize the Arg220Glu and Arg(169,220)Ala TvDAAO mutants. For this purpose, the

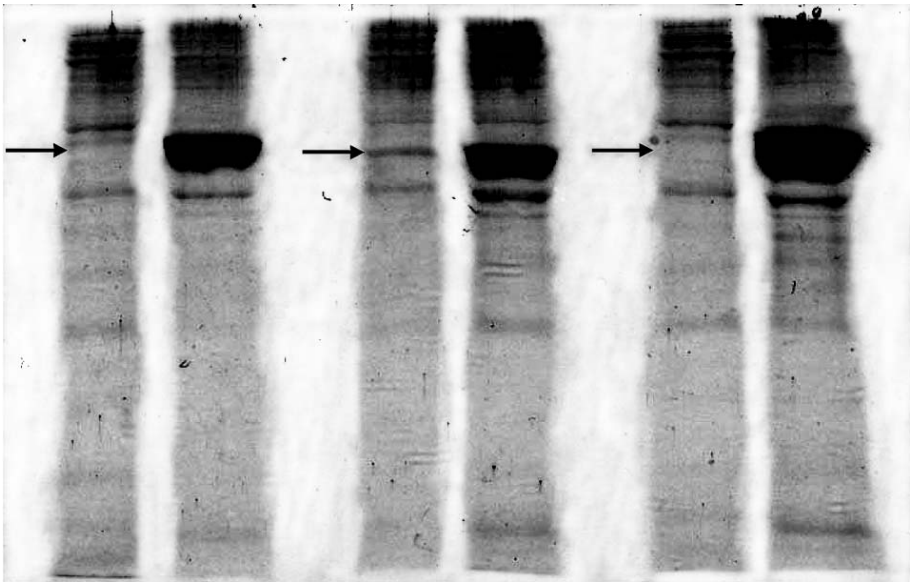

Fig. 3. Protein electrophoresis under denaturing condition of the Arg(169,220)Ala TvDAAO mutant (lanes *1* and *2*), wild-type TvDAAO (lanes *3* and *4*), and the Arg220Glu TvDAAO mutant (lanes *5* and *6*). The soluble and insoluble fractions are labeled by odd and even numbers, respectively. The positions of the mutant TvDAAO and the wild-type enzyme on the gel are indicated by arrows.

precipitate of the cell suspension, which was prepared by sonication followed by removal of the supernatant, was treated with non-ionogenic surfactants (Twin 20, Twin 40, Twin 80, Twin 100, Triton X-100, and Triton X-350) and with low concentrations of urea. However, in these cases we also failed to obtain the enzyme in the soluble form. Since the precipitate of the cell suspension exhibited activity, we decided to measure the kinetic parameters of the membrane-bound enzyme.

Determination of the kinetic parameters of the Arg220Glu and Arg(169,220)Ala TvDAAO mutants. The results of determination of the kinetic properties of the Arg220Glu and Arg(169,220)Ala mutants of TvDAAO are given in Table 1. It was impossible to determine the absolute values of the catalytic constant k_{cat}, because there are no methods that can be used to determine the concentration of the active sites in the membrane-bound mutants of TvDAAO. Hence, the activities for different D-amino acids are given relative to the activity with respect to D-methionine, which is the best substrate for wild-type TvDAAO, as well as for its mutants.

As can be seen from Table 1, the introduction of the Arg220Glu and Arg(169,220)Ala mutations into the TvDAAO molecule leads to a sharp change in the substrate specificity profile. In particular, the enzyme lost the activity with respect to D-Leu, D-Thr, D-Tyr, and D-Ala. In addition, the Arg220Glu TvDAAO mutant does not exhibit activity with respect to D-Ser and D-Phe. The largest changes in the kinetic parameters were observed for the Arg220Glu TvDAAO mutant. The Michaelis constants of the latter mutant with respect to most of the D-amino acids under study are substantially higher. For some of the substrates, the almost linear dependence of the reaction rate on the concentration of the D-amino acid was observed up to the maximum solubility of the latter. Hence, only the ratio V_{max}/K_m but not the individual parameters can be determined.

For the Arg(169,220)Ala TvDAAO double mutant, the Michaelis constants with respect to some D-amino acids, in particular, D-valine, D-asparagine, and D-lysine, increased several-fold.

Thermal stability of Arg220Glu and Arg(169,220)Ala TvDAAO. The Arg220Glu and Arg(169,220)Ala mutants of TvDAAO are much less stable than the wild-type enzyme. For wild-type TvDAAO, T_M (the temperature at which the initial activity decreased by 50% in 30 min) is 54 °C, whereas T_M for the Arg220Glu and Arg(169,220)Ala mutants are 28 and 20 °C, respectively, *i.e.*, by 26 and 34 degrees lower than that for the wild-type enzyme. Hence, the kinetics of thermal inactivation of the Arg220Glu and Arg(169,220)Ala mutants of TvDAAO was measured in the temperature range of 32—40 and 20—28 °C, respectively.

Previously, the investigation of the thermal denaturation of wild-type TvDAAO showed that the time dependence of the residual activity of the enzyme is described by the sum of two exponentials, and the inactivation occurs at least in two steps.[14] The reversible dissociation of the dimer into monomers as the first step is followed by the irreversible inactivation. The time dependences of the residual activity (A/A_0) of the Arg220Glu TvDAAO mutant for the lower and upper limits in the temperature range of measurements (32 and 40 °C, respectively) are shown in Fig. 4. The inactivation curve at 32 °C is well described only by the sum of two exponentials. At 40 °C, the two-exponential character of the inactivation curve is less evident. Similar results were obtained also for the Arg(169,220)Ala TvDAAO mutant (not shown in Fig. 4). Hence, Fisher's criterion was used for the quantitative es-

Table 1. Kinetic parameters of wild-type TvDAAO and its Arg169Ala/Arg220Ala and Arg220Glu mutants

Amino acid	TvDAAO Arg169Ala/Arg220Ala			TvDAAO Arg220Glu			Wild-type TvDAAO		
	K_M /mmol L^{-1}	$\frac{V^{max}}{V_{max}^{Met}}$ (%)	$\frac{V_m/K_m}{(V_m/K_m)^{Met}}$ (%)	K_M /mmol L^{-1}	$\frac{V^{max}}{V_{max}^{Met}}$ (%)	$\frac{V_m/K_m}{(V_m/K_m)^{Met}}$ (%)	K_M /mmol L^{-1}	$\frac{V^{max}}{V_{max}^{Met}}$ (%)	$\frac{V_m/K_m}{(V_m/K_m)^{Met}}$ (%)
D-Met	3.0±0.9	100	100	0.78±0.5	100	100	0.24	100	100
D-Ser	105±33	62	2	*	*	*	28.7	15	0.1
D-Val	**4.3±0.9****	69	48	>100	***	17.7	17.7	89	1.2
D-Phe	38±5	100	8	*	*	*	0.40	40	24
D-Asn	**5.6±0.7**	85	42	30±4	74	2	15.8	59	0.9
D-Lys	**27±7**	25	3	>100	**	49.4	49.4	39	0.2
D-Leu	*	*	*	*	*	*	0.78	34	10
D-Thr	*	*	*	*	*	*	10	5	0.1
D-Tyr	*	*	*	*	*	*	0.73	38	13
D-Ala	*	*	*	*	*	*	0.3	30	24
D-Leu	*	*	*	*	*	*	0.78	34	10

* The reaction does not proceed.

** The kinetic parameters of the mutant enzyme that exceed those of wild-type TvDAAO are in the boldface type.

*** Data are absent.

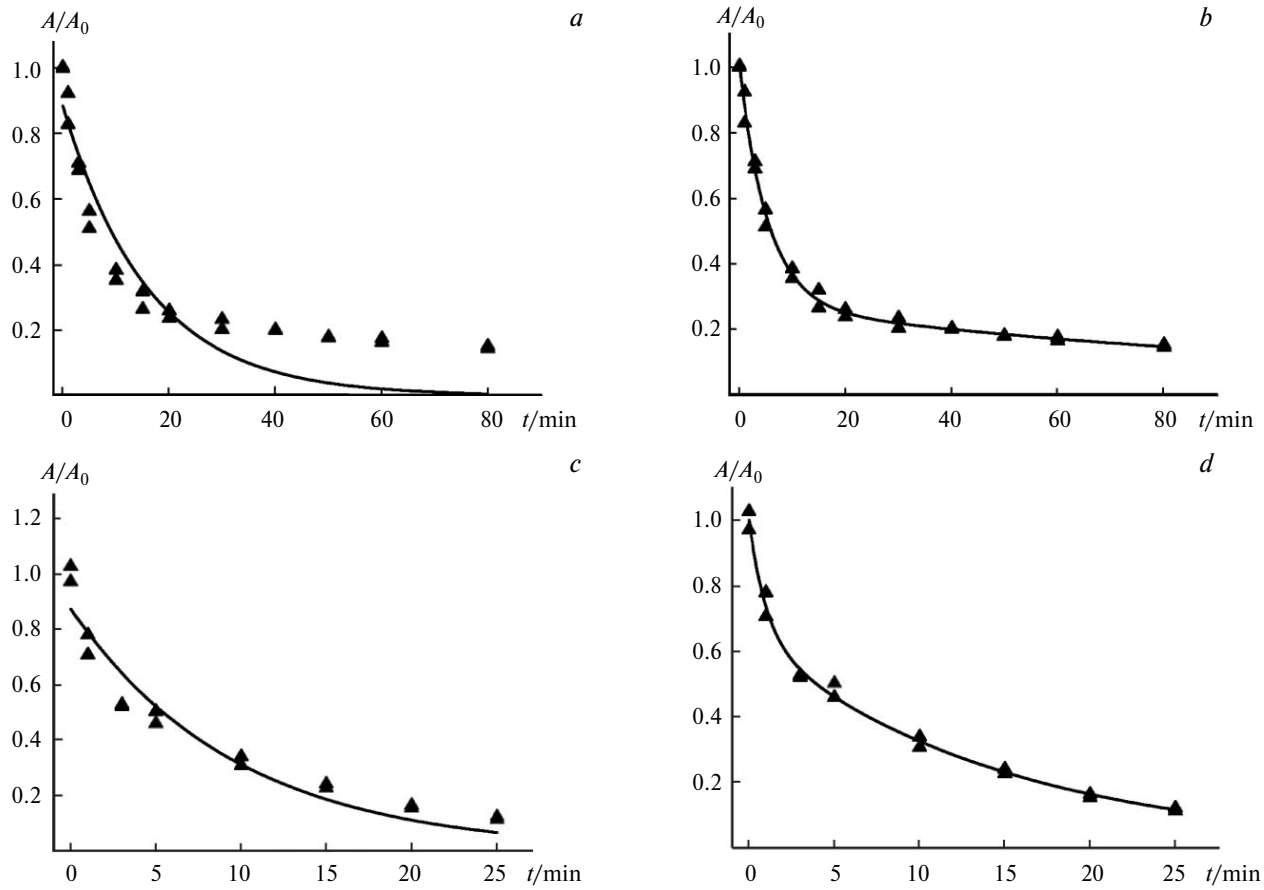

Fig. 4. The inactivation curves of the Arg220Glu TvDAAO mutant at 32 (*a*, *b*) and 40 °C (*c*, *d*) described by one exponential (*a*, *c*) and by the sum of two exponentials (*b*, *d*) (0.1 *M* phosphate buffer, pH 8.0).

timation of the validity of the two-exponential model for the description of the inactivation curves of both mutants throughout the temperature range of measurements. According to this criterion, the model proposed for the approximation of the experimental data is adequate if calculated Fisher's coefficient (F_{exp}) is smaller than the theoretical value (F_{calc}). Table 2 exemplifies the results of calculations of F_{exp} for one- and two-exponential models for the description of the inactivation curves of the Arg220Glu and Arg(169,220)Ala mutants of TvDAAO at 40 and 28 °C, respectively, at which the two-exponential model is less evident. The theoretical coefficient F_{exp} was taken from Fisher's table for the confidence probability of 0.95. As can be seen from Table 2, in the case of the simple exponential model, calculated Fisher's coefficient is much larger than the theoretical value. This means that this model is not adequate for the description of experimental data. In the case of the approximation by the sum of two exponential functions, calculated Fisher's criterion indicates that the model is adequate. Hence, only the approximation of the experimental data on the inactivation of both the Arg220Glu and Arg(169,220)Ala mutants of TvDAAO by the sum of two exponential functions is adequate and statistically significant. Therefore, the results of calculations indicate that, as in the case of the wild-type enzyme, the inactivation of both mutant TvDAAO occurs at least in two steps. Hence, it can be concluded that both mutant enzymes also exist as dimers.

Table 3 gives the numerical values of the calculated effective rate constants for inactivation for the first and second steps in the temperature range under study. As is

Table 2. Estimation of the validity of the one- (I) and two-exponential (II) models for the description of the kinetics of inactivation of the Arg220Glu and Arg(169,220)Ala TvDAAO mutants at 40 and 28 °C, respectively

Mutant enzyme	Parameter	Model*	
		I	II
TvDAAO Arg(169,220)Ala	F_{exp}	21.15	2.24
	F_{calc}	2.45	2.62
TvDAAO Arg220Glu	F_{exp}	134.45	1.96
	F_{calc}	2.32	2.43

* I: $y = A \cdot \exp^{-kx}$, II: $y = A \cdot \exp^{-k_1 \cdot x} + B \cdot \exp^{-k_2 \cdot x}$

Table 3. Effective rate constants for inactivation of R220E TvDAAO and R169A/R220A TvDAAO at different temperatures

Mutant enzyme	T/ °C	k_1	k_2
		min^{-1}	
TvDAAO R220E	32	0.184±0.023	0.0078±0.0019
	34	0.253±0.08	0.020±0.005
	36	0.490±0.11	0.039±0.008
	38	0.730±0.18	0.047±0.013
	40	1.000±0.26	0.068±0.015
TvDAAO R169A/R220A	20	0.250±0.004	0.0096±0.0024
	22	0.480±0.10	0.032±0.005
	24	0.510±0.12	0.044±0.008
	26	0.770±0.15	0.061±0.110
	28	0.960±0.17	0.076±0.016

evident from the above-considered data, the effective rate constant k_1 characterizing the dissociation of the dimer more slowly increases with temperature than the effective rate constant k_2 characterizing the rate of inactivation of the monomer, *i.e.*, the energy of activation for the second step is higher than that for the first step. This is in good agreement with our model of inactivation of TvDAAO, because the energy of activation for the dissociation of the dimer into individual subunits should be lower than that necessary for the unfolding of the protein globule in the step of inactivation of the monomer.

The above-considered data indicate that the arginine residues in positions 169 and 220 play a considerable role in the formation of the quaternary structure and ensuring the stability of TvDAAO. The substitution of other amino acid residues for these residues results in that the enzyme is synthesized in the active but insoluble form. The introduction of the Arg220Glu mutation into the TvDAAO molecule substantially impairs the kinetic parameters with different substrates and leads to a decrease in stability of the enzyme. In the case of the double substitution Arg169Ala/Arg220Ala, the higher constants K_M with respect to a series of D-amino acids were observed in spite of the fact that the kinetic properties were studied for the cell wall-bound enzyme. It should be noted that substantial changes in the substrate specificity profile were caused by the substitutions of the amino acid residues that are not involved in the active site of the enzyme. An analogous effect was observed in the case of the Cys108Phe, Cys108Ala, and Cys108Ser single mutations.[13] The first mutation leads to an increase in thermal stability of TvDAAO, whereas the second and third mutations cause a decrease in thermal stability.

The results of our experiments also indicate that the existence of TvDAAO in the dimeric form is more critical for its stability compared to the enzyme from the yeast *R.gracilis*. In the latter case, the monomeric mutant enzyme was obtained in the active and soluble form, whereas the removal of hydrogen bonds involved in the intersubunit contact leads to the transformation of TvDAAO in the completely insoluble form. We plan to perform amino acid substitutions that would enhance the intersubunit contact in the TvDAAO molecule and lead to an increase in thermal stability of the enzyme.

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